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新たなバイオテクノロジーを用いて得られた食品の安全性確保とリスクコミュニケーションのための研究

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「新たなバイオテクノロジーを用いて得られた食品の安全性確保と
リスクコミュニケーションのための研究」

総括研究報告書

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研究要旨：
ゲノム編集技術を利用した作物（ゲノム編集作物）から作られる新たな食品の研究開発が国内外で活発に行なわれている。しかし、安全性審査が必要な従来の遺伝子組換え食品とは異なり、導入遺伝子は存在しない。ゲノム編集食品の届出制度が平成31年度（令和元年）10月に開始となり食品衛生法上の取扱いも明確化された。今後制度実施にあたり、そこに至った科学的背景等を整理しておくことが必要である。また、安全性確認を科学的なエビデンスをもって行うための手法開発整備も重要な課題である。本研究では、種々の手法による遺伝子改変の影響、ゲノム編集作物の開発状況や規制状況の情報収集を行い施策に反映するとともに、安全性確認で必要な項目や問題点を明らかにした。また、ゲノム編集技術や合成生物学など新たなバイオテクノロジー技術を用いた新開発食品の安全性を確認するために必要な新たな手法の開発検討を行った。ゲノム編集ではオフターゲットが課題になっていることから、配列類似性によらないバイアスのないゲノム全体の DNA 切断部位を検出する方法の開発、非アレルゲンタンパクのアミノ酸情報も加味し、既知アレルゲンタンパクとの相同性に依存しない人工知能を用いた全く新たなタンパクアレルゲン性予測アルゴリズムの開発、新開発食品試料中に出現する未知成分の質量分析インフォマティクスを用いた同定手法の開発、の検討を行った。その結果、ゲノム解析では、SITE-seq 法を出発点にしたオフターゲット検出法を確立するとともに、web ツールを開発した。新規アレルゲン性予測では、アレルゲンタンパクにのみ出現するアミノ酸配列パターンを抽出、データセットの改良を行いながらアレルゲン性予測が従来よりも精度が高いことが確認できた。アレルゲン性とも関連するタンパクの分解性試験について、pH、酵素濃度について細かく設定して検討した。その結果、分解されやすい　タンパクにおいてもペプシン濃度よりも pH 変化が分解性に大きく影響することが分かった。質量分析インフォマティクスでは、基になる高品質な質量分析スペクトル情報が必要なため公共データベース、および標品測定からスペクトル情報を取得することでデータベース化するとともに、Python プログラム等を用いて結果をネットワーク化して可視化できた。さらに、ゲノム編集マダイ、トラフグ開発において、ゲノム編集食品の事前相談・届出制度にある必要項目に沿った十分なデータを取得してその安全性を確認した。

A. 研究目的

ゲノム編集技術を応用した新たな食品（ゲノム編集食品）の研究開発が国内外で活発に行なわれている。ゲノム編集食品では、従来の遺伝子組換え食品のような外来遺伝子を導入することはなく、もともとの性打つが有する内在性遺伝子の配列を数塩基欠失により機能欠失させることで新たな形質（もち性向上、筋肉量増加、GABA 量増加など）を付与することが期待されている。そのため、国民受容の改善の点でも大きく期待されている。また、合成生物学を利用した物質生産も米国を中心に活発に研究されている。酵母などの微生物に、新たな物質生産に必要な多数の遺伝子を導入することで、その生物が元来合成できない化合物の生

産が可能になっている。

ゲノム編集食品では、安全性評価の対象は内在性遺伝子改変に伴う塩基配列変化とゲノム編集時の意図しない変化（いわゆる、オフターゲット）となると考えられる。一方で、合成生物学利用作物では、生合成経路に関わる多数の遺伝子を導入するため、安全性評価対象は導入した遺伝子群とその影響であるが、組換え範囲が大きいため従来の遺伝子組換え前後の比較による実質的同等性の考え方が適用できないことも想定される。

従来の遺伝子組換え食品における安全性確認の基本的な考え方は、組換えをする前後の作物を用いた比較解析からの実質的同等性（リスクが組換え前と比較して同等かそれ以下）で判断している。すなわち、導入遺伝子に関する分子生物学的特性、ヒトによる長期間にわたる安全な食経験、構成成分変化、使用方法等について同等性を失っていないかである。しかし、改変されるのは内在性遺伝子上における塩基の挿入・欠失であり、標的部位（オンターゲット）での変化が十分解析されていることが重要で、その上で潜在的なリスクは意図しない改変であるオフターゲットの影響である。オン・オフターゲット部位での変化によって生じるリスクは、新たな毒性・アレルゲン性タンパクの生成である。ゲノム解析が進んだ現在においても、ゲノム配列のみから毒性タンパクやアレルゲン性タンパクが生成しないことを明らかにするのは容易ではない。また、意図しない有害成分産生の可能性があったとしても、現在の質量分析を用いた解析では未知ピークの同定や推定は困難である。さらに、タンパクアレルゲン性の確認も、現在実行可能な *in silico* 解析は既知のアレルゲンタンパク質との相同性比較のみであり、相同性がない新規アレルゲン性タンパク質の予測や非天然型アミノ酸から構成されるタンパクのアレルゲン性を予測することは極めて難しい。このような状況を鑑みて、ゲノム編集食品の開発状況情報収集をもとにしたケーススタディーや開発者との連携で申請側の問題点を明らかにするとともに、上記のゲノム編集食品や合成生物学利用食品の安全性確認のために必要な評価手法の新たな開発が急務と考えられた。

本研究では、手法開発において、標的配列と類似した配列のオフターゲット検索しかできない点を克服すべく、全ゲノム解析をすることなく潜在的なDNA２本鎖切断部位を網羅的に検出する手法、新たな成分が産生した場合の質量分析インフォマティクスを用いた成分同定あるいは基本構造推定手法、人工知能を活用して相同性がないアレルゲン性タンパクの予測や非天然型アミノ酸から構成されるタンパクのアレルゲン性を予測する手法、の開発検討を行う。また、諸外国の規制・ゲノム編集・合成生物学に関する情報収集を行い、その結果から仮想的モデル生物を用いたケーススタディーを行い、安全性確認に必要なデータや問題点を明らかにすることとした。また、平成 31 年度（令和元年）10 月に、ゲノム編集食品の届出・事前相談制度が開始されたことから、それに伴う科学的知見の整理に必要な文献情報を整理した。リスクコミュニケーションにおいては、ゲノム編集技術に関する知識がほとんど無い層をターゲットにした、チラシ、パンフレットの作成を行う。

B. 研究方法

（１）ゲノム編集に関する情報収集解析、ケーススタディーおよびアレルゲン分解性の検討

植物・動物（細胞）を主な対象に、自然変異、放射線による突然変異誘導、ゲノム編集による変異誘導について、定量的な解析がされた科学論文を中心に調査整理した。

（２）リスクコミュニケーションに関する研究

専門的な知識を持たない一般の人の、遺伝子組換え食品やゲノム編集食品の疑問や不安が大きいため、疑問点の整理、専門家と一般の人での認識の違いを調査しながらパンフレットや小冊子の作成を行った。

（３）ゲノム網羅的に DNA２本鎖切断部位を検出する手法とツールの開発検討

現在、ゲノム編集技術を用いた時のオフターゲットについては、標的部位（オンターゲット）と類似したゲノム上の場所を *in silico* に検索することしかできない。そのため、オンターゲットと配列類似性がない部位でのオフターゲットやその影響は検出把握できない。これを解決するために、既報である SITE-Seq 法を出発点に動物や植物に適用可能で簡便かつ再現性の高い、ゲノムワイドな DNA２本鎖切断部位解析手法とそれを利用者が使用する環境ツールの開発検討を行った。

（４）質量分析インフォマティクスによる化合物

同定

化合物の質量スペクトル（フラグメントスペクトル）類似度をもとにして、食品中の未知化合物の検出と構造推定を行うシステムの構築を行った。データ解析のワークフローとして、安全と考えられる食品試料（非ゲノム編集体）と分析対象の試料（ゲノム編集体等）の液体クロマトグラフィー質量分析データから得られる化合物イオンの比較定量値およびフラグメントスペクトルを解析データとして用いた。化合物の構造・クラス推定に不可欠なスペクトルデータの取得拡充に努めて、独自の代謝物の標準品 300 種類を質量分析装置 Q-exactive HF（Thermo Fischer Science）で分析したライブラリを含めた統合フラグメントスペクトルデータベースを構築した。データ解析には、プログラミング言語:Python を用い、ケモインフォマティクスライブラリ：RDKit、データ可視化ライブラリ：Plotly 等と連携させることでデータ解析環境の構築を行った。

（５）アレルゲンデータベース ADFS のアップデート、および新規タンパクアレルゲン性予測に必要な情報の作製

現アレルゲンデータベース ADFS の情報更新のため、アレルゲン情報の追加と 2018 年 6 月から 2019 年 5 月までの 1 年間に NCBI PubMed に収載された論文からのエピトープ情報を追加した。また、ADFS サイトの脆弱性対策のために、JAVA、MySQL などのバージョンアップの他スクリプトを改訂した。

新規アレルゲン予測手法の検討のために、アレルゲンデータベース Compare からアレルゲンタンパク情報を、非アレルゲンタンパク情報は、Uniprot からアレルゲン情報を除くことによって構築した。

（６）機械学習を用いた新規タンパクアレルゲン性予測手法とツールの開発

既知のアレルゲンタンパクのアミノ酸配列のみに依存しない、新たなアレルゲン予測システム構築のために、既知アレルゲンタンパクのほかに非アレルゲンタンパクのアミノ酸配列情報を加えたデータセットをもとに検討を行うこととした。

使用するデータセットについて、食品および非食品タンパク質を追加して検討した。アレルゲンタンパクに特徴的なパターンの抽出について、データマイニング分野の技術（系列マイニング）を利用して、アレルゲンにのみ出現する部分アミノ酸配列を検索した。また、アレルゲンには食品・非食品タンパクを含むため、非アレルゲンデータにも食品・非食品タンパクを含むデータを構築することを検討した。予測システムの構築は上述の Leave-Food-Out クロスバリデーションを利用した教師あり学習によって行った。さらに、2019 年度は、さらに、抽出されたパターンの生物学的な考察として、既存のエピトープとの一致度の確認や、結合性の確認なども行った。

（７）ゲノム編集生物作製における現象解析と規制の進め方

ミオスタチン遺伝子破壊マダイおよびトラフグ各３系統、レプチン受容体遺伝子破壊トラフグ２系統、メラノコルチン４型受容体遺伝子トラフグ１系統について、アレルゲン性、オフターゲット、外来遺伝子残存性および継代安定性について検討した。改変部位での予想される全アミノ酸配列、新生ペプチドとその直上１０アミノ酸部分、および、塩基欠失部位を挟んだ両側の終止コドン内で予想されるペプチドを用い、web 上のアレルゲン検索サイトによりアレルゲン性の有無を検討した。オフターゲットおよび外来遺伝子残存性は、レプチン受容体遺伝子破壊トラフグの全ゲノム解析をもとに解析した。

（８）人材育成（統計学、バイオインフォマティックス、AI 分野）

分担研究者および協力研究者と共同で行うことで、インフォマティクス関連技術の取得に努めた。

C. 研究結果および考察

（１）ゲノム編集に関する情報収集解析、ケーススタディーおよびアレルゲン分解性の検討

2018 年途中までの新規育種技術（NBT)を用いた動物および植物について調査した結果、動物では食品用途（食用）は全 39 報中 20 報であった。使用技術はほとんど CRISPR/Cas9 およびその改変型であり、主な獲得形質はブタの筋肉量増大やウイルス抵抗性である。植物でも使用技術はほとんど CRISPR/Cas9 で、食用は全 122 報中 42 報、研究用は 76 報であった。食用では、トマトの保存性向上や種子がなくても果実ができるもの、コムギの光合成能向上やうどんこ病抵抗性、イネの除草剤耐

性、イネでは収量の増加のほかウイルス抵抗性キュウリなどがある。詳細は分担報告書を参照のこと。ケーススタディーでは、開発直近の筋肉量増大マダイやフグ、もち性向上トウモロコシの実際の事例からとゲノム編集技術で仮想の農作物等を設定して、確認すべき事項や問題点を明らかにした。詳細は分担報告書に記載しているので参照のこと。

（２）リスクコミュニケーションに関する研究

一般の人 4,000 人を対象にした Web 調査の結果から、ゲノム編集技術に関して知らないかあまり知らないが 8 割に上り、ゲノム編集食品を食べたい人は 1 割程度、またゲノム編集食品の安全性についての専門家の意見を信頼できるとしたのは 2 割に満たないことが分かった。社会受容についてリスクの程度や対応、発生確率などが重要な事項と考えていることが伺える結果となった。

詳細は分担報告書及び別添資料に記載している。

（３）ゲノム網羅的に DNA２本鎖切断部位を検出する手法とツールの開発検討

標的部位と類似していない箇所のオフターゲットの検出が可能な unbias な手法を、SITE-Seq 法をもとに、イネもとに詳細に検討した。ALS 遺伝子を標的にした実験結果から、標的部位が切断された実験条件（Cas9 濃度が 64、256 nM）で検出されたオフターゲット部位は 9 か所あった。このうち 4 つは PAM 配列も存在することからオフターゲットの候補と考えられた。一方で、ミスマッチ数は 4 塩基以上で確率的にはかなり小さいと推察された。このうち、PAM を含む 2 つのオフターゲット候補について切断効率を算出すると、オンターゲット 74%に対して、13%（4 塩基ミスマッチあり）および 6%(7 塩基ミスマッチあり)であった。これらは通常のオンラインツール（CRISPRdirect や Cas-OFFinder など）の通常の検索条件では検査されない。同様に、ほかの 6 か所についても同様の結果であった。以上の結果は、オフターゲットのオンライン検索ではすべてのオフターゲットを検出することはできないことを示している。今回の実験結果は、主に抽出 DNA を用いた結果であり *in vivo* においては DNA 修復されて検出されない可能性も考えられるが、ゲノム編集技術の一つ CRISPR/Cas9 は技術が持つ本質的性質として、ミスマッチが多くても切断されることを示している（この場合でも 3’側 10 塩基で見れば 1、2 塩基ミスマッチが主である)。最終的に検出されるかどうかは、用いる生物の DNA 修復能力に大きく依存するため、評価においてはその点も考慮に入れて行うべきと考えられた。

詳細は分担報告書に記載している。

（４）質量分析インフォマティクスによる化合物同定

本研究では、化合物の質量スペクトル類似度をもとに試料中の未知化合物の検出と構造推定を行う解析システムの構築を行うための検討を行った。既存のスペクトルライブラリに加え標準品・標準試料から大量の質量スペクトルライブラリを取得すること統合スペクトルライブラリの拡充を行うとともに、比較定量情報を反映した可視化機能の実装など解析ツールの高機能化を行った。

代表的な食品・モデル植物 30 種（大豆、トマト、ジャガイモ等）に関しては低分子化合物の抽出と実際の測定によるスペクトルデータ取得を行った。生物種－代謝物関係データベース（KNApSACK）から試料ごとの代謝物情報を抽出し、スペクトルデータと照合することで標準試料由来のスペクトルデータベースを構築した。今後、実際の試料（遺伝子組換え前後の試料）などを使用して、変化のある成分の同定、推定がどの程度可能か検討する。

（５）アレルゲンデータベース ADFS のアップデート、および新規タンパクアレルゲン性予測に必要な情報の作製

アレルゲン情報は、AllergenOnline の登録アレルゲンと統合するためアップデートを行った。エピトープ配列は、キーワード検索により抽出した 20 報について、アレルゲン・エピトープ情報が記載されている 10 報についてピアレビューを行った。その結果、7 報の論文から 7 種のアレルゲンについて、総数 22 のエピトープ情報を新たに追加した。

新規予測法のために、アレルゲンデータベース Compare からアレルゲンタンパク情報 2038 種を入手し、アレルギー表示が義務付けされている特定原材料 7 品目並びに推奨されている原材料のうち 4 品目の非アレルゲンタンパク配列情報について UniProt から 10577 種を取得した。非アレルゲン学習データの種類を増やして解析ができるよう

に、アレルゲンとして登録された全ての種について情報を取得し解析できるよう調整したが、Uniprot から取得したデータからすべてのアレルゲン情報を削除して、非アレルゲン情報を作成するのは困難と考えられた。

（６）機械学習を用いた新規タンパクアレルゲン性予測手法とツールの開発

特定の食物に頻出するアミノ酸部分配列を誤ってアレルゲン特異的パターンとして抽出してしまうリスクを避けるために、順に一つの作物を除き残りの食物で訓練する Leave-Food-Out クロスバリデーションを行った。抽出したアレルゲンパターンについて、アレルゲン性タンパク質が多くのアレルゲン特異的パターンを含んでいることが確認できた。実際、これらのアレルゲン特異的パターンの生物学的特徴を調べたところ、既知のエピトープと類似していることが確認されている。従来の予測方法に比べて、本研究で構築した方法ではおおむねすべての場合において最もよい判定・予測性能を示していることが確認できた。次年度はさらにほかのアプローチとの比較も行うことで本システムの有効性の実証を行う。

（７）ゲノム編集生物作製における現象解析と規制の進め方

ミオスタチン遺伝子破壊したマダイおよびトラフグでは、E-value<0.05 以下においていずれの系統もアレルゲン性が疑われるアミノ酸配列は検出されなかった。レプチン受容体遺伝子破壊トラフグにおいても、E-value<1 ではイネの α－アミラーゼと相同性が認められたが、E-value<0.05 以下においていずれの系統もアレルゲン性が疑われるアミノ酸配列は検出されなかった。

レプチン受容体遺伝子破壊トラフグにおいてオフターゲットおよび外来遺伝子残存性について解析した結果、オフターゲットによる欠失は観察されなかった。また、残存性については NGS による全ゲノム解析データからのリードを用いたベクターにマッピングさせることで検索した結果、ベクターのバックボーン配列の一部（ColE1 ori）が検出されたがゲノム編集魚および野生型魚の両者で観察されていることから、トラフグゲノム内に一般的に侵入した細菌断片であると思われる。それ以外の外来遺伝子とその断片配列は認められなかった。その他に可食部のメタボローム解析を実施した。詳細は分担報告書に記載している。

D. 結論

自然変異、放射線による突然変異育種、ゲノム編集技術の各技術について放射線による突然変異育種は、後代交配を経ることで最終的な変異は、考えられていたよりも小さく数塩基の変異が中心であるのに対して、ゲノム編集により変異は同様に小さいものの、その頻度は非常に高いため、相対的に意図しない変異頻度も高いと考えられた。ゲノム編集作物では、イネの研究が非常に活発であるほかに多様な植物で研究開発が進んでいることが分かった。

リスクコミュニケーションでは、専門的知識がない人に向けての新しいパンフレット（厚労省用）を作成した。各種の説明会や web 形式アンケートから、ゲノム編集食品を知っているのは 2 割で十分に知られていない。また、専門家の意見について信頼できるとした割合も 2 割以下など、一般の人への理解や信頼性が低く、これを改善することが不可欠である。対象別にきめ細かいコミュニケーションが必要である。

網羅的なオフターゲット検出法 SITE-Seq は、イネでも有効であることを確認した。SITE-Seq 法は、オンラインオフターゲット予測ツールでは予測不可能であったオフターゲットの予測が可能であり、ゲノム編集食品の安全性評価時に有用な手法と考えられる。

意図しない新たな代謝物を同定予測するための手法について、質量分析インフォマティクスを用いて試料間比較による代謝物の変動と連携した化合物のクラス推定・可視化という解析フレームワークを用いて確立した。

既存の ADFS データベースの情報更新を行うとともに、新規アレルゲン性予測に活用するためにタンパク情報を整備した。これらのデータセットを用いて、アレルゲンに特徴的なアミノ酸モチーフを検索した。これまでに構築したアレルゲン性判定・予測システムのプロトタイプの問題点を抽出し、改良を加えた結果、訓練データベースの大規模化と高精度化、アレルゲン特異的パターンの信頼性向上、判定・予測システムの精度向上が可能となった。

ミオスタチンゲノム編集マダイ、トラフグ、レプチン受容体ゲノム編集トラフグ、メラノコルチン４型受容体ゲノム編集トラフグにおいて、予想

されるタンパク質およびペプチドはアレルゲン性を示さないこと、オフターゲット・外来遺伝子残存性もないことが示され、届出・事前相談における必要項目をカバーするデータの取得ができた。

E. 業績

論文、学会発表、説明会、リスコミ開催などの業績詳細は、各分担報告書に記載。

厚生労働科学研究費補助金（食品の安全確保推進研究事業）
「新たなバイオテクノロジーを用いて得られた食品の安全性確保と
リスクコミュニケーションのための研究」
分担研究報告書

ゲノム編集に関する情報収集解析、ケーススタディーおよびアレルゲン分解性

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研究要旨：
ゲノム編集食品の届出制度運営にあたり、安全性審査が必要でなく届け出になったこと、届け出制度内での安全性確認について、判断根拠となる科学的背景が技術について整理されていることが重要である。本研究では、今年度は自然変異、放射線や変異原物質を用いた突然変異誘導法、およびゲノム編集法の特性について科学文献をもとに調査整理した。その結果、放射線や変異原物質を用いた突然変異誘導法によるイネの研究では　変異頻度は(23.1±1.5)×10^{-8} per/bp と自然変異の数十倍で、大部分が数塩基（+1 bp～-4 bp）の Indel であった。一方、ゲノム編集では変異頻度は高いものでは数十%に達し、場合によりオフターゲットも数%になることもある。また、ゲノム編集では DNA２本鎖切断後もゲノム上の標的配列から遊離しないため、修復過程に影響することが考えられ、その遊離を促進することができれば意図しない変化を一層低減できるのではないかと示唆された。
ゲノム編集技術を用いた研究の文献調査では、2018 年から 2019 年を調査した。研究報告が多かった順に、rice 130 件、tomato 45 件、wheat 21 件、maize 20 件のほか grape 3 件、apple 3 件、banana 2 件、melon 1 件などであった。病害虫耐性、高塩耐性、生産性向上などの形質が多かった。
アレルゲン評価に用いる資料の一つであるタンパク分解性について、pH 変化による影響を実験的に検討した。ピーナッツの主要アレルゲンの一つである Ara h1 をピーナッツより精製して、異なる pH、pepsin 濃度で分解性試験を行った。その結果から、これまで比較的分解しやすいと考えられている Ara h1 は pH 2 では速やかに分解されるものの、pH3.3 ではほとんど分解されずに残っていることが分かり、タンパク分解性は胃（の酸性度）の状態により大きく異なり、アレルゲン性評価にはより詳細な検討が必要と考えられた。EFSA においても、同様の検討がされており継続して行う必要があると考えられた。

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A. 研究目的

ゲノム編集技術を利用した作物（ゲノム編集作物）から作られる新たな食品の研究開発が国内外で活発に行なわれている。ゲノム編集作物では、従来の遺伝子組換え作物のような外来遺伝子を導入することはなく、内在性遺伝子の配列を数塩基欠失により機能欠失させて新たな形質（もち性向上、筋肉量増加、GABA 量増加など）を付与できる。しかしながら、最終的に外来遺伝子が存在しないゲノム編集作物は、規制上（食品衛生方法上）どう扱われるか、また、ゲノム編集食品の安全性を確認するために新たに必要とされる分析手法は何か、などは十分に議論されてきたとは言えない。一方で、ゲノム編集技術を利用した食品（ゲノム編集食品）の届出・事前相談制度が平成 31 年 10 月から開始された。そこでは、安全性審査が不要であるとする根拠として、従来から安全に用いられてきた突然変異育種（放射線など）と起きる変化が同等であることとされた。ゲノム編集食品の届出制度を運営するにあたり、突然変異育種との同等性を考える判断根拠となる科学的背景やゲノム編集技術の特性、応用例について、十分に調査研究することが必要である。

本研究では、ゲノム編集作物の開発状況の情報収集を PubMed、Scifinder などデータベースを用いて文献等調査を行い、自然変異、放射線による突然変異、ゲノム編集技術による変異誘導について調査整理を行った。また、タンパクのアレルゲ

ン性とも関連するタンパク分解性試験について、EU ではヒトの実際に合わせた細かい条件での検討が推奨されているため、国内においてもその影響を考える必要が生じている。そのため、人工胃液による分解性試験条件、酵素濃度・pH について細かく設定して検討して分解性に与える影響を調査した。

B. 研究方法

1. ゲノム編集食品の届出制度での安全性確認に関わる判断の科学的背景・技術特性調査

植物・動物（細胞）を主な対象に、自然変異、放射線による突然変異誘導、ゲノム編集による変異誘導について、定量的な解析がされた科学論文を中心に調査整理した。

2. 研究開発に関する文献調査

データベースとして PubMed、SciFinder を主に用いて 2018 年から 2019 年前半について検索した。検索キーワードは、表 1 の A 群と B 群から 1 つずつ選んだ物を組み合わせて利用した。検索によりヒットした文献から、タイトル・書誌情報とアブストラクトの情報をもとにヒットした文献についてリストを作成した。リストの項目を以下に示す。(文献 ID（通し番号)、生物種（動物、植物)、種名（ブタ、ウシ等)、用いた技術、雑誌名、タイトル、発表年、巻・号・ページ、著者名、所属機関、国、Pubmed ID, DOI, ターゲット遺伝子名)。

3. 人工消化液によるタンパク分解性試験（酵素濃度・pH 条件の影響）

ピーナッツアレルゲン Ara h1 の精製は、ピーナッツ 10.5 g から既報に基づいて硫安沈殿、イオン交換クロマトグラフィーにより行い、N 末端アミノ酸分析および質量分析により、Ara h1 であることを同定確認した。最終的に 3.1 mg の Ara h 1 を得た。

タンパク分解性試験は、EFSA の意見書に基づいて、ペプシン濃度（高濃度、低濃度）と pH（2.0, 3.3, 5.5）で、ペプシン濃度は高濃度の条件として、ペプシン：テストタンパク質＝10 U：1 mg とした。ペプシンの低濃度の条件として 1000 U/mL とした。インキュベーションの条件は 37℃で、1, 2, 5, 10, 30, 60, 120 分として実験を行った。

C. 研究結果および考察

1. ゲノム編集食品の届出制度での安全性確認に関わる判断の科学的背景・技術特性調査

自然変異、放射線や変異原物質を用いた突然変異誘導法（遺伝子組換え規制外)、およびゲノム編集法の特性や応用例について科学文献をもとに調査整理した。

まず、自然に起きる突然変異についてのシロイヌナズナを 30 世代観察した結果から、1 世代/場所当たりの発生頻度（$\times 10^{-9}$）は

塩基置換では　5.9-7.1±0.6-0.7

挿入欠失では　0.6±0.2

で、G:C→A:T 変異が主であった。

次に、突然変異導入による突然変異頻度についての放射線を用いた突然変異誘導法によるイネの研究の例から、変異頻度（$\times 10^{-8}$）は

トータルで　23.1±1.5

と自然変異の数十倍で、大部分が数塩基（+1 bp～-4 bp）の挿入欠失であった。24 の変異のうち、15 変異は小さな欠失（1～16 bp)、4 変異は大きな欠失（9.4～130 kb)、3 変異は 1 塩基置換であり、中間の大きさの欠失（100 bp～8 kb）はなかった。

ゲノム編集では、変異頻度は高いものでは数十%に達し、場合によりオフターゲットも数%になることもある。また、ゲノム編集では DNA２本鎖切断後もゲノム上の標的配列から遊離しないため、修復過程に影響することが考えられ、その遊離を促進することができれば意図しない変化を一層低減できるのではないかと示唆された。

まとめたものを別添にした。

2. 研究開発に関する文献調査

ゲノム編集技術を用いた研究開発の文献 2018 年から 2019 年について調査した。その結果、動物ではゲノム編集技術を用いたものが 853 件、植物ではゲノム編集のほか新育種技術を含めて 1,924 件抽出された。

食品となる植物で研究報告が多かったものは順に、rice 130 件、tomato 45 件、wheat 21 件、maize 20 件、brassica 属 20 件、soybean 19 件、strawberry 4 件、grape 3 件、apple 3 件、coffee 3 件、carrot 3 件、banana 2 件、barley 2 件、kiwi 2 件、lettuce 2 件、peanuts 2 件、Chinese kale 2 件、peanuts 2 件、melon1 件、blueberry 1 件、papaya 1 件、cucumber1 件、pear 1 件などであった。それ以外の植物では Arabidopsis と

nicotiana がそれぞれ 76 件と 42 件が主で研究用と考えられた。

一方、食品となる動物で研究報告が多かったものは順に、chicken 22 件、cow 16 件、sheep 12 件、salmon 4 件、pig 2 件、shrimp 2 件などであった。それ以外の動物では、zebrafish 168 件および medaka 11 件、が主であった（表２）。目的形質としては、病害虫耐性、高塩耐性、生産性向上などの形質が多かった。なお、開発動向調査結果はページ数が多いため各分担報告書の後に表３として加えた。

また、これまでにない傾向として、タンパクをコードする遺伝子ではなく、それらを制御する低分子 RNA の一つである miRNA を標的にした応用例が、研究用生物 zebrafish などで見られた。今後、遺伝子制御因子を標的にした事例が増加する可能性も考えられた。

3. 人工消化液によるタンパク分解性試験（酵素濃度・pH 条件の影響）

ピーナッツアレルゲン Ara h1 を pH2.0 でペプシン濃度を変えて（高濃度、低濃度）120 分間人工胃液で消化させたところ、両条件で 1 分以内に SDS-PAGE 上からバンドが消失したことから速やかに分解されたが、1～6 kDa 付近にバンドが生成した（図１、２）。原理的には、この程度の大きさでもエピトープとして機能しうることから患者血清を用いた検討を行ったところ、患者血清とは反応しなかった。一方、pH3.3 の条件ではほとんど分解されなかった。未分解の Ara h1 のバンドは、ピーナッツ患者血清に反応した。ヒトの胃内環境は、食事によって大きく変化する。例えば、pH は 2 から 6 程度まで大きく変化することが判っている。また、制酸剤を服用している人も pH は 2 より大きい。今回の検討から、pH 条件が 2.0 から 3.3 に変化しただけで Ara h1 の人工胃液による分解性は劇的に変化した。また、pH 2.0 条件下で低分子の分解物が認められた。患者血清とは反応しなかったが、分解条件により抗原性が保持されている場合も想定される。以上の検討から、タンパクアレルゲン性評価資料の一つになっている分解性試験について、その条件を含めて詳細に検討することが、今後のアレルゲン性評価に重要であると考えられた。

D. 結論

自然変移、放射線を利用した積極的変異誘導、ゲノム編集技術を利用した変異誘導による育種に関する調査から、放射線育種では後代交配選抜によって得られた植物に誘導された変異は数塩基と小さいものが主であることが明らかになった。この育種過程では、後代交配が重要な役割を果たしていると考えられた。ゲノム編集技術による変異誘導も小さな変異が主であるが、少ない確率でオフターゲットが起きることに加えて、何世代にもわたる後代交配を行わないこと、DNA 修復にあたえる影響など、未知の部分も存在していることから、安全性の観点から更なる研究が必要であると考えられた。

ゲノム編集技術を用いた品種開発では、植物においては特に多くの作物種で研究が行われていることが分かった。なかでも、イネの研究は非常に多く、今後の動向に注目する必要がある。動物ではニワトリなどが多いが、事例数は植物に比べて少ない。

タンパク分解性試験では Ara h 1 においては、pH による大きな差が認められ、Ara h 1 の性質についての有益な情報が得られた。他のアレルゲンでもこのような研究から有用な情報が得られる可能性があると考えられた。

E. 業績

1. 論文発表

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2) Soga, K., Nakamura, K., Ishigaki, T., Kimata, S., Ohmori, K., Kishine, M., Mano, J., Takabatake, R., Kitta, K., Nagoya, H., Kondo, K. Data representing applicability of developed growth hormone 1 (GH1) gene detection method for detecting Atlantic salmon (Salmo salar) at high specificity to processed salmon commodities. *Data in*

Brief, 104695, 2019

3) Soga, K., Nakamura, K., Ishigaki, T., Kimata, S., Ohmori, K., Kishine, M., Mano, J., Takabatake, R., Kitta, K., Nagoya, H., Kondo, K. Development of a novel method for specific detection of genetically modified Atlantic salmon, AquAdvantage, using real-time polymerase chain reaction. *Food Chemistry*, 305, 125426, 2020

2. 学会発表

1) 中村公亮、木俣真弥、成島純平、志波優、秋本智、曽我慶介、権藤崇裕、明石良、近藤一成: ゲノム編集生物に残留する意図せざる DNA 切断の予測・検出法の評価、日本薬学会、第 140 年会、京都、2020 年 3 月

2) 成島純平、中村公亮、木俣真弥、志波優、秋本智、曽我慶介、権藤崇裕、明石良、近藤一成 : 2017 年中に発表されたゲノム編集イネのオフターゲッ ト効果に関する評価、日本農芸化学会、2020 年度福岡大会、福岡、2020 年 3 月

3) Kazunari Kondo, Kozue Sakata, Reiko Kato, Akio Noguch. Identification of toxic plants that cause severe food poisoning using real-time PCR. Recent Advances in Food Analysis Prague, Czech Republic, Nov.5-8 (2019)

3. 講演会、説明会等の社会貢献

1) ゲノム編集技術を利用して得られた食品等に関する意見交換会、近藤一成、厚労省・消費者庁・農水省主催　2019 7.4（東京）2019 7.12（福岡）

2) ゲノム編集技術を利用した食品等とその取扱い、近藤一成、日本食品工業倶楽部　月例会（2019.6.27、東京）

3) ゲノム編集技術応用食品の現状と課題、北嶋聡、近藤一成、日本食品化学会　第 35 回食品化学（2019.11.8、東京）

4) 第 17 回食品安全フォーラム「ゲノム編集技術を利用した食品の安全性確保の取組み」、近藤一成、日本薬学会主催（2019.11.29、東京）

5) 食品衛生学会特別シンポジウム、近藤一成、日本食品衛生学会主催（2020.2.13、東京）

F. 知的財産権の出願・登録状況

該当なし

表1　研究開発に関する文献調査の検索キーワード

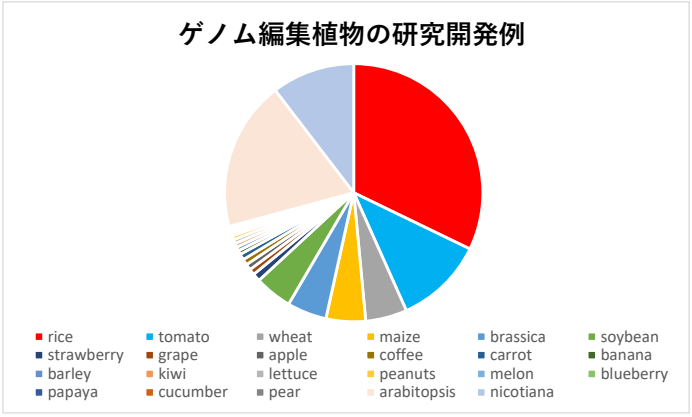
i）動物の場合（ゲノム編集のみが対象）

	キーワード	備考
A 群	zinc finger nuclease, ZFN, TALEN, TAL effector, CRISPR, Cas9, Cpf1	ゲノム編集
B 群	pig, cow, chicken, fish, sheep, goat	動物の種類

ii）植物の場合（ゲノム編集を含む新育種技術全体が対象）

	キーワード	備考
A 群	zinc finger nuclease, ZFN, TALEN, TAL effector, CRISPR, Cas9, Cpfl	ゲノム編集
	ODM, oligonucleotide-directed mutagenesis, targeted nucleotide exchange, TNE	オリゴヌクレオチド指定突然変異
	Cisgenesis	シスジェネシス
	Intragenesis	イントラジェネシス
	RdDM, RNA-depending DNA methylation	RNA 依存性 DNA メチル化
	Transgrafting, transgraft, graft & siRNA	接ぎ木
	Reverse breeding	逆育種
	Agroinfiltration	アグロインフィルトレーション
B 群	plant	

表２　ゲノム編集技術を用いた研究動向調査の結果



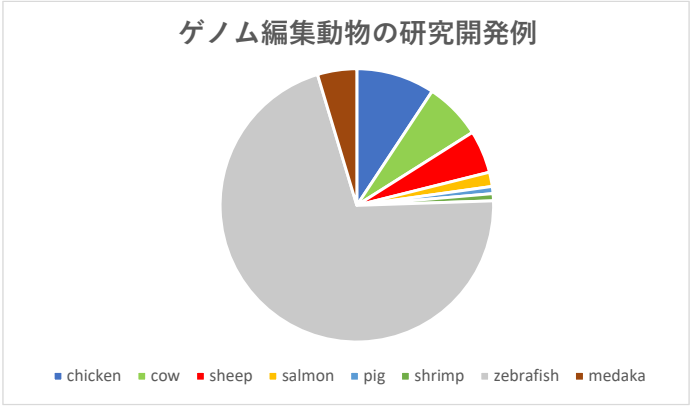
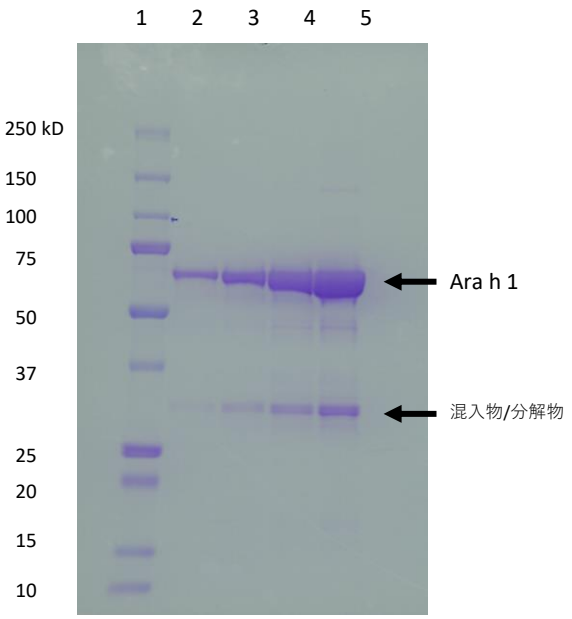


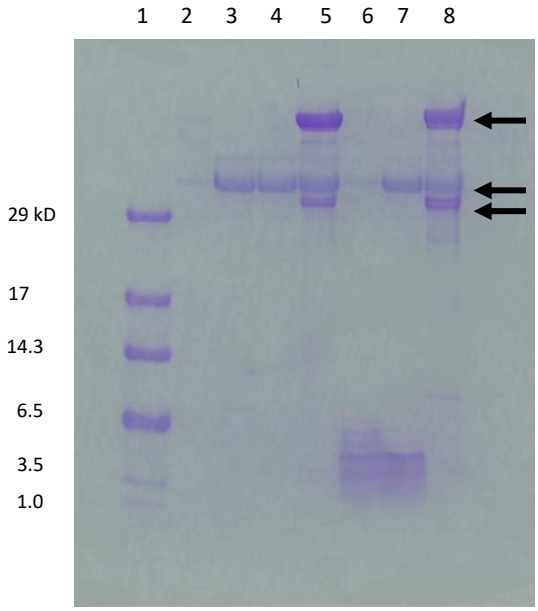
表３　（電子版に掲載）

図1 精製したAra h 1の純度確認



レーン１：分子量マーカー
レーン２－５：精製したAra h 1，0.6, 1.3, 2.5, 5.0 μg

図2 Ara h 1の分解性試験



レーン１：分子量マーカー
レーン２－４：Ara h 1無し（コントロール）
レーン５－８：Ara h 1有り
レーン２，６：pH 2.0，低ペプシン濃度，120分
レーン３，７：pH 2.0，高ペプシン濃度，120分
レーン４，８：pH 3.3，高ペプシン濃度，120分
レーン５：0分

ゲノム編集技術と関連する情報の収集と解析、ケーススタディー

1．自然突然変異の割合（植物および動物細胞の例）

1－1　動物細胞（ヒト細胞）

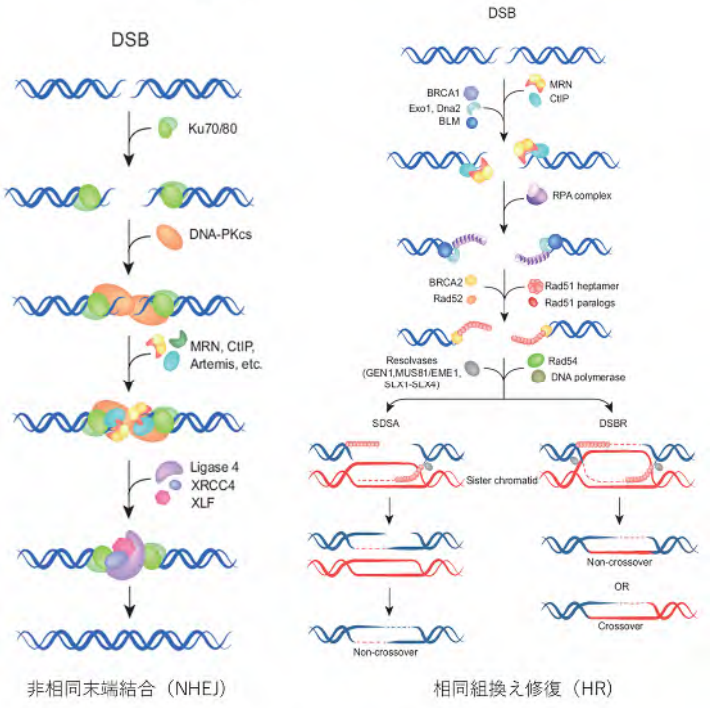
生物種：ヒト B 細胞株（thymidine kinase mutant（*TK*$^{-/-}$）TK6）
引用論文：Schwartz *et al*, Mutagenesis, **19**, 477-482 (2004)

内容：TK 遺伝子変異に対する自然突然変異による復帰を指標にした生育頻度を基に、自然変異頻度を算出している。一遺伝子に起きる自然突然変異の頻度は次のようである。

$(2.73\pm0.78)\times10^{-6}$ /cell であった
これは、他の遺伝子(*HPRT**) での解析結果の、**$1\sim10\times10^{-6}$ /cell**　と同じであった。
(Jones *et al*, Cancer Epidemiol. Biomakers Prev., **2**, 249-260 (1993), Park *et al*, Radiat. Res. **141**, 11-18 (1995))

*HPRT, hypoxanthine phosphoribosyl transferase

(関連する論文から)
TK6 細胞を改変して *TK* locus に I-SceI 認識配列を挿入した、TSCE5, TSCE2 細胞で検討
DNA2 本鎖切断後の修復は、大部分が非相同末端結合（NHEJ, Non-homologous end joining）によるもので小さな欠失が起きる。一方で、修復ミスが起こりにくい相同組換え（HR, Homologous recombination）は NHEJ の 1/270 の確率でしか起きない。
Honma *et al*, Environ. Mol. Mut., **42**, 288-298 (2003)

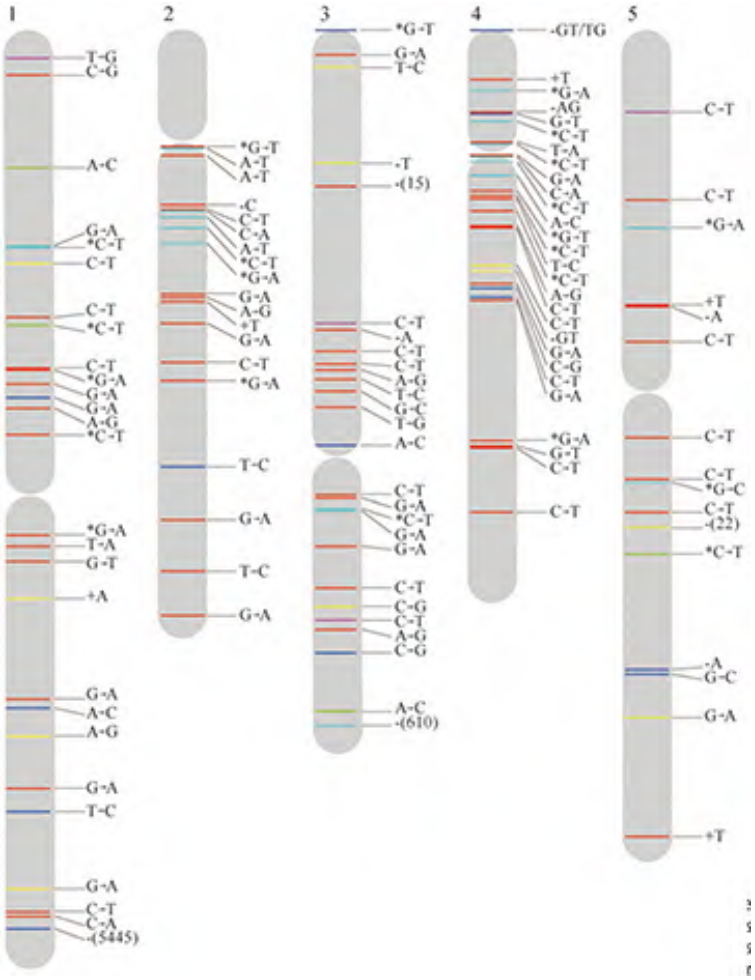


Translational Cancer Res., 2, 163-177 (2013)より

１－２　植物（細胞）

生物種：シロイヌナズナ（*Arabidopsis thaliana*）
引用論文：Ossowski *et al*, Science, **327,** 92-94 (2010)

内容：2000 年にゲノム解析された系統 Col-0 の単一種子から 30 世代経て得られた 5 つの系統のゲノムを解析した。
遺伝子変異頻度は、塩基置換　**(5.9-7.1±0.6-0.7)×10^{-9} per site/generation**
欠失挿入　**(0.6±0.2)×10^{-9} per site/generation**
また、変異導入場所については、55%が intergenic（遺伝子間領域）、17%がトランスポゾンなどで、アミノ酸変異を誘導する non-synonymous mutation は 11%である。G:C→A:T 変異が他よりも 10 倍高い。
また、ゲノム上の変異分布は以下に示した。セントロメア付近に多くみられるが全体に分布している



なお、Weng et al, Genetics, 211, 7030714 (2019)でも自然変異発生は同様の報告である
6.95×10^{-9} per site/generation

２．突然変異誘導（放射線、変異原物質）

２－１　放射線による突然変異誘導による変化

生物種：イネ（*Oryza sativa* L.）
引用論文：Genes Genet Syst, **84,** 361-370 (2009)

実験条件
種子、花粉、植物体 100～300 Gy(10～50Gy/h)でγ-rays を照射、解析は TAIL-PCR で行った。
24 の変異のうち、15 変異は小さな欠失（1～16 bp)、4 変異は大きな欠失（9.4～130 kb)、3 変異は 1 塩基置換であり、中間の大きさの欠失（100 bp～8 kb）はなかった。
また、2つの逆位が認められ、その範囲は 1,285 kb, 3208 kb であったことから、ガンマ線照射では小さな変異とともに変色体レベルの大きな変異も観察された。
具体的には、200 Gy (10 Gy/h)で照射した系統 YM15 と 02g200Gy は japonica rice 由来である。

文献中には、放射線照射後に、目的形質で選抜した後の変異体を解析しているが、何世代継代したものを解析しているのかの記載がない。解析している配列は、目的形質に関わる遺伝子のみであり、NGS が普及する前であるため全ゲノム解析は行っていない。

（解析結果は、次ページに表を示す）

No.	Allele	Mutation	Size	Sequence (5' - 3')	Position of mutation
1	*cao-g1*	deletion	1 bp	TATGCAAAGAACA TATGCAA–GAACA	AC087599: 42208/42209/42210
2	*cao-g2*	deletion	3 bp	TTTGCCAAGGGTT TTTGC–––GGGTT	AC087599: 43797–43799
3	*cps-g1*	deletion	1 bp	GGCCACCTGCAC GGCCAC–TGCAC	AP004872: 48297/48298
4	*ga3ox-g1*	deletion	1 bp	AGGAAGGGGAGAAG AGGAAGGG–AGAAG	AP002523: 125385/125386/125387/125388
5	*ga3ox-g2*	deletion	3 bp	GGTCGCCGACGTT GGTCG–––ACGTT	AP002523: 124879–124881
6	*gid1-g1*	deletion	1 bp	GAGGAGGGGGCGGCG GAGGAGGGG–CGGCG	AC137928: 53651/53652/53653/53654/53655
7	*gid2-g1*	deletion	42.2 kbp	TGAGATG•••TTACATG TGAGA–•••••–ACATG	AP006161: 95795–137978
8	*glb1*	deletion	62.8 kbp	ATAATATA•••TGAAAAA ATAATA–••••••–AAAA	AC113332: 15191–77983
9	*glu1*	deletion	129.7 kbp	TACCTCG•••AATTTA TACCT–••••–^{G}TTTA	AP005428: 44130- AP005875: 18726
10	*gluA1-g1*	deletion	1 bp	TGACCGAAAGT TGACC–AAAGT	AP003274: 112346
11	*gluA2-g1*	deletion	1 bp	GAATTGGCTCAA GAATTG–CTCAA	AC021891: 82815/82816
12	*gluA2-g2*	base substitution	1 bp	GCGTTCAACGC GCGTT$_{A}$AACGC	AC021891: 82000 (C/G → A/T)
13	*kao-g1*	deletion	4 bp	CCTCCCTCCGCCGC CCTCC––––GCCGC	AP002805: 59563–59566/59567–59570
14	*kao-g2*	deletion	16 bp	CGCCGGC•••CGGCCACA CGCCG–••••••–CCACA	AP002805: 59964–59979
15	*pla1-g1*	deletion	5 bp	GAAGGCGTGGACGAG GAAGG––––––ACGAG	AE017091: 254226–254230
16	*pla1-g2*	base substitution	1 bp	GCGTCTCCTTC GCGTC$_{A}$CCTTC	AE017091: 254549 (T/A → A/T)
17	*pla2-g1*	deletion	5 bp	GGTGCCGCCGCCGCC GGTGCC––––––CGCC	AP006531: 92089–92093
18	*wx-g1*	deletion	2 bp	TCCGCCACGGGT TCCGCC––GGGT	AP002542: 91149–91150/91150–91151
19	*wx-g2*	deletion	5 bp	GGTGCTCACCGTGAG GGTGC––––––GTGAG	AP002542: 92735–92739
20	*wx-g3*	deletion	6 bp	CAGCCTTCTTTGCCAG CAGCC––––––GCCAG	AP002542: 91976–91981
21	*wx-g4*	deletion	9.4 kbp	AGTCAAA•••ATATCGA AGTCA–••••••–TCGA	AP002542: 88234–97663
22	*wx-g5*	base substitution	1 bp	TCTGGTATAAT TCTGG$_{A}$ATAAT	AP002542: 93203 (T/A → A/T)

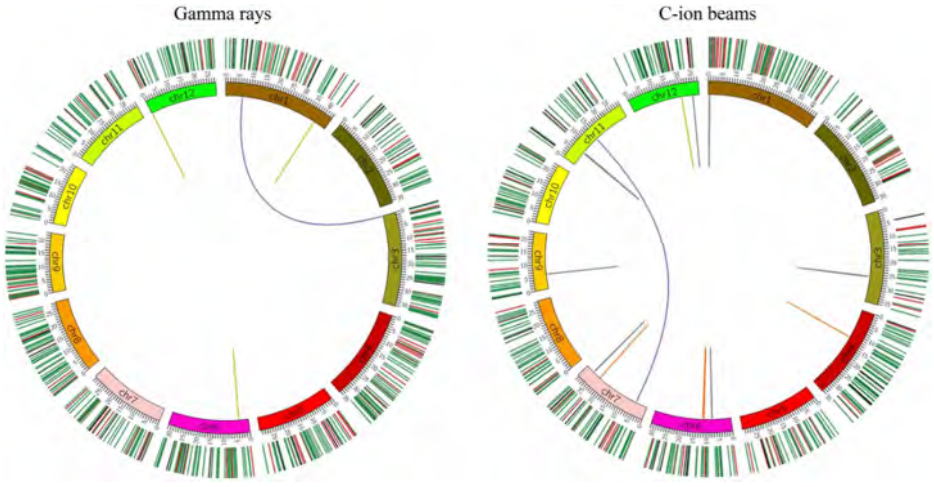
Allele name, mutation type and size were shown.
Sequence: Upper line shows the wild type, lower line shows the mutant. Hyphens in the lower sequence line represent deleted bases. Subscript and superscript letters show substituted and inserted bases, respectively. Underlining indicates microhomology.
Position of mutation: The location where the mutation occurred is shown as the position in the genomic clone.

Genes Genet Syst, **84,** 361-370 (2009)の Table2 より

生物種：イネ（*Oryza sativa* L.）
引用論文：G3 Genes Genomes Genetics, **9,** 3743-3751 (2019)

実験条件
(1) γ-rays from150 to 450 Gy with a dose rate of 10 Gy/h (250 Gy がイネで一般的で LD_{30} 相当)
(2) 220 MeV C ions from10 to 60 Gy　の条件で照射した。
M_6 progeny までを得る。
解析は、M_6 mutant line それぞれ 7 株を NGS 解析。uSeq DNA Sample Preparation Kit (Illumina Inc.), and paired-end (2X150 bp) sequencing was performed on Illumina HiSeq X Ten to determine genomic sequences with about 30-fold depth for each line.

変異誘導結果（頻度とパターン）
M_5 における変異結果として
γ-rays の結果では、1 塩基変異、欠失、挿入が平均 57.0, 17.7, 5.9 個
変異頻度は、　**$(23.1 \pm 1.5) \times 10^{-8}$ per bp** であった。
C ions の結果では、1 塩基変異、欠失、挿入が平均 43.7, 13.6, 5.3 個
変異頻度は、　**$(18.3 \pm 3.4) \times 10^{-8}$ per bp** であった。
この結果から、直接比較することは難しいが、自然変異より 10 倍程度大きいと考えられる。
また、変異パターンは、G/C から A/T 変移が最も多かった
（43.0±3.4% in γ-rays and 46.7±6.6% in C ions)。
InDel では、+1 bp～-4 bp が最も多く 72.8±7.4% in γ-rays and 60.4±12.2% in C ions だった。
100 bp 以上の欠失や重複、転移などの構造的変異（SV）は C ions の方が多かった。



2　CRISPR/Cas システム　（種類、構造、作用機構、起こる変化）

2－1　分類

最も利用されているものは、class 2 に属する Cas9, Cas12, Cas13 などである。

Table 1. Cas class and type				
	type	subtype #	endonuclease	target
Class 1	I	7	Cas3	DNA
	III	4	Cas10	DNA/RNA
	IV	1	--	--
Class 2	II	3	Cas9	DNA
	V	3	Cas12	DNA
	VI	3	Cas13	RNA

Type II の Cas の代表的なものを下に記した。

Casの種類（DNA切断型）

代表的なtype II型 Cas9

最も研究されている spCas9

最も小さい cjCas9

Cas ortholog	PAM	size (a.a)
Francisella novicida	NGG	1,628
Streptococcus pyogens	NGG	1,368
Staphylococcus aureus	NNGRRT	1,053
Brevibacillus laterosporus	NNNNCNDD	1,092
Neisseria meningitidis	NNNNGATT	1,081
Campylobacter jejuni	NNNVRYAC	984

spCas9の改良型：e-spCas9, spCas9-HF, HiFi-Cas9, sniper-Cas9, etc

Cas9以外のCas

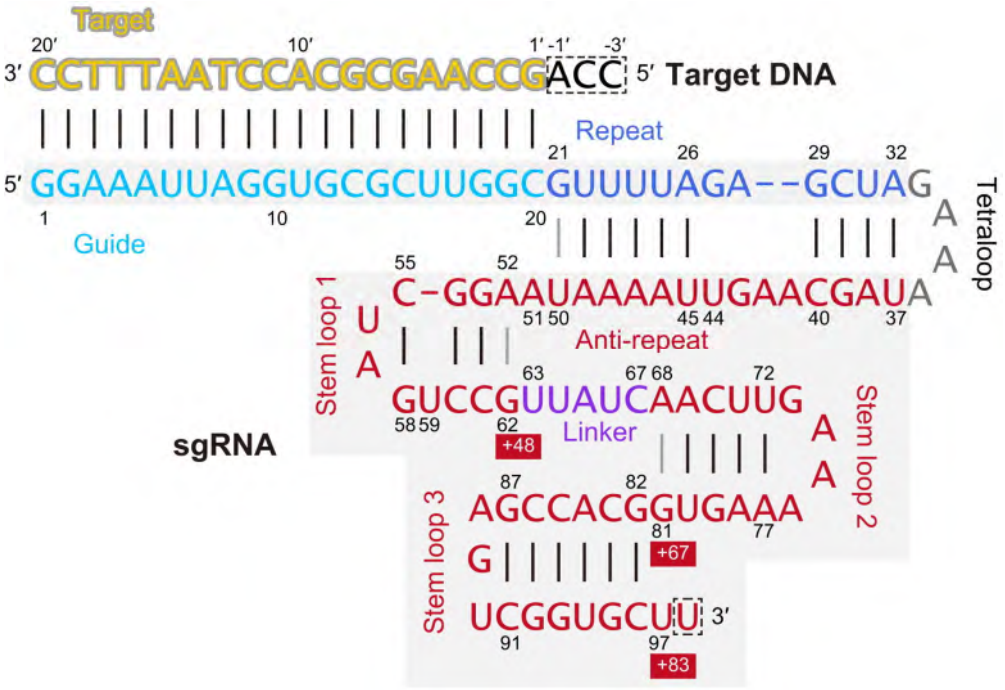
Cas12a(cpf1), 12b, 12c, 12g, 12h, 12i,…. Yan et al, Science (2019)

Cas13a(C2C2), 13b, 13c, 13d,,…. Abandayyeh et al, Science (2016)

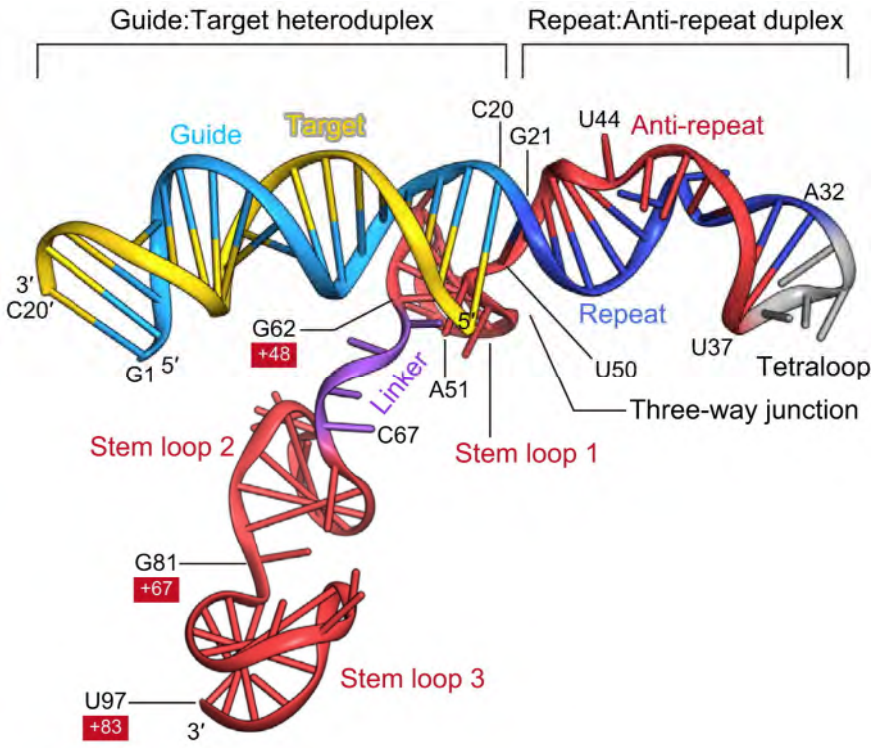
Cas14,…. Horringtonet al, Science (2018)

2－2　構造

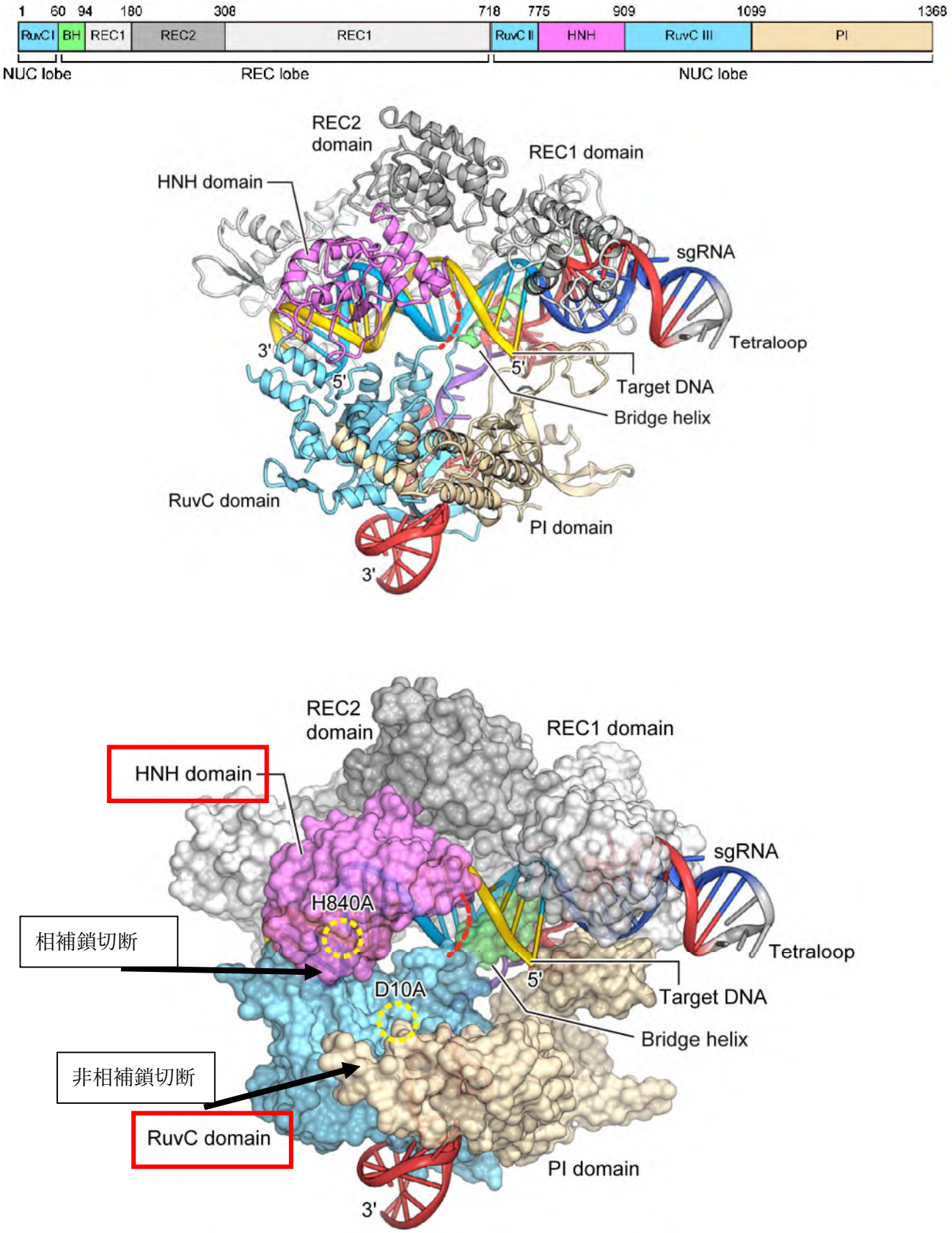
1）標的 DNA と gRNA との相補結合（平面図）



2）標的 DNA と gRNA との相補結合（立体図）



図は *Cell*, **156** 935-949 (2014)より



図は *Cell*, **156** 935-949 (2014)より一部改変

２－３　作用機構

１）標的配列のスキャン（Cas9 がどのように標的配列を検索して結合するか？）

（DNA curtain）脂質 2 重膜に直鎖上の DNA2 本鎖（図では 48 kb λDNA）をカーテンのように固定して並べる（図１）。
ここに、ピンクに標識した（YOYO）Cas9 を緑で標識した DNA 上をスキャンする様子
g RNA が存在しないと、PAM をごく短時間スキャンしながら動くが、gRNA(λ2 標的)が存在するとより長い時間、強く結合する（図 2A, B）。
（*Nature*, **507**, 62-67 (2014)より）

図１

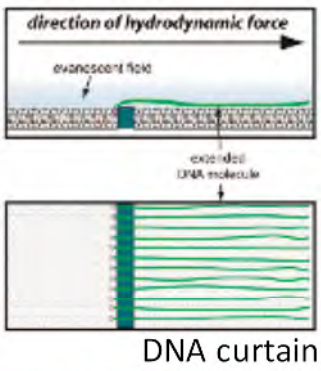
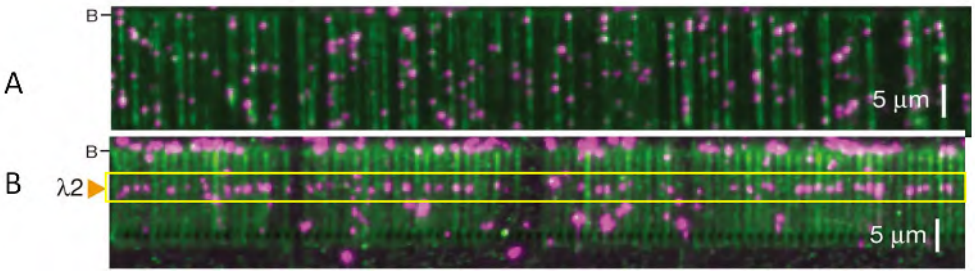
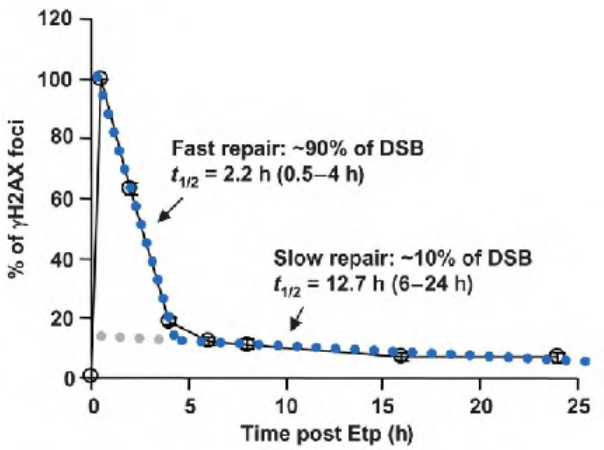


図２

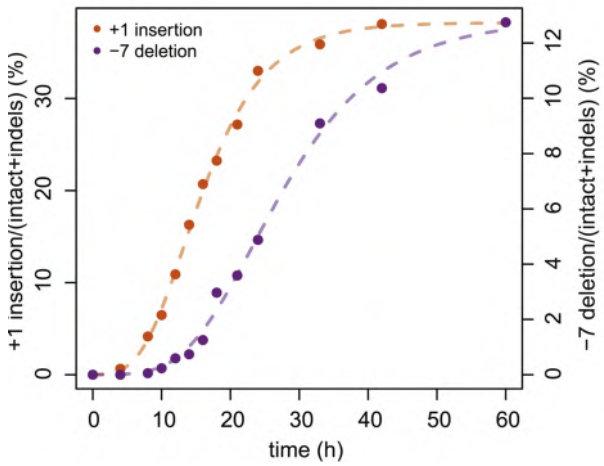


２）速度論的解析（どれくらいの時間、結合修復に要するか）

放射線（5 Gy）による DNA 切断後の再結合実験では、再結合は 2 相性で早い再結合では 18.0～36.4 min、遅い再結合では 1.5～5.1 h である（*Br J Cancer,* **71**, 311-316 (1995)）。
その他の報告でも同様である（10～60 min）。
DNA2 本鎖マーカーγ-H2AX を指標にした実験では、照射によって生じたγ-H2AX は 1 ～ 3 h で消失した。また、50 μM Etp(エトポシド)処理した細胞では、90%が 0.5～4 h で、残り 10%は 6～24 h で生じたγ-H2AX は消失した（下図）。　　（*EMBO J,* **30**, 1079-1092 (2011)より）



DNA2 本鎖切断の修復は、細胞周期にも依存するが大部分は非相同末端結合（NHEJ, MMEJ）により、残りは相同組換え(HR)の機構により起こる。NHEJ では小さな indel が起き,MMEJ ではそれよりは大きい deletion が起きる。。ここで、非相同末端結合による修復を詳しく見ると MMEJ (micro-homology mediated end-joining)による deletion (-7 bp)は c-NHEJ（canonical non-homologous end-joining）による indel (+1 bp)よりも時間を要する。
NHEJ 軽油を阻害すると MMEJ 経路が増加する



（Cas9 による切断修復、*Mol Cell* **70**, 801-813 (2018)より）

50%修復時間は、サイトにより異なり 1,4～10.7 h であり、放射線による DNA 切断修復の 1 h 以内に比べて長い（Cas9-RNP で誘導した場合)。

Cas9 が DNA から遊離する速度は、PAM-distal 部位で $1.0 \pm 0.2 \times 10^{-5}$/s、PAM-proximal 部位で $6.0 \pm 0.8 \times 10^{-6}$/s であった（*Nat Biotech*, **34**, 340-345 (2016))。

Cas9 と gRNA の複合体の結合力は、抗体（mAb）の解離定数 nM レベルよりもかなり強く 10 pM である（*PNAS* **112**,2984-2989 (2015))。

Table 1.
Equilibrium dissociation constants for protein–sgRNA interactions
* Three independent experiments were performed for each condition, and the values represent the mean ± SEM.

Protein	Equilibrium dissociation constant (K_d) for indicated sgRNA*		
	Full-length	Δ Hairpins1-2	Δ Spacer–nexus
WT Cas9	10 ± 2 pM	0.86 ± 0.12 nM	16 ± 2 pM
α-Helical lobe	0.75 ± 0.12 nM	0.70 ± 0.13 nM	>100 nM
Nuclease lobe	0.30 ± 0.07 nM	>100 nM	0.17 ± 0.06 nM
Split-Cas9	0.23 ± 0.04 nM	1.05 ± 0.05 nM	0.17 ± 0.07 nM

最後に、変異導入効率は、手法、標的配列によりさまざまであり、0.1%以下から数十%までである。これは、自然変異や放射線などの突然変異育種よりもかなり高いが、標的部位のみに変異が導入されるのであれば、特に懸念することないものと考えられる。

2．開発動向・ケーススタディー

現在、ゲノム編集分野におけるもっとも重要な課題は、いかにオフターゲット変異を抑制するか、および、設計する上での配列上の制約を可能な限りなくす、の 2 点に集約される。

前者については、当初の DNA2 本鎖切断を誘導しない塩基置換（base editing）が活発に研究されているが、C->T 変異を誘導する CBE、A->G 変異を誘導する ABE いずれにおいても、当初想定外であったオフターゲット変異が多いことが判明して、それらの改良法が報告されている。また、CBE と ABE による塩基置換は、SpCas9 が認識する PAM から~15±2 nt に位置し、かつプロトスペーサー上の狭いウィンドウ内に位置する塩基に限定されてしまう制約も存在する。現状では、DNA2 本鎖を切断しないことからオフターゲット切断を回避できる期待されたが、意図しない変異の問題は解決されていない。

後者について、ゲノム編集の主流である CRISPR/Cas9 では 20 塩基（nt）の標的配列の他に、その 3’側に PAM と呼ばれる 3 nt の NGG 配列が必要である。この制約を緩めるために、NGG の制約を、オフターゲットを最大限抑制しながら NG にした変異体（spCas9-NG）開発されている。さらに、この spCas9-NG に塩基編集を適用した spCas9-NG-AID が解析されているが、これらの意図しない変異については十分に検討されているとは言えない。spCas9-NG は、すでにイネとシロイヌナズナに応用されている。

その他に、Cas12a や Cas13a は、標的 DNA または標的 RNA の切断時に一本鎖 DNA または一本鎖 RNA を非特異的に切断する性質を有する。

厚生労働科学研究費補助金（食品の安全確保推進研究事業）
「新たなバイオテクノロジーを用いて得られた食品の安全性確保と
リスクコミュニケーションのための研究」

リスクコミュニケーションに関する研究

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研究要旨：
ゲノム編集食品に関する複数回のリスクコミュニケーション活動、新聞報道等の解析あるいはWEB による大規模調査より、リスクコミュニケーションの対象として「ゲノム編集技術に関する知識がほとんど無い層」、「ゲノム編集食品に関してある程度知識を持つが、食品の安全性や表示の有無に関して懐疑的な層」が重要であり、前者と後者には異なるリスクコミュニケーションの手法が必要であると考えた。今年度は前者に使用することを念頭に平易な冊子を作成した。

A.　研究目的

遺伝子組換え食品あるいはゲノム編集技術応用食品（以下、ゲノム編集食品）といった新たなバイオテクノロジーを用いて得られた食品に関して国民、中でも一般の人（専門家でない人）、の疑問や不安が大きいと考えられる。特に平成 31 年/令和元年度にはゲノム編集食品に関する安全性や表示の取扱いルールが決まり、厚生労働省による意見交換会も行われた。また、ゲノム編集食品に関連する報道も多く見られた。しかし、十分なリスクコミュニケーションが行われ、一般の人の疑問や懸念が解消されたかどうかは定かでない。疑問あるいは懸念の内容や専門家と非専門家の間の認識の差も明確でない。

このような状況を考慮し、主にゲノム編集食品を対象としてリスクコミュニケーションが適切に行えるように、1）一般の人の疑問や懸念の内容や、その程度の調査、2）専門家と非専門家間のゲノム編集食品に対する認識の違い、3）意見交換会等で使えるゲノム編集食品に関するチラシの作成、4）リスクコミュニケーションのための小冊子の作成、を行い、リスクコミュニケーションに必要な情報を収集するとともにリスクコミュニケーションに必要な題材を作成することを目的とした。

また、効果的なリスクコミュニケーションを行うために対象を大まかに分類分けするとともに、提供すべき情報について考察した。

B.　研究方法

1）　研究分担者が平成 31 年/令和元年度に行った複数のサイエンスカフェや公開講座等におけるリスクコミュニケーション活動（別添資料 1）での参加者の発言やアンケート結果から代表的な意見に加え、厚生労働省の案に対するパブリックコメント*から代表的な意見を抽出した。また、ネガティブな意見に加えてゲノム編集食品に対するポジティブな意見にも着目した。各イベントで提供した情報は質的、量的に異なるため一概に比較はできないが、主観的に代表的な意見をピックアップした。また、日本科学未来館において来館者に意識調査もおこなった。一般の人への影響が大きく、一般の人の代弁者の役割を果たすように見えるマスメディア（新聞）の報道傾向についても、定性的ではあるが調査した（別添資料 2）。あくまで例示になるが一般の人からの共感を得るための情報提供者の姿勢についても考察した。

* https://search.e-gov.go.jp/servlet/PcmFileDownload?seqNo=0000192458

2）　WEB 調査を実施した。別添資料 3 に示す質問事項に関して一般モニター4,000 人と研究者約 200 人（令和 2 年 3 月 10 日現在実施中）を対象とした。一般モニターは調査会社に登録している多様な年齢層であり、属性は様々である。研究者は各種 ML を通じて回答者を募った。

3）　今年度に加えてこれまで行ってきたコミュニケーション活動における経験から提供すべき最低限の情報を精査した。行政担当者との意見交換も行った。

4）　3）の資料と比べ情報量を多くする一方で中学生、主婦などでも理解でき、リスクコミュニケーションの題材となる平易な内容の冊子を作ること

を念頭に、専門家、行政担当者、一般の人及びサイエンスコミュニケーターから意見を聞いた。

C. 研究成果

1) ゲノム編集食品に対しては、新聞報道も含め主観的ではあるが、別添資料 4 に示すような意見として整理した。
2) 令和 2 年 3 月 10 日現在、調査結果を解析中である。一般の人向けの調査は終了しており、予備的な解析結果を別添資料 5 に示す。
3) 別添資料 6 のチラシを作成した。
4) 別添資料 7 の冊子を作成した。

本年度に決まったルール作りの経緯と上記の研究結果を以下にまとめた。

ルール作りとゲノム編集食品に対する意識

平成 31 年/令和元年度には以下の時系列でゲノム編集食品に関するルール作りが進んだ。

- 平成 31 年 3 月 27 日　薬事・食品衛生審議会食品衛生分科会 新開発食品調査部会 報告書「ゲノム編集技術を利用して得られた食品等の食品衛生上の取扱いについて」令和元年 6 月 27 日～7 月 26 日「ゲノム編集技術応用食品及び添加物の食品衛生上の取扱要領（案)」の発表とパブリックコメントの募集
- 上記の期間中に全国 5 箇所で農林水産省、厚生労働省および消費者庁による意見交換会が実施された（7 月 12 日）
- 令和元年 9 月 19 日　ゲノム編集技術応用食品及び添加物の食品衛生上の取扱要領
- 令和元年 10 月 1 日　ゲノム編集食品の届出制の運用開始

こうした行政の動きに関連する報道とともに学会、消費者団体（生協を含む)、関心を持つ個人がパブリックコメントを行った。本研究ではこうした意見の内容を踏まえゲノム編集食品に対する意識について考察した。別添資料 1 に示した意見交換の機会では、専門性が高くない一般の人の意見を聴いた。別添資料 2 に代表例を示したがマスメディアの論調も適宜調査した。主観的ではあるが、これらの調査結果から見えることを、別添資料 4 に示した。また 4,000 人を対象とした WEB 調査で一般の人の意見も聴取した（別添資料 5)。別添資料 4 と 5 の結果についても相関がみられた。

情報提供ツール（チラシと冊子）の作成

チラシは上述の意見交換会での使用を念頭において作成した。結果的には使用されなかったが、その後、体裁を整えて完成版のチラシとした（資料 6)。今後の意見交換会で利用されることが期待される。冊子（別添資料 7）も、まだ使用していない。リスクコミュニケーションの場で効果的に使用できるかどうかは今後の課題である。

D. 考察

平成 31 年/令和元年度にはゲノム編集食品に関するルールが決まった。端的に言えば、遺伝子の変異が従来育種で起る変異と区別できない場合、遺伝子組換え食品に課している安全性審査を必要としない。しかし、消費者の懸念を考慮し遺伝子組換えに相当しない場合にも届出による情報提供を開発者・販売者には求める。届出には事前相談が必要で情報提供の内容を厚生労働省と協議する。届出は義務ではないが、届出せずにゲノム編集食品を流通させた場合、事業者名が WEB 上で公表されるという一種のペナルティがある。リスクコミュニケーションの重要性も言われている。

令和 2 年 3 月現在届出が公表された事例はなく、ゲノム編集食品は流通していない。従って、ゲノム編集食品が上市された後にリスクコミュニケーションがどのように実施されるかは明確でない。本研究で調査した範囲ではゲノム編集食品のリスクコミュニケーションには課題がある。重要なことは対象別のリスクコミュニケーション方法の確立であると考える。勿論、厳密に対象をグループ分けすることは出来ないが、以下のように対象を大別し、どのようなコミュニケーションが効果的かを考察した。

(ア) ゲノム編集についてほぼ知らない。言葉を聞いたことが無い、あるいは聞いたことはあるが内容は知らない。定義は難しいが、本研究で意図するいわゆる一般の人である。この中には家庭科教員などインフルエンサーになる可能性がある層も一部含まれる。
この集団にはゲノム編集技術の原理や開発事例などを説明することから説明を始める必要がある。この知識が無ければ次の段階には進むことが難しい。この層は技術に対する理解

は限られていても、「届出が義務でない」、といった自分達にとってネガティブと思われる情報には敏感である。特にインフルエンサーへの情報提供は工夫が必要である。

(イ)ゲノム編集についてそれなりの知識、問題意識を持っている。具体的にはメディア（主に新聞記者）や消費者団体（生協など）が該当する。もっとも科学的知識には限界がある。この層は技術の概要や開発事例についてはかなり情報を持っている。しかし、技術の内容と施策の整合性に関して疑問を持っている。届出あるいは表示を義務化出来ないことが納得できない。社会的検証による届出の義務化を求めることもある。オフターゲットに関しても気にする傾向が見られる。
この層に関しては丁寧な技術の説明が求められる。オフターゲットはきちんと説明すれば疑問が解消されることが多い。しかし、技術が理解できても届出、表示が義務化できないことは、なかなか受け入れられない。多くの新聞報道や生協が公表しているパブリックコメントを見ても、選択の権利のための義務化の必要性に関する主張が多い。従って、技術の説明を十分にした上でその流通等の運用におけるルール作りの考え方を丁寧に説明する必要がある。その際、科学的根拠だけに基づいてルールが決まっているわけでないことを説明することが重要と考える。

(ウ)ゲノム編集の技術については良く知っている。つまり科学者。しかし、必ずしもゲノム編集食品の実用化に関するルールに関しては詳しくない。ルールが厳しすぎると考える傾向もみられる。この層についても、科学的根拠以外の要因が考慮されていることを説明することが求められる。

(エ)あくまでゲノム編集食品を否定する層。ゲノム編集技術の理論を理解しても流通をほぼ肯定しない。産物（いわゆるプロダクト）ベースでの判別が困難なことは理解しているが、社会的検証（トレーサビリティ）を求める。コミュニケーションは難しいが社会的検証のコストとゲノム編集食品のリスクについての説明が必要ではないか。中には完全に議論がかみ合わない人たちが一定数存在し、生産的なリスクコミュニケーションは難しい。

上述のようにリスクコミュニケーションは多様な層を対象に行う必要がある。全ての層に対応することは不可能だし、多様な層を一度に対象とすることもある。しかし、上に示したア）～エ）の層を念頭としたリスクコミュニケーション手法の確立は重要と思われる。特に圧倒的に数としてはマジョリティと考えられるア）及び社会的影響力の大きいイ）の層とのコミュニケーションを優先して行うことが効果的である。Q&A を含むそれぞれのマニュアルの作成が必要と考えられる。本研究で作成した冊子（別添資料７）は、ア）での使用を念頭に置いている。

また、上記のいずれの場合も、理解（信頼）が得られやすい態度が重要である。当然のことと言えるが包み隠さず誠実に対等に接することでコミュニケーションが進む。同時に、答えらえないことがあると信頼感が低下する。話題提供者には様々な疑問に答えられるように十分な知識を持っていることが求められる。また、場面によるが分かり易い用語での説明も求められる。

E. 結論

ゲノム編集食品の安全性評価の考え方は難しい。従来育種と産物に差異が認められない限り純粋な自然科学の観点からはゲノム編集食品は、従来育種の産物が規制の対象でないことを考えれば、規制の対象とはなり得ない。しかし、実際には事前相談を経へ届出を行うというステップがある。届出は消費者の懸念を念頭に置いたものであるが、消費者には必ずしも正しく伝わっていない。科学的に届出を義務化できないことが、理解されず、義務でないことがかえって不信感を生んでいる。これはパブリックコメント、新聞報道、意見交換あるいは WEB 調査などの結果から明らかである。表示に関しても同様である。自然科学の技術で差異を検証できないのであれば社会的な検証（トレーサビリティシステムの構築）を行うべきという意見もあるが、その信頼性、コストに関する考えは十分とは言えない。

「施策は科学的根拠のみよって決まるのではない」ことが十分に伝わっていないため、多くの国民(科学者を含む)が混乱しているように見える。リスクは従来育種を超えるとは考えられないため、

自然科学の考え方では規制は不要である。しかし、それでは消費者が不安を抱く。それで事前相談・届出という考え方が生まれたと思われる。つまり、施策は消費者の懸念に考慮しているが、そのことが却って消費者の不安の要因になっている可能性がある。科学的情報に加えてルール作りの内情を公開したリスクコミュニケーションが効果的と考える。

ゲノム編集食品に対する関心の高さや知識に応じたリスクコミュニケーションも求められる。余り関心が高くなく、知識も少ない層にはまずは基本情報の提供を行わざるを得ない。一方、ある一定の知識を持ち、関心を持っている層には、その社会的影響力も考慮して、上述の施策との関連も含めたリスクコミュニケーションを十分に行う必要がある。そのためには科学的知識とルール作りの在り方の両方の知識が必要となり、その資料の整備も今後求められる。ゲノム編集技術・食品に非常にポジティブな層（研究者）やネガティブな層（一部の消費者団体等）とのコミュニケーションの在り方についても検討する必要がある。

F. 健康危険情報

なし

G. 業績

1. 論文発表

1) Mishiba, K.-I., Iwata, Y., Mochizuki, T., Matsumura, A., Nishioka, N., Hirata, R., & Koizumi, N. (2019). Unfolded protein-independent IRE1 activation contributes to multifaceted developmental processes in Arabidopsis. *Life Sci Alliance*, 2, e201900459.

2) Hirata, R., Mishiba, K.-I., Koizumi, N., & Iwata, Y. (2019). Deficiency in the double-stranded RNA binding protein HYPONASTIC LEAVES1 increases sensitivity to the endoplasmic reticulum stress inducer tunicamycin in Arabidopsis. *BMC Res Notes*, 12, 580.

3) Hirohata, A., Sato, I., Kaino, K., Iwata, Y., Koizumi, N., & Mishiba, K.-I. (2019). CRISPR/Cas9-mediated homologous recombination in tobacco. *Plant Cell Reports*, 38, 463-473.

2. 学会発表・講演

1) 小泉　望（大阪府立大）「科学技術の社会実装のためのコミュニケーション　ゲノム編集食品のルール作りを例に」、日本サイエンスコミュニケーション協会、三鷹ネットワーク大学、令和元年 12 月 7 日

2) 小泉　望（大阪府立大）「ゲノム編集食品の現状と課題」、日本食品微生物学会、令和元年 12 月 12 月 20 日、I-site なんば（招待講演）

3) 小泉　望（大阪府立大）「ゲノム編集技術の基礎から食品への応用およびその現状と課題」、日本食品衛生学会、令和２年２月 28 日、立命館大学（招待講演）※新型コロナウイルスのため延期

4) 小泉　望（大阪府立大）「ゲノム編集食品に関するパンフレット」、日本植物生理学会、令和２年３月 19 日、大阪大学（ポスター発表）※新型コロナウイルスのため中止（学会としては成立）

H. 知的財産権の出願・登録状況

該当なし

別添資料１　主なリスクコミュニケーション活動の概要

年月日	活動内容	対象
2019 年 4 月 23 日	村中氏（大阪大学）によるゲノム編集ジャガイモの開発に関する話題提供とパネルディスカッション	一般の人 （生協理事）
2019 年 5 月 10 日	小泉によるゲノム編集食品に関する話題提供（サイエンスカフェ）	一般の人（専門学校生など）
2019 年 6 月 24 日	山口氏（大阪府大）ならび三原氏（大阪いずみ市民生協）によるゲノム編集食品のルール作りに関する話題提供	一般の人 （高校生）
2019 年 7 月 17 日	木下氏（京都大学)、村中氏（大阪大学）らによるゲノム編集食品に関する話題提供と表示に関する議論	専門家およびサイエンスコミュニケーター
2019 年 9 月 25 日	山口氏（大阪府大）による話題提供	一般の人 （主としてシニア）
2019 年 10 月 2 日	住吉氏（サナテックシード）による話題提供とパネルディスカッション	一般の人 （主としてシニア）
2019 年 11 月 5 日	村中氏（大阪大学)、山川氏（東京大学)、山口氏（大阪府大）らによる話題提供	専門家、生協理事等
2019 年 11 月 17 日	村中氏（大阪大学)、江面氏（筑波大学）らによる話題提供とワークショップ及び科学未来館スタッフによる一般の人とのコミュニケーション	一般の人、専門家およびサイエンスコミュニケーター
2019 年 12 月 12 日	村中氏（大阪大学）による話題提供と双方向コミュニケーション	一般の人 （高校生）
2020 年 2 月 8 日	四方氏（農研機構）による話題提供とパネルディスカッション	一般の人 （生協職員）
2020 年 2 月 8 日	木下氏（京都大学）による話題提供とワークショップ	専門家

別添資料２　主な新聞記事の抜粋（NHK クローズアップ現代を含む）
（報道日、新聞名、見出し、URL、書き出し部分、を記載）

2019.05.09　農業協同組合新聞
意図しない変異も　安全性の検証議論不足 実用化に国の規制必要
https://www.jacom.or.jp/nousei/rensai/2019/05/190509-37973.php
北海道大学　石井哲也教授に聞く
狙った遺伝子を効率よく改変するゲノム編集技術を使って開発された食品の一部について、厚生労働省は 3 月に従来の育種による品種改良と同じだとして、遺伝子組み換え食品のような安全審査を必要とせず、開発者が国に必要な情報を「届け出」すれば食品として販売できる方針を決めた。前回はこの技術の可能性に焦点をあてて識者の考えを中心に紹介した。そこではゲノム編集技術は従来の品種改良より、短期間で、消費者・生産者にとってより有用な品種が開発できることが強調された。しかし、一方で安全性や消費者・生産者が自ら選択する表示制度などをめぐる議論などが不足したまま実用化へ向かっているとの心配の声も多い。今回はこうしたゲノム編集技術についての問題点と課題を整理してみた。

2019.5.23　毎日新聞
ゲノム編集食品の表示　消費者庁が意見聴取を開始　夏ごろ流通へ最終手続き
https://mainichi.jp/articles/20190523/k00/00m/040/207000c
遺伝子を効率よく改変する「ゲノム編集技術」を使った食品の表示のあり方について、消費者庁は 23 日、内閣府消費者委員会の食品表示部会の委員らのヒアリングを始め、検討が本格化した。ゲノム編集食品の市場流通に向けた最後の手続きとなり、夏ごろ運用が開始される見通し。

2019.6. 5　毎日新聞
ゲノム編集食品「食べたくない」4 割　東大調査　今夏にも解禁
https://mainichi.jp/articles/20190605/k00/00m/040/193000c
生物の遺伝子を効率よく改変できるゲノム編集技術を使い開発した農作物について、東京大の研究チームが一般市民を対象に意識調査をしたところ、「食べたくない」と答えた人が 4 割を超えた。畜産物では 5 割を超え、抵抗感を持つ人が多い現状が浮かんだ。東京都内で開催された日本ゲノム編集学会で 5 日、報告された。

2019.6.20　毎日新聞
ゲノム編集食品、表示義務化見送りへ
https://mainichi.jp/articles/20190620/k00/00m/040/189000c

遺伝子を効率良く改変する「ゲノム編集」の技術を使った食品を巡り、編集表示の義務化が見送られる見通しになった。消費者庁は 20 日、内閣府消費者委員会の食品表示部会（部会長＝受田浩之・高知大教授）で「従来の農産物との違いを科学的に検証できず、義務違反の特定は困難」とする考えを示し、部会の委員から意見を聞いた。任意表示については検討し、8 月末をめどに表示のあり方を公表する。

2019.6.27　日本経済新聞
ゲノム編集食品、届け出にはアレルギー物質など確認を 厚労省が要項案
https://www.nikkei.com/article/DGXMZO46675260X20C19A6000000/
厚生労働省は 27 日、狙った遺伝子を効率よく改変する「ゲノム編集」技術で開発した食品を販売する前に、届け出が必要となる項目を示した要項案を公表した。利用した技術の詳細のほか、アレルギーの原因物質や有害物質が含まれていないかなどを報告しなければならない。7 月 26 日まで一般の意見を募り、8 月にも届け出の受けつけが始まる見通しだ。

2019.6.30　毎日新聞
ゲノム食品　情報開示に課題
https://mainichi.jp/articles/20190630/ddm/013/040/012000c
肉厚のマダイやアレルギー物質が少ない卵など、遺伝子を改変する「ゲノム編集技術」で開発した食品の流通に向け、消費者庁は８月にも製造・販売業者に対する表示のルールを決める。今月２０日には「表示の義務化は困難」という考えを示し、事業者の任意とされる見通し。早ければ年内にもゲノム編集食品が店頭に並び始めるが、消費者が安心して買い求められる仕組みになるのだろうか。

2019.7.3　朝日新聞（論座）
ゲノム編集食品を自発的・積極的に表示しよう
https://webronza.asahi.com/science/articles/2019062600010.html
遺伝子を効率よく改変できるゲノム編集技術で生まれた食品の表示をどうするかが大きな関心を集めている。消費者庁は 6 月下旬、「表示の義務化は困難」との見方を示した。従来の品種改良で生まれた食品との違いを科学的に検証できないというのが理由だ。各新聞もそのように報じている。本当にそうだろうか。実は特定のゲノム編集食品に絞れば、検証可能なケースはある。いったいどういうことか。「表示と検証」の重大な意味と意義を考えてみた。

2019.7.11　朝日新聞
（社説）ゲノム編集食品　「選べる」ことが必要だ
https://www.asahi.com/articles/DA3S14091541.html

これでは消費者の利益よりも、役所の都合を優先させているとしか思えない。
　肉厚のマダイや栄養成分を強化したトマトなど、ゲノム編集技術を使って遺伝子を操作した食品について、改変した旨の表示を見送る方向で検討が進んでいる。消費者庁が先月、「義務化は困難」との見解を示し、近く最終決定するという

2019.9.14　朝日新聞
肉厚マダイ、血圧抑制トマト…ゲノム編集食品いつ食卓に
https://www.asahi.com/articles/ASM9F56J4M9FULBJ01D.html
ゲノム編集技術を使って野菜や魚の遺伝情報を変えた食品について厚生労働省は１３日、事業者からの届け出を１０月１日から受け付けると発表した。国内ではすでに血圧を抑える成分が多いトマトや肉厚なマダイなどの開発が進んでいる。食卓に上るのは早くても年末以降になる見通しだ。

2019.9.19　朝日新聞
ゲノム編集食品、届け出したら表示を　義務化は見送り
https://www.asahi.com/articles/ASM9L62D3M9LUTFL00L.html
遺伝情報を効率よく変えられる「ゲノム編集」を使った食品をめぐり、消費者庁は１９日、狙った遺伝子を壊して変異を起こす手法の場合は表示を義務化しないと発表した。厚生労働省に届け出があった食品については表示などの情報提供をするよう求める通知を出した。

2019.9.19　産経新聞
ゲノム食品、表示義務なし　消費者庁「判別不可能」　年内にも流通
https://www.sankei.com/life/news/190919/lif1909190029-n1.html
　消費者庁は１９日、ゲノム編集技術で品種改良した農水産物の大半について、生産者や販売者らにゲノム編集食品であると表示することを義務付けないと発表した。ゲノム編集食品は特定の遺伝子を切断してつくられるが、外部から遺伝子を挿入する場合と挿入しない場合があり、現在開発が進む食品の大半は挿入しないタイプという。厚生労働省は、同タイプの販売について安全性審査を経ずに届け出制にするとしており、今回の消費者庁の発表で流通ルールの大枠が決まった。

2019.9.23　毎日新聞
社説　ゲノム編集食品　消費者が選べるルールに
https://mainichi.jp/articles/20190923/ddm/005/070/075000c
新技術のゲノム編集で品種改良した食品について、消費者庁は特定の遺伝子を壊しただけの食品には表示を義務付けないと決めた。

2019..9.24 NHK クローズアップ現代
解禁！“ゲノム編集食品”　～食卓への影響は？～
https://www.nhk.or.jp/gendai/articles/4331/index.html
生命の設計図を改変して作られる“ゲノム編集食品”。今月、国内で解禁され、近く販売が可能になる。ゲノム編集の技術を使えば、肉厚のマダイや栄養価が高いトマトなどを短期間で開発することができ、私たちの食卓に大きな影響を及ぼす可能性がある。果たしてその安全性は？そして、従来の遺伝子組み換え食品との違いは？日本に先行し、すでにゲノム編集食品の流通が始まっているアメリカの動向も取材。あらたな技術との向き合い方を探る。

2019.9.27　読売新聞
社説　ゲノム編集食品　消費者の選択に資する表示を
https://www.yomiuri.co.jp/editorial/20190926-OYT1T50347/
食品に対する消費者の関心は高い。遺伝子改変で開発された食品を流通させる場合には、消費者が納得して購入できるよう、適切な情報提供が求められよう。
　消費者庁が、ゲノム編集技術で特定の遺伝子を壊す操作をした食品の表示について、事業者の任意とすることを決めた。

2019.10.4　産経新聞
【主張】ゲノム編集食品　風評禍の阻止に取り組め
https://www.sankei.com/life/news/191004/lif1910040003-n1.html
ゲノム編集技術を使った食品について、流通と販売の届け出制度が 1 日から始まった。厳正な安全審査と適切な情報開示に資する制度としなければならない。
　ゲノム編集食品であることの表示に関しては義務化が見送られた。既存の品種改良との区別が技術的に困難であることがその理由である。

2020.1.23　毎日新聞
ゲノム編集食品第 1 号「血圧下げるトマト」は売れるか
https://mainichi.jp/premier/business/articles/20200120/biz/00m/020/012000c
血圧を下げる機能があることが報告されている天然のアミノ酸「GABA」（γ－アミノ酪酸、読み方は「ギャバ」）。ゲノム編集技術で、この GABA を多く含むトマトがいよいよ市場に登場しそうだ。ただし、GABA の効用をうたう食品は多い。はたして消費者に受け入れられるのだろうか。

128202：食品に関するアンケート

Rakuten Insight

■質問カウント数　本調査　31問

調査票出力日時：2020/02/26 11:18

ウント	質問カ			
			本調査	
0問	任意			
			●本調査は、厚生労働省からの委託調査の一環として、日本における ついて考えるための資料とするために行うものです。 ●設問の一部に、立ち入ったことなどをおたずねする内容もございますが、いずれも研究のために大切な質問です。 可能な範囲で構いませんので、ご協力いただけましたら幸いです。 ●結果の公表では、回答結果は集計値としてまとめられ、あなた様のお名前や個人的な情報が外部へ出ることはございません。また、結果を研究・教育目的以外に使用することもありません。 以上の内容についてご確認の上ご協力の同意をいただけましたら、ご回答をお願い申し上げます。 このアンケートへのご回答をもって、ご協力の同意をいただけたものと判断させていただきます。 ご多忙中誠に恐れ入りますが、ご協力くださいますようお願い申し上げます	
	X1	フリーフォーム	(質問文非表示)	
			改ページ	
1問	必須			
			■「ゲノム編集」についてお伺いします。 遺伝子をピンポイントかつ高精度に改変する技術として「ゲノム編集」の研究が進められています。 遺伝子組換え食品とは異なる新しいゲノム編集技術を活用して作成された食品を、 「ゲノム編集食品」といいます。現在、無毒なジャガイモや、成長の早い鯛などをつくる研究が 進んでいます。	
	Q1	SA	ゲノム編集という言葉を聞いたことがありますか。	
			1 聞いたことがあり、内容も知っている 2 聞いた事はあるが、内容はよく知らない 3 聞いたことはない	
			改ページ	
1問	必須			
	Q2	SA	あなたは、ゲノム編集食品についてどの程度知っていると思いますか。	
			1 よく知っている 2 知っているほうである 3 少し知っている 4 どちらとも言えない 5 あまり知らない 6 ほとんど知らない 7 聞いたことも無い	
			改ページ	
1問	必須			
	Q3	SA	ゲノム編集食品をご自身が食べることについてあなたの意見に近いものはどれですか。	
			1 強く賛成 2 賛成 3 どちらかというと賛成 4 どちらとも言えない 5 どちらかというと反対 6 反対 7 強く反対	
			改ページ	
1問	必須			
	Q4	SA	ゲノム編集食品は安全だという専門家の意見について、あなたはどの程度信頼できますか。	
			1 強く信頼できる 2 信頼できる 3 やや信頼できる 4 どちらとも言えない 5 あまり信頼できない 6 信頼できない 7 全く信頼できない	
			改ページ	
1問	必須			
	Q5	SA	ゲノム編集食品は今後日本社会で受け容れられていくと思いますか。	
			1 かなり受け容れられると思う 2 ある程度、受け容れられる 3 少し受け容れられると思う 4 どちらとも言えない 5 あまり受け容れられない 6 受け容れられない 7 全く受け容れられない	
			改ページ	
1問	必須			
	Q6	MA	ゲノム編集食品が社会に受け容れられるかどうかについて、どのような事柄が重要だと思いますか。 下の項目について、重要度の高いと思うものを3つ選んで回答してください。 （必ず3つ）	
			1 科学的な面白さ	

			2	科学的妥当性
			3	社会が規制して、その科学や技術の誤用・悪用を防ぐことができるかどうか
			4	その科学や技術が社会にとって必要かどうか
			5	起こり得るリスクの深刻さ
			6	起こり得るリスクの発生確率の高さ
			7	起こり得るリスクに対応できるかどうか（リスク対策）
			8	大学、国、企業などの科学や技術を開発・利用する主体が信頼できるかどうか
			9	責任の所在がはっきりしているかどうか
			10	将来、その科学や技術によって社会に何が起こるか予測できるかどうか
			11	あてはまるものはない(排他)
		回答数制限		
		あり（3以上3まで）		
	改ページ			
1問	必須			
	Q7	MA		あなたはゲノム編集食品について、どんなことを知りたいと思いますか。 下の項目について、知りたいと思うものを5つ選んで回答してください。 （必ず5つ）
			1	ゲノム編集技術のメカニズム・しくみ
			2	ゲノム編集食品のベネフィット（利点・良い点）
			3	ゲノム編集食品のリスク
			4	ゲノム編集食品の値段
			5	ゲノム編集食品の必要性
			6	ゲノム編集食品の産業としての可能性
			7	ゲノム編集食品の安全性確保の手段について
			8	ゲノム編集技術の倫理問題について
			9	ゲノム編集食品の表示方法について
			10	ゲノム編集技術の今後の研究活動のスケジュールについて
			11	ゲノム編集食品による風評被害が発生した場合の対応について
			12	ゲノム編集食品によって生じるネガティブな影響への対応について
			13	ゲノム編集食品をめぐる国の政策・制度の現状について
			14	ゲノム編集食品に関する規制作りの今後のスケジュールについて
			15	ゲノム編集食品を巡る国際的な制度の状況
			16	その他：[FA](回答必須)(入力制限なし)(200文字まで)
			17	特に知りたいことはない(排他)
		回答数制限		
		あり（5以上5まで）		
	改ページ			
1問	必須			
	Q8	マトリクス→		以下にあてはまるものをそれぞれお選びください。 ※この設問は、それぞれ横方向（→）にお答えください。
				【質問アイテム】
	SA		1	人間は、ゲノム編集食品が人体に悪い影響を与えないように上手に利用することができると思いますか 必須
	SA		2	人間は、ゲノム編集食品が環境に悪い影響を与えないように上手に利用することができると思いますか 必須
	SA		3	人間は、ゲノム編集食品が経済に悪い影響を与えないように上手に利用することができると思いますか 必須
				【選択肢】
			1	強くそう思う
			2	そう思う
			3	やどちらかというとそう思う
			4	どちらとも言えない
			5	どちらかというとそう思わない
			6	そう思わない
			7	全くそう思わない
	改ページ			
1問	必須			
	Q9	SA		ゲノム編集食品について、日本ではゲノム編集食品の食品への表示に関する議論が進んできています。 ゲノム編集食品の表示に関してお答えください。
			1	ゲノム編集食品は、常に表示すべきである
			2	ゲノム編集食品は、栄養成分が変化した場合に表示すべきである
			3	ゲノム編集食品の表示は企業の自主判断に任せるべきである
			4	ゲノム編集食品について、表示は不要である
			5	その他：[FA](回答必須)(入力制限なし)(200文字まで)
			6	わからない
	改ページ			
1問	必須			
	Q10	SA		あなたはいつ頃ゲノム編集食品が実際にスーパーで売られるようになると思いますか。 以下の項目から一つ選んでください。
			1	もうすぐ
			2	1年
			3	数年
			4	早くて10年ぐらい
			5	早くて20年ぐらい
			6	さらに長い期間
			7	わからない

				改ページ		
2問	必須					
	Q11		マトリクス→	ゲノム編集食品に関する以下の事柄について、あなたの意見に近いものを選んでください。 ※この設問は、それぞれ横方向（→）にお答えください。		
				【質問アイテム】		
	SA		1	食料の安定供給に役に立つ	必須	
	SA		2	人々の健康のために役に立つ	必須	
	SA		3	人々の健康に対してリスクを与える	必須	
	SA		4	長期的にみたときにリスクが顕在化する	必須	
	SA		5	日本の経済に良い影響がある	必須	
	SA		6	植物や昆虫の生態系が変化する	必須	
	SA		7	安全性の確認が不十分である	必須	
	SA		8	予期せぬリスクがある	必須	
	SA		9	技術が悪用される可能性がある	必須	
	SA		10	生命倫理上の問題を感じる	必須	
	SA		11	規制が上手くいかない可能性が高い	必須	
	SA		12	ゲノム編集食品の利用について社会的な合意が取れていないと思う	必須	
	SA		13	ゲノム編集食品で何か問題が起きたときに政府は、上手く対処できない	必須	
	SA		14	ゲノム編集食品で何か問題が起きたときに企業は、上手く対処できない	必須	
	SA		15	ゲノム編集食品で何か問題が起きたときに専門家は、上手く対処できない	必須	
	SA		16	ゲノム編集食品で何か問題が起きたときに政府は責任を取らないと思う	必須	
	SA		17	ゲノム編集食品で何か問題が起きたときに専門家は責任を取らないと思う	必須	
	SA		18	ゲノム編集食品で何か問題が起きたときに企業は責任を取らないと思う	必須	
	SA		19	良く理解できずなんとなく怖さを感じる	必須	
				【選択肢】		
			1	強くそう思う		
			2	やや強くそう思う		
			3	どちらでもない		
			4	余りそう思わない		
			5	そうは思わない		
				改ページ		
1問	必須					
	Q12		SA	ゲノム編集食品の規制に関する考え方について一番近いものはどれですか。		
			1	リスクが限りなくゼロに近いような基準で規制すべき		
			2	科学的・技術的に妥当な基準で規制すべき		
			3	経済効率を重視し、必要最低限の基準で規制すべき		
			4	規制を行う必要はない		
				改ページ		
1問	必須					
	Q13		SA	ゲノム編集食品についていろいろおたずねしましたが、全体としてあなたのお考えに近いものを次の中から1つ選んでください。		
			1	安全性には配慮する必要があるが、ゲノム編集食品を推進するのがよい		
			2	安全性には多少不安があるが、ゲノム編集食品を利用していくことはやむを得ない		
			3	ゲノム編集技術の食品利用よりも、他の農林水産技術に注力するほうがよい		
			4	ゲノム編集食品は利用すべきではない		
			5	わからない		
				改ページ		
1.5問	必須					
	Q14		長文FA	ゲノム編集食品のリスクについて、イメージするものをご自由にお書きください。 ※キーワードだけでも構いません。		
				[FA] (文字数制限なし)		
				改ページ		
2問	必須					
	Q15		マトリクス→	以下の項目それぞれについて、意見に近い度合いを選んでください。 ※この設問は、それぞれ横方向（→）にお答えください。		
				【質問アイテム】		
	SA		1	地元の食材をなるべく食べたい	必須	
	SA		2	栄養バランスの取れた食事をしたい	必須	
	SA		3	地元らしさを活かした農業をしてほしい	必須	
	SA		4	遺伝子組み換え食品でないものを食べたい	必須	
	SA		5	環境保全に気を使った農業をしてほしい	必須	
	SA		6	食事・食品を選ぶ際に旬や季節感は大事にしたい	必須	
	SA		7	食品を選ぶ際に傷んでないか等、見た目でわかる品質は大事にしたい	必須	
	SA		8	農業と消費者の関係を強くしたい	必須	
	SA		9	生産履歴、栽培履歴が分かる食品を選びたい	必須	
	SA		10	6次産業化や輸出などによって農業が儲かるようになることは好ましい	必須	
	SA		11	どんな農家が作ったかが見て分かるような販売を増やしてほしい	必須	
	SA		12	農産物をできるだけ多く生産できるようにしていてほしい	必須	
	SA		13	自然食品・無添加・オーガニックなどの食品をなるべく多く食べたい	必須	
	SA		14	自然に近い方法で農業をしてほしい	必須	
	SA		15	食事・食品を選ぶ際に価格は大事にしたい	必須	

	SA		16	新しい技術を取り入れた農業をしてほしい	必須	
	SA		17	消費者が低価格で買えるような生産方法の農業が望ましい	必須	
	SA		18	加工品やお惣菜、お弁当、外食などをうまく使って食事をしたい	必須	
	SA		19	有名なブランド化している食品を選びたい（例：夕張メロン、松坂牛、他）	必須	
				【選択肢】		
			1	強くそう思う		
			2	やや強くそう思う		
			3	どちらでもない		
			4	余りそう思わない		
			5	そうは思わない		
				改ページ		
2問	必須					
	Q16		マトリクス→	次の文章が正しい内容であるか誤った内容であるか、お答えください。 ※この設問は、それぞれ横方向（→）にお答えください。		
				【質問アイテム】		
	SA		1	我々が呼吸に使っている酸素は植物から作られたものである	必須	
	SA		2	赤ちゃんが女の子になるかどうかを決める遺伝子は、母親の持っている遺伝子である	必須	
	SA		3	抗生物質はバクテリア同様ウィルスも殺す	必須	
	SA		4	現在の人類は原始的な動物種から進化したものである	必須	
	SA		5	放射能に汚染された牛乳は沸騰させれば安全である	必須	
	SA		6	バクテリアの中には排水の中でも生きているものが存在する	必須	
	SA		7	妊娠2～3ヶ月で、赤ちゃんがダウン症候群になるかどうか見つけ出すことが可能である	必須	
	SA		8	ビールを醸造するイースト菌は生きている微生物からできている	必須	
	SA		9	人の遺伝子の半分以上はチンパンジーのものと同じである	必須	
	SA		10	遺伝子組み換え果物を食べることによって、人の遺伝子もまた組み換えられる	必須	
	SA		11	生物のクローン（複製）を作ることは、遺伝的に同一の子孫を生み出すことである	必須	
	SA		12	ふつうのトマトは遺伝子を含まないが、遺伝子組み換えトマトは遺伝子を含んでいる	必須	
	SA		13	遺伝子組み換え動物は、常にふつうの動物よりも大きい	必須	
	SA		14	動物の遺伝子は植物には導入できない	必須	
				【選択肢】		
			1	正しい内容		
			2	誤った内容		
				改ページ		
4問	必須					
	Q17		マトリクス→	以下の態度や考え方について、あなたはどの程度あてはまると思いますか。 4つの選択肢から選んでください。 ※この設問は、それぞれ横方向（→）にお答えください。		
				【質問アイテム】		
	SA		1	新聞をよく読むほうだ	必須	
	SA		2	テレビ・ラジオをよく見る・聞くほうだ	必須	
	SA		3	本をよく読むほうだ	必須	
	SA		4	インターネットをよく使うほうだ	必須	
	SA		5	家族・友人とよく情報交換するほうだ	必須	
	SA		6	普段から、わからないことや生活に必要なことは、よく調べるほうだ	必須	
	SA		7	社会で話題になっていることについて知りたいときに、よく調べるほうだ	必須	
	SA		8	身近な人が病気になったとき、自分でも治療法や原因などを調べる	必須	
	SA		9	博物館、科学館や市民講座にはよく行くほうだ	必須	
	SA		10	人の話を信じやすい	必須	
	SA		11	周りの雰囲気に流されない	必須	
	SA		12	解決しなければならない問題について、人と話し合って上手に解決できる	必須	
	SA		13	問題を解決するときには、「まるくおさめる」よりも、「筋を通すこと」が重要だ	必須	
	SA		14	社会問題など、公共的な問題についての話し合いに興味がある	必須	
	SA		15	地域活動や市民活動に積極的に参加するほうだ	必須	
	SA		16	選挙にはできるだけ行くほうだ	必須	
	SA		17	科学技術の評価活動に市民として参加したい	必須	
	SA		18	超能力のような超自然現象は存在する	必須	
	SA		19	壊れたものの修理・修繕が得意だ	必須	
	SA		20	新しい科学技術を使った電化製品が販売されるとすぐに欲しくなる	必須	
	SA		21	ものづくり（料理、園芸、手芸なども含む）が好きだ	必須	
	SA		22	新しい電子機器をすぐに使いこなせる	必須	
	SA		23	科学技術についての知識は豊かなほうだ	必須	
	SA		24	科学技術についてもっと知りたい	必須	
	SA		25	地球環境のために貢献したい	必須	
	SA		26	ペットボトルやビンはリサイクルに出すほうだ	必須	
	SA		27	すこし値段が高くても、廃棄物にならないものや、電力・燃料の消費量が少ないものを買うほうだ	必須	
	SA		28	スポーツが得意だ	必須	
	SA		29	音感・リズム感が良い	必須	
	SA		30	展開図（平面）から立体像を予想することが得意だ	必須	
	SA		31	地図を読むのが得意だ	必須	
	SA		32	長い文章や講義などの要点をつかむのが得意だ	必須	
	SA		33	論理的にものを考えることが得意だ	必須	
	SA		34	ものの共通点をとらえるのが得意だ	必須	
	SA		35	自分の判断・決定について振り返って考えることがよくある	必須	
				【選択肢】		
			1	強くそう思う		
			2	やや強くそう思う		

			3	どちらでもない		
			4	余りそう思わない		
			5	そうは思わない		
				改ページ		
2問	必須					
	Q18		マトリクス→	科学や社会に関する以下の意見・考えについて、あなたはどのように思いますか。 4つの選択肢からそれぞれ選んでください。 ※この設問は、それぞれ横方向（→）にお答えください。		
				【質問アイテム】		
	SA		1	科学・技術に関する理解は日常生活に役立つ	必須	
	SA		2	科学には有用性だけではなく、知的な楽しみとしての価値もある	必須	
	SA		3	科学的な発見や新技術の開発は、社会や人間を豊かにする	必須	
	SA		4	国が国際的な発展を遂げるためには科学技術の発達が必要だ	必須	
	SA		5	科学技術は社会や人間に悪い影響をもたらす	必須	
	SA		6	科学技術のあり方に社会・市民の視点が反映される必要がある	必須	
	SA		7	社会の中に科学的な考え方が浸透すると良い	必須	
	SA		8	技術が発達すれば、社会的に悪影響を与えない製品やものを作ることができる	必須	
	SA		9	科学の装いをした間違った考え方や製品には厳しい目を向けるべきだ	必須	
	SA		10	科学者・技術者は私たちの生活を良くしようと考えて研究している	必須	
	SA		11	科学者・技術者は信頼できる	必須	
	SA		12	科学・技術は科学者・技術者に任せてよい	必須	
	SA		13	政治家・行政機関は信頼できる	必須	
	SA		14	政治・行政は政治家・行政機関に任せてよい	必須	
				【選択肢】		
			1	強くそう思う		
			2	やや強くそう思う		
			3	どちらでもない		
			4	余りそう思わない		
			5	そうは思わない		
				改ページ		
1問	必須					
				■最後に、あなたご自身のことについて、お伺いします。		
	Q19		SA	あなたのお子さまについて教えてください。		
			1	子どもなし		
			2	子どもあり（妊娠中含む）：[FA](回答必須)(数字小数不可)(制限あり:1以上100以内)人（お子様の人数をご記入ください） ※半角数字でご記入ください。		
				改ページ		
1問	必須	(Q19 or 2)				
	Q20		短文FA	■前問で「2 子どもあり」を選んだ方にお伺いします■ 一番下のお子様の年齢について教えてください。 （半角数字でご記入ください）		
			1	年齢：[FA][必須](数字小数不可)(制限あり:0以上99以内)歳		
				回答矛盾制御		
			優先順位	条件名	条件式	発動条件
			1	Q20エラー	((年齢 val) <= (Q20_1 val))	成立
				改ページ		
1問	必須					
	Q21		SA	あなたの世帯全体の昨年の収入（年収・額面）をお答えください。		
			1	300万円未満		
			2	300～400万円未満		
			3	400～600万円未満		
			4	600～800万円未満		
			5	800～1,000万円未満		
			6	1,000～1,200万円未満		
			7	1,200～1,500万円未満		
			8	1,500～2,000万円未満		
			9	2,000～3,000万円未満		
			10	3,000万円以上		
				改ページ		
1問	必須					
	Q22		SA	あなたが最後に卒業された学校は次のどれですか。		
			1	中学校		
			2	旧制中学		
			3	高校		
			4	旧制高校		
			5	専門学校		
			6	高専・短大		
			7	大学		
			8	大学院		
			9	その他　具体的に：[FA](回答必須)(入力制限なし)(200文字まで)		
				改ページ		
1問	必須					

	Q23		SA	あなたが一番学んだと思う領域についてお答えください。	
			1	理系	
			2	文系	
			3	その他：[FA](回答必須)(入力制限なし)(200文字まで)	
	改ページ				
1問	必須				
	Q24		MA	あなたの現在就いているご職業について、専門領域を分けるとするとどちらになりますか。	
			1	理系	
			2	文系	
			3	農学・食品	
			4	その他：[FA](回答必須)(入力制限なし)(200文字まで)	
			5	わからない／働いていない(排他)	
	アンケート終了				

別添資料４　ゲノム編集食品に対する立場等

受容度（食べてもいいか、食べたくないか）：

東大の調査では 4 割が食べたくないとなっているが、サイエンスアゴラでの調査では食べたい、食べても良いが半数を大きく超える。もっとも母集団が東大の調査とは比べ物にもならないほど小さく（約 100 分の１）、日本科学未来館のスタッフが解説をしているので一概には比較できない。また複数おこなった意見交換会でも、対象や情報提供の方法、内容が異なるため正確な数字は出せない。一言でいえばケースバイケースである。必ずしも絶対受け入れないという意見は少ないが、何となく不安という意見が多い。遺伝子組換え食品の場合もそうであるが、子供や孫には食べさせたくないという意見も多い。基本的に遺伝子組換え食品に対する態度と似ている。絶対に受け入れないという層はある一定程度存在する。

技術に対する理解度（特に遺伝子組換え食品との違いが分かっているか）：

アンケート調査等の結果を見る限り、一般の人のゲノム編集技術に対する理解度は低い。遺伝子組換えとの違いを理解している人も少ない。DNA、遺伝子、ゲノムの違いの説明を出来ない人が圧倒的に多いように感じる。生物をある程度習っている高校生は説明するとそれなりに理解できる。生協を含む消費者団体になると遺伝子組換えとの違いは理解しており、団体により差はあるがゲノム編集技術に対する理解は進む。マスメディアは記者によるが、理解度は余り高くないのではないか。勉強熱心な生協に必ずしも優るとは言えない。オフターゲットに関しては、ほとんど正しく理解されていないと思われる。多くの新聞がオフターゲットの起こる可能性があるからゲノム編集食品に想定外のリスクがあると報じている。

施策の決定について：

多くの新聞報道の論調はルール作りに十分の時間をかけていないことに対する批判である。政府の方針あるいは米国への配慮から国民への十分な説明がなされないままにルール作りが進められたという内容の記事も少なくない。拙速という論調は多く、厚生労働省が公にルール作りを始めたのは 2018 年の夏で、届出のルールが決まったのは 2019 年の 9 月である。約 1 年が短いかどうかの判断は難しい。

施策の内容（表示を含む）について：

届出に事前相談という情報提供のステップがあることが余り知られていないようである。届出が任意であることに対する批判は非常に多い。消費者庁の管轄であるが表示が義務でないことに対する批判は今も根強い。パブリックコメント、リスクコミュニケーションでの意見、新聞報道どれをとっても選択の権利は強調されている。

厚生労働省

『新たなバイオテクノロジーを用いて得られた食品の安全性確保とリスクコミュニケーションのための研究』

リスクコミュニケーション手法の開発・一般意識調査・集計結果一次報告

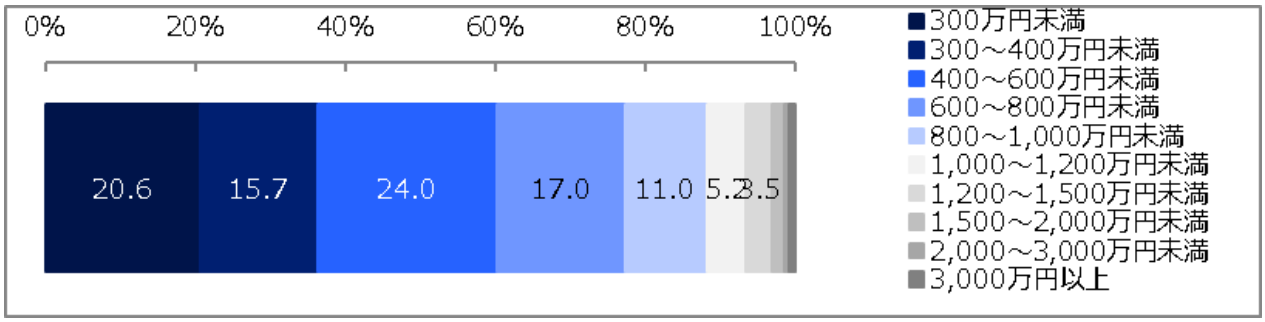
2020年3月10日

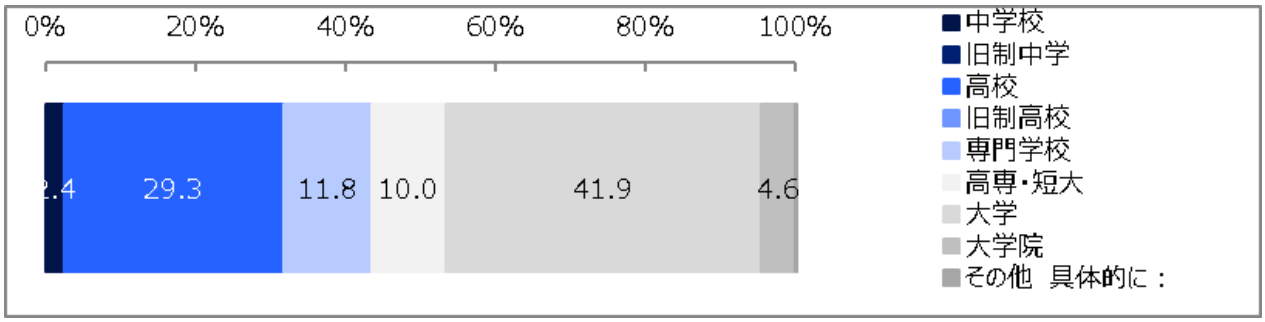
回答者基本情報

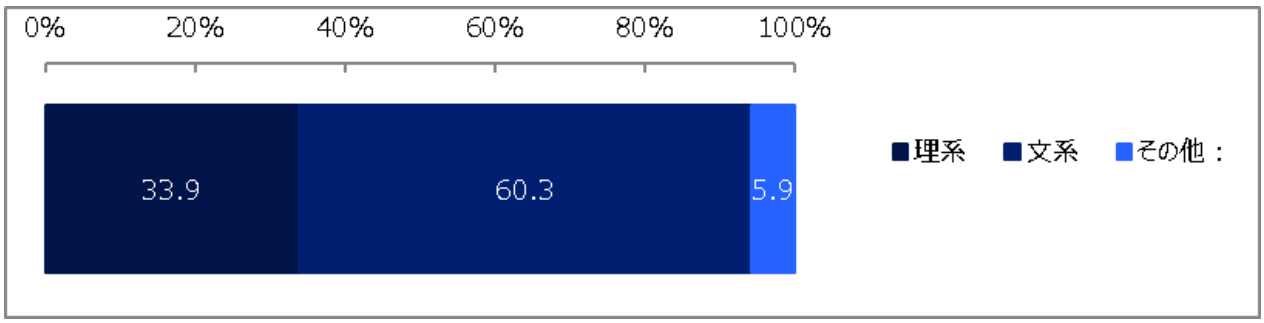
回答者数：4000人（楽天インサイトを経由したインターネットモニターを用いたWeb質問紙調査）
調査実施時期：2020年2月21日～2月26日
回答者年齢平均：49.31歳
回答者割付条件：
20～70代まで10歳ごと6階層均等割り付け
性別・男女で均等割り付け

	n	%
全体	4000	100.0
男性 20代	333	8.3
男性 30代	334	8.4
男性 40代	334	8.4
男性 50代	333	8.3
男性 60代	333	8.3
男性 70代	333	8.3
女性 20代	333	8.3
女性 30代	334	8.4
女性 40代	334	8.4
女性 50代	333	8.3
女性 60代	333	8.3
女性 70代	333	8.3

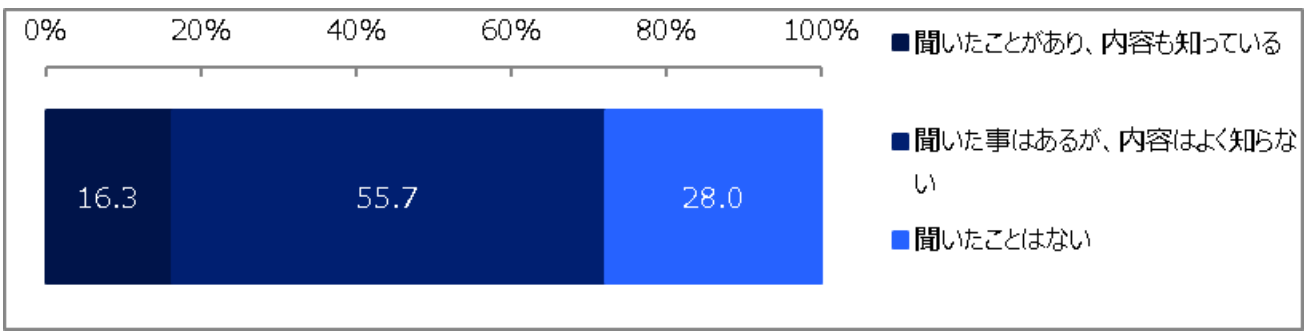
年収・学歴・教育専攻分野回答



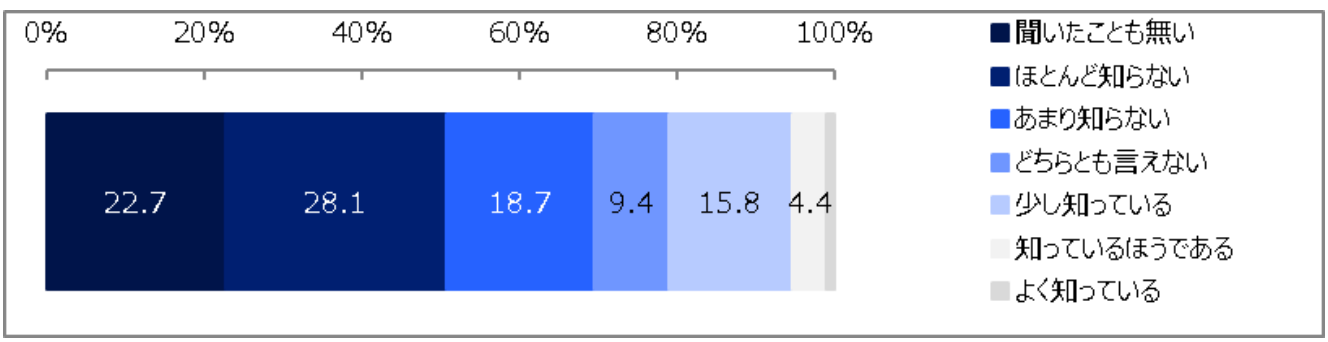




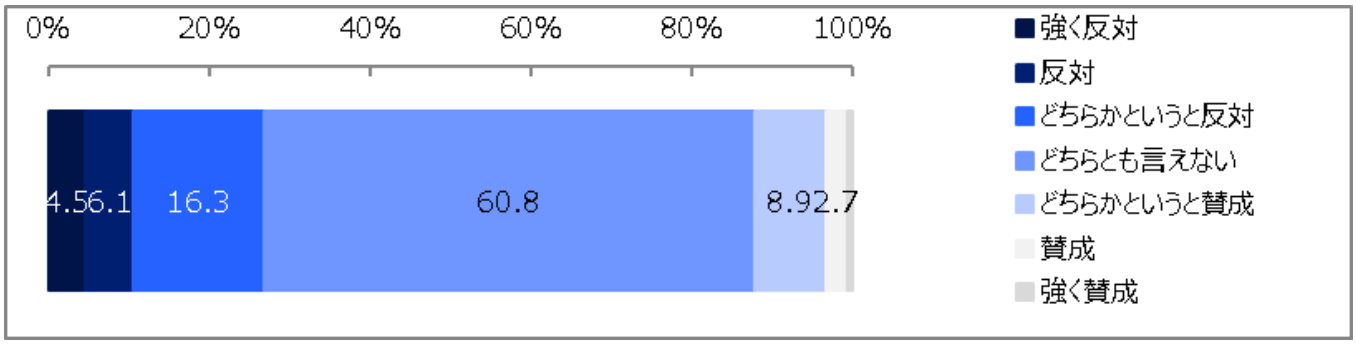
Q1.ゲノム編集という言葉を聞いたことがありますか。



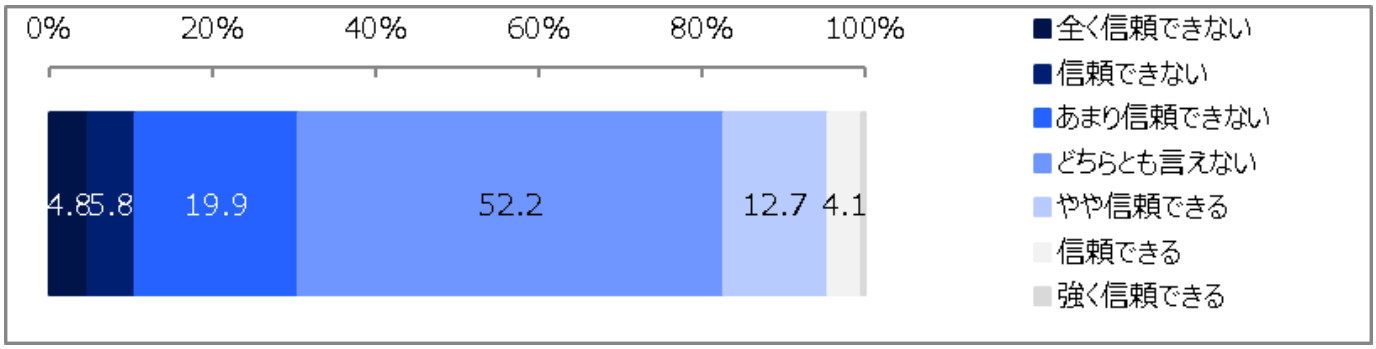
Q2.あなたは、ゲノム編集食品についてどの程度知っていると思いますか。



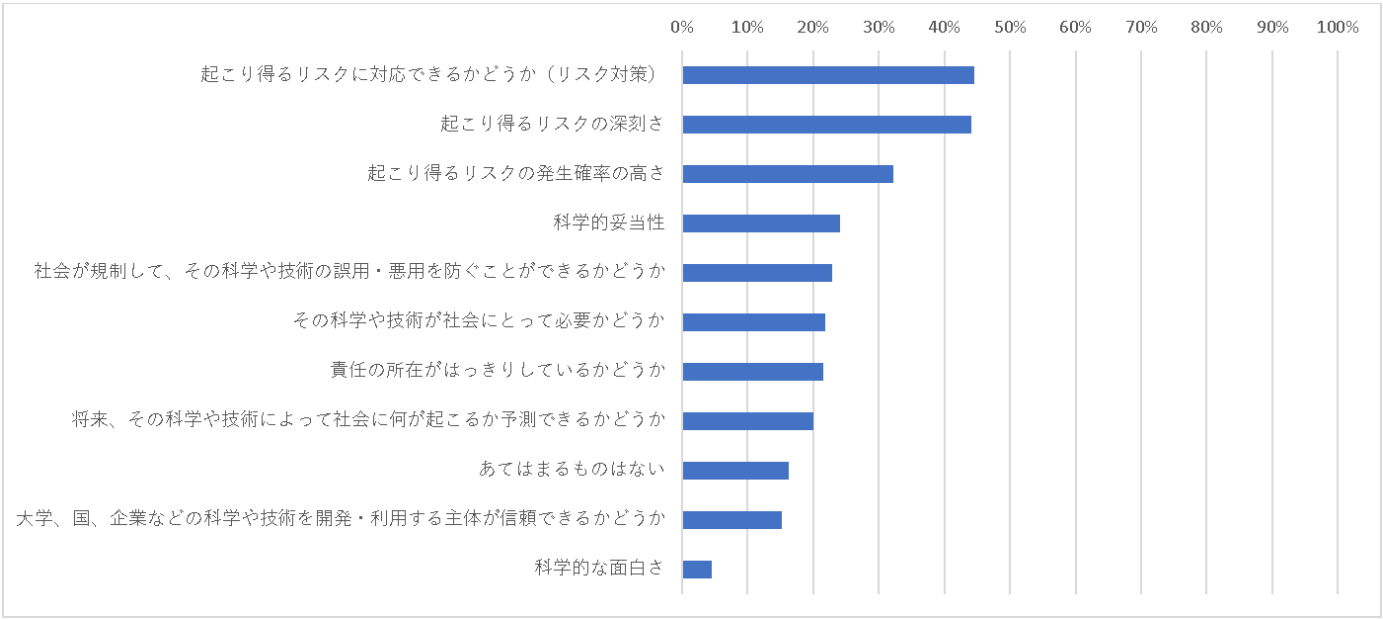
Q3.ゲノム編集食品をご自身が食べることについてあなたの意見に近いものはどれですか。



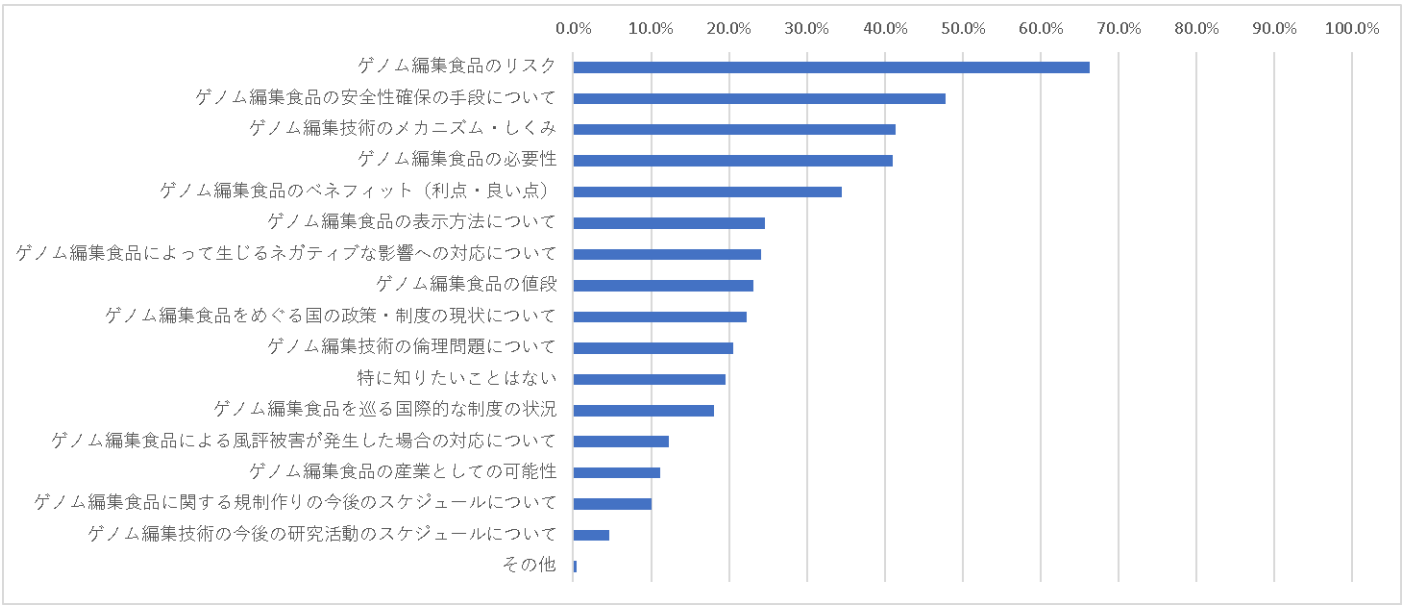
Q4.ゲノム編集食品は安全だという専門家の意見について、あなたはどの程度信頼できますか。



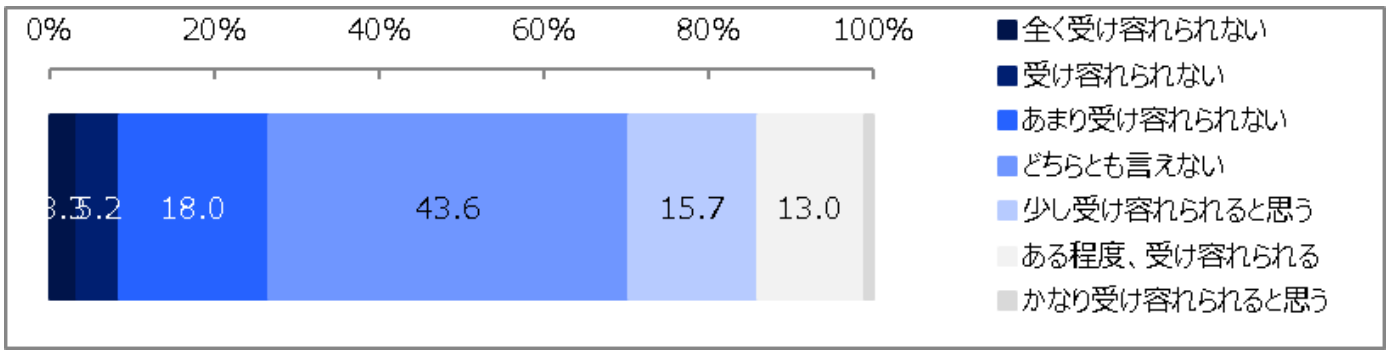
Q6.ゲノム編集食品が社会に受け容れられるかどうかについて、どのような事柄が重要だと思いますか。下の項目について、重要度の高いと思うものを3つ選んで回答してください。（必ず3つ）



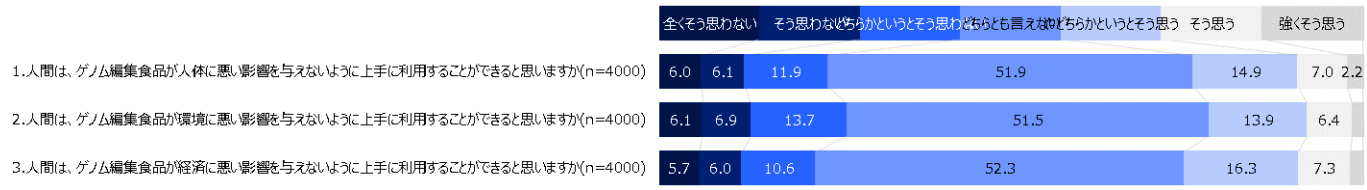
Q7.あなたはゲノム編集食品について、どんなことを知りたいと思いますか。下の項目について、知りたいと思うものを5つ選んで回答してください。（必ず5つ）



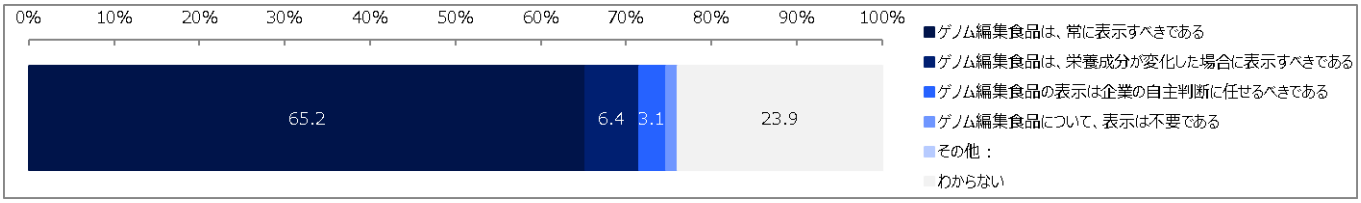
Q5.ゲノム編集食品は今後日本社会で受け容れられていくと思いますか。



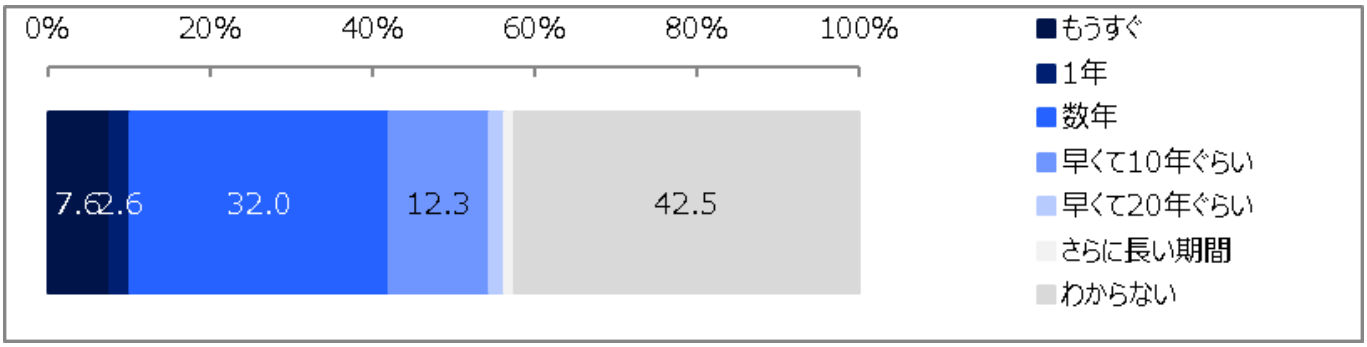
Q8.以下にあてはまるものをそれぞれお選びください。
※この設問は、それぞれ横方向（→）にお答えください。



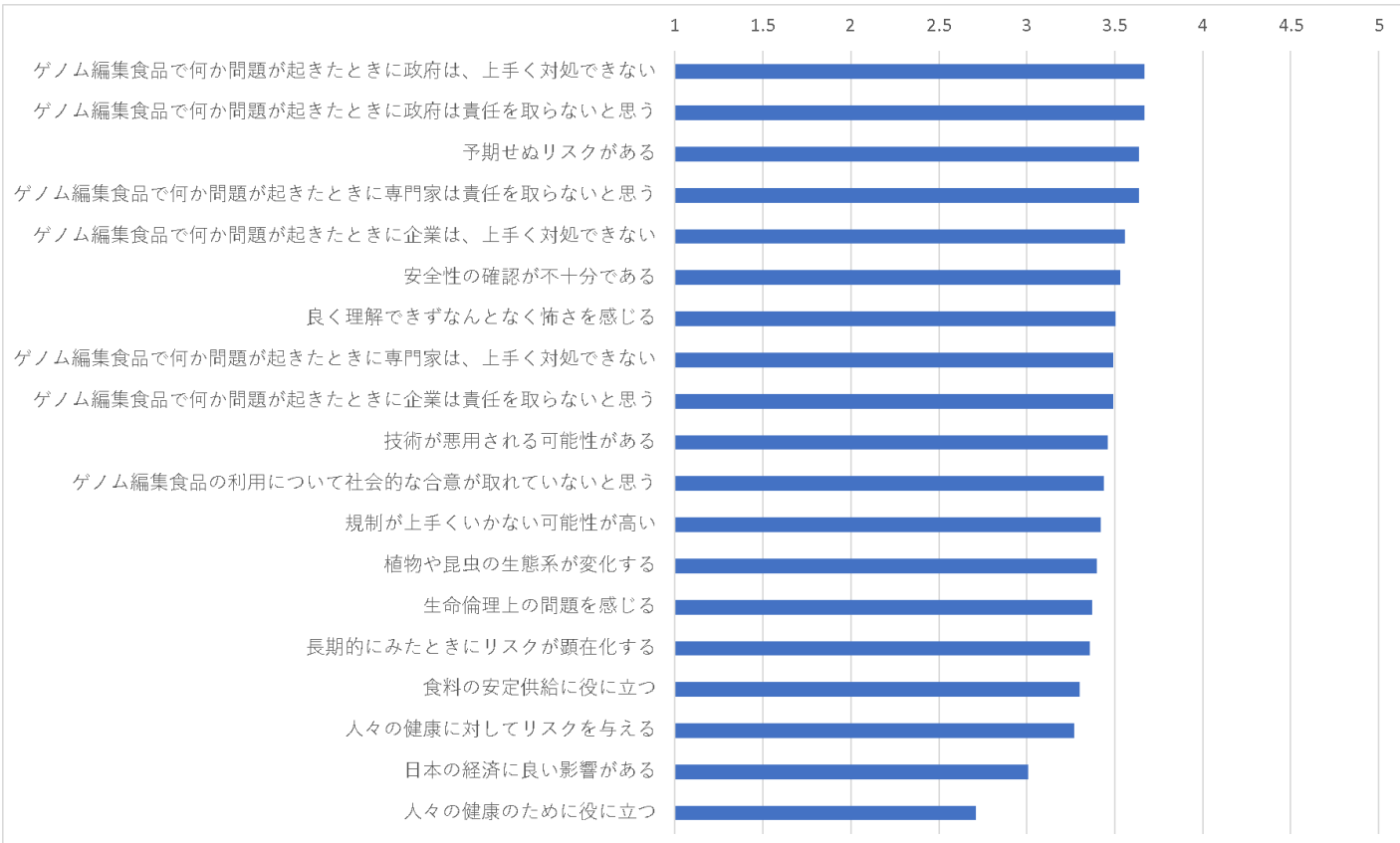
Q9.ゲノム編集食品について、日本ではゲノム編集食品の食品への表示に関する議論が進んできています。ゲノム編集食品の表示に関してお答えください。



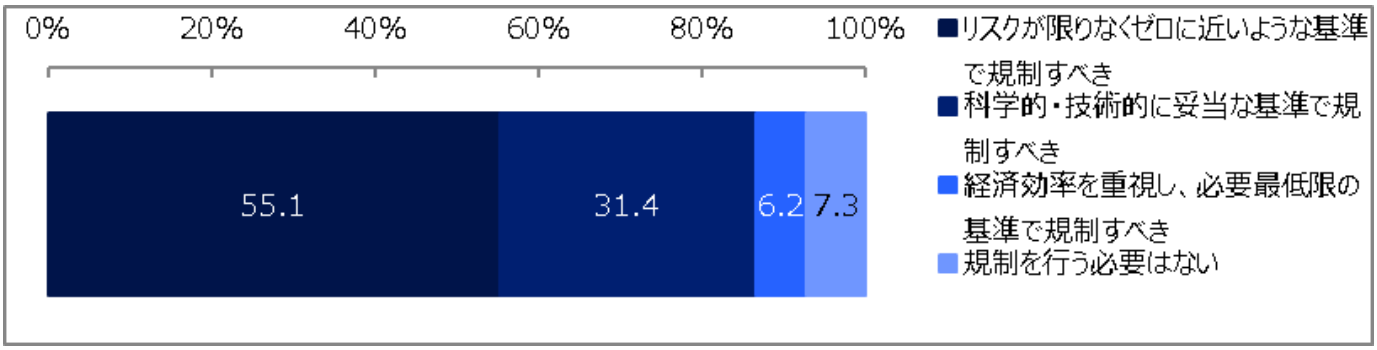
Q10.あなたはいつ頃ゲノム編集食品が実際にスーパーで売られるようになると思いますか。以下の項目から一つ選んでください。



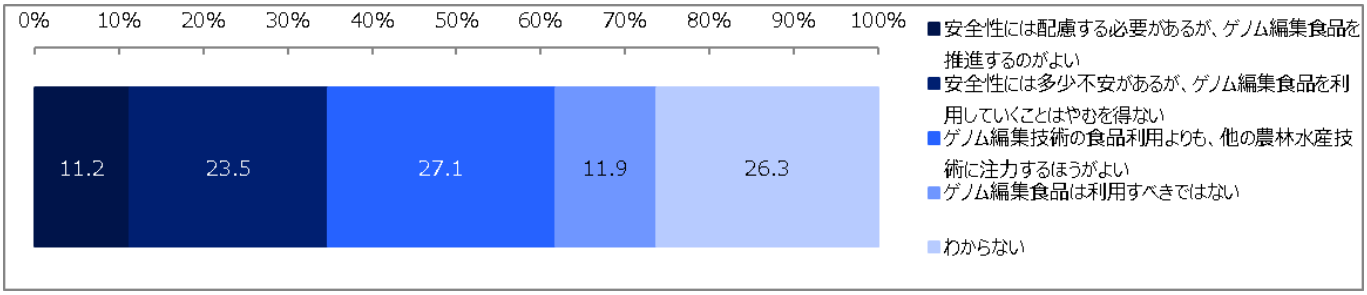
Q11.ゲノム編集食品に関する以下の事柄について、あなたの意見に近いものを選んでください。※この設問は、それぞれ横方向（→）にお答えください。（5：強くそう思う ⇔ 1：そうは思わない）



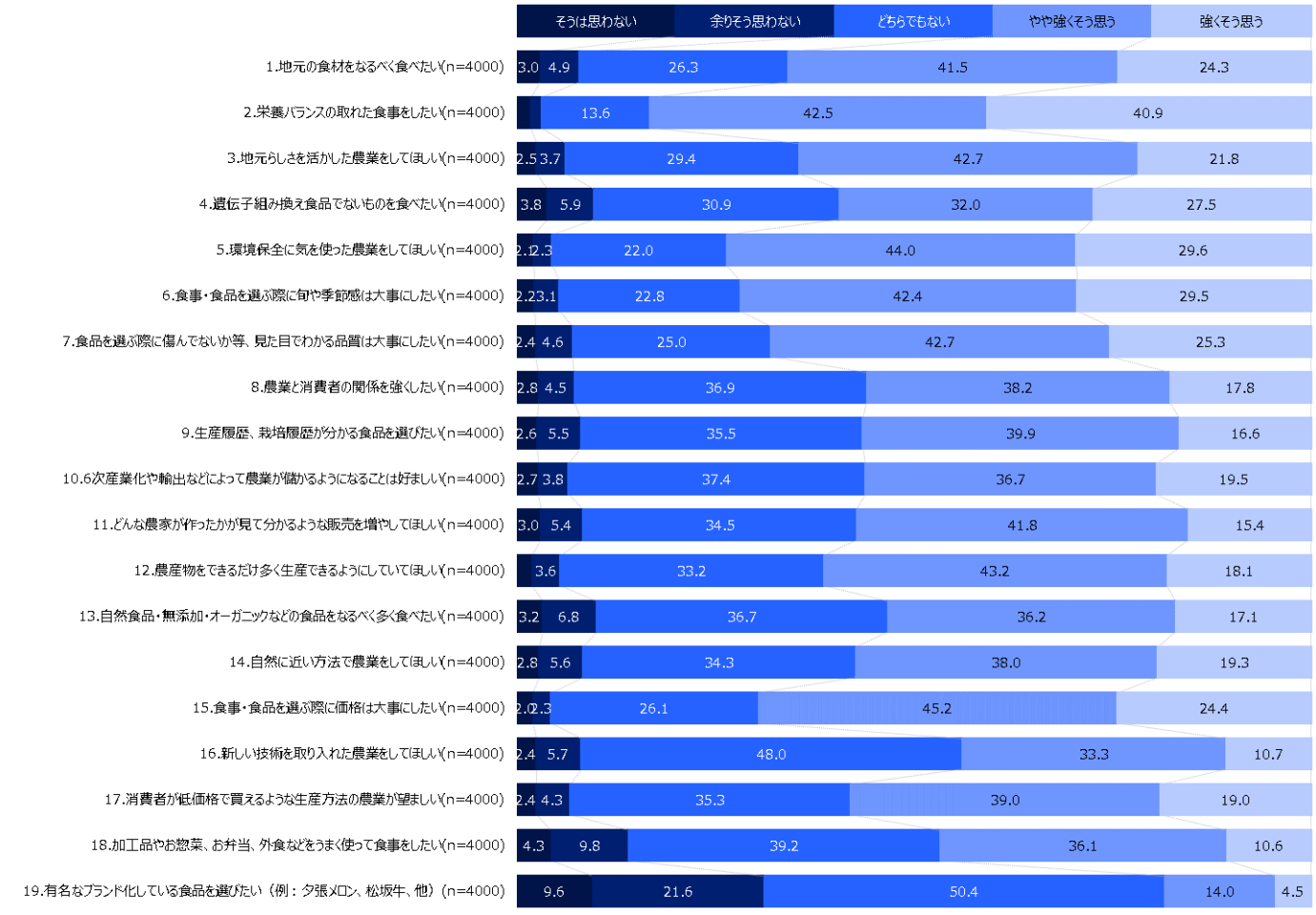
Q12.ゲノム編集食品の規制に関する考え方について一番近いものはどれですか



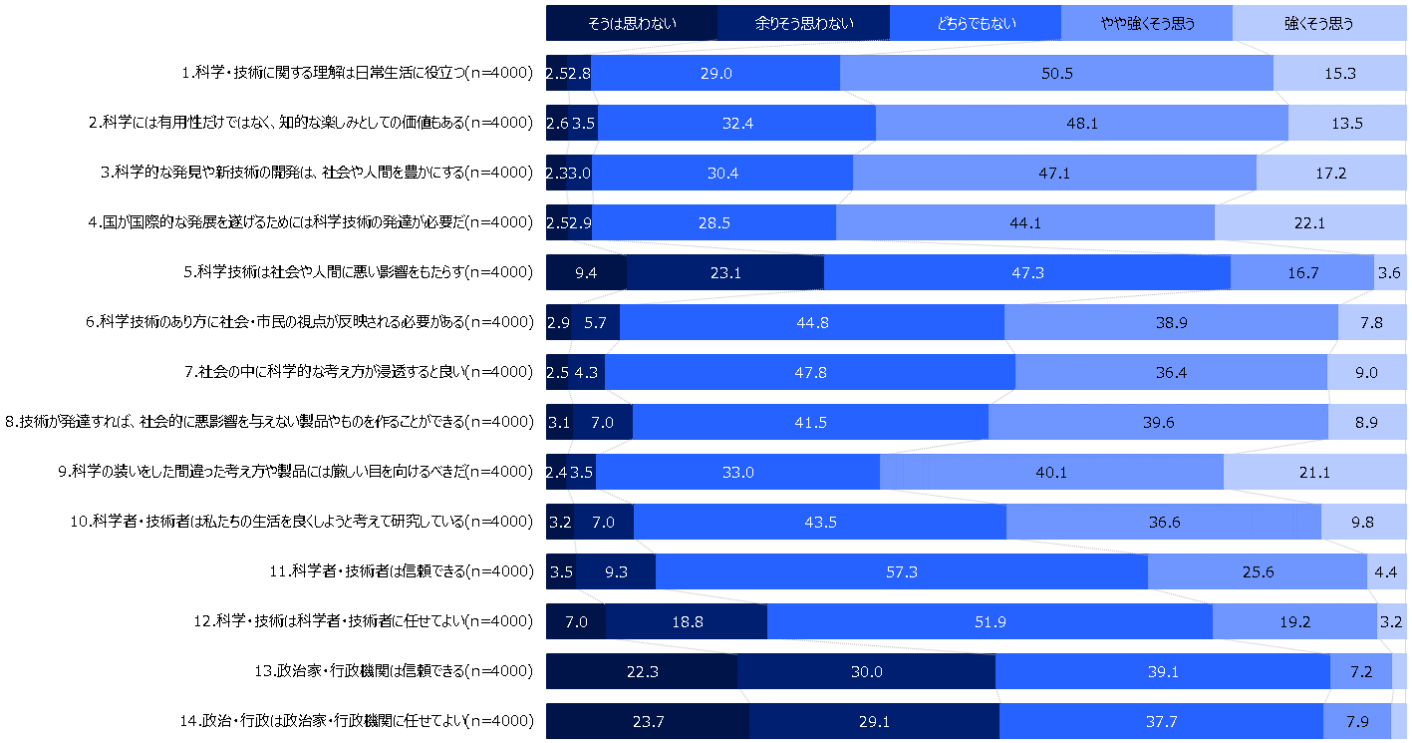
Q13.ゲノム編集食品についていろいろおたずねしましたが、全体としてあなたのお考えに近いものを次の中から1つ選んでください。



Q15.以下の項目それぞれについて、意見に近い度合いを選んでください。
※この設問は、それぞれ横方向（→）にお答えください。



Q18.科学や社会に関する以下の意見・考えについて、あなたはどのように思いますか。4つの選択肢からそれぞれ選んでください。※この設問は、それぞれ横方向（→）にお答えください。

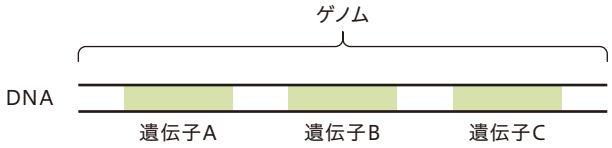


ゲノム編集技術応用食品を適切に理解するための6つのポイント

近年、農作物などの新しい育種技術として研究開発が進められている“ゲノム編集技術”と、この技術によって作られる食品の食品衛生上の取り扱いについて、適切に理解するための６つのポイントを説明します。

Point 1 ゲノムとは？

生物を構成する１つ１つの細胞には、DNA（デオキシリボ核酸）と呼ばれる遺伝物質が含まれています。DNAは、ACGTで表現される４種類の塩基が連なった構造をとっています。DNAの中で、機能を持つ部分を遺伝子と呼びます。ゲノムとは、遺伝子でない部分も含むDNA全体を指します。



Point 2 組換えDNA技術とは？

「組換えDNA技術」（いわゆる「遺伝子組換え技術」）とは、ある生物から取り出したDNAを細胞外で操作した後、細胞の中のDNAに組み込む技術です。この技術は、既に育種技術として応用されていますが、「組換えDNA技術応用食品」（いわゆる「遺伝子組換え食品」）の利用には、安全性審査が義務付けられています。

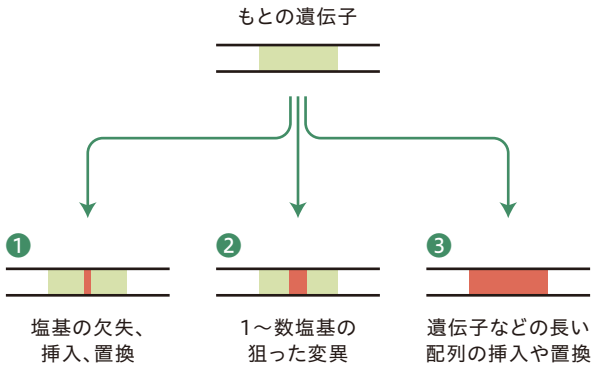
Point 3 ゲノム編集技術とは？

自然界では、放射線などによりDNAの切断が起こることがあります。生物はDNAの修復機能を持ちますが、正しく修復されないと、塩基の挿入、欠失や置換といった変異が起こります。従来の育種技術では、こうした変異の頻度を上げることで、多様な性質を持つ品種を作りますが、変異はランダムに起こります。

ゲノム編集技術では、特定の塩基配列を認識する酵素を細胞の中で働かせ、その塩基配列上の特定部位の切断を行います。その後、生物のDNAの持つ修復機構が働き、

❶自然界においても起こり得る塩基の欠失、挿入、置換
❷１～数塩基の狙った変異
❸遺伝子などの長い配列の挿入や置換

といったDNA配列の変化が起こります。この技術を用いて得られた食品が「ゲノム編集技術応用食品」となります。



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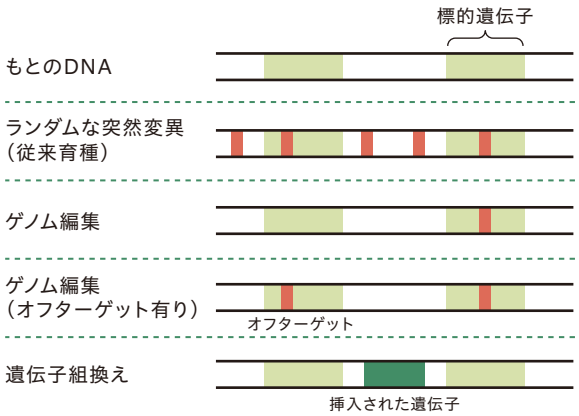
Point 4 ランダム変異とゲノム編集におけるオフターゲットとは？

交配や自然発生または人為的に誘発した突然変異を利用した従来育種では、変異がランダムに起こります。そのため、標的の遺伝子が変異する確率は非常に低いのに比べ、「ゲノム編集技術」では、高い確率で特異的に標的遺伝子に変異を起こすことができます。それでも意図しない変異が起こることがあり、その変異は「オフターゲット」と呼ばれています。遺伝子組換えでは新たに遺伝子が挿入されます。



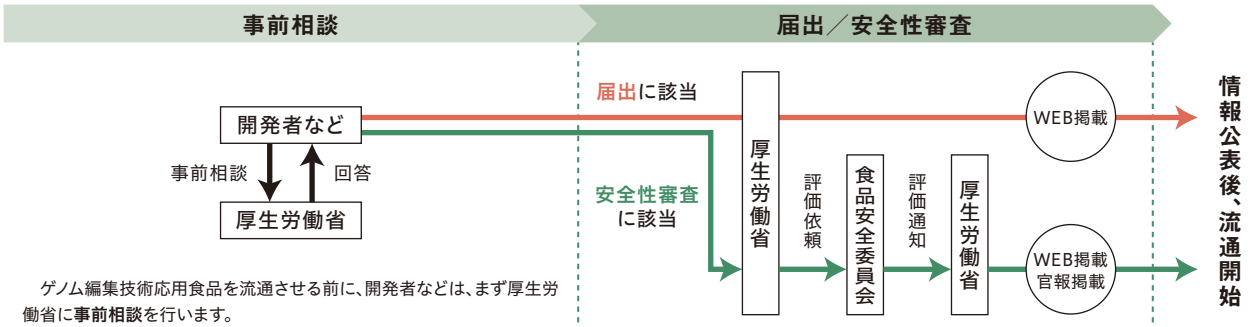
Point 5 育種過程とは？

農作物は、自然発生または人為的に誘発した突然変異を利用し、それらを掛け合わせることで品種改良が進められてきました。従来育種では、多くの意図しない変異が起こりますが、都合の悪い性質は育種過程（交配・選抜）で除かれ、優れた性質を持つ品種となります。「ゲノム編集技術応用食品」においても、交配・選抜を経ることで、ゲノム編集で生じる「オフターゲット」は取り除くことが可能です。

Point 6 ゲノム編集技術応用食品の基本的な取り扱い

薬事・食品衛生審議会食品衛生分科会新開発食品調査部会で取りまとめられた報告書を踏まえ、ゲノム編集技術応用食品等の届出等の食品衛生上の取り扱いに関する制度は、次のとおりです。

【ゲノム編集技術応用食品の届出制度等に関するフロー図】



ゲノム編集技術応用食品を流通させる前に、開発者などは、まず厚生労働省に**事前相談**を行います。

厚生労働省では、当該食品が届出または安全性審査のいずれかの対象に該当するか否かを専門家の意見を聴いた上で判断します。

最終的に自然界または従来の育種技術でも起こっている範囲内の遺伝子変化のものは届出、それを超える遺伝子変化のものは安全性審査の対象となります。

※必要に応じてその取り扱いなどについて、食品安全委員会へ諮問する場合があります。

届出と判断された場合、開発者などは厚生労働省に対し、届出を行います。厚生労働省は、開発者などから届出された情報の一部を厚生労働省のウェブサイトで公表します。

安全性審査と判断された場合（ほとんどが「組換えDNA技術応用食品」に該当）、食品安全委員会での食品健康影響評価を経ることになります。

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Ministry of Health , Labour and Welfare

【問い合わせ先】
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別添資料 7

新バイオ食品冊子　ver.5.11

厚生労働省医薬・生活衛生局食品基準審査課

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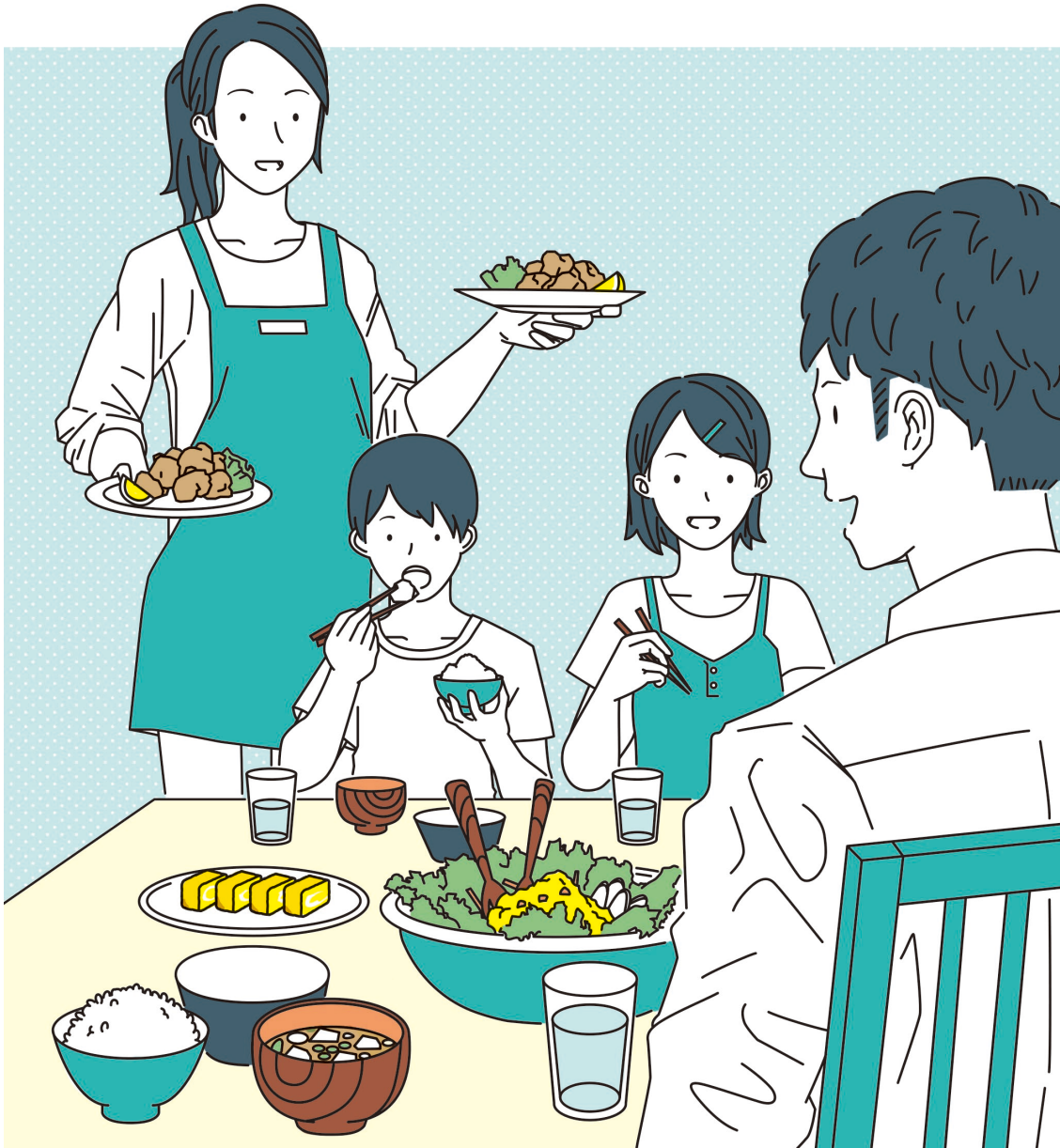
新しいバイオテクノロジーで作られた食品について



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1 | はじめに

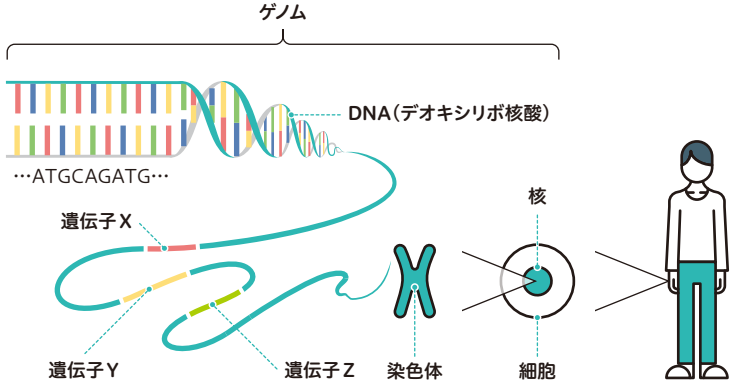
おいしいお米に甘いトマト。私たちの生活はさまざまな食品によって成り立っています。こうした食品の材料となる作物や家畜の多くは、人間の手によって育種（品種改良）されてきたものです。交配や突然変異といった従来の方法に加え、遺伝子組換え技術も使われています。また、最近ではゲノム編集技術が登場しました。しかし、これらの技術を用いた「遺伝子組換え食品」や「ゲノム編集技術応用食品（ゲノム編集食品）」に疑問を抱く人が少なくないようです。
このパンフレットは、こうした新しいバイオテクノロジーで作られた食品への疑問に答えるために作られました。

新しいバイオテクノロジー

しょう油やお酒を発酵によって造ることもバイオテクノロジーの一種です。そうした昔ながらのバイオテクノロジーと区別するため、このパンフレットでは「遺伝子組換え技術」と「ゲノム編集技術」を「新しいバイオテクノロジー」と呼びます。

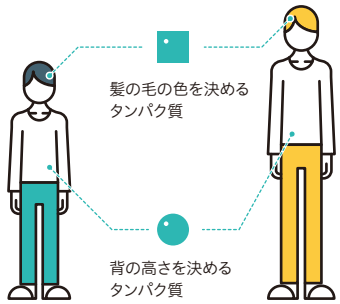
2 | DNAとゲノムと遺伝子

全ての生物の細胞の中にはDNA（デオキシリボ核酸）という物質があります。DNAはACGTで表現される4つの物質がたくさんつながってできています。このDNAの全ての情報をゲノムと呼びます。ゲノムの中でも生物の性質を決める部分を遺伝子と呼びます。
育種の過程では、遺伝子の変化によって生物の性質が変わります。



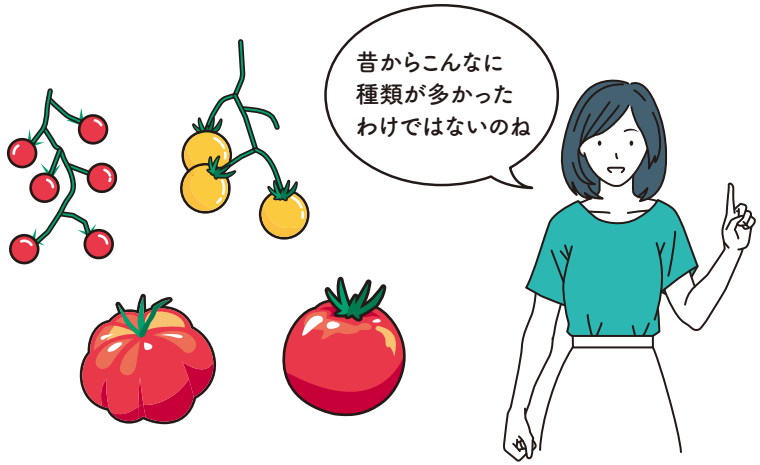
働くのはタンパク質！

生物の性質を決定するのは遺伝子ですが、実際に働くのはタンパク質です。遺伝子のDNA配列（ACGTの並び方）でタンパク質の性質が決まるので、DNAの配列が変わると、タンパク質の性質が変化したり、タンパク質が出来なくなります。その結果、生物の性質が変化します。



3 | 育種過程での遺伝子の変化

育種の過程では人間が人工的に作物や家畜の遺伝子を変化させ、新しい性質を持つものを作り出してきました。例えば、トマトの野生種は毒を持った小さい実しかつけませんが、長い年月をかけた育種の結果、おいしく、栽培しやすいさまざまなトマトが生まれました。



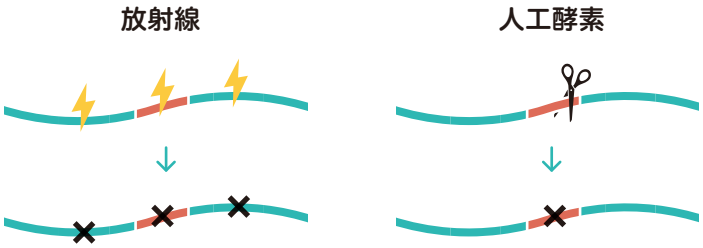
DNA の配列と突然変異

DNA の配列が変わることは突然変異と呼ばれ、育種において重要な役割を果たします。突然変異は自然界でも起きますが、放射線の照射などにより人工的に起こすこともあります。もっとも、どの配列が変わるかは偶然に頼るので、育種を行う上で都合の悪い突然変異が起こることもありますが、そうした突然変異はその後の交配、選抜により取り除くことができます。

4 | ゲノム編集技術

細胞の中のDNAは自然界の、あるいは人工的な放射線などにより切断されることがあります。生物は切断されたDNAを修復する仕組みを持っていますが、修復に失敗するとDNAの配列が変わって突然変異が起こります。ゲノム編集技術は、DNAを切断する人工酵素を使ってDNAに突然変異を起こす技術です。

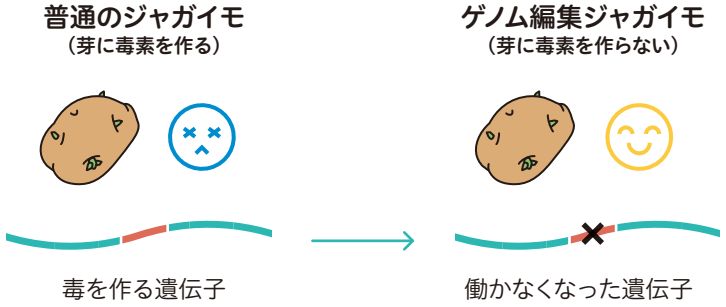
放射線によるDNAの切断はランダムに起こるので、計画的に突然変異を起こすことはできません。一方、ゲノム編集では、決まったDNAの配列を切断できる人工酵素を細胞の中で働かせるので、狙った遺伝子に突然変異を起こすことができます。



5 | ゲノム編集食品

毒素のないジャガイモ

ジャガイモの芽や緑色の部分には天然毒素が含まれています。ゲノム編集により、毒素を作る遺伝子を働かなくさせ、毒素を作らないジャガイモを効率的に作ることができます。



この他、日本国内では、下の例のようなゲノム編集作物や水産物の研究開発が行われています。

**血圧降下作用が期待される
GABAを多く含むトマト**

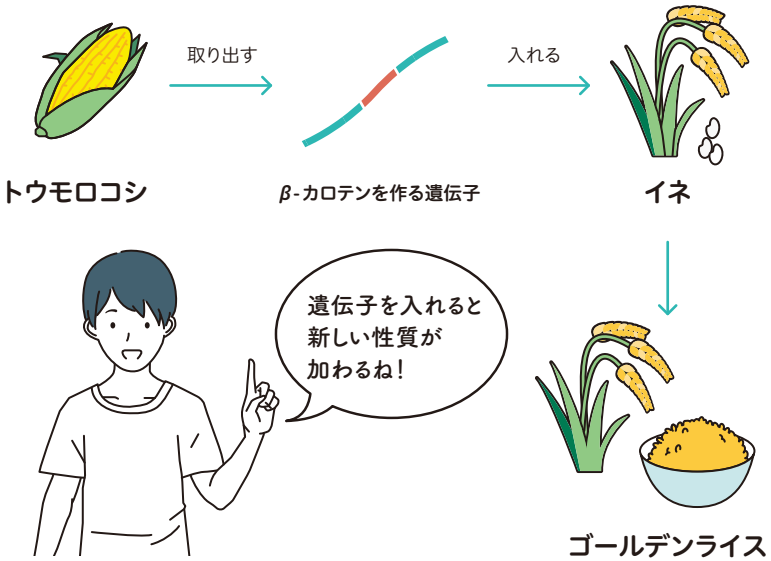
筋肉量を増やしたタイ

国外では

アメリカでは変色しにくいマッシュルームやオレイン酸を多く含む大豆などが開発されています。

6 遺伝子組換え技術

遺伝子組換え作物は、ほかの生物から取り出した遺伝子をゲノムに組み込むことで作られます。その結果、その作物は新しい性質を持つようになります。
特定の除草剤に強い作物や害虫に強い作物などがこの方法で開発され、海外では1996年から実用化されています。



ゴールデンライス

トウモロコシから取り出した遺伝子を組み込んで作られたイネ（ゴールデンライス）は、ビタミンAの素となるβ-カロテンをコメに多く含みます。ゴールデンライスは、発展途上国で問題となっているビタミンA欠乏症を解決するために開発されました。

7 遺伝子組換え食品

現時点において日本国内では、遺伝子組換え作物の商業栽培は行われていませんが、アメリカなどから除草剤に強い作物や害虫に強い作物が、加工用や飼料用として輸入されています。
輸入食品を監視する検疫所では、安全性が確認されていない遺伝子組換え食品が市場に出回らないように監視や指導が行われています。

国内で主に流通・消費されている遺伝子組換え作物

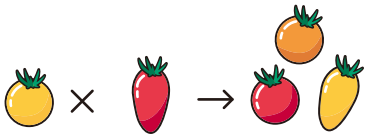
	主な性質	主な用途
大豆	●除草剤に強い	●大豆油 ●飼料
とうもろこし	●害虫に強い ●除草剤に強い	●コーン油　●飼料 ●異性化糖　●デンプン
なたね	●除草剤に強い	●なたね油
わた	●害虫に強い	●綿実油

8 さまざまな育種技術

人類は交配や突然変異による育種でさまざまな作物を生み出してきました。ゲノム編集や遺伝子組換えなどの新しいバイオテクノロジーも育種技術のひとつです。

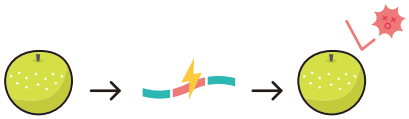
交配

異なる品種をかけ合わせることで、ゲノムが混じり合いさまざまな性質が得られる。



従来の突然変異

自然あるいは放射線照射などによる突然変異によって、異なる性質が得られる。



ゲノム編集による突然変異

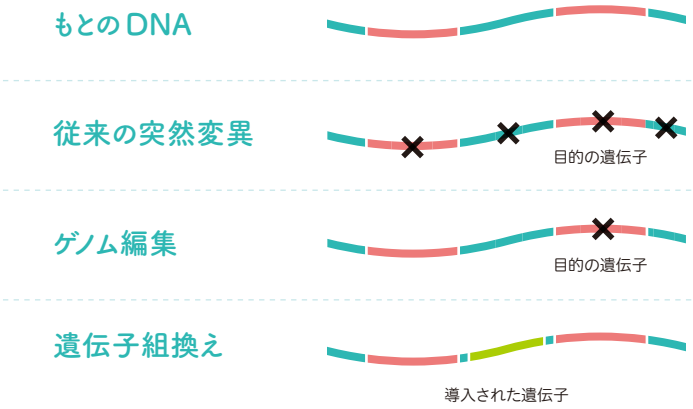
人工酵素を使って、狙ったDNA配列に突然変異を起こし、計画的に性質を変える。

遺伝子組換え

他の生物の遺伝子をゲノムに組み込み、計画的に性質を変える。

9 育種技術とDNA配列の変化

用いた技術によって、起こるDNAの配列の変化は異なります。放射線照射では目的の遺伝子以外にもランダムに突然変異が起こります。ゲノム編集による変異では目的の遺伝子を効率的に変化させることができます。遺伝子組換えでは他の生物の遺伝子のDNA配列が組み込まれます。



Q ゲノム編集の際に予期せぬ変異（オフターゲット変異）は起こりませんか？

これまでの育種ではランダムに突然変異が起こるので、多くの予期せぬ変異が起こっています。しかし、都合の悪い性質は交配と選抜によって取り除かれてきました。ゲノム編集の場合も同様に、都合の悪い形質を持つ変異は交配と選抜を経て取り除くことができるので、健康への悪影響が問題になる可能性は非常に低いと考えられています。

10 安全性確保の手続き

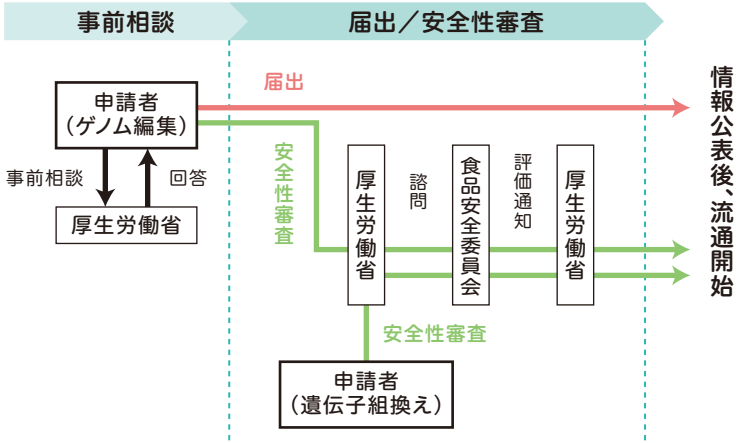
食品が市場に出る前には、安全性を確保するための仕組みが備えられています。

従来の放射線照射などを用いて突然変異を誘導して育成された品種の場合、特別な安全性の確認はしていません。

ゲノム編集食品については基本的に、厚生労働省への届出を経て、安全性に関する情報の公表の手続きが行われます。ただし、遺伝子を組み込むなどした場合は遺伝子組換え食品と同様の手続きが求められます。

遺伝子組換え食品については、安全性審査を経て安全性に問題がないと判断された食品のみが流通します。この場合、厚生労働省は専門家で構成される食品安全委員会に安全性の評価を依頼し、食品安全委員会は安全性の評価（食品健康影響評価）を行います。

評価の結果、安全性に問題がないと判断した食品を厚生労働省が公表し、流通します。



11 安全性のチェックポイント

ゲノム編集食品を流通する際の届出については、下記のようなポイントをチェックします。

- 新たなアレルギーの原因（アレルゲン）が作られていないか、有害物質などが作られていないか。
- （毒素をなくす、ある成分を増やすなどの改変をした場合）食品中の栄養素などがどう変化したか

遺伝子組換え食品を流通する際の安全性審査では下記のようなポイントをチェックしています。

- 組み込む前の作物（既存の食品）、組み込む遺伝子、ベクター（遺伝子の運び屋）などはよく解明されたものか、ヒトが食べた経験はあるか。
- 組み込まれた遺伝子はどのように働くか。
- 組み込んだ遺伝子からできるタンパク質はヒトに有害でないか、アレルギーを起こさないか。
- 組み込まれた遺伝子が間接的に作用し、有害物質などを作る可能性はないか。
- 食品中の栄養素などが大きくかわらないか。

これらについて科学的なデータをもとに評価し、総合的に安全性を判断しています。

また、新たな科学的知見が生じた場合は再評価を行います。

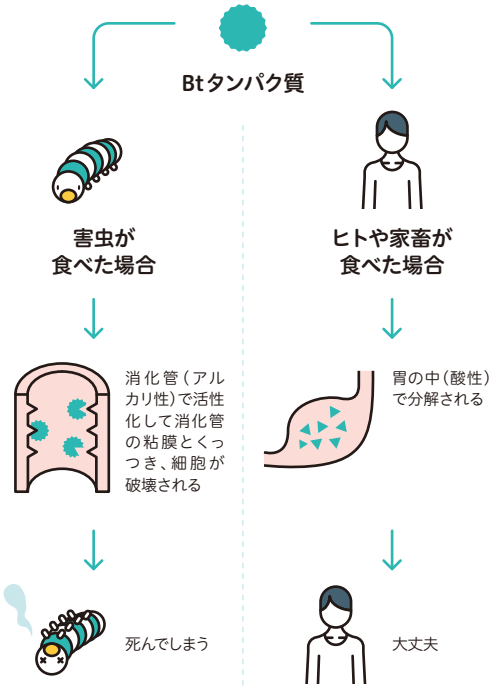
12 | Q&A

Q1 遺伝子組換えに相当するゲノム編集とは何ですか？

ゲノム編集では人工酵素で、決まったDNA配列を切断し、そこに遺伝子を組み込むことも可能です。この方法によって従来の遺伝子組換えより正確に遺伝子を組み込むことができます。この場合は、遺伝子組換えとして取り扱われます。

Q2 害虫に強い作物を害虫が食べると死ぬそうですが、人が食べても大丈夫なのですか？

害虫に強い作物には殺虫性タンパク質（Btタンパク質）が含まれています。このタンパク質はヒトや家畜には無害なので食べても問題ありません。今まで害虫に強い作物が食品や飼料としてたくさん消費されてきましたが、健康被害は確認されていません。



Btタンパク質：バチルス・チューリンゲンシスと呼ばれる細菌が作るタンパク質で、殺虫性があります。生物農薬として有機栽培への使用が認められています。

Q3 遺伝子組換え食品を食べ続けても健康被害は起こりませんか？

さまざまなデータに基づき、組み込んだ遺伝子によって作られるタンパク質の安全性や遺伝子が間接的に作用し、有害物質などを作る可能性がないことが確認されていますので、食べ続けても問題はありません。

Q4 ゲノム編集食品には安全性評価が義務付けられず、届出も義務ではないのはなぜですか？

ゲノム編集でDNAに起こる変化は自然界や従来の品種改良でも起こり得る変化です。従って、安全性もそれらと同程度と考えられ、安全性審査は必要ないと判断されましたが、新たな技術であることや消費者への配慮も必要なため、届出と一定の情報の公表を求めることとしました。

Q5 日本におけるゲノム編集食品や遺伝子組換え食品の安全について教えてください。

厚生労働省のホームページをご覧ください。

https://www.mhlw.go.jp/stf/seisakunitsuite/bunya/kenkou_iryou/shokuhin/bio/index_00013.html

Q6 遺伝子組換え食品やゲノム編集食品の表示の制度について教えて下さい。

消費者庁のホームページをご覧ください。

●遺伝子組換え食品についてはこちら

https://www.caa.go.jp/policies/policy/consumer_safety/food_safety/food_safety_portal/genetically_modified_food/

●ゲノム編集食品についてはこちら

https://www.caa.go.jp/policies/policy/food_labeling/quality/genome/

厚生労働科学研究費補助金（食品の安全確保推進研究事業）
「新たなバイオテクノロジーを用いて得られた食品の安全性確保と
リスクコミュニケーションのための研究」
分担研究報告書

人工ヌクレアーゼの特異性を調べる *in vitro* アッセイツールの開発

研究分担者　中村 公亮　　（国立医薬品食品衛生研究所）

研究要旨:
本研究では、ゲノム編集食品の安全性評価法の一つとしてオフターゲット部位を網羅的に推定する SITE-Seq 法の有用性について、イネを用いて検証を行った。本研究結果より、オフターゲット予測は、各種オンラインオフターゲット予測ツールを使った *in silico* 解析だけでは不十分であることが確認された。相同性データベースを用いた解析は高確率で生じるオフターゲットを予測することができるが、予測された候補配列は SITE-Seq の予測を完全にカバーすることができず、実際に起こったオフターゲットの切断を見落とす可能性が示唆された。したがって、ガイド RNA を設計する際には、まず *in silico* 解析により最もユニークな配列を選抜した後、そのガイド RNA の潜在的なオフターゲットを SITE-Seq 法で生化学的に検証し、ゲノム編集作物で当該箇所の変異を確認する必要がある。この一連の解析について、高い再現性を有する試験法を確立することで、ゲノム編集作物の開発や安全性評価に役立つことが期待され、また、Cas9 の特異性に関する情報集積とその制御に貢献できると考える。

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協力研究者　木俣 真弥　（国立医薬品食品衛生研究所）
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A. 研究目的

2019年10月よりゲノム編集食品の届出制度が開始され、国内での流通・販売が可能となった。ゲノム編集技術を応用することで、従来の育種方法より短期間で有用品種を作出することができる一方で、意図しない切断（オフターゲット）による影響を予測することは難しく、食品としての安全性を懸念する声がある。CRISPR/Cas9 システム利用時の簡便なオフターゲット予測としては、各種オンラインツールが汎用されており、予測された箇所についてサンガーシーケンスをして変異を確認するといった事例も見られるが、オフターゲット箇所を網羅できているかは不明である。そこで本研究では、NGS を使ってオフターゲット箇所を網羅的に検知する手法として、Selective enrichment and identification of tagged genomic DNA ends by sequencing （SITE-Seq）法 [1] について、ゲノム編集イネを検査対象とした場合の有効性について検討を行った。

B. 研究方法

１．サンプル

実験に使用したイネ（*Oryza sativa* L. cv. Nipponbare)は農研機構農業生物資源ジーンバンクより分与して頂いた。その種子を発芽、培養し、1 週齢の幼植物体から DNA を抽出した。

幼植物体の培養法

もみ殻を除き、70%エタノールで 1 分間洗浄後、次亜塩素酸（有効塩素濃度 6%）溶液で 30 分間振とうして種子表面を滅菌後、クリーンベンチ内にて滅菌済み超純水でよくすすぎ、滅菌済み丸シャーレ(中に滅菌済み超純水で湿らせた滅菌済みのキムワイプを敷く）に静置した。3 日ほどして発芽した種子を MS 培地 [2] に移植して 1 週間無菌培養を行った。発芽と培養条件は 28℃で 16 時間明期、8 時間暗期とした。

２．DNA の抽出と精製

DNA 抽出には幼植物体のシュート部のみを使

用した。ハサミで根本付近を切断後すぐさま液体窒素にて凍結させ、-80℃にて保存した。

CTAB 法

ZAP 処理済みの乳鉢・乳棒を用いてサンプルを破砕し、1.5% CTAB 溶液を 2.5 mL/サンプル 1 g 加えて 56℃で 20 分間インキュベートした。その後、CIA (Chloroform/Isoamyl alcohol, 24:1) を等量加えて室温で 20 分間転倒混和し、スイングローターを用いて 3,000 rpm、15 分間、室温で遠心分離した。遠心分離後のサンプルを優しく取り出し、上の水層を別のチューブへ移し、0.7 倍量のイソプロパノールを加えて転倒混和後、アングルローターにて 4℃、10,000 xg、10 分間遠心した。チューブ内壁に見えるペレットを吸わないよう上清を捨て、70%エタノールでペレットを洗浄後、超純水 100 µL でペレットを溶解し、DNase free RNase A (10 mg/mL) を 10 µL 添加、37℃、30 分間インキュベートして RNA を分解した。そこへ 44 µL SPRISelect (Beckman Coulter, Inc. USA, cat. No. B23317) を添加し、優しく混和後、マグネットスタンドを用いてビーズを集めて上清のみ破棄し、85%エタノールを 180 µL でビーズを 3 回洗浄し、風乾後に超純水を 30 µL 加えて 5 分間静置して DNA を溶解させ、マグネットスタンドを使って上清のみ回収した。そこへ RNase secure (Thermo Fisher Scientific, Inc., Waltham, MA, USA, cat. NO. AM7005) を 1.2 µL 加えて 60℃、10 分間インキュベートして RNase A を失活させた後、室温になってから Nanodrop ND-1000 (Thermo Fischer Scientific, Inc., Waltham, MA, USA) を用いて濃度測定を行った。また、アガロースゲル電気泳動を行い、品質を評価した。DNA の保存は 4℃で行い、調製後 24 時間以内に使用した。

３．ガイド RNA の選定

ガイド RNA については、既に報告されている論文の中から選定した。Acetolactate synthase (ALS, EC2.2.1.6) については、W548L および S627I 変異によりスルホニルウレア系除草剤耐性を獲得することが報告されており[3]、これら 2 か所を CRISPR/Cas9 システムにより改変した文献[4]に記載されているガイド RNA を用いた。また、2017 年に発表されたジャポニカイネ (*Oryza sativa* L. subsp. japonica) を対象に CRISPR/Cas9 で２本鎖切断を行っている論文の中から 4 報[5-8]を選定し、それ等に用いられた 5 つのガイド RNA を試験に供した。ガイド RNA の配列は表 1 に記載した。

４．ガイド RNA の合成と精製

ガイド RNA の合成と精製には、Agilent SureGuide gRNA Synthesis Kit を用いた。付属プロトコルに従い、本検討では、長鎖型の (extended backbone) シングルガイド RNA を合成した。まず、ガイド RNA 合成の鋳型となる２本鎖 DNA を作成するため、次のプライマーを合成した。

Primer-Forward: 5’-CG ATG TAA TAC GAC TCA CTA TAG GXX XXX GTT TTA GAG CTA TGC TGA AA-3’;
Primer-Reverse: 5’-AAG CAC CGA CTC GGT GCC ACT TTT TCA AGT TGA TAA CGG ACT AGC CTT ATT TTA ACT TGC TAT GCT TTT CAG CAT AGC TCT AAA ACY-3’
(「X」は標的とする 20 塩基;「X」と「Y」は相補塩基とする;下線はオーバーラップする領域)。

表 1 に記載したプライマーを使用し、以下の反応液 (50 µL/反応) を調製した:5x Herculase II reaction buffer (10 µL)、2.5 mM each dNTPs (4 µL)、10 µM primer-forward (5 µL)、10 µM primer-reverse (5 µL)、Herculase II Fusion DNA polymerase (1 µL)、DW (28 µL)。この反応液を 95℃で 2 分間、次いで 60℃で 1 分間、最後に 72℃で 3 分間インキュベートした。合成された鋳型 DNA (130 bp) は、Wizard SV Gel and PCR Clean-up System (Promega) を用いて精製し、吸光度測定結果をもとに、1 µM に再調製した。

続いて調製した 2 本鎖 DNA を鋳型にガイド RNA を合成するために、次の反応液 (20 µL/反応) を調製した:DPEC water (7.5 µL)、5x Transcription buffer (5 µL)、rATP (1 µL)、rCTP (1 µL)、rGTP (1 µL)、rUTP (1 µL)、0.75 M DTT (1 µL)、Yeast Pyrophosphatase (0.5 µL)、RNase Block (1 µL)、T7 RNA polymerase (1 µL)。 この反応液に 1 µM 鋳型 DNA を 5 µL 添加し、混和後、37℃で 4～16 時間インキュベートした。その後 RNase-free DNase を 1 µL 添加し、37℃で 20 分間インキュベートした。十分量のガイド RNA を得るため (50 µM 以上)、一種類のガイド RNA あたり、4 反応分 (100 µL) 調製し、キット付属のプロトコルに従い精製した。ただし、次の工程を改良した:2 反応液分のガイド RNA を 1 つのカラムに吸着させ、25 µL

の nuclease-free water で溶出させた。ガイド RNA 量は、Nanodrop ND-1000 で測定し、濃度はその分子量（108 bp）をもとに算出した。ガイド RNA は、使用するまで-80℃に保存した。

５．ゲノム DNA の Cas9 切断

ゲノム DNA の２本鎖切断は、様々な濃度のガイド RNA-Cas9 複合体（Ribonucleoprotein, RNP）で処理した。オフターゲットの出現場所と頻度はガイド RNA の配列だけでなく、ゲノム編集の際に使用される Cas9 の添加濃度とも関連することが報告されている [9]ことから、本検討では、Cas9 の終濃度を 1、64、256、1,024 nM に設定し、ガイド RNA 濃度はその 15 倍添加した。酵素反応は、1x CCB（Cas9 Cleavage buffer; 20 mM HEPES、150 mM KCl、10 mM $MgCl_2$、5% glycerol、pH 7.4）で行った。まず、RNP 複合体を形成させるため、15 µL の Cas9（3.3x CCB に溶解）と 15 µL のガイド RNA（DW に溶解）を 1 : 15 の濃度比となるように混合し（例：213 nM Cas9 : 3.2 µM ガイド RNA）、37℃で 10 分間インキュベートした。ガイド RNA は、95℃で 2 分間加熱後、室温で 5 分間静置してから使用した。ここに、20 µL のゲノム DNA（150 ng/µL）を添加し、37℃で 16 時間インキュベートすることで、十分にゲノム DNA を切断した。その後、RNA 分解溶液 6.3 µL（10 mg/mL RNase A、4.4 µL; 5x CCB、1.4 µL; DW、0.5 µL）を添加し、37℃で 20 分間インキュベートした。さらに、proteinase K（20 mg/mL）を 0.5 µL 添加し、55℃で 20 分間インキュベートして Cas9 を失活させた。次の工程まで、氷中で一時的に保管した。

６．シーケンスライブラリーの調製

SITE-Seq 法でオフターゲット切断部位を特定するため、Illumina MiSeq システムに対応した DNA ライブラリーを以下の通り作成した。

上記で Cas9 処理したゲノム DNA（50 µL 全量）は、エタノール沈殿法により精製し、25 µL の DW で溶解させた。Cas9 切断面末端にアデニンを付加するため、DNA 溶液（25 µL）に 10x NEB2（5 µL）、10 mM dATP（5 µL）、Klenow Exo-（3 µL）、DW（12 µL）を添加し、37℃で 30 分間反応させた。この A 突出末端へのビオチンアダプター（Adapter 1）の結合は、100 µM Adapter 1 For（1 µL）、100 µM Adapter 1 Rev（1 µL）、DW（8 µL）、2x annealing buffer（20 mM Tris、100 mM NaCl、2 mM EDTA、pH 7.5）（10 µL）を混合し 95℃で 5 分間インキュベートした後、室温で 45 分間放置し、この２本鎖化した Adapter 1（2 µL）と dA 付加した DNA（38 µL）、NEB 10x T4 DNA ligase buffer（5 µL）、NEB Quick Ligase（5 µL）を混合した溶液を 20℃で 30 分間、次いで 16℃で 16 時間インキュベートすることで完了した。

ビオチンアダプター付き DNA は、マグネットビーズ型のサイズ別 DNA 回収試薬 SPRISelect を用いて、付属のプロトコルに従い精製した。DNA 溶液 50 µL に対して、0.5 倍量の SPRISelect 試薬（25 µL）を加え、よく混合した。室温で 5 分間放置した後、マグネットスタンドを用いてビーズ（DNA）と上清を分離し、上清を破棄した。ビーズを 85%エタノール 175 µL で 30 秒間洗浄した。この洗浄は二回繰り返した。完全にエタノールを取り除き、ビーズが乾燥する前に、50 µL の DW を加え、十分に懸濁した。室温で 10 分間静置した後、DW に溶出した DNA 45 µL を回収した。

上記 DNA（40 µL）と NEB 10x dsFragmentase buffer v2（5 µL）、NEB dsFragmentase Enzyme（1.5 µL）、DW（3.5 µL）を混合し、37℃で 1 時間反応させ、DNA の断片化を行った（時間厳守；長時間のインキュベートは DNA を過度に分解させる）。12.5 µL の 0.5 M EDTA を添加し触媒を停止させ、37.5 µL の DW を添加した。その直後、0.9x SPRISelect 処理により、200～1000 bp の DNA 断片を 45 µL 回収した。切断末端は、断片化 DNA（27.7 µL）と NEB 10x End-repair reaction buffer（3.3 µL）、NEB End-repair enzyme mix（1.5 µL）、DW（0.5 µL）を混合した反応液を 20℃、30 分間に次ぐ 65℃、30 分間のインキュベートで修復した。修復面へのアダプター（Adapter 2）の結合は、100 µM Adapter 2 N7 For（1 µL）と 100 µM Adapter 2 N6 For（1 µL）、100 µM Adapter 2 N5 For（1 µL）、100 µM Adapter 2 Rev（3 µL）、2x annealing buffer（6 µL）を混合し 95℃で 5 分間インキュベートした後、室温で 45 分間放置し、この２本鎖化した Adapter 2（1.25 µL）と末端修復 DNA（32.5 µL）、NEB Blunt/TA Ligase Master Mix（7.5 µL）、NEB Ligase enhancer（0.5 µL）を混合した溶液を 20℃で 30 分間、次いで 16℃で 16 時間反応させることで完了した。

Cas9 で切断された DNA の選択的な回収は、ビオチン-ストレプトアビジン相互作用を利用した。

まず、1 反応あたり、25 µL の Dynabeads（Invitrogen、ベリタス社）を 125 µL の 1x BW buffer（5 mM Tris、1 M NaCl、0.5 mM EDTA、pH 7.5）で 5 分間、回転させながら洗浄した。これを２回繰り返した後、41 µL の 2x BW buffer でビーズを再懸濁した。ここに等量の DNA 試料（41 µL）を添加し、室温で 30 分間、溶液を混合した。マグネットで上清を破棄し、200 µL の 1x BW buffer で 30 秒間洗浄した。これを２回繰り返し、さらに、同様の洗浄を 10 mM Tris-HCl、pH 8.5 を用いて行った。DNA が吸着したビーズは、20 µL の 10 mM Tris-HCl で再懸濁した。

DNA ライブラリーへのインデックス付加は、リカバリーPCR 後に行った。上記で得た DNA-ビーズ混合液（20 µL）と 10 µM Recovery PCR For primer（2.5 µL）、10 µM Recovery PCR Rev primer（2.5 µL）、NEB 2x Phusion Master Mix（25 µL）を混合し、以下の温度サイクルで DNA を増幅させた：[98℃, 30 秒] x 1、[98℃, 10 秒；61℃, 30 秒；72℃, 2 分] x 12、[72℃, 2 分] x 1、[4℃, ∞] x 1。ビーズと上清とをマグネットを用いて完全に分離し、上清 30 µL を回収した。その上清 3 µL と DW 148.5 µL を混合し、次のインデックス PCR の鋳型として用いた。鋳型 DNA（12 µL）と NEB 2x Phusion Master Mix（20 µL）、5 µM Index primer For（4 µL）、5 µM Index primer Rev（4 µL）を混合し、以下の温度サイクルで DNA を増幅させた：[98℃, 30 秒] x 1、[98℃, 10 秒；60℃, 30 秒；72℃, 2 分] x 12、[72℃, 2 分] x 1、[4℃, ∞] x 1。

目的サイズの DNA 断片（200～800 bp）は、複数回の SPRISelect 処理で精製した。上記と同様に 0.7x 処理で DNA を精製した後、別法により 1,000 bp 以上の断片を排除し、さらに 0.7x 処理（１回目と同様）により僅かに残存したプライマーダイマー（～200 bp）を完全に除去した。以下に 1,000 bp 以上の DNA 断片を排除する SPRISelect 別法を記載する。ここでは、目的外の DNA サイズ断片をビーズに吸着させ、必要な DNA サイズ断片を含む上清を回収する点に注意する。まず、DNA 溶液に 0.5 倍量の SPRISelect 試薬を混合し、室温で 5 分間静置した。マグネットを用いて上清を全量回収し、この上清に 1.3 倍量の SPRISelect 試薬を追加した（以下、通常法に準ずる）。室温で 5 分間静置した後、マグネットを用いて上清を破棄し、1 mL の 85%エタノールで２回洗浄した。廃液を完全に取り除き、1 倍量の DW で DNA を溶出した。

調製した DNA ライブラリーの品質は、Agilent Bioanalyzer High Sensitivity DNA chip を用いて評価した。プライマーダイマーが存在しないこと、また、>1,000 bp の DNA 断片が多量に含まないことを確認した。DNA 濃度を見積もるため、ライブラリーの平均サイズ値を記録した。本方法で作製されるライブラリーの平均 DNA サイズは、約 650 bp である。２本鎖 DNA の量は Qubit HS で測定し、この数値(ng/µL)と平均 DNA 分子量値(X-bp x 660 g/mol）から DNA ライブラリーの濃度を算出した。異なるインデックス配列が付加された各試料の DNA 濃度を同値に再調整した後、これらを等量混合し分析試料とした。

７．MiSeq を用いたシーケンス解析

DNA ライブラリーの変性は、サンプルと等量の 0.2 N NaOH を添加し、室温で 5 分間静置して完了した。変性後、DNA は直ちに氷中に移行させた。MiSeq Reagent Kit v3（150 サイクル）に付属の緩衝液 HT1 を用いて、ライブラリーを 10 pM に希釈した。この時、NaOH の終濃度は 0.001 N 以下とした。10 pM ライブラリー 600 µL を試薬カートリッジ（同キット）の 17 ポートにロードし、フローセル（同キット）、PR2 試薬（同キット）とともに MiSeq に取り付け、解析を開始した。フローセルは、取り付け前に超純水で塩を洗い流し、エタノールで汚れと曇りをふき取った。

解析のワークフローは、Illumina Experimental Manager（IEM, v1.16.1）で作成した。本ソフトは Illumina 社のサイトから無償でダウンロード可能である。
[http://jp.support.illumina.com/sequencise/sequencing_software/experiment_manager/ddownload.html?langsel=/jp/]
IEM を用いたサンプルシートの作成方法を以下に示す。まず、Illumina Experimental Manager を起動し、「Create Sample Sheet」を選択する。「MiSeq」をクリックし、「Next」ボタンを押す。次ページで、Select Category は「Other」、Select Application は「FASTQ Only」を選択し、「Next」を押す。ワークフローのパラメーター以下の画面の通りに設定した（UD index が選択される任意の work flow prep を選択する）。画面右の specific settings のチェックはすべて外した。

Sample selection の記入例を以下に示す（注意点：sample ID は異なる名前にすること）。

「Finish」ボタンを押すと、エクセル上で内容を確認する。このファイル名はカートリッジに記載されている Cartridge Barcode として保存する。MiSeq 装置本体に解析データを保存する場合、このサンプルシートは MiSeq 装置の特定の場所に保存した（[Computer]<[Data]<[Illumina]<[MiSeq Control Software]<[Sample sheets]）。

８．DSB 箇所のリファレンスゲノムへのマッピングとクリフ判定

様々な DNA 断片種が収容されたライブラリーを Cas9 切断面側からシーケンスし、得られたリードは、オン・オフターゲット部位を特定する情報とした。MiSeq より得られたシーケンスファイル（Fastq）は、公開されているイネリファレンスゲノムにマッピングし、Cas9 で切断された位置候補の特定、切断位置のゲノム上の情報、切断効率の情報取得を行った。マッピングソフトウェア bowtie2（バージョン 2.3.4.2）を用い、リファレンスゲノムは IRGSP-1.0 を用いた。[https://rapdb.dna.affrc.go.jp/download/irgsp1.html] 切断位置候補は、原著論文内の切断位置検出スクリプト（Python）にて解析した。切断位置が遺伝子のコーディング領域内に位置するかは、公開されているアノテーションファイルと bedtools（バージョン 2.27.0）にて参照した。また、個々の切断された位置情報は、IGV（Integrative Genomics Viewer バージョン 2.4）を使用しマッピング状況の確認は目視で行なった。

９．Galaxy を使用した DSB 判定ツールの開発

上記「8. DSB 箇所のリファレンスゲノムへのマッピングとクリフ判定」の中で使用した各種ソフトウェアは、BioContainers（Docker）を用いて、データ解析プラットフォーム Galaxy（https://galaxyproject.org/）に実装させた SITE-Seq 解析用ワークフローを作成しツールを開発した。

１０．リアルタイム PCR を用いた切断確認

SITE-Seq 法が示すオフターゲット切断の妥当性は、リアルタイム PCR を用いて検討した。ガイド RNA および Cas9 で切断されたゲノム DNA と、未処理のゲノム DNA（ネガティブコントロール;NC）を鋳型として、後述のプライマーを用いてリアルタイム PCR を行い、Cq_{sample}-Cq_{NC} から ΔCq を算出した。ΔCq=n の時、鋳型 DNA の量は 2n 倍であることから、ネガティブコントロールの DNA 量を 100%とし、何%の鋳型 DNA が減少したかを、切断効率（%、推定値）とした（例：ΔCq=1 の場合、ネガティブコントロールの鋳型 DNA の量は、切断処理した鋳型 DNA の 2 倍であるため、切断効率は 50%である）。Cas9 による切断処理は、「5. ゲノム DNA の Cas9 切断」に準じたが、proteinase K で Cas9 を失活させた後、95℃で 10 分間加熱することで proteinase K を失活させる工程を加えた。また比較として、ガイド RNA および Cas9 無添加のネガティブコントロールを切断サンプル同様に処理し、リアルタイム PCR に供した。

<u>プライマーの設計</u>

SITE-Seq 法により予測されたカットサイトにまたがるようにプライマーを設計した。その際、プライマーの 3’エンドのミスアニーリングによる増幅を避けるため、カットサイトが Fwd もしくは Rev プライマー配列の 3’エンドから 3～5nt になるように設計した。

<u>反応溶液の調製</u>

ガイド RNA および Cas9 で切断処理した DNA 溶液は、1 ng/μL となるように超純水で希釈を行った。PCR 反応液は一反応当たり 25 μL として、12.5 μL の 2x FastStart Universal SYBR Green Master (ROX)、各 0.4 μL の 50 μM プライマー対、6.7 μL の超純水と 5 μL の鋳型 DNA を含めた。

<u>使用機器及び分析の設定</u>

分析には LightCycler 480 (Roche 社) を用い、分析モードは、SYBR Green I / HRM Dye を使用した。PCR の条件は以下の通りに設定した。ステップ 1 (pre-incubate) : [95℃, 10 分間] ×1 サイクル、ステップ 2 (amplification) : [95℃, 15 秒間]、[60℃, 1 分間] ×45 サイクル、ステップ 3 (cooling) : [40℃, 30 秒間] ×1 サイクルを分析プロトコルとし、ステップ 2 の 60℃インキュベート時に蛍光シグナルを回収した。核酸増幅曲線に由来する Cq 値（quantification cycle）は

2nd derivative max 法で算出した。試験は全て 2 ウェル併行で行い、Cq 値の比較には 2 ウェルの平均値を用いた。

C. 研究結果

１．イネからの DNA 抽出

ゲノム編集で生じた２本鎖切断位置を解析するに当たり、ゲノム DNA をいかに物理的な剪断なしにインタクトな状態で精製するかが、SITE-Seq 法の特異性を上げるため最も重要となる。本研究では、CTAB 法による幼植物体からのゲノム DNA の精製を行い、得られたゲノム DNA の品質を 1%（w/v）アガロースゲル電気泳動で評価した。0.5～1.0 µg DNA をアガロースゲルのウェルにロードし、観察したところ、幼植物体から抽出された高分子量 DNA に相当するバンドの他に、<500 bp のバンドが確認された（図 1）。そのため CTAB 法による DNA 抽出後に SPRISelect によるビーズ精製を行うことで 1 kbp 以下の DNA を除き、これを鋳型 DNA として SITE-Seq 解析に供試した。

２．ALS target1 解析結果

CRISPR/Cas9 システムを用いてゲノム編集を行う場合、従来の遺伝子組換えとは異なり、indel によるフレームシフトを伴うノックアウト、もしくは塩基置換は規制の対象外となる可能性がある。ALS のように塩基置換により有用形質を獲得するケースは、食品への利用が十分考えられため、本研究では、まず ALS をターゲットとして解析を行った。

<u>エクソン内オフターゲット数の傾向</u>

ALS の W548 をターゲットとしたガイド RNA（ALS target1）を用いた結果、Cas9 濃度 64 nM および 256 nM ではオンターゲットの切断が確認された。一方で、1 nM および 1,024 nM では切断が確認されず（図 2, 5）、この傾向は昨年度ヒト・ブタ DNA を用いた場合の傾向と一致した。

各濃度でのオフターゲット数としては、1 nM では 62 か所（エクソン内では 10 か所）、64 nM では 347 か所（エクソン内では 50 か所）、256 nM では 535 か所（エクソン内では 53 か所）、1,024 nM では 836 か所（エクソン内では 106 か所）となっており、切断処理する Cas9 濃度が上がるにつれて、オフターゲット箇所も増加する傾向にあった（図 3）。また、エクソン内のオフターゲット箇所の内、異なる濃度で共通して検出された箇所についても調査を行った。その結果、64 nM と 256 nM で共通して検出されたオフターゲット箇所が 4 か所、64 nM と 1,024 nM で共通して検出されたオフターゲット箇所が 1 か所、256 nM と 1,024 nM で共通して検出されたオフターゲット箇所が 7 か所で、これらの内、1 nM、64 nM、256 nM の 3 濃度で共通して検出されたオフターゲット箇所が 1 か所、64 nM、256 nM、1,024 nM で共通して検出されたオフターゲット箇所が 1 か所であった。4 濃度で共通して検出されたオフターゲット箇所は 0 か所であった（図 4）。異なる Cas9 切断処理濃度で共通して検出された 8 か所について、カットサイト付近の配列とガイド RNA 配列を比較すると（図 5）、オフターゲット②、③、⑥、⑨は近接した PAM 配列が存在し、ミスマッチ数が順番に 10、6、4、7 塩基であった。さらにオフターゲット②に関しては 1 塩基の DNA バルジ（2 本鎖の核酸において、相補的な塩基が存在しない場合に生じる二次構造で、片方の鎖が膨らむ構造）が 1 か所、オフターゲット③に関しては 1 塩基の RNA バルジが 1 か所、オフターゲット⑨に関しては 1 塩基の DNA バルジが 1 か所生じていた。オフターゲット①、④、⑤、⑦、⑧に関しては近接した PAM 配列が存在しなかった。

<u>リアルタイム PCR を用いた妥当性確認</u>

ゲノム DNA を ALS target1 ガイド RNA と Cas9（256 nM）で 37℃16 時間切断処理後、カットサイトにまたがるように設計したプライマーを用いてリアルタイム PCR を行い、SITE-Seq 法の妥当性について検証を行った。RNP 無処理のネガティブコントロールと比較して、Cq_{Sample}-Cq_{NC} から ΔCq 値を算出した。オフターゲットについてはリード数の多い 2 か所（オフターゲット⑥、⑨）でリアルタイム PCR を実施した。その結果、オンターゲットにおいては ΔCq=1.88（切断効率 74.09％）、オフターゲット⑥においては ΔCq=0.20（切断効率 13.04%）、オフターゲット⑨においては、ΔCq=0.08（切断効率 5.66%）であった（図 6）。これらの結果から、ALS target1 に関しては特異性の高いガイド RNA であることが示唆された。

３．ALS target2 解析結果

<u>エクソン内オフターゲット数の傾向</u>

ALSのS627をターゲットとしたガイドRNA(ALS target2) を用いた結果、Cas9 濃度 64 nM、256 nM および 1,024 nM でオンターゲットの切断が確認され、1 nM では切断が確認されなかった（図 7, 10)。

各濃度でのカットサイト数としては、1 nM では 86 か所（エクソン内では 6 か所)、64 nM では 87 か所（エクソン内では 10 か所)、256 nM では 537 か所（エクソン内では 52 か所)、1,024 nM では 1,065 か所（エクソン内では 156 か所）となっており、切断処理する Cas9 濃度が上がるにつれて、カットサイトも増加する傾向だった（図 8)。また、エクソン内のカットサイトの内、異なる濃度で共通して検出された箇所についても調査を行った。その結果、64 nM と 256 nM で共通して検出されたオフターゲット箇所が 3 か所、64 nM と 1,024 nM で共通して検出されたカットサイトが 2 か所、256 nM と 1,024 nM で共通して検出されたカットサイトが 6 か所で、これらの内、64 nM、256 nM、1,024 nM で共通して検出されたカットサイトが 2 か所であった。4 濃度で共通して検出されたカットサイトは 0 か所であった（図 9)。異なる Cas9 切断処理濃度で共通して検出された 7 か所（オンターゲット含む）について、カットサイト付近の配列とガイド RNA 配列を比較すると（図 10)、オフターゲット①、③、⑤、⑥は近接した PAM 配列が存在し、ミスマッチ数が順番に 8、6、7、8 塩基であった。さらにオフターゲット③に関しては 2 塩基の DNA バルジが 1 か所、オフターゲット⑤に関しては 1 塩基の DNA バルジが 2 か所、オフターゲット⑥に関しては 1 塩基の DNA バルジおよび RNA バルジが 1 か所ずつ生じていた。オフターゲット②、④に関しては近接した PAM 配列が存在しなかった。

リアルタイム PCR を用いた妥当性確認

オンターゲットにおいては ΔCq=1.19（切断効率 56.14%)、オフターゲット⑥においては ΔCq=0.48（切断効率 28.06%）であった（図 11)。このことから、ALS target2 に関しては特異性の高いガイド RNA であることが示唆された。

４．SBE1 解析結果

SBE1 ガイド RNA を用いた結果、Cas9 濃度 64 nM、256 nM および 1,024 nM の全ての濃度下で切断が確認されなかった（図 12, 15)。

各濃度でのカットサイト数としては、64 nM では 199 か所（エクソン内では 4 か所)、256 nM では 324 か所（エクソン内では 10 か所)、1,024 nM では 990 か所（エクソン内では 137 か所）となっており、切断処理する Cas9 濃度が上がるにつれて、カットサイトも増加する傾向だった（図 13)。また、エクソン内のカットサイトの内、異なる濃度で共通して検出された箇所についても調査を行った。その結果、64 nM と 256 nM で共通して検出されたカットサイトが 4 か所、64 nM と 1,024 nM で共通して検出されたカットサイトが 3 か所、256 nM と 1,024 nM で共通して検出されたカットサイトが 7 か所で、これらの内、64 nM、256 nM、1,024 nM で共通して検出されたカットサイトが 3 か所であった（図 14)。異なる Cas9 切断処理濃度で共通して検出された 8 か所について、カットサイト付近の配列とガイド RNA 配列を比較すると（図 15)、オフターゲット②は近接した PAM 配列が存在し、ミスマッチが 6 塩基（特に 3’側 10 塩基ではミスマッチが 1 塩基)で比較的相同性は高かった。オフターゲット⑥に関してはカットサイトから 5～7 塩基離れたところに PAM 様配列が存在するものの、ミスマッチが 13 塩基で 2 塩基の DNA バルジが存在し、相同性は低かった。オフターゲット①、③、④、⑤、⑦、⑧に関しては近接した PAM 配列が存在せず、相同性も低い箇所であった。

５．SBE3 解析結果

SBE3 をターゲットとしたガイド RNA を用いた結果、Cas9 濃度 64 nM、256 nM および 1,024 nM の全ての濃度で切断が確認された（図 16, 19)。

各濃度でのカットサイト数としては、64 nM では 148 か所（エクソン内では 5 か所)、256 nM では 279 か所（エクソン内では 9 か所)、1,024 nM では 387 か所（エクソン内では 9 か所）であった（図 17)。また、エクソン内のオフターゲット箇所の内、異なる濃度で共通して検出された箇所についても調査を行った。その結果、64 nM と 256 nM で共通して検出されたカットサイトが 4 か所、64 nM と 1,024 nM で共通して検出されたカットサイトが 2 か所、256 nM と 1,024 nM で共通して検出されたカットサイトが 4 か所で、これらの内、64 nM、256 nM、1,024 nM で共通して検出されたカットサイトが 2 か所であった（図 18)。異なる Cas9 切断処理濃度で共通して検出された 5 か所（オン

ターゲット含む) について、カットサイト付近の配列とガイド RNA 配列を比較すると (図 19)、オフターゲット①は近接した PAM 配列が存在したものの、ミスマッチが 11 塩基で相同性は低かった。オフターゲット②、④に関してはカットサイトから8～10塩基離れたところにPAM様配列は存在したものの、ミスマッチが 10 塩基で、2 塩基の DNA バルジが存在し、相同性は低かった。しかしオフターゲット②、④はカットサイト付近の配列が同一であり、共にクリフとして検出されたということは、ガイド RNA とゲノム DNA の相同性が低くとも切断する何らかの規則性があることが示唆された。なお、ガイド RNA および Cas9 による切断を行わないで SITE-Seq 解析に供したネガティブコントロールではオフターゲット②、④は検出されないことを確認しており、DNA 抽出やライブラリー調製時の DNA の物理的な剪断によるものではないと考えられる。

６．BSR1 解析結果

BSR1 をターゲットとしたガイド RNA を用いた結果、Cas9 濃度 64 nM、256 nM および 1,024 nM の全ての濃度で切断が確認されなかった (図 20, 23)。

各濃度でのカットサイト数としては、64 nM では 218 か所 (エクソン内では 3 か所)、256 nM では 433 か所 (エクソン内では 8 か所)、1,024 nM では 281 か所 (エクソン内では 9 か所) であった (図 21)。また、エクソン内のカットサイトの内、異なる濃度で共通して検出された箇所についても調査を行った。その結果、64 nM と 256 nM で共通して検出されたカットサイトが 1 か所、64 nM と 1,024 nM で共通して検出されたカットサイトが 1 か所、256 nM と 1,024 nM で共通して検出されたカットサイトが 4 か所で、これらの内、64 nM、256 nM、1,024 nM で共通して検出されたカットサイトが 1 か所であった (図 22)。異なる Cas9 切断処理濃度で共通して検出された 4 か所について、カットサイト付近の配列とガイド RNA 配列を比較すると (図 23)、オフターゲット①、③、④では近接する PAM 配列が存在し、オフターゲット①に関してはミスマッチが 6 塩基、1 塩基と 2 塩基の DNA バルジがそれぞれ 1 か所であった。オフターゲット③に関してはミスマッチが 8 塩基、1 塩基の DNA バルジが 1 か所であった。オフターゲット④に関してはミスマッチが 9 塩基、また 1 塩基の DNA バルジが 1 か所であった。オフターゲット②に関してはガイド RNA との相同性は低かった。

７．HAK1 解析結果

HAK1 をターゲットとしたガイド RNA を用いた結果、Cas9 濃度 64 nM、256 nM および 1,024 nM の全ての濃度で切断が確認されなかった (図 24, 27)。

各濃度でのカットサイト数としては、64 nM では 199 か所 (エクソン内では 4 か所)、256 nM では 324 か所 (エクソン内では 10 か所)、1,024 nM では 990 か所 (エクソン内では 137 か所) となっており、切断処理する Cas9 濃度が上がるにつれて、カットサイトも増加する傾向だった (図 25)。また、エクソン内のカットサイトの内、異なる濃度で共通して検出された箇所についても調査を行った。その結果、64 nM と 256 nM で共通して検出されたカットサイトが 4 か所、64 nM と 1,024 nM で共通して検出されたカットサイトが 3 か所、256 nM と 1,024 nM で共通して検出されたカットサイトが 7 か所で、これらの内、64 nM、256 nM、1,024 nM で共通して検出されたカットサイトが 2 か所であった (図 26)。異なる Cas9 切断処理濃度で共通して検出された 10 か所 (オンターゲット含む) について、カットサイト付近の配列とガイド RNA 配列を比較すると (図 27)、オフターゲット①～⑨全てで近接する PAM 配列が存在し、オフターゲット①に関してはミスマッチが 6 塩基、また 3 塩基の RNA バルジおよび 2 塩基の DNA バルジがそれぞれ 1 か所であった。オフターゲット②～⑨に関しては、ミスマッチの数が順番に 9、11、9、11、9、12、9、10 塩基であった。さらにオフターゲット⑦は 1 塩基の DNA バルジが 1 か所、オフターゲット⑨に関しては 4 塩基の DNA バルジが 1 か所であった。

８．FH15 解析結果

エクソン内カットサイト数の傾向

FH15 をターゲットとしたガイド RNA を用いた結果、Cas9 濃度 64 nM、256 nM および 1,024 nM の全ての濃度でオンターゲットの切断が確認された (図 28, 31)。

各濃度でのカットサイト数としては、64 nM では 219 か所 (エクソン内では 4 か所)、256 nM では 484 か所 (エクソン内では 9 か所)、1,024 nM

では 425 か所（エクソン内では 139 か所）であった（図 29）。また、エクソン内のカットサイトの内、異なる濃度で共通して検出された箇所についても調査を行った。その結果、64 nM と 256 nM で共通して検出されたカットサイトが 1 か所、64 nM と 1,024 nM で共通して検出されたカットサイトが 1 か所、256 nM と 1,024 nM で共通して検出されたカットサイトが 7 か所で、これらの内、64 nM、256 nM、1,024 nM で共通して検出されたカットサイトが 1 か所（オンターゲット）であった（図 30）。

SITE-Seq 法とオンラインオフターゲット予測ツールの比較

異なる Cas9 切断処理濃度で共通して検出された 9 か所のうち、オンターゲットを除く 8 か所のオフターゲットについて、イネのリファレンスゲノムが用意されている 5 つのオンラインオフターゲット予測ツール（Cas-OFFinder[10]、CHOPCHOP[11]、CRISPOR[12]、CRISPRdirect[13]、CRISPR-P v2.0[14]）で予測されるか検証を行った（図 31）。その結果、Cas-OFFinder では 8 か所中 2 か所を予測したが、他のオンラインオフターゲット予測ツールでは全て予測されなかった。Cas-OFFinder では NGG もしくは NAG を PAM として認識し、ミスマッチが 9 塩基まで、また 2 塩基までの DNA もしくは RNA バルジを含む箇所を予測することが可能である。オフターゲット③に関しては NAG の近接した PAM 配列が存在し、ミスマッチが 3 塩基、DNA バルジが 2 塩基であり、オフターゲット④に関しては NGG の PAM が存在し、ミスマッチが 2 塩基、RNA バルジが 2 塩基と比較的相同性の高い箇所であったため、予測がなされたと考えられる。一方でオフターゲット①、⑤、⑦、⑧に関しては近接した PAM 配列が存在せず、オフターゲット②、⑥に関してはミスマッチが 12 塩基と多かったため、予測がなされなかったと考えられる。

SITE-Seq 法で予測されたカットサイトの妥当性確認

SITE-Seq 法で予測されたカットサイトにまたがるように設計したプライマーを用いてリアルタイム PCR を行い、Cq 値をネガティブコントロールと比較することで SITE-Seq 法の妥当性評価を行った（図 32）。切断処理は SITE-Seq 解析時と同様に、Cas9 濃度 64 nM、256b nM、1,024 nM で行った。その結果、オンターゲットでは 64 nM では ΔCq 値が 6.48、256 nM では 6.49、1,024 nM では 6.89 であり、いずれの濃度でも切断効率 98%以上と、高い切断効率であることが推測された。オフターゲットについては、リード数の多い上位 3 か所（オフターゲット③、④、⑦）についてリアルタイム PCR を実施した。その結果、オフターゲット③では 64 nM では ΔCq 値が 0.03（切断効率: 1.96%）、256 nM では 0.34（21.26%）、1,024 nM では 1.15（54.95%）であった。オフターゲット④では 64 nM では ΔCq 値が 1.11（53.70%）、256 nM では 4.53（95.67%）、1,024 nM では 4.62（95.93%）であった。オフターゲット⑦では 64 nM では ΔCq 値が 0.28（17.36%）、256 nM では 1.64（67.95%）、1,024 nM では 3.71（92.36%）であったことから、FH15 ガイド RNA は特異性の低いガイド RNA であることが示唆されると同時に、SITE-Seq 法によるオフターゲット予測の妥当性が確認された。

D. 考察

ゲノム編集技術の食品への利用において、オフターゲット作用によるタンパク質の改変に伴うアレルゲンや有害タンパク質等の生成の可能性がある以上、オフターゲットの網羅的な検知とその影響の詳細な検証は必須である。SITE-Seq 法は Cas9 で切断されたゲノム DNA を選択的に濃縮し、網羅的にシーケンスすることで、より多くのオフターゲット部位の情報を得ることができる点で、本研究の目的である新たなバイオテクノロジーを用いた場合の食の安全性確保とマッチしている。

FH15 ガイド RNA を用いた際のリアルタイム PCR による切断確認では、3 か所のオフターゲットに関して、特にオフターゲット④、⑦では 1,024 nM では切断効率 90%以上と、高い切断効率を示した。オフターゲット⑦に関しては、近接した PAM 配列（NGG または NAG）が存在せず、Cas-OFFider をはじめ、オンラインオフターゲット予測ツールでは予測不可能であったにも関わらず、比較的高い切断効率を示したことから SITE-Seq 法は既存のオフターゲット予測ツールでは予測できないオフターゲットを網羅的に検知するのに有効であることが示唆された。またオフターゲット③、④に関して、Cas-OFFinder を用いることで予測

はされるものの、オフターゲット③（NAG もしくは NGG の PAM 配列、ミスマッチ 3 塩基、DNA バルジサイズ 2 塩基)で予想されるカットサイト総数は 742 か所、オフターゲット④(NGG の PAM 配列、ミスマッチ 2 塩基、RNA バルジサイズ 2 塩基）の総数は 209 か所と膨大であり、それらの変異解析を行う手間や費用を鑑みても SITE-Seq 法は有用であると考えられる。

現在、FH15 ガイド RNA に関しては CRISPR/Cas9 ベクターに導入し、実際に日本晴イネの形質転換を実施した。SITE-Seq 法により予測されたオフターゲットに変異が導入されるかを *in vivo* モデルを用いて検証することを考えている。形質転換体の作成に成功した場合には、RNA を抽出し、逆転写して得られた cDNA を鋳型に、リアルタイム PCR を実施するする予定である。

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5) Sun Y, et al., *Frontiers in Plant Science* 8, 298, 2017
6) Kanda Y, et al., *Bioscience, Biotechnology, and Biochemistry* 81, 1497-1502, 2017
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E. 結論

本研究結果より、ゲノム編集食品の安全性評価法の一つとしてオフターゲットを網羅的に推定する SITE-Seq 法について、イネにおいても有効であることを確認した。SITE-Seq 法は、オンラインオフターゲット予測ツールでは予測不可能であったオフターゲットの予測に成功し、ゲノム編集食品の安全性評価時に有用な手法の一つと考えられる。来年度は、過去 2 年間に発表された学術論文に記載されているガイド RNA について、オフターゲットの有無の検証を行う予定である。

F. 業績

1. 論文発表

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3) Soga, K., Nakamura, K., Ishigaki, T., Kimata, S., Ohmori, K., Kishine, M., Mano, J., Takabatake, R., Kitta, K., Nagoya, H., Kondo, K. Development of a novel method for specific detection of genetically modified Atlantic salmon, AquAdvantage, using real-time polymerase chain reaction. *Food Chemistry*, 305, 125426, 2020

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G. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

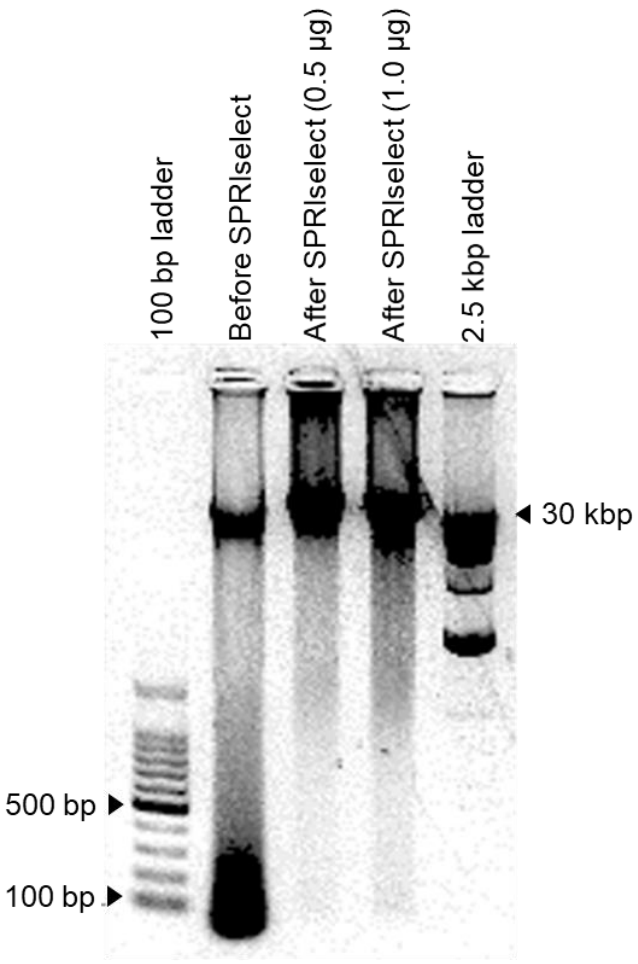
なし

3. その他

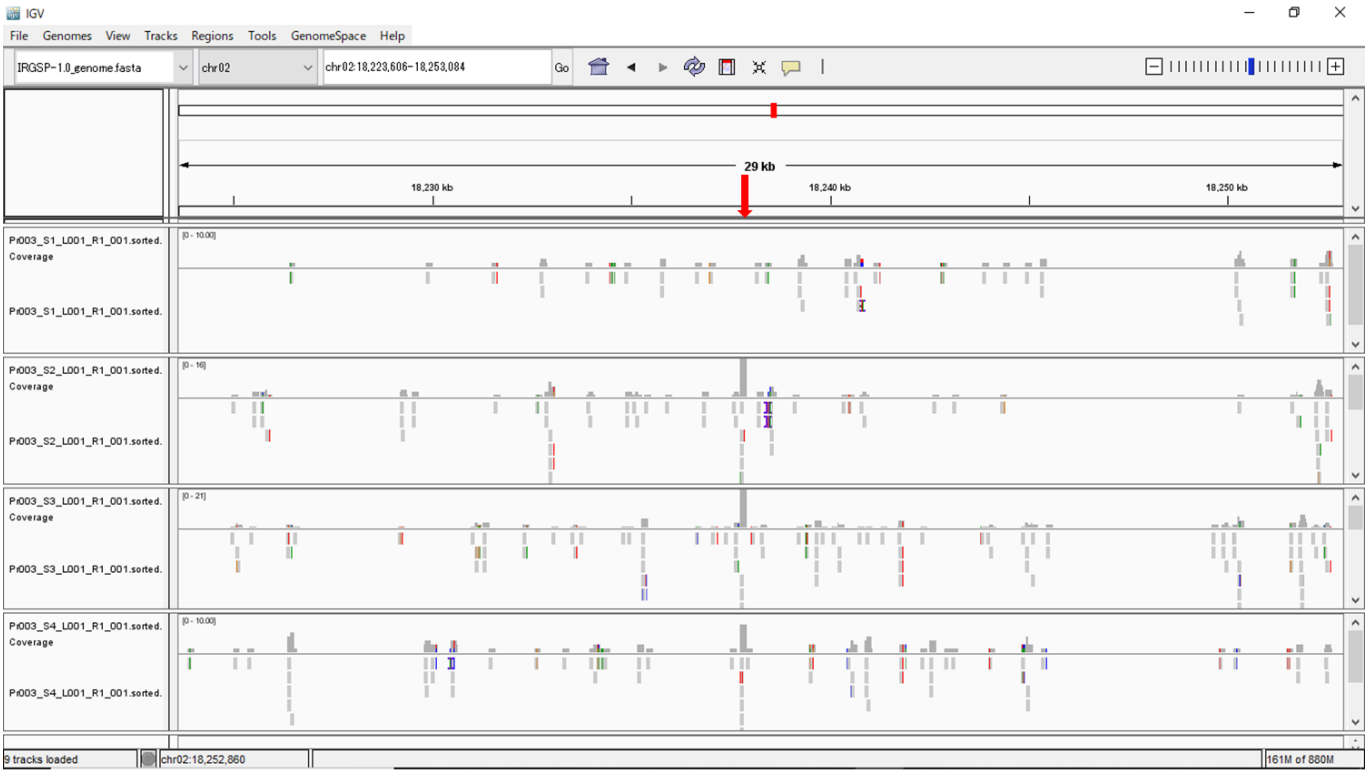
なし

表1. 本研究で使用した2016～2017年に発表されたイネにおけるCRISPR/Cas9用のガイドRNA配列、および合成に使用したオリゴヌクレオチド一覧

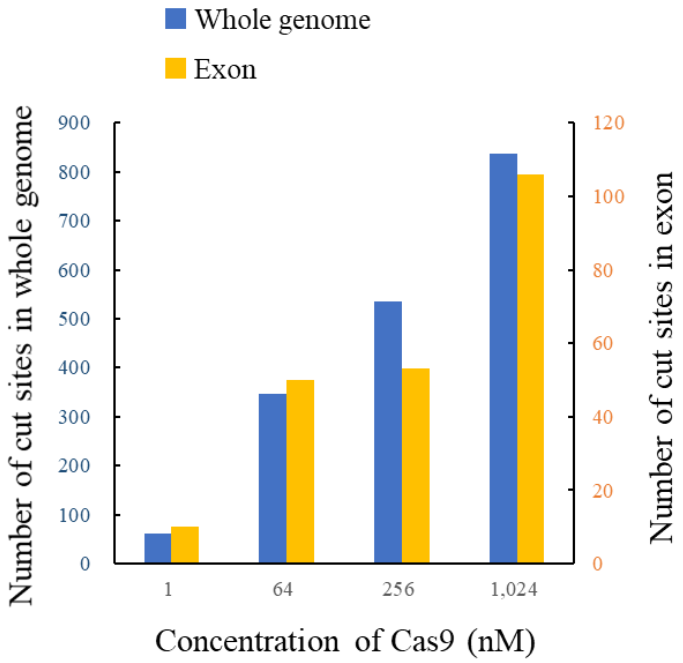
標的遺伝子	ガイドRNA配列 (緑字はPAM配列を示す)	ガイドRNA合成に使用したヌクレオチド（5'-3'） （大文字は固有の配列、小文字は共通の配列を示す）	引用文献
Acetolactate synthase, ALS	GGGTATGGTGGTGCAATGGGAGG	Fwd: cgatgtaatacgactcactataggGGGTATGGTGGTGCAATGGGgttttagagctatgctgaaa Rev: aagcaccgactcggtgccacttttttcaagttgataacggactagccttattttaacttgctatgcttttcagcatagctctaaaacC	Sun Y, et al., *Molecular Plant* 9, 628-631, 2016
Acetolactate synthase, ALS	CCTATGATCCCAAGTGGGGGCGC	Fwd: cgatgtaatacgactcactataggGCGCCCCCACTTGGGATCATgttttagagctatgctgaaa Rev: aagcaccgactcggtgccacttttttcaagttgataacggactagccttattttaacttgctatgcttttcagcatagctctaaaacA	Sun Y, et al., *Molecular Plant* 9, 628-631, 2016
Starch branching enzyme 1, SBE1	CCGCGCCCGCTCCGCTCCTTCCC	Fwd: cgatgtaatacgactcactataggGGGAAGGAGCGGAGCGGGCGgttttagagctatgctgaaa Rev: aagcaccgactcggtgccacttttttcaagttgataacggactagccttattttaacttgctatgcttttcagcatagctctaaaacC	Sun Y, et al., *Frontiers in Plant Science* 8, 298, 2017
Starch branching enzyme 3, SBE3	CCAGCCTTAGATGATGAATTAAG	Fwd: cgatgtaatacgactcactataggCTTAATTCATCATCTAAGGCgttttagagctatgctgaaa Rev: aagcaccgactcggtgccacttttttcaagttgataacggactagccttattttaacttgctatgcttttcagcatagctctaaaacG	Sun Y, et al., *Frontiers in Plant Science* 8, 298, 2017
Broad-Spectrum Resistance 1, BSR1	TCCAAGAGCAAGGAATCGTCGGG	Fwd: cgatgtaatacgactcactataggTCCAAGAGCAAGGAATCGTCgttttagagctatgctgaaa Rev: aagcaccgactcggtgccacttttttcaagttgataacggactagccttattttaacttgctatgcttttcagcatagctctaaaacG	Kanda Y, et al., *Bioscience, Biotechnology, and Biochemistry* 81, 1497-1502, 2017
Plant high-affinity K+ 1, HAK1	CAGAGCGTGGGCATCATCTACGG	Fwd: cgatgtaatacgactcactataggCAGAGCGTGGGCATCATCTAgttttagagctatgctgaaa Rev: aagcaccgactcggtgccacttttttcaagttgataacggactagccttattttaacttgctatgcttttcagcatagctctaaaacT	M N Cordones, et al., *The Plant Journal* 92, 43-56, 2017
A formin class I protein, FH15	AGCATCCAAGAATGGAGTCAAGG	Fwd: cgatgtaatacgactcactataggAGCATCCAAGAATGGAGTCAgttttagagctatgctgaaa Rev: aagcaccgactcggtgccacttttttcaagttgataacggactagccttattttaacttgctatgcttttcagcatagctctaaaacT	Sun T, et al., *Scientific Reports* 7, 6538, 2017



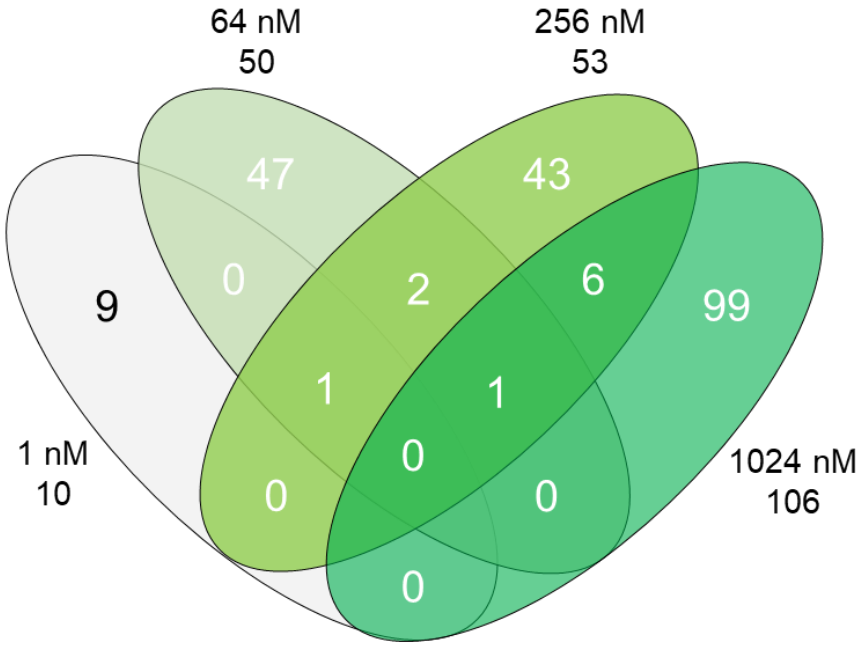
「図1」CTAB法によるイネゲノム抽出後の1%（w/v）アガロースゲルでの電気泳動写真
左から、100 bp DNAラダーマーカー、ビーズ精製前のDNA、ビーズ精製後のDNA（0.5 µg）、ビーズ精製後のDNA（1 µg）、2.5 kbp DNAラダーマーカー



「図2」 IGVにおけるALS target1のオンターゲット付近のマッピングの様子。
上から、1 nM, 64 nM, 256 nM, 1,024 nMのCas9でゲノムDNAを切断処理した。



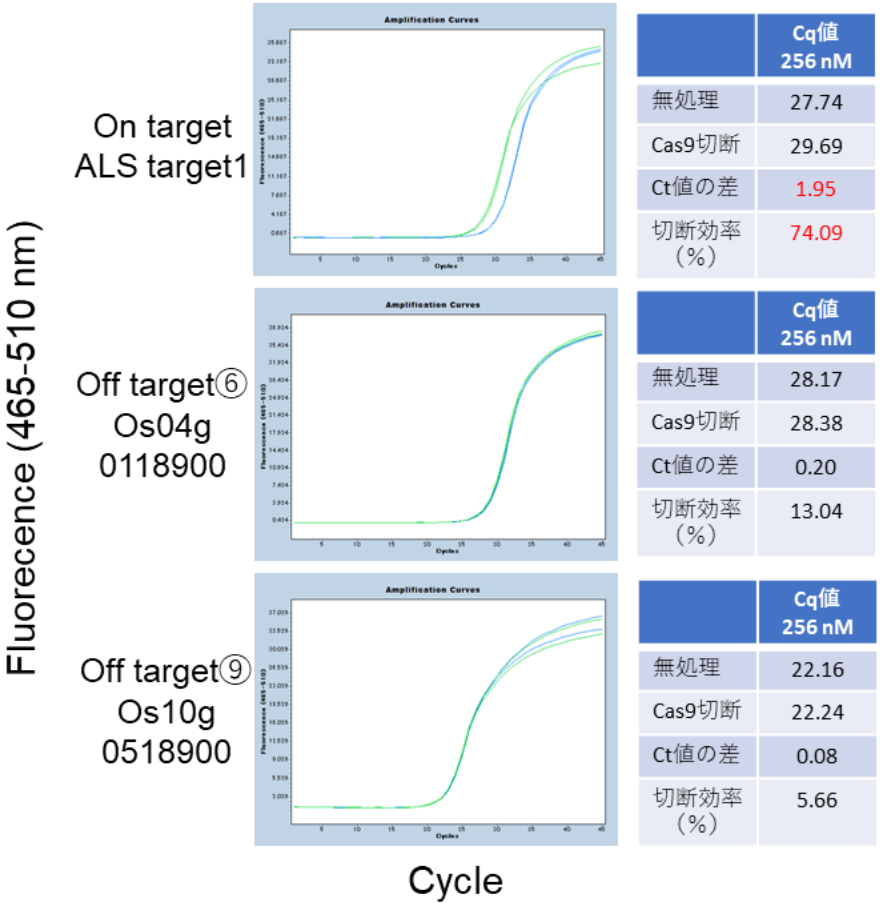
「図3」ALS target1ガイドRNAを用いた際の、各Cas9濃度における全ゲノムおよびエクソン内のカットサイト数



「図4」ALS target1ガイドRNAを用いた際のエクソン内カットサイトの濃度間比較

gRNA gene name	On/ Off	Locus	Gene ID	Sequence adjacent to cut sites and predicted PAM (NGG or NAG)	Concentration of Cas9 occur the digestion (nM)			
					1	64	256	1,024
ALS target1	On	chr02: 18237761	Os02g 0510200 (ALS)	----GGGTATGGTGGTGCAATGGG---------- ATTTGGGTATGGTGGTGCAATGGGAGGATAGGTTTTACAAG **********	-	+	+	-
	Off ①	chr01: 18826979	Os01g 0525500	--------GGGTATGGTGGTGCAATGGG---------- GGTCGCTTGGGCCTACACAAGCAAATTGTTGATAAAACTCC *** * **** *	-	+	+	-
	Off ②	chr02: 126032	Os02g 0102300	----------CCCATT-GCACCACCATACCC-- ATAAAAATAAACAATCCAACTATTAGGGGTGCCAAAGCGGG * *** * *** * *	-	-	+	+
	Off ③	chr02: 28109503	Os02g 0686300	---GGGTATGGTGGTGC-AATGGG---------- ACTGAAGGTAGGGGTGCTAATGGGTGGAAATCCAATCCATT * * * ***** ******	-	-	+	+
	Off ④	chr03: 2304950	Os03g 0141800	----------CCCATTGCACCACCATACCC TTTTTATAAATTTAGTCGCCTGTTTACTTAGATCATCATAT-- ** * ** ****	-	+	+	+
	Off ⑤	chr03: 33540032	Os03g 0803800	--------CCCATTGCACC-ACCATACCC---------- GTATAATTCCTTTAACATTTACCATGGATGCGAGCGGGACT ** * ** *****	-	-	+	+
	Off ⑥	chr04: 1121474	Os04g 0118900	----------CCCATTGCACCACCATACCC--- TCTCGCCTACCATAGCCACCTCTTGCACCATCATACCTGTC ** ******** ******	-	-	+	+
	Off ⑦	chr04: 24669355	Os04g 0493300	CCCATTGCACCACCATACCC---------- CCCCCCCTCCCGCGAAAACCAAGGCATGATTACAACAAGAG *** ** * * * **	+	+	+	-
	Off ⑧	chr10: 10862009	Os10g 0356000	--CCCATTGCACCACCATACCC---------- TTCCTATTGTAATGCATGACTACTTAACCGGGGGATTCACC ** **** * * **	-	-	+	+
	Off ⑨	chr10: 20071038	Os10g 0518900	---GGGTATGGTGGTGCA-ATGGG---------- AGCTGCTTTGATGGGGGACATGGAAGGATTGAAATAACAAG * * ** *** * * ****	-	-	+	+

「図5」図４に示した異なる濃度で重複して検出された10ヶ所のカットサイト付近のシークエンスとガイドRNAのホモロジーを示した。
赤線はカットサイトを、緑枠は推定PAM配列を表している。

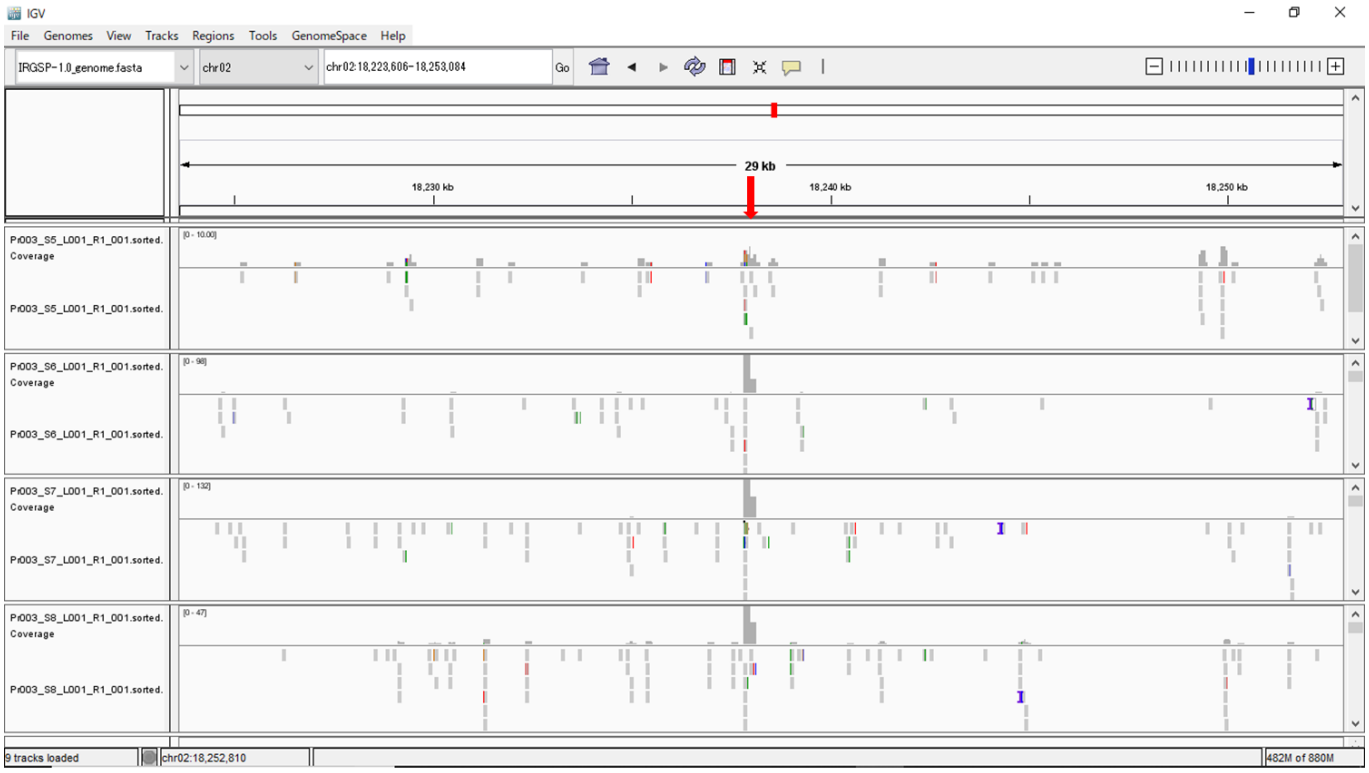


	Cq値 256 nM
無処理	27.74
Cas9切断	29.69
Ct値の差	1.95
切断効率（%）	74.09

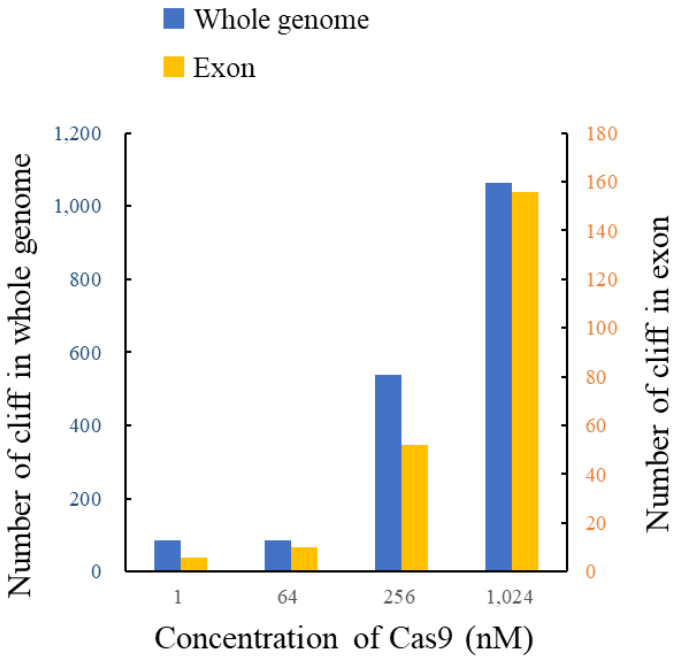
	Cq値 256 nM
無処理	28.17
Cas9切断	28.38
Ct値の差	0.20
切断効率（%）	13.04

	Cq値 256 nM
無処理	22.16
Cas9切断	22.24
Ct値の差	0.08
切断効率（%）	5.66

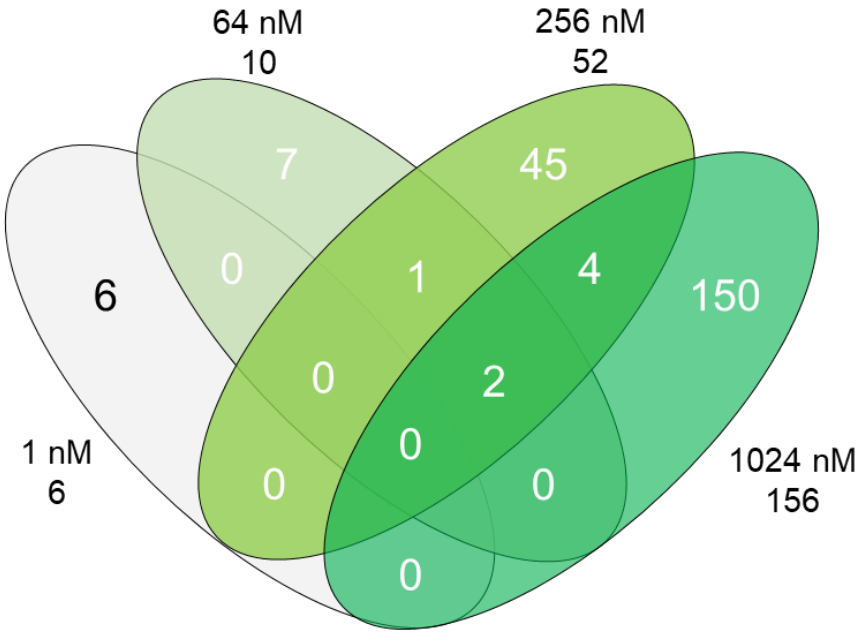
「図6」リアルタイムPCRを用いた各カットサイトの切断確認
ゲノムDNAをALS target1ガイドRNAとCas9（64, 256, 1,024 nM）で37℃16時間切断処理後、カットサイトをまたぐように設計したプライマーを用いてリアルタイムPCRを行った。ガイドRNAとCas9無添加のネガティブコントロールとCq値を比較をし、ΔCq値を求めた。またΔCq値から鋳型DNA量の差を算出し、ネガティブコントロールの鋳型DNA量を100%として、何%が切断されたかを切断効率（%、推定値）として記載した。



「図7」 IGVにおけるALS target2のオンターゲット付近のマッピングの様子。
上から、1 nM, 64 nM, 256 nM, 1,024 nMのCas9でゲノムDNAを切断処理した。



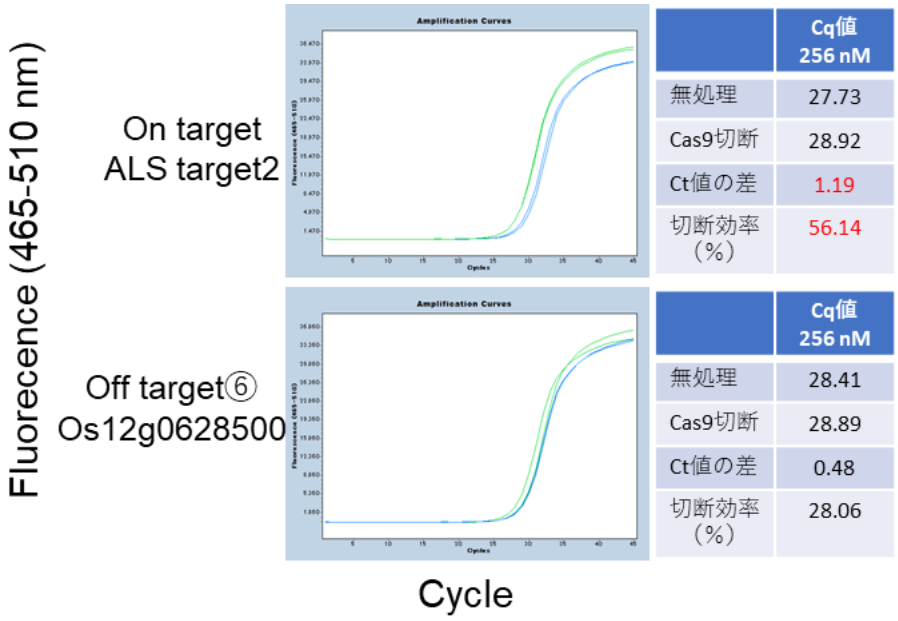
「図8」 ALS target2ガイドRNAを用いた際の、各Cas9濃度における全ゲノム、またはエクソン内のカットサイト数



「図9」ALS target2ガイドRNAを用いた際のエクソン内カットサイトの濃度間比較

gRNA gene name	On/ Off	Locus	Gene ID	Sequence adjacent to cut sites and predicted PAM (NGG or NAG)	Concentration of Cas9 occur the digestion (nM)			
					1	64	256	1,024
ALS target2	On	chr02: 18237991	Os02g 0510200 (ALS)	----------ATGATCCCAAGTGGGGGCGC--- CAGGAGCATGTGCTGCCTATGATCCCAAGTGGGGGCGCATT **********	-	+	+	+
	Off ①	chr04: 6444455	Os04g 0193950	----GCGCCCCCACTTGGGATCAT---------- TGCGAAGCAACAAGCTAGGATCATAAGGTGTTGGAGAAGGC ** * * * *******	-	-	+	+
	Off ②	chr04: 24669355	Os04g 0493300	GCGCCCCCACTTGGGATCAT---------- -CCCCCCCTCCCGCGAAAACCAAGGCATGATTACAACAAGAG * ***** * * ** *	-	+	+	+
	Off ③	chr04: 31448732	Os04g 0619200	--GCGCCCCCACT--TGGGATCAT---------- CTGCATTCTGACCAATGGGATCATCGGTTATTACTGGTAGT ** * ** *********	-	-	+	+
	Off ④	chr10: 14535611	Os10g 0415600	---------ATG-ATCCCAAGTGGGGGCGC---------- CGAATTGATATGTATTCGAATCTTAGGAGCTGGTAATCACT *** ** * ** ** **	-	-	+	+
	Off ⑤	chr12: 20650092	Os12g 0525300	---GCGCCCCCACTTGG-GATCAT---------- TGACAGACCGCACTCAGAGACCATTTGGAAAGAATAGCAGC * ** **** * ** ***	-	+	+	-
	Off ⑥	chr12: 26909858	Os12g 0628500	-----GCGCCCCCACTTGGGATCAT---------- CTATCATGCTAT-GTATGGGATCATATGGCGGAATCATCAGA ** *********	-	-	+	+

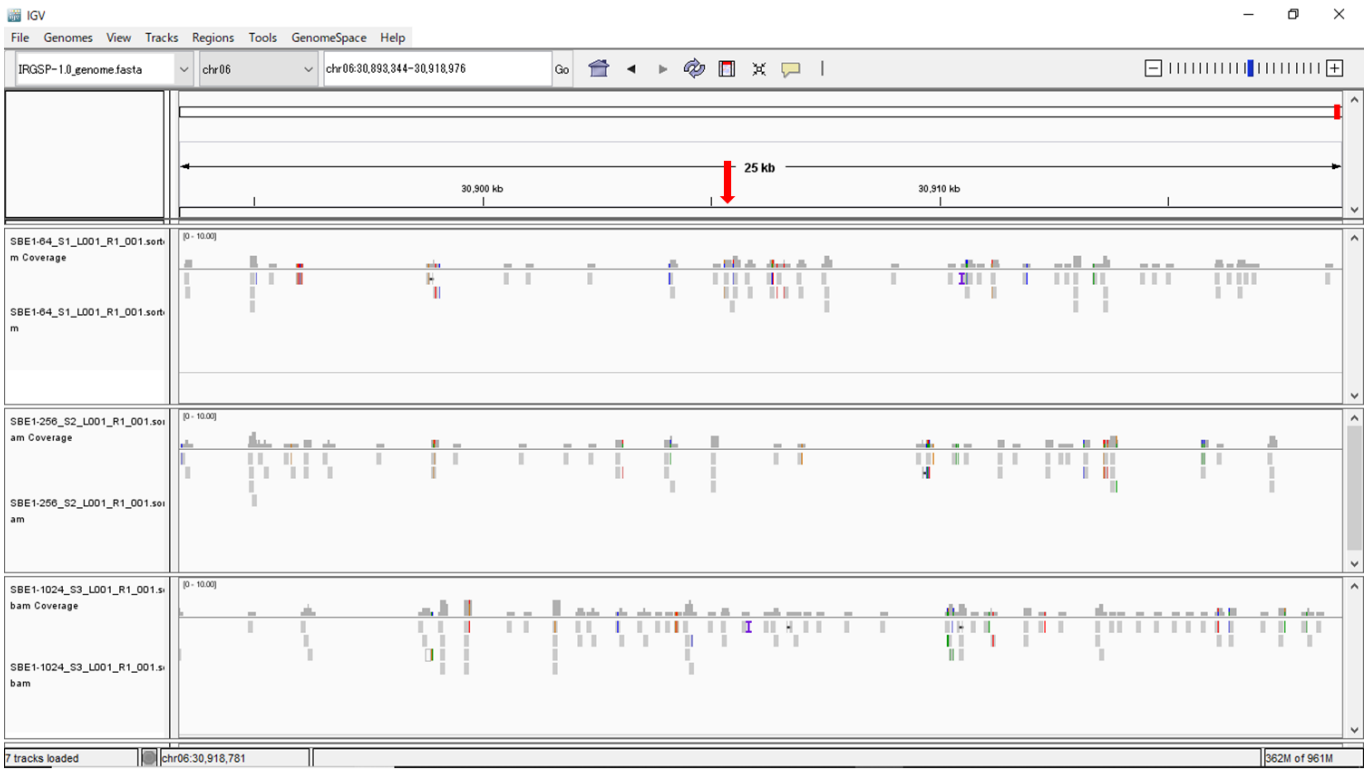
「図10」図9に示した異なる濃度で重複して検出された7ヶ所のシークエンスとガイドRNAのホモロジーを示した。赤線はカットサイトを、緑枠は推定PAM配列を表している。



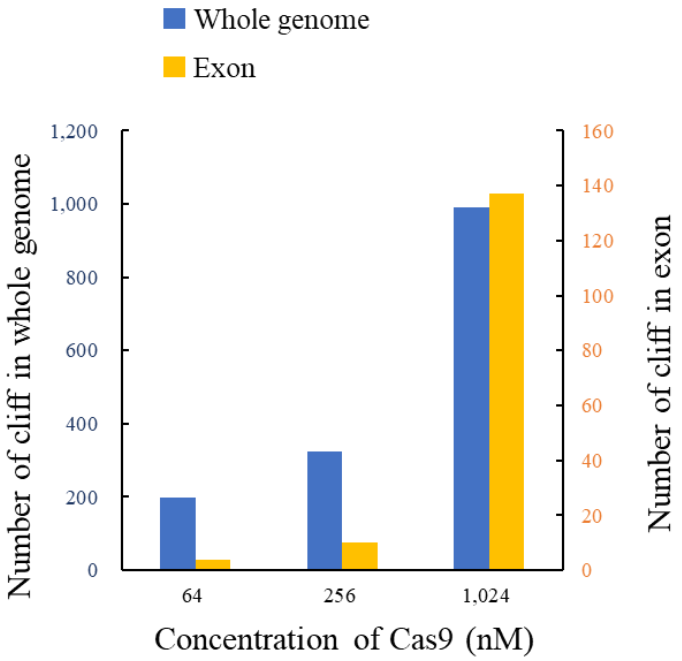
	Cq値 256 nM
無処理	27.73
Cas9切断	28.92
Ct値の差	1.19
切断効率（%）	56.14

	Cq値 256 nM
無処理	28.41
Cas9切断	28.89
Ct値の差	0.48
切断効率（%）	28.06

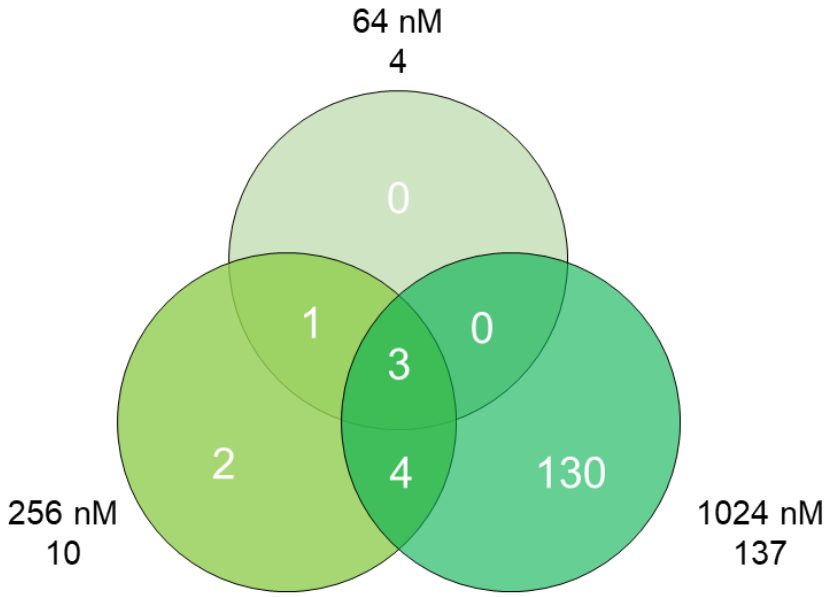
「図11」リアルタイムPCRを用いた各カットサイトの切断確認
ゲノムDNAをALS target2ガイドRNAとCas9（64, 256, 1,024 nM）で37℃16時間切断処理後、カットサイトをまたぐように設計したプライマーを用いてリアルタイムPCRを行った。ガイドRNAとCas9無添加のネガティブコントロールとCq値を比較をし、ΔCq値を求めた。またΔCq値から鋳型DNA量の差を算出し、ネガティブコントロールの鋳型DNA量を100%として、何%が切断されたかを切断効率（%、推定値）として記載した。



「図12」 IGVにおけるSBE1のオンターゲット付近のマッピングの様子。
上から、64 nM, 256 nM, 1,024 nMのCas9でゲノムDNAを切断処理した。



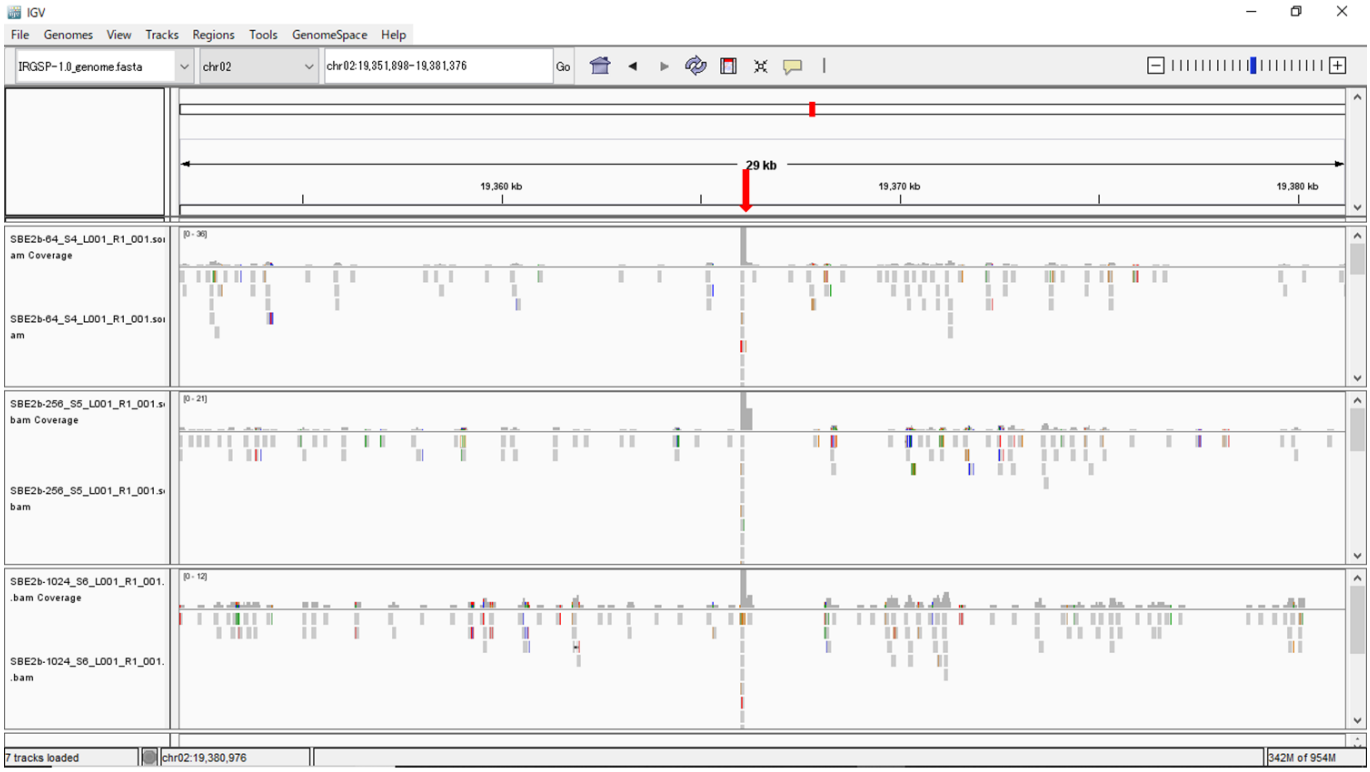
「図13」 SBE1ガイドRNAを用いた際の、各Cas9濃度における全ゲノムおよびエクソン内のカットサイト数



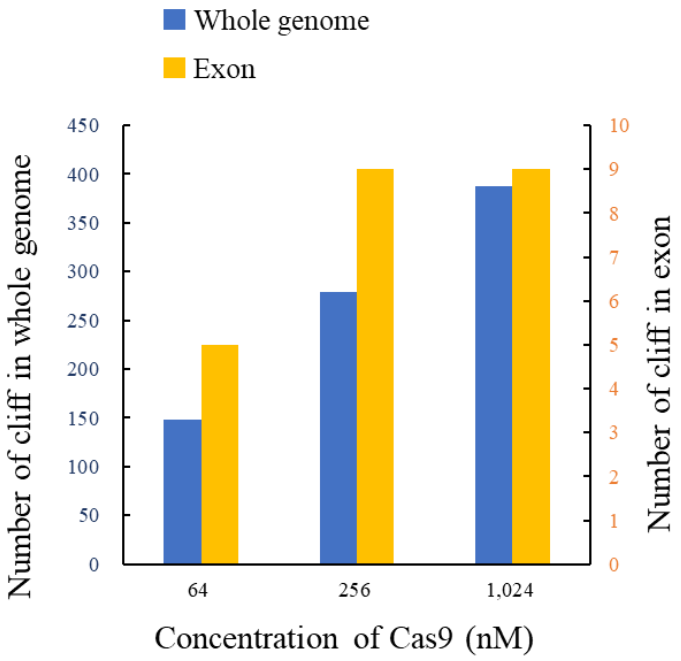
「図14」SBE1ガイドRNAを用いた際のエクソン内カットサイトの濃度間比較

gRNA gene name	On/ Off	Locus	Gene ID	Sequence adjacent to cut sites and predicted PAM (NGG or NAG)	Concentration of Cas9 occur the digestion (nM)		
					64	256	1,024
SBE1	On	chr06: 30905652	Os06g 0726400 (SBE1)	----GGGAAGGAGCGGAGCGGGCG---------- GAGAGGGAAGGAGCGGAGCGGGCCCGGAGGAGGAAGAGGAG **********	-	-	-
	Off ①	chr01: 33534133	Os01g 0791033	-------CGCCCGCTCCGCTCCTTCCC---------- AATACAGAATCATCTCCAAAGATTTCGGTCAGAGCTGGCAT * **** ** *	+	+	+
	Off ②	chr02: 35675910	Os02g 0829800	----GGGAAGGAGCGGAGCGGGCG---------- GCGGAGAGGAGAGCGGAGAGGGCGCGGTCGGAGGGCGGGGT * ******** *****	-	+	+
	Off ③	chr03: 26894918	Os03g 0679100	-CGCCCGCTCCGCTCCTTCCC---------- GTACTTGAGAGTTTCCTACCTCATACGGCTCAGAAATTGCT * * **** **	+	+	+
	Off ④	chr04: 19888440	Os04g 0401700 (HAK1)	----------CGCCCGCTCCG-CTCCTTCCC-- GTTCCAGAGCGTGGGCATCATCTACGGCGACATCGGCACGT * * * ** * * * *	-	+	+
	Off ⑤	chr09: 20181243	Os09g 0517600 (FH15)	----------GGGAAGGAGCGGAGCGGGCG--------- GGAGAGCATCCAAGAATGGAGTCAAGGAGGCAAATGCAGCT * * **** ** ***	-	+	+
	Off ⑥	chr10: 10859498	Os10g 0355800	--GGGAAGGAGCGGAGCGGGCG---------- TTGCAAAGAACCCATTTCTGTACTAAGAGTAGGTTGATAAC * *** * * *	-	+	+
	Off ⑦	chr10: 10859868	Os10g 0355800	--GGGAAGGAGCGGAGCGGGCG---------- TTGATTAATTCCATGATGAGTACTGTTTTACCTACTCCAGC * * * * *	+	+	+
	Off ⑧	chr10: 10862382	Os10g 0356000	----------GGGAAGGAGCGGAGCGGGCG------ TATGCCAGCTCTGACCGAAATCTTTGGAGATGATTCTGTAT * ** **** *	+	+	-

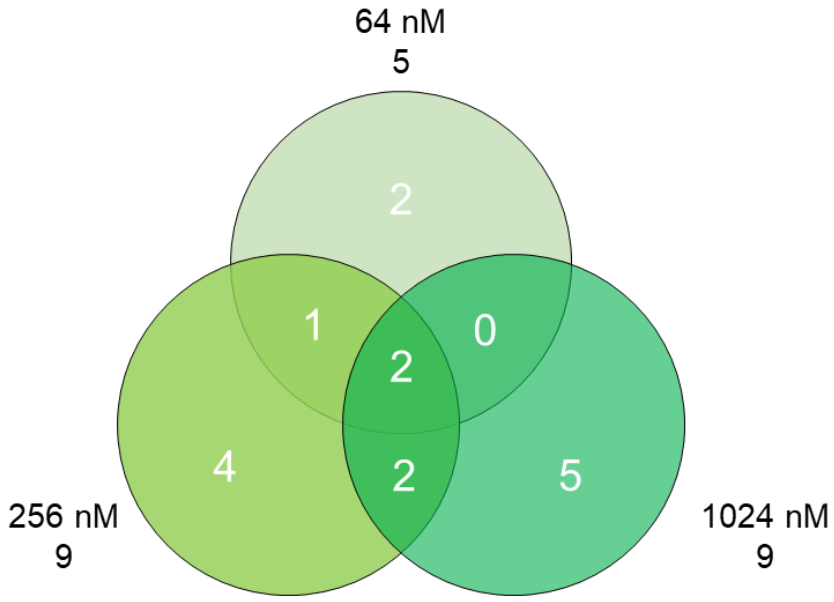
「図15」図14に示した異なる濃度で重複して検出された8ヶ所のカットサイト（いずれの濃度でも検出されなかったオンターゲットも参考までに加えた）付近のシークエンスとガイドRNAのホモロジーを示した。赤線はカットサイトを、緑枠は推定PAM配列を表している。



「図16」 IGVにおけるSBE3のオンターゲット付近のマッピングの様子。
上から、64 nM, 256 nM, 1,024 nMのCas9でゲノムDNAを切断処理した。



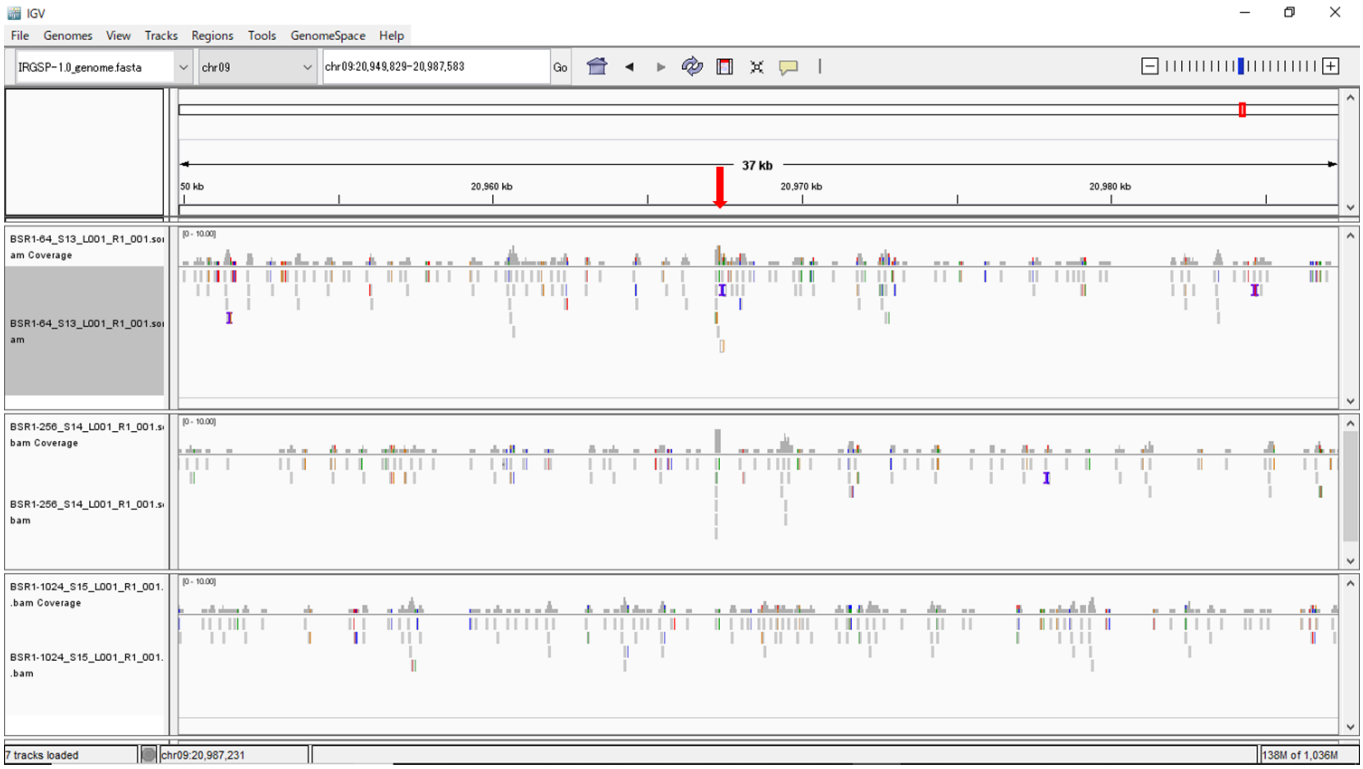
「図17」 SBE3ガイドRNAを用いた際の、各Cas9濃度における全ゲノムおよびエクソン内のカットサイト数



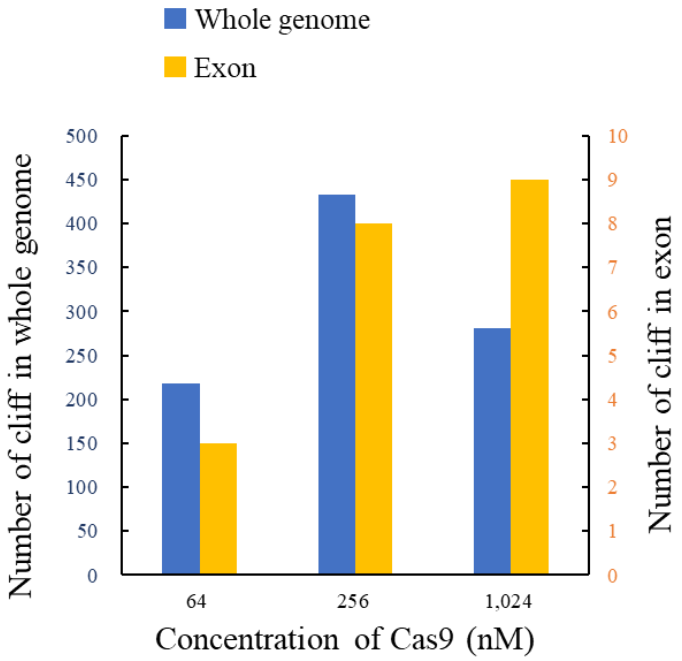
「図18」SBE3ガイドRNAを用いた際のエクソン内カットサイトの濃度間比較

gRNA gene name	On/ Off	Locus	Gene ID	Sequence adjacent to cut sites and predicted PAM (NGG or NAG)	Concentration of Cas9 occur the digestion (nM)		
					64	256	1,024
SBE3	On	chr02: 19366161	Os02g 0528200 (SBE3)	----CTTAATTCATCATCTAAGGC---------- CGTGCTTAATTCATCATCTAAGGCTGGCAACTACAACAATG **********	+	+	+
	Off ①	chr03: 26894918	Os03g 0679100	----------GCCTTAGATGATGAATTAAG---- GTACTTGAGAGTTTCCTACCTCATACGGCTCAGAAATTGCT *** * * * * **	+	+	+
	Off ②	chr10: 10862196	Os10g 0356000	----------GCCTTAGATGATGAATTAAG----- TCATATCCACGCTGGTACAGTAGTAGGTAAGTTAGAAGGGG * *** * * * ***	+	+	-
	Off ③	chr10: 10862418	Os10g 0356000	----------CTTAATTCATCA--TCTAAGGC--------- TGTATTGCAATTTGGTGGAGGAACTTTAGGACATCCTTGGG ** * * * * ** * *	-	+	+
	Off ④	chr12: 5615131	Os12g 0207600	----------GCCTTAGATGATGAATTAAG----- TCATATCCACGCTGGTACAGTAGTAGGTAAGTTAGAAGGGG * *** * * * ***	-	+	+

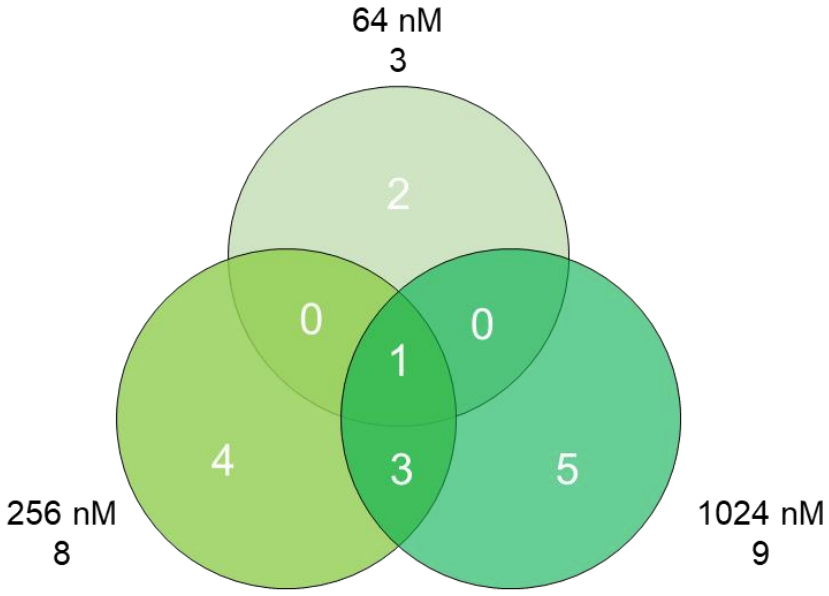
「図19」図18に示した異なる濃度で重複して検出された5ヶ所のカットサイト付近の配列とガイドRNA配列を比較した。赤線はカットサイトを、緑枠は推定PAM配列を表している。



「図20」 IGVにおけるBSR1のオンターゲット付近のマッピングの様子。
上から、64 nM, 256 nM, 1,024 nMのCas9でゲノムDNAを切断処理した。



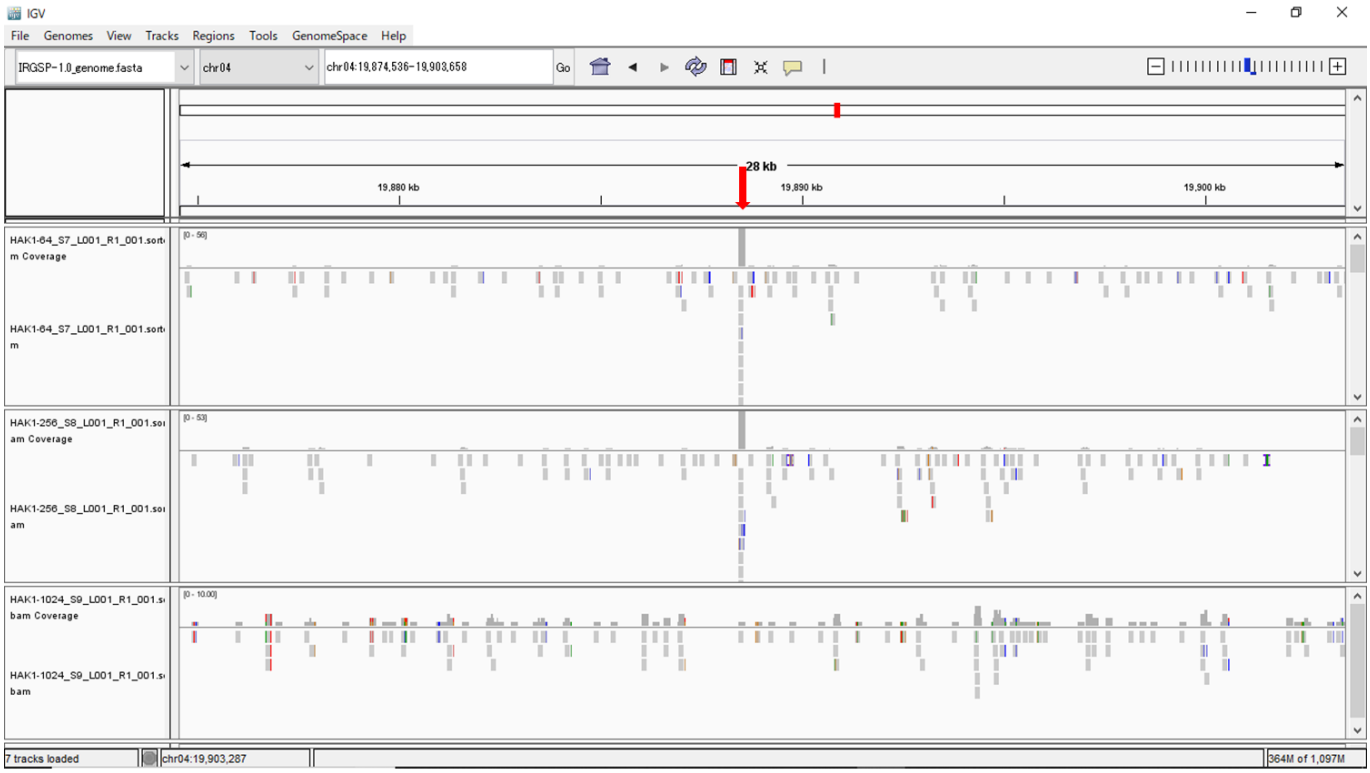
「図21」 BSR1ガイドRNAを用いた際の、各Cas9濃度における全ゲノムおよびエクソン内のカットサイト数



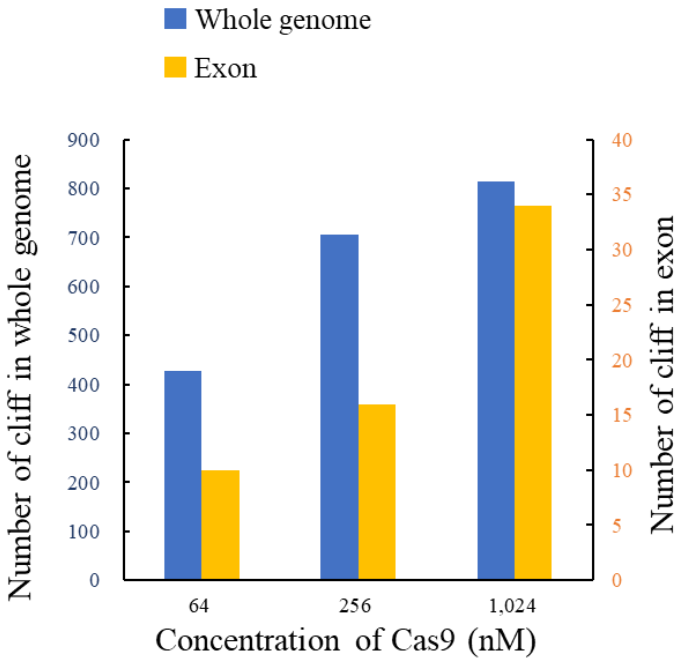
「図22」BSR1ガイドRNAを用いた際のエクソン内カットサイトの濃度間比較

gRNA gene name	On/ Off	Locus	Gene ID	Sequence of adjacent to cut sites and predicted PAM (NGG or NAG)	Concentration of Cas9 occur the digestion (nM)		
					64	256	1,024
BSR1	On	chr09: 20967356	Os09g 0533600 (BSR1)	----TCCAAGAGCAAGGAATCGTC---------- GTCGTCCAAGAGCAAGGAATCGTCGGGGAGGCGGGGCTCGA **********	-	-	-
	Off ①	chr04: 19888440	Os04g 0401700 (HAK1)	-TCCAAGAGCAAGGA-ATCGTC---------- GTTCCAGAGCGTGGGCATCATCTACGGCGACATCGGCACGT * * ***** ** *** **	-	+	+
	Off ②	chr09: 20181243	Os09g 0517600 (FH15)	--------TCCAAGAG------CAAGGAATCGTC------- GGAGAGCATCCAAGAATGGAGTCAAGGAGGCAAATGCAGCT ******* ****** *	-	+	+
	Off ③	chr10: 10859065	Os10g 0355800	-------TCCAAGAGCAAGGAATCGTC---------- TAGTTTCTGCAAGACCAACATACTTTCCCGGAGAACCGGTA * ***** *** .* **	-	+	+
	Off ④	chr12: 20542985	Os12g 0524201	----------GACGATTCCTTGCTCTTGGA- TACAGATTCGGCAACCCTAGGAGGATTCTTTCTAAAAGGTA ** ***** ** **	+	+	+

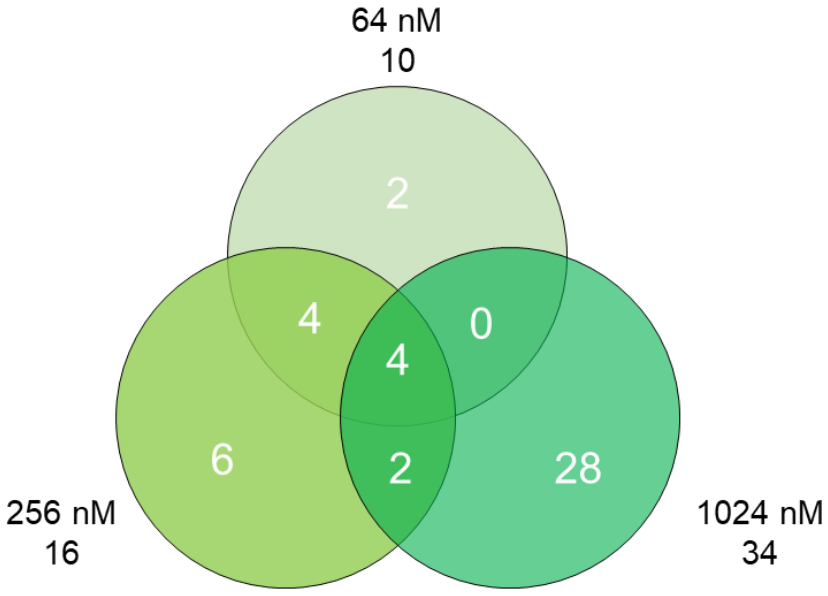
「図23」図22に示した異なる濃度で重複して検出された4ヶ所のカットサイト（いずれの濃度でも検出されなかったオンターゲットも参考までに加えた）付近のシークエンスとガイドRNAのホモロジーを示した。赤線はカットサイトを、緑枠は推定PAM配列を表している。



「図24」 IGVにおけるHAK1のオンターゲット付近のマッピングの様子。
上から、64 nM, 256 nM, 1,024 nMのCas9でゲノムDNAを切断処理した。



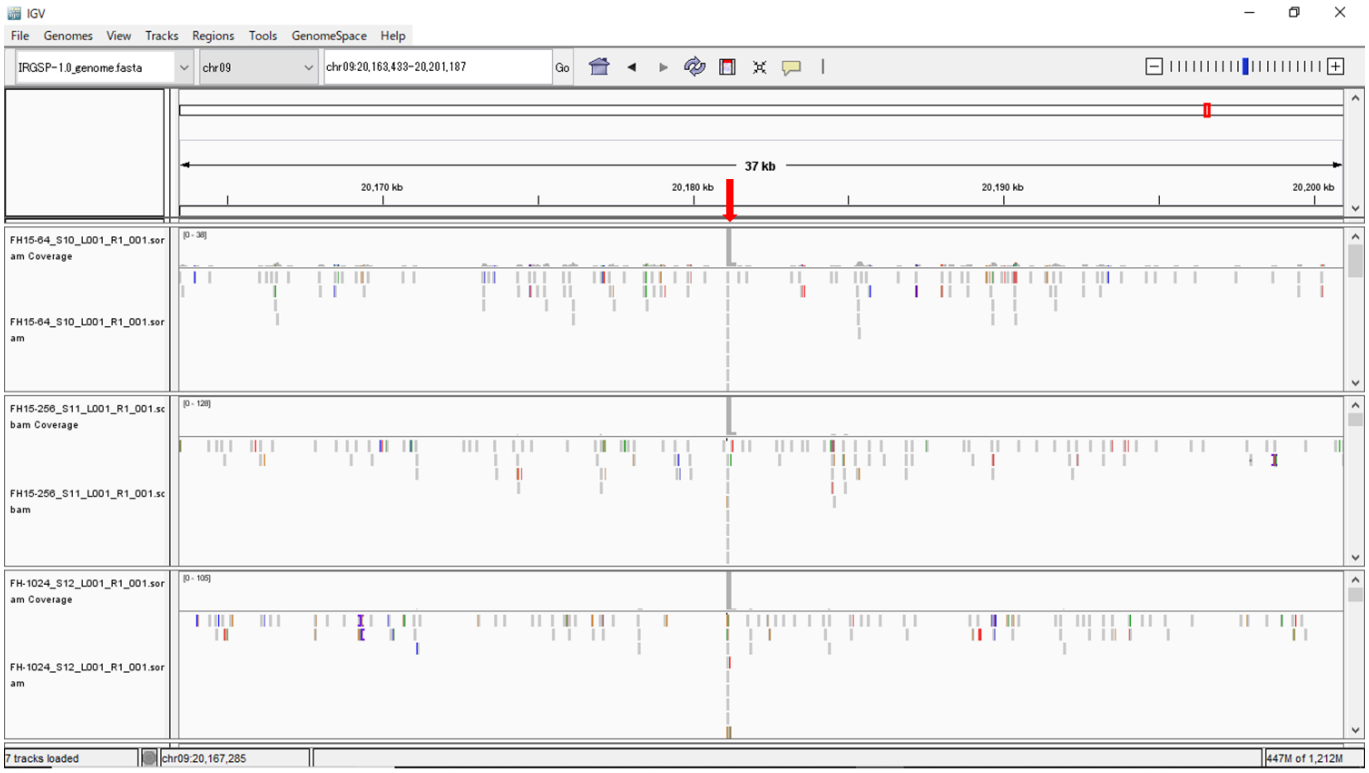
「図25」 HAK1ガイドRNAを用いた際の、各Cas9濃度における全ゲノムおよびエクソン内のカットサイト数



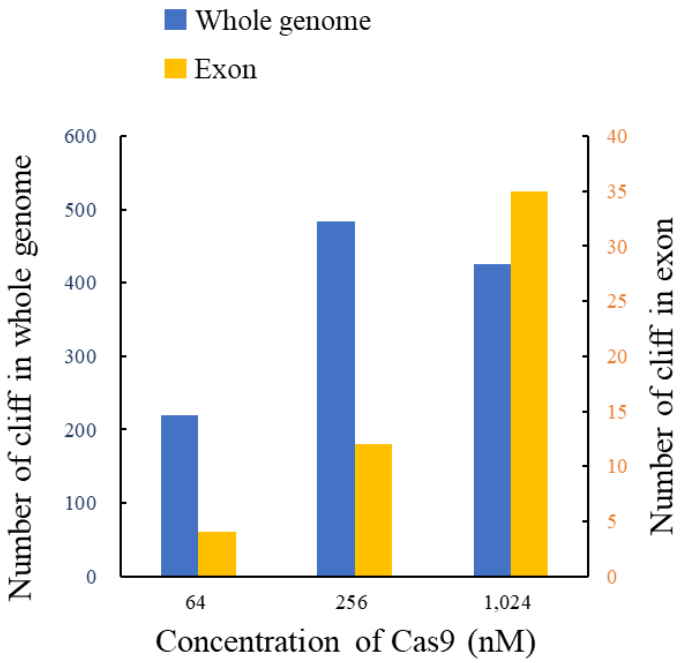
「図26」HAK1ガイドRNAを用いた際のエクソン内カットサイトの濃度間比較

gRNA gene name	On/ Off	Locus	Gene ID	Sequence adjacent to cut sites and predicted PAM (NGG or NAG)	Concentration of Cas9 occur the digestion (nM)		
					64	256	1,024
HAK1	On	chr04: 19888440	Os04g 0401700 (HAK1)	----CAGAGCGTGGGCATCATCTA---------- GTTCCAGAGCGTGGGCATCATCTACGGCGACATCGGCACGT **********	+	+	-
	Off ①	chr01: 33534343	Os01g 0791033	----------TAGATGATGCCC--ACGCTCTG---- ACCAGGTGCATTACCA---AGGATGTCCTAAAGTTCCTCCAC * **** ** * * **	-	+	+
	Off ②	chr03: 1131231	Os03g 0120501	----------TAGATGATGCCCACGCTCTG--- GGAGGCCCTCAATGACCATAGATCGAACCTATCCTATTTTT ***** ** * ** *	-	+	+
	Off ③	chr03: 25801489	Os03g 0659266	----------TAGATGATGCCCACGCTCTG--- TGAAGCTCGATCTCCCCCAGATGAACCATATAGCCAAGAG ****** * * *	+	+	-
	Off ④	chr10: 10859302	Os10g 0355800	----CAGAGCGTGGGCATCATCTA---------- GTAACATAGTTGAGGTTGAATCTAAAGGATCTACTGTAGGA ** ** ** *****	+	+	+
	Off ⑤	chr10: 10859729	Os10g 0355800	----------TAGATGATGCCCACGCTCTG--- AGCTACCTTTGATTCCTCAAGATTTTTTTCATTAATTACTC **** * ** *	+	+	+
	Off ⑥	chr10: 10861808	Os10g 0356000	----------TAGATGATGCCCACGCTCTG--- GTGGACTTGATTTTACCAAAGATGATGAAAACGTAAACTCA ******** ***	+	+	+
	Off ⑦	chr10: 10862621	Os10g 0356000	----------TAGATGATGCCCACGCTCTG--- AATTCGAGTTCGAGCCGGTAGATAAACTAGATAGCTAGACT ***** * * *	+	+	-
	Off ⑧	chr12: 5614743	Os12g 0207600	----------TAGATGATGCCCACGCTCTG--- GTGGACTTGATTTTACCAAAGATGATGAAAACGTAAACTCA ******** ***	+	+	+
	Off ⑨	chr12: 13410523	Os12g 0424300	----------TAGATGATGCCCACGCTCTG GCAAGTGATCTAGCTCCTGAGATATATGACATTCTTTATCC- ** **** * **	+	+	-

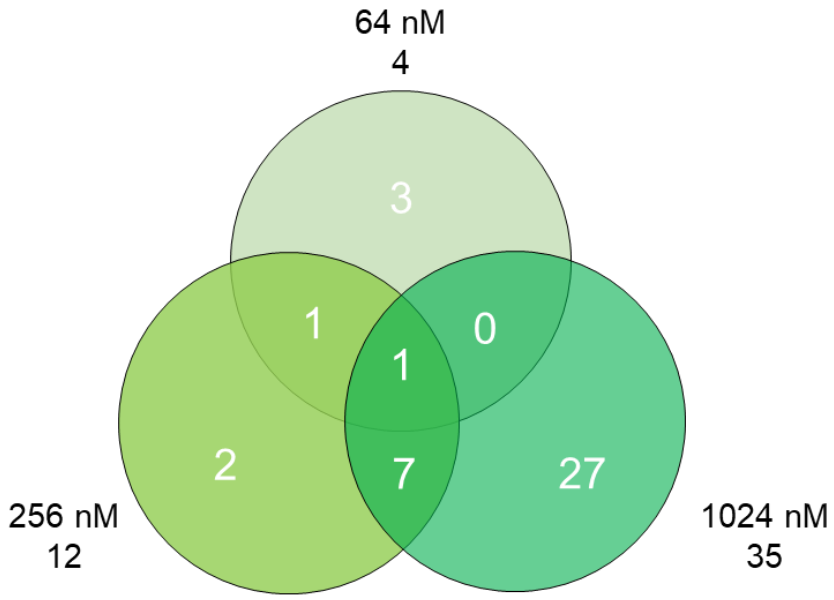
「図27」図26に示した異なる濃度で重複して検出された10ヶ所のカットサイト付近のシークエンスとガイドRNAのホモロジーを示した。赤線はカットサイトを、緑枠は推定PAM配列を表している。



「図28」 IGVにおけるFH15のオンターゲット付近のマッピングの様子。上から、64 nM, 256 nM, 1,024 nMのCas9でゲノムDNAを切断処理した。



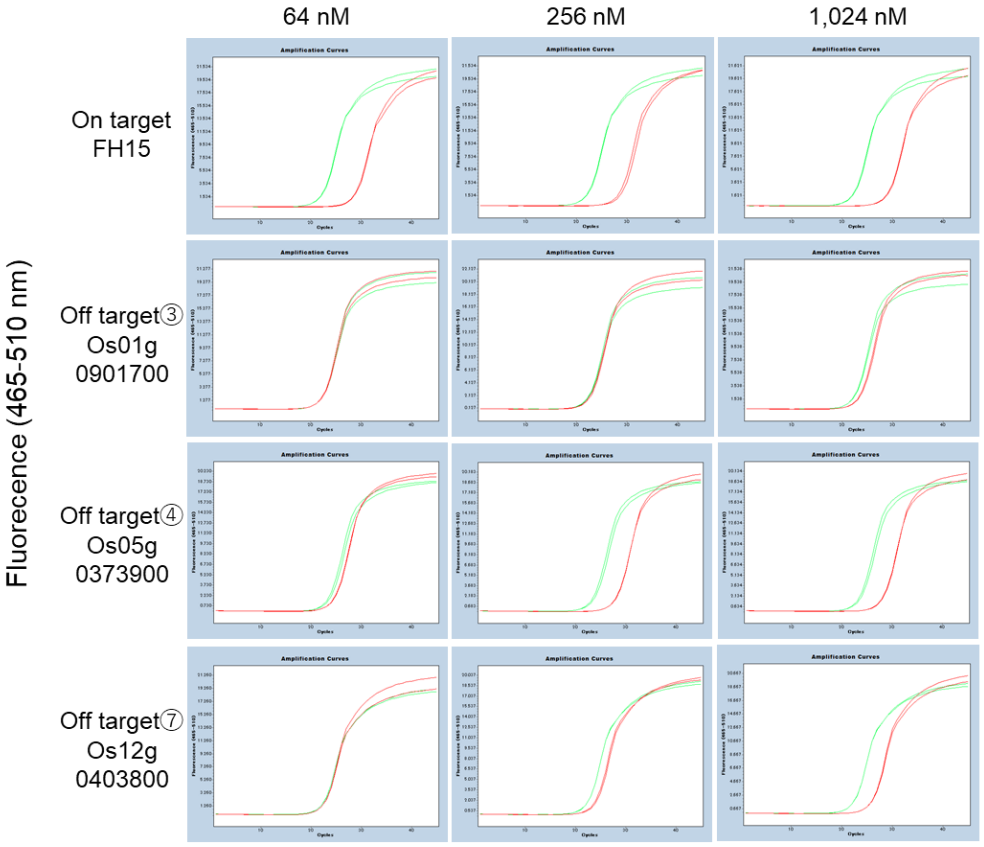
「図29」 FH15ガイドRNAを用いた際の、各Cas9濃度における全ゲノムおよびエクソン内のカットサイト数



「図30」FH15ガイドRNAを用いた際のエクソン内カットサイトの濃度間比較

					SITE-Seq (本研究)			オフターゲット予測ツール				
gRNA gene name	On/ Off	Locus	Gene ID	Sequence adjacent to cut sites and predicted PAM (NGG or NAG)	Concentration of Cas9 occur the digestion (nM)			Cas-OFFinder	CHOP CHOP	CRISPOR	CRISPR direct	CRISPR P
					64	256	1,024					
FH15	On	chr09: 20181243	Os09g 0517600 (FH15)	----AGCATCCAAGAATGGAGTCA---------- GGAGAGCATCCAAGAATGGAGTCAAGGAGGCAAATGCAGCT **********	+	+	+					
	Off ①	chr01: 18688154	Os01g 0523401	--AGCATCCAAGAATGGAGTCA---------- GAAACTTAAGAAACTCCAACCAGACTTTAAGAAACAACAAT * * * * * * * **	-	+	+	×	×	×	×	×
	Off ②	chr01: 33534133	Os01g 0791033	----------TGACTCCATTCTTGGATGCT-- AATACAGAATCATCTCCAAAGATTTCGGTCAGAGCTGGCAT ** * * ** * **	-	+	+	×	×	×	×	×
	Off ③	chr01: 39263530	Os01g 0901700	----------TGACTCCATTCTTGGAT--GCT- GTATTGCACTTCACTCTCTGGCTCCATTCTTGGTTCAGTTG ** ********** * * *	-	+	+	○	×	×	×	×
	Off ④	chr05: 18020045	Os05g 0373900	-----AGCATCCAAGAATGGAGTCA---------- ATAGGAGCATC--AGAAGGGAGCCATGGTTGCTTAAGAACATC ****** **** **** **	-	+	+	○	×	×	×	×
	Off ⑤	chr06: 15796609	Os06g 0473100	----------TGACTCCATTCTTGGATGCT----- ACTACCATTCCGCGTATTCGACTTC--TATTAGTTTCTTTTCT **** * * ** * * **	-	+	+	×	×	×	×	×
	Off ⑥	chr10: 10862382	Os10g 0356000	---AGCATCCAAGAATGGAGTCA---------- TATGCCAGCTCTGACCGAAATCTTTGGAGATGATTCTGTAT ** * ** * * **	-	+	+	×	×	×	×	×
	Off ⑦	chr12: 12080780	Os12g 0403800	----------TGACTCCATTCTTGGATGCT--- TAACAATTTGATATACAGTGACTCCATTCTTGTATGATTTC ********** *** *	-	+	+	×	×	×	×	×
	Off ⑧	chr12: 20542434	Os12g 0524201	-----AGCATCCAAGAATGGAGTCA---------- TCAGAAGGGCTAGACCCTGGGACTCCGCTTTGTTGCTAGCT ** * ***	+	-	+	×	×	×	×	×

「図31」図30に示した異なる濃度で重複して検出された9ヶ所のカットサイト付近のシークエンスとガイドRNAのホモロジーを示した。赤線はカットサイトを、緑枠は推定PAM配列を表している。また5種のオフターゲット予測ツールを用いて、SITE-Seq法で予測されたオフターゲットが予測されるかを検証した。



	64 nM	Cq値 256 nM	1,024 nM
無処理	21.85	21.85	21.85
Cas9切断	28.33	28.34	28.74
Ct値の差	6.48	6.49	6.89
切断効率（%）	98.88	98.89	99.16

	64 nM	Cq値 256 nM	1,024 nM
無処理	22.06	22.06	22.06
Cas9切断	22.09	22.40	23.21
Ct値の差	0.03	0.34	1.15
切断効率（%）	1.96	21.26	54.95

	64 nM	Cq値 256 nM	1,024 nM
無処理	23.23	23.23	23.23
Cas9切断	24.34	27.76	27.85
Ct値の差	1.11	4.53	4.62
切断効率（%）	53.70	95.67	95.93

	64 nM	Cq値 256 nM	1,024 nM
無処理	21.82	21.82	21.82
Cas9切断	22.10	23.46	25.53
Ct値の差	0.28	1.64	3.71
切断効率（%）	17.36	67.95	92.36

「図32」リアルタイムPCRを用いた各カットサイトの切断確認
ゲノムDNAをFH15ガイドRNAとCas9（64, 256, 1,024 nM）で37°C16時間切断処理後、カットサイトをまたぐように設計したプライマーを用いてリアルタイムPCRを行った。ガイドRNAとCas9無添加のネガティブコントロールとCq値を比較をし、ΔCq値を求めた。またΔCq値から鋳型DNA量の差を算出し、ネガティブコントロールの鋳型DNA量を100%として、何%が切断されたかを切断効率（%）として記載した。

厚生労働科学研究費補助金（食品の安全確保推進研究事業）
「新たなバイオテクノロジーを用いて得られた食品の安全性確保と
リスクコミュニケーションのための研究」

質量分析インフォマティクスによる化合物同定
～解析フレームワークの確立・スペクトルライブラリの拡充～

研究分担者　早川 英介　　（沖縄科学技術大学院大学）

研究要旨：
本研究では化合物の質量スペクトルの類似度をもとに試料中の未知化合物の検出と構造推定を行う解析システムの構築を行う。本年度は既存のスペクトルライブラリに加え標準品・標準試料から大量の質量スペクトルライブラリを取得すること統合スペクトルライブラリの拡充を行うとともに、比較定量情報を反映した可視化機能の実装など解析ツールの高機能化を行った。本年度の開発より試料間比較による代謝物の変動の検出と連携した、広範囲の化合物のクラスの迅速な推定・可視化という解析フレームワークの根幹が確立されたと言える。

A.　研究目的

ゲノム編集作物はターゲット遺伝子以外にも想定外の代謝への影響などによる未知の化合物や毒性の増加など安全性の問題が危惧されている。従来の質量分析による分析ではターゲットを特定の化合物に絞った“ターゲット分析”が一般的であるが、想定外の代謝経路の変動やその結果生得る未知の化合物を分析するためには有効な手法ではない。近年、分析ターゲットを限定しない“ノンターゲット分析”が提唱されているものの、未知の化合物を迅速に検出・構造推定することは未だに困難である。そこで本研究では、化合物の質量スペクトルの類似性に着目したデータ解析法により、「想定外の未知化合物」の迅速な検出と構造推定を可能にすることを目的としている。

B.　研究方法

本研究では化合物の質量スペクトル（フラグメントスペクトル）の類似度をもとに食品中の未知化合物の検出と構造推定を行うシステムの構築を行った。データ解析のワークフローとしては安全と考えられる食品試料（非ゲノム編集体）と分析対象の試料（ゲノム編集体等）の液体クロマトグラフィー質量分析データから得られる化合物イオンの比較定量値およびフラグメントスペクトルを解析データとして用いる。

試料から検出された化合物の構造・クラス推定のために、標準物質のフラグメントスペクトルの組織的な取得を行った。一般に公開されているフラグメントスペクトルデータベースとともに、標準物質を我々が実際に分析したデータ、さらに食品試料から抽出した代謝物のスペクトルデータも追加した統合フラグメントスペクトルデータベースを構築した。

試料由来のデータと統合フラグメントスペクトルデータベースを用い、網羅的にフラグメントスペクトル類似度を計算することで化合物の類似度を反映したネットワーク構造を創出するデータ解析フレームワークの構築を行った。（図１）

C.　研究成果

本研究では「B. 研究方法」で述べたデータ解析フレームワークに関し、プログラミング言語:Python を用い、ケモインフォマティクスライブラリ：RDKit、データ可視化ライブラリ：Plotly 等と連携させることでデータ解析環境の構築を行った。一般に広く用いられる質量分析データ処理フリーソフト（Mzmine2）からのフラグメントスペクトルおよび比較定量データのインポートを実装し、より広範囲の研究者が容易に利用可能になるデータ解析環境を実現した。

フラグメントスペクトルデータベースとしては従来の MassBank に加え､天然物に特化した GNPS、理研 ReSpect ライブラリを追加し、各化合物の化合物クラス情報等構造情報を付加したうえで統合データベース化した。さらに、独自で代謝物の標準品 300 種類を Q-exactive HF（Thermo Fischer

Science）で分析し in house ライブラリを作成した。

さらに、代表的な食品・モデル植物 30 種（大豆、トマト、ジャガイモ等）に関しては低分子化合物の抽出と Q-exactive HF によるスペクトルデータ取得を行った。生物種－代謝物関係データベース（KNApSACK）から試料ごとの代謝物情報を抽出し、スペクトルデータと照合することで標準試料由来のスペクトルデータベースを構築した。

この大規模なフラグメントスペクトルライブラリにより、全化合物スーパークラスを網羅し、広範囲の化合物クラスとその類似物質の検出が可能となった。（図２）この標準物質及び標準試料の質量スペクトルデータ取得は今後も継続することで、スペクトルデータベースの拡充を図る。

D. 考察

本研究の目的はゲノム編集作物等で想定外の質・構造的変化を生じる化合物の迅速な検出と構造の推定である。本年度では解析フレームワークの根幹の確立のためにフラグメントスペクトルライブラリの拡充にとくに力を入れることで、広範囲な化合物クラスの検出が可能となった。さらに質量分析定量データ解析ツール Mzmine2 と連携することで、試料間での代謝物の量的変動と統合した上でのデータ可視化が可能となり、他に類をみないユニークな解析ワークフローが確立できた。

検出された化合物が未知の化合物であることを想定した場合、実際の構造推定を行う上で構造類似性やフラグメントの構造の推定など、より詳細な構造情報をケモインフォマティクスとの連携により実現する必要があり、これは次年度の課題のひとつである。

また、本年度では一般的に広く使われている質量分析データ解析ツール Mzmine からの出力ファイルを読み込む機能を実装したことで、ユーザーが利用する上での技術的ハードルは低くなった。一方でデータの可視化と化合物クラス推定等の部分は Python スクリプトの編集が必要となるなど、現状では一般ユーザーには困難な部分が多い。次年度では GUI を実装するなど、より容易に利用可能なツールとして確立する予定である。

E. 結論

本年度の研究により、試料間比較による代謝物の変動と連携した化合物のクラス推定・可視化という解析フレームワークの根幹が確立されたと言える。本解析法では標準物質のフラグメントスペクトルライブラリが量・質的にも重要であり、今後も継続して拡充を進めていく。次年度の課題としては未知化合物の構造推定のための厳密な構造推定のためのアルゴリズムの先鋭化と利用が容易になるユーザーインターフェースの開発である。

F. 健康危険情報

なし

G. 業績

1. 論文発表

なし

2. 学会発表・講演

なし

H. 知的財産権の出願・登録状況

該当なし

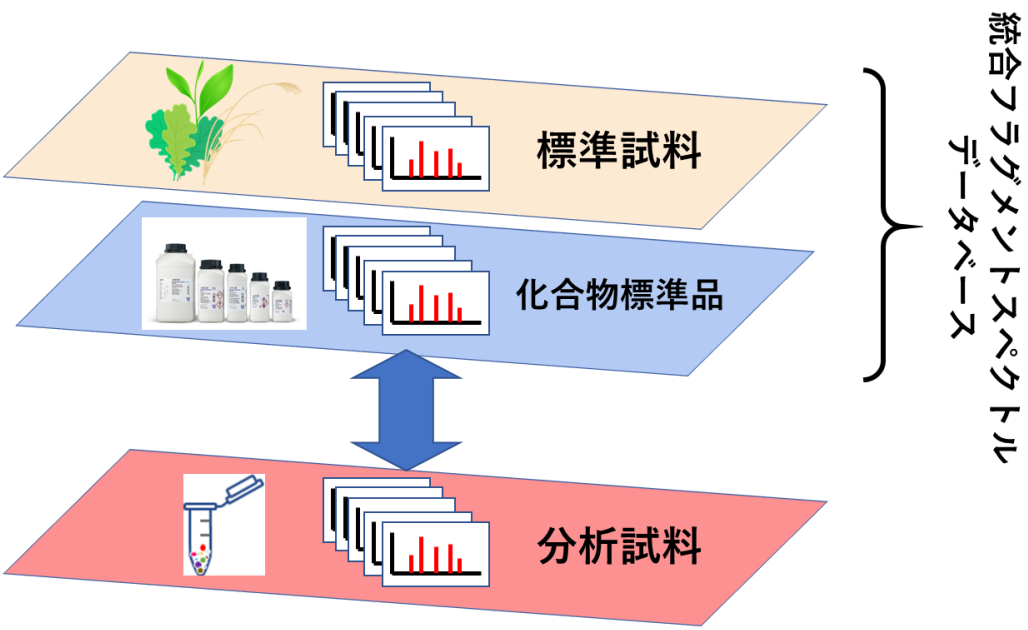


図 1　解析データ構造の模式図

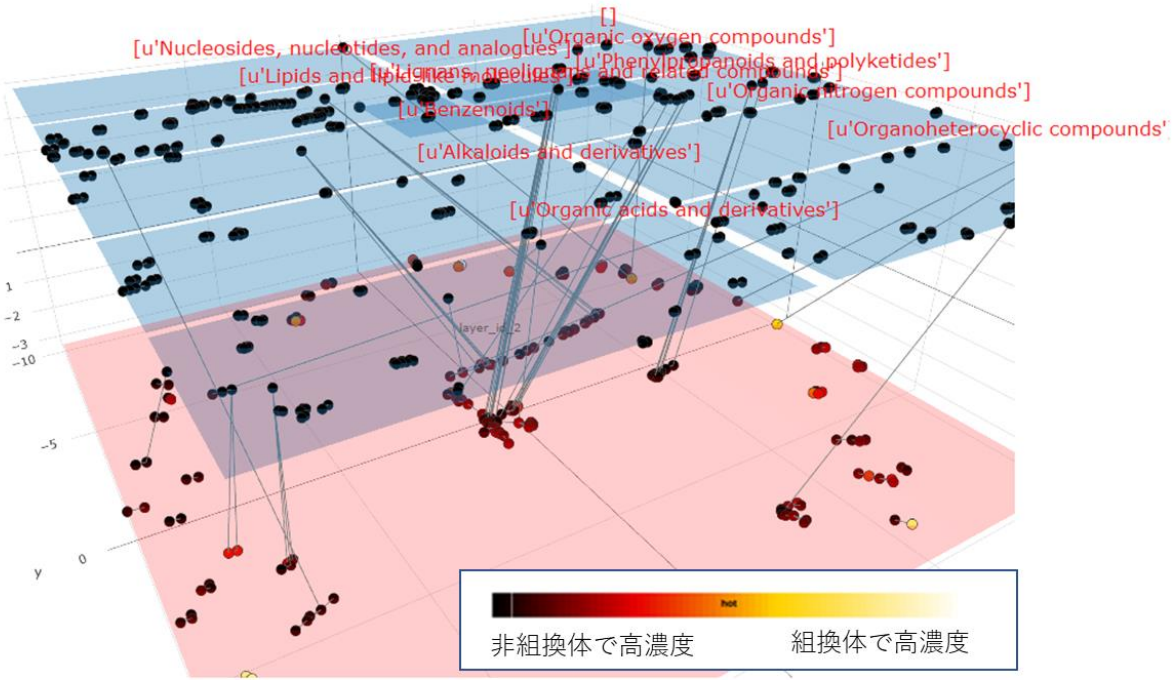


図 2　標準物質の大豆の組み換え体・非組み換え体の比較定量（下層）と
標準物質のフラグメントスペクトル（上層）

厚生労働科学研究費補助金（食品の安全確保推進研究事業）
「新たなバイオテクノロジーを用いて得られた食品の安全性確保と
リスクコミュニケーションのための研究」
分担研究報告書

高精度アレルゲン性予測システムの構築に必要な情報の収集

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研究要旨：
本研究では、バイオテクノロジーを用いて得られた食品のリスク管理に関する研究の一環として、アレルゲン性予測解析法の１つとして運用・公開しているアレルゲンデータベース(ADFS; Allergen Database for Food Safety)に、過去一年間で新たに報告されたアレルゲン及びエピトープ情報を追加し、データベースの更新作業を行った。その結果、アレルゲン及びイソアレルゲンのアミノ酸配列情報 40、及び、7 種のアレルゲンについて総数 21 のエピトープ情報が追加された。本年度の更新作業により、アレルゲン及びイソアレルゲンのアミノ酸配列情報は 2325 となり、エピトープ既知のアレルゲン数は 242 であった。また、アレルゲン予測評価システムに AI を搭載し予測精度を向上させることを目的として、使用する学習情報の追加収集、及び整理を行った。一方、ADFS サーバーのクロスサイトスクリプティング脆弱性について、昨年度に改善しきれなかった部分を改修するため、プログラミング言語を Jave から php に変更し、システムの全面的な再構築を行った。

研究協力者　安達 玲子　（国立医薬品食品衛生研究所）

A. 研究目的

現在、様々な遺伝子組換え食品が、生産性の向上や栄養付加を目的として開発されている。組換え食品の分野では、植物だけでなく、動物を宿主とした開発も進んでおり、また最近では、遺伝子組換え植物同士を交配して、付与された機能をスタックすることにより得られるスタック品種も開発されている。しかし、これらのようにバイオテクノロジーを利用して得られた品種について、どのような意図しない形質変化が出現するかを研究している例は少ない。したがって、新たに得られる遺伝子組換え生物について、非意図的な影響等を考慮し、安全性評価の方法等を検討する必要がある。

バイオテクノロジー技術を用いて開発された遺伝子組換え食品のリスクの 1 つの可能性として、アレルゲン性増大が考えられる。本研究では、アレルゲン性解析法の１つとして国立医薬品食品衛生研究所で管理・公開している、アレルゲン性の予測機能を装備したアレルゲン・エピトープ情報データベース(ADFS; Allergen Database for Food Safety)に関して、その情報内容を更新し、充実させることにより、遺伝子組換え食品のリスク管理の上で必須であるアレルゲン性評価系に関する研究を行う。また、アレルゲン予測システムに AI を搭載する事で、予測精度の飛躍的向上を試みる。

B. 研究方法

登録アレルゲン（アミノ酸配列情報）のアップデート

米国ネブラスカ大学リンカーン校が運営しているアレルゲンデータベース（AllergenOnline）における登録アレルゲンのアップデート内容を、ADFS に反映させた。

エピトープ情報の追加

2018 年 6 月から 2019 年 5 月までの 1 年間に NCBI PubMed に収載された論文から、キーワード検索により、エピトープ配列決定に関するものを抽出した。キーワードとしては、IgE、epitope、linear、conformational、sequence、recognition 等々のワードを使用し、これらを複数組み合わせて 6 通りの検索式を作成して検索を行った。この

検索により抽出されてきた論文についてピアレビューを行った。その結果エピトープ情報を報告していると判断された論文について、そのエピトープ情報を整理し、アレルゲンデータベース(ADFS)のデータに追加した。

C. 研究結果

登録アレルゲン（アミノ酸配列情報）のアップデート

米国ネブラスカ大学リンカーン校が運営しているアレルゲンデータベースである AllergenOnline は、登録アレルゲンの全てが国際的なアレルギーの専門家チームによるピアレビューを経ており、登録タンパク質がアレルゲンであるというエビデンスの信頼性が非常に高いデータベースである（但しエピトープ情報は含まない）。ADFS における登録アレルゲンは平成 20 年度に AllergenOnline の登録アレルゲンと統合し、その後も AllergenOnline のアップデートに伴って ADFS 登録アレルゲンのアップデートを行っている。令和元度においても引き続きこのアップデート作業を実施した。

エピトープ情報の追加

エピトープ配列に関しては、キーワード検索により抽出された論文は 20 報であった。要旨を確認し、その中からアレルゲン・エピトープ情報が記載されていると思われる 10 報を選択し、ピアレビューを行った。その結果、7 報の論文（表１）から 7 種のアレルゲンについて、総数 22 のエピトープ情報を新たに追加した（表２）。

上記のアレルゲン及びエピトープ情報更新作業により、ADFS のアレルゲン及びイソアレルゲンのアミノ酸配列情報は 2325、エピトープ既知のアレルゲン数は 242、構造既知のアレルゲン数は 163、糖鎖付加アレルゲン数は 131 となった。

AI 学習用データセットの準備

昨年度に、米国環境保健科学研究所が組織する HESI が公開している包括的な既知あるいは推定アレルゲン蛋白配列のレポジトリ“COMprehensive Protein Allergen REsource (COMPARE)”から、アレルゲンタンパク情報（2038 種）を入手し、わが国で加工食品へのアレルギー表示が義務付けされている特定原材料 7 品目（卵、乳、落花生、そば、小麦、えび、かに）並びに推奨されている原材料のうち 4 品目（いくら、さけ、キウイフルーツ、大豆）の非アレルゲンタンパク配列情報について UniProt から 10577 種を入手した。これらのアレルゲン・非アレルゲンデータを学習させる際、より効率的な各パターンのマイニングを実行するため、生物種によって分類できるよう目情報を追加し、情報を整理した。また、非アレルゲン学習データの種類を増やして解析ができるように、アレルゲンとして登録された全ての種について情報を取得し解析できるよう調整した。

ADFS 脆弱性の対応

ADFS は、「OpenBugBounty」のウェブサイト上において、不正なスクリプトを挿入することが出来る環境にあり、エンドユーザーは不正スクリプトを利用してサイバー攻撃（クロスサイトスクリプティング）を受ける可能性があると昨年末に公表された。そこで、当初より予定していた Java ソースのリコンパイルと Java フレームワークの改良、そして Mysql 等のミドルウェアのバージョンアップに加え、apache や tomcat に対して SLL 設定を実施し、インターネット上でのデータ通信を暗号化し、クロスサイトスクリプティングに対応したウェブアプリケーションにすべく緊急対応したが、脆弱性を根本的に解決するにはプログラミングの再構築が必要である事が明らかとなった。そこで本年度は、ADFS のプログラミングを java から php に変更し、OS は最新の RedHat8 にバージョンアップした。また相同検索ツールのプログラム（Blast、FASTA、PfTools）も全て最新のバージョンに更新した。加えて、HTML5 準拠に使用を変更し、来年度以降に行うデザインの改修（図１）についても対応できるよう準備した。

D. 考察

令和元年度においては、アレルゲン及びイソアレルゲンのアミノ酸配列情報を 40 種追加、また、7 種のアレルゲンについて総数 22 個のエピトープ情報を ADFS に追加した。本研究により、遺伝子組換え食品のアレルゲン性に関する評価・予測系を充実させることができ、現在までに既に開発されている遺伝子組み換え食品、及び多様化するバイオテクノロジー技術により今後作製される新規遺伝子組換え食品のアレルゲン性を、より高

い精度で評価・予測することが可能となっている。また、本年度の改修により昨年末から危惧されていた脆弱性については対策を整え、ユーザーの ADFS 利用に際してのセキュリティを向上させることができた。

E. 業績

1. 論文発表
   なし

2. 学会発表
   なし

F. 知的財産権の出願・登録状況

該当なし

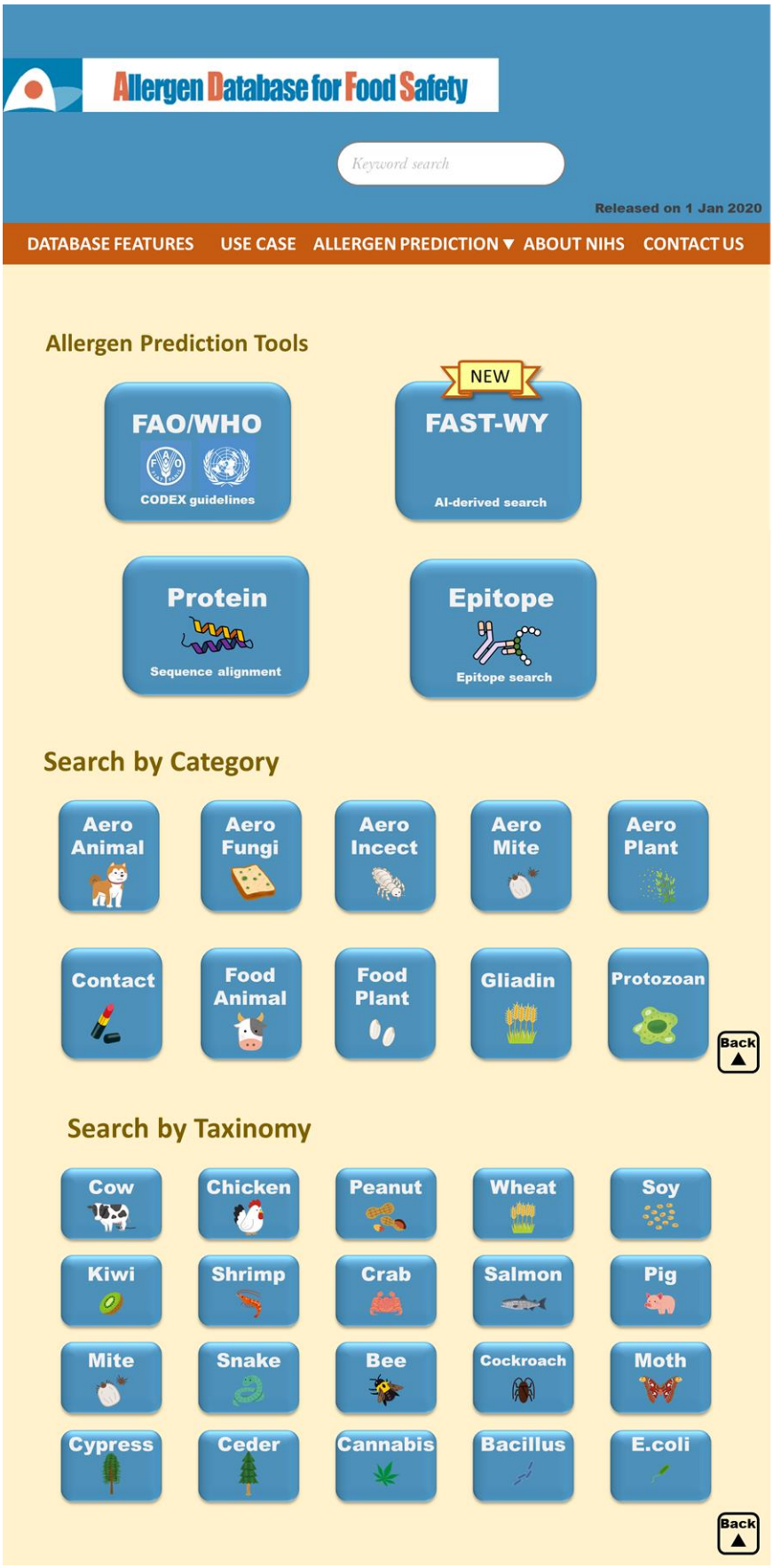
表１　令和元年度ピアレビューによりエピトープ情報を収集した論文

1.	Liu GY, Mei XJ, Hu MJ, Yang Y, Liu M, Li MS, Zhang ML, Cao MJ, Liu GM. Analysis of the Allergenic Epitopes of Tropomyosin from Mud Crab Using Phage Display and Site-Directed Mutagenesis. *J Agric Food Chem.* 2018 Aug 29;66(34):9127-9137. PMID:30107732
2.	He S, Zhao J, Elfalleh W, Jemaà M, Sun H, Sun X, Tang M, He Q, Wu Z, Lang F. In Silico Identification and in Vitro Analysis of B and T-Cell Epitopes of the Black Turtle Bean (Phaseolus Vulgaris L.) Lectin. *Cell Physiol Biochem.* 2018;49(4):1600-1614. PMID:30223257
3	Kern K, Havenith H, Delaroque N, Rautenberger P, Lehmann J, Fischer M, Spiegel H, Schillberg S, Ehrentreich-Foerster E, Aurich S, Treudler R, Szardenings M. The immunome of soy bean allergy: Comprehensive identification and characterization of epitopes. *Clin Exp Allergy.* 2019 Feb;49(2):239-251. PMID:30267550
4	Lahiani S, Dumez ME, Bouaziz A, Djenouhat K, Khemili S, Bitam I, Gilis D, Galleni M. Immunodominant IgE Epitopes of Der p 5 Allergen. *Protein Pept Lett.* 2018;25(11):1024-1034. PMID:30430936
5.	Cai ZL, Chen JJ, Zhang Z, Hou YB, He YS, Sun JL, Ji K . Identification of immunodominant IgE binding epitopes of Der p 24, a major allergen of *Dermatophagoides pteronyssinus*. *Clin Transl Allergy.* 2019 May 23;9:28. PMID:31139345
6.	Yamamoto K, Ishibashi O, Sugiura K, Ubatani M, Sakaguchi M, Nakatsuji M, Shimamoto S, Noda M, Uchiyama S, Fukutomi Y, Nishimura S, Inui T. Crystal structure of the dog allergen Can f 6 and structure-based implications of its cross-reactivity with the cat allergen Fel d 4. *Sci Rep.* 2019 Feb 6;9(1):1503. PMID:30728436
7.	Fang L, Li G, Zhang J, Gu R, Cai M, Lu J . Identification and mutational analysis of continuous, immunodominant epitopes of the major oyster allergen Crag 1. *Clin Immunol.* 2019 Apr;201:20-29 PMID: 29319884

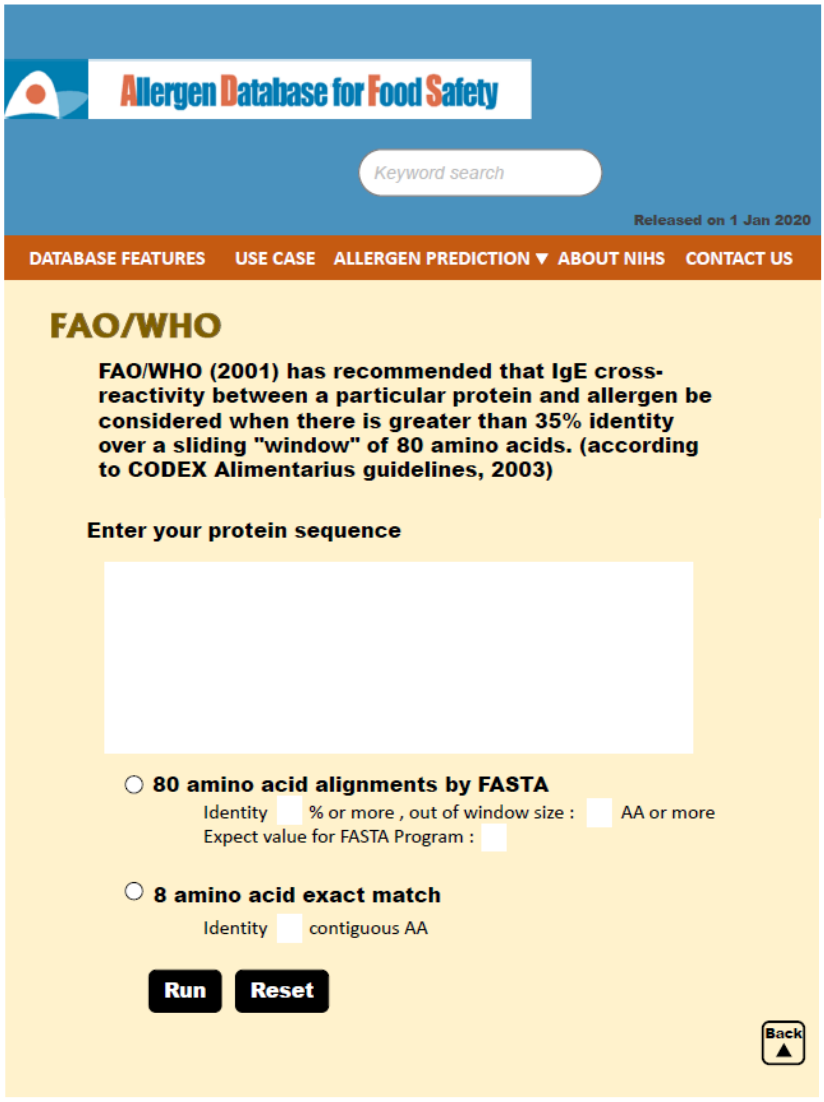
表２　令和元年度新たに ADFS に追加したエピトープ情報

	Name	start	end	Sequence	Method	CTYPE	Reference	UniProt acc.No
001	Scy s 1	44	55	ATQKKMQQVEN	Phage display/ Dot blotting/ ELISA	L	PMID 30107732	A7L5V2
	Scy s 1	105	112	RLNTATTK	Phage display/ Dot blotting/ ELISA	L	PMID 30107732	A7L5V2
	Scy s 1	133	140	RSLSDEER	Phage display/ Dot blotting/ ELISA	L	PMID 30107732	A7L5V2
	Scy s 1	143	152	ALENQLKEAR	Phage display/ Dot blotting/ ELISA	L	PMID 30107732	A7L5V2
	Scy s 1	199	206	VVGNNLKS	Phage display/ Dot blotting/ ELISA	L	PMID 30107732	A7L5V2
	Scy s 1	253	264	VDRLEDELVNEK	Phage display/ Dot blotting/ ELISA	L	PMID 30107732	A7L5V2
	Scy s 1			R90 ,E164, Y267	Phage display/ Dot blotting/ ELISA	C	PMID 30107732	A7L5V2
002	Pha v ?	55	66	NVNDNGEPTLSS	ELISA/ lymphocyte proliferation/ cytokine profile analyses	L	PMID 30223257	V5QN77
	Pha v ?	116	125	VGSEPKDKGG	ELISA/ lymphocyte proliferation/ cytokine profile analyses	L	PMID 30223257	V5QN77
	Pha v ?	133	141	NNYKYDSNAHT	ELISA/ lymphocyte proliferation/ cytokine profile analyses	L	PMID 30223257	V5QN77
	Pha v ?	149	160	LYNVHWDPKPRH	ELISA/ lymphocyte proliferation/ cytokine profile analyses	L	PMID 30223257	V5QN77
	Pha v ?	95	103	FNIDVPNNS	ELISA/ lymphocyte proliferation/ cytokine profile analyses	L	PMID 30223257	V5QN77
	Pha v ?	39	47	LQRDATVSS	ELISA/ lymphocyte proliferation/ cytokine profile analyses	L	PMID 30223257	V5QN77
003	Gly m 2	21	27	QVVVQTE	Peptide phage display/peptide microarray	L	PMID 30267550	Q07502
004	Der p 5	90	108	DRLMQRKDLDIFEQYNL EM	peptide microarray/ alanine scanning mutagenesis	L	PMID 30430936	P14004
005	Der p 24	1	32	MVHLTKTLRFINNPGFR KFYYGLQGYNKYGLY	peptide microarray	L	PMID 31139345	A0A0K2GUJ4
006	Can f 6	28	59	DISKISGDWYSILLASDIK EKIEENGSMRVFV	ELISA/ alanine scanning mutagenesis	L	PMID 30728436	H2B3G5
007	Cra g 1	44	49	TSLQKK	ELISA/ amino acid substitution	L	PMID 30807831	B7XC66
	Cra g 1	69	85	TKLEEAEKTASEAEQEI	ELISA/ amino acid substitution	L	PMID 30807831	B7XC66
	Cra g 1	99	108	MERSEERLQT	ELISA/ amino acid substitution	L	PMID 30807831	B7XC66
	Cra g 1	134	144	NNASEERTDVL	ELISA/ amino acid substitution	L	PMID 30807831	B7XC66
	Cra g 1	209	224	VQNDQASQREDSYEET	ELISA/ amino acid substitution	L	PMID 30807831	B7XC66

図１．ADFS サイトデザイン案



（続）図１．ADFS サイトデザイン案



（続）図１．ADFS サイトデザイン案

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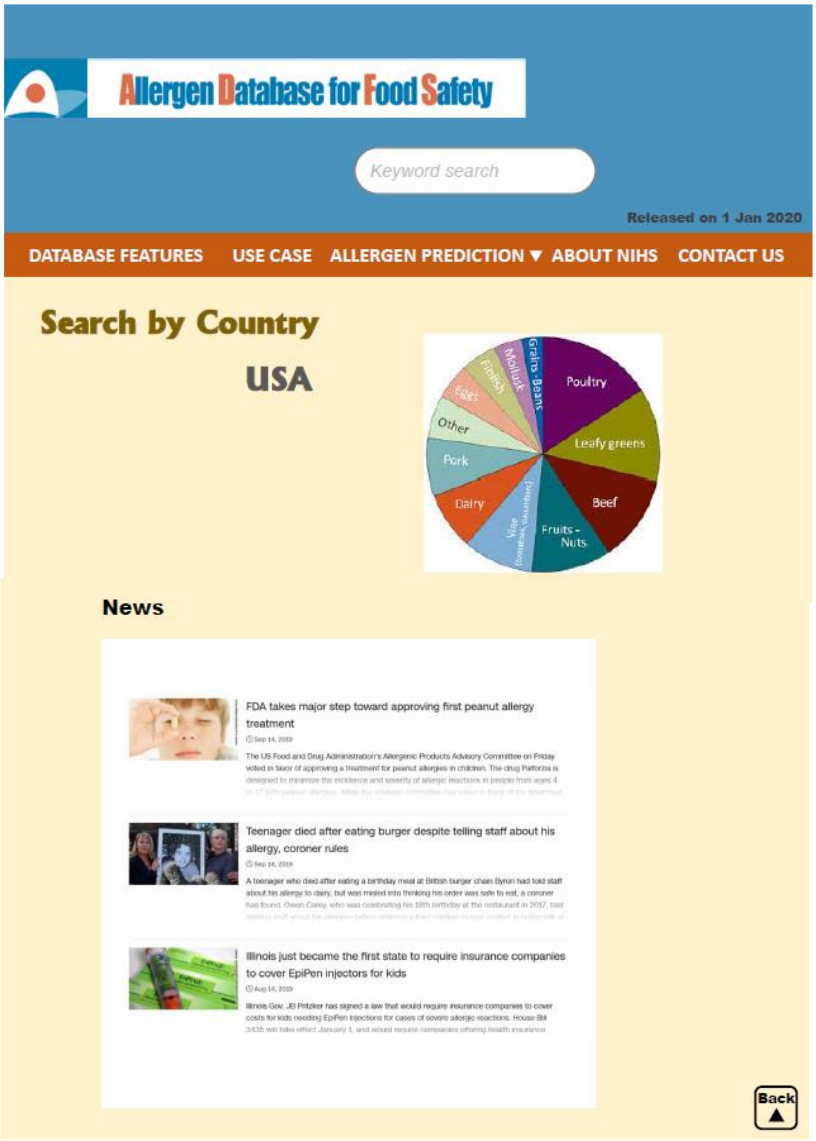

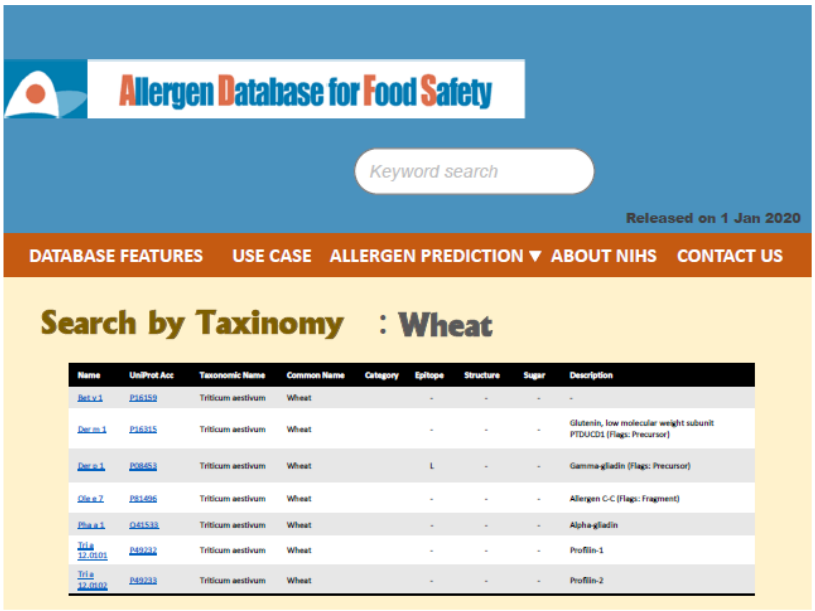

厚生労働科学研究費補助金（食品の安全確保推進研究事業）
「新たなバイオテクノロジーを用いて得られた食品の安全性確保と
リスクコミュニケーションのための研究」
分担研究報告書

人工知能を用いたアレルゲン性評価のためのアルゴリズム開発

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研究要旨：
ゲノム編集技術などを用いて人工的に生成した食品のアレルゲン性を確認する方法は明らかになっていない。アレルゲン性の主要な識別子とされる単一の因子は知られておらず、複数の因子が複雑に関連することでアレルゲン性を持つことが示唆されている。また、人工的に生成された食品のアレルゲン性を都度に実験的に検証するのは様々なコストがかかり、現実的でない。そこで、本課題では、人工知能やデータ科学のアプローチを用い、食品のアレルゲン性を高精度、高信頼度で汎用的に判定・予測できるシステムを開発することを目指す。これまでに、様々なアレルゲン性を判定・予測のための分析ツールが開発されてきた。残念ながら、これら既存の方法には様々な問題点が存在する。国際連合食糧農業機関/世界保健機関によるガイドラインはアミノ酸配列の類似性に基づく規準であり、精度が低く、大規模データの分析には適していない。また、既知の IgE エピトープに基づく規準、タンパク質構造の物理化学的表現に基づく規準、アミノ酸/ジペプチド組成物に基づく規準など、タンパク質に関する生物科学的・物理化学的な知見に基づく単一、もしくは少数の因子を採用したツールが提案されているが、これらはアレルゲン物質の多様性を十分に反映できるものとなっていない。本研究では、以下の３つの課題に取り組む：(課題 1) 既存のデータベースを拡張し、アレルゲンタンパク質と非アレルゲンタンパク質のデータベースを作成する。(課題 2) 課題 1 で作成したデータベースをもとに、アレルゲン特異的なパターン（アミノ酸配列）を統計学的手法により抽出する。(課題 3) 課題 1 で作成したデータベースと課題 2 で抽出したパターンをもとにアレルゲン性判定モデルを人工知能・機械学習手法により作成する。課題 1 に関して、2018 年度は食品種目のアレルゲン性、および、非アレルゲン性タンパク質を含むデータベースを構築したが、2019 年度はこれに非食品アレルゲン性タンパク質を追加し、データベースの大規模化、高精度化を行った。課題 2 に関して、2018 年度はアレルゲンタンパク質と非アレルゲンタンパク質それぞれに特異的なパターンを抽出していたが、2019 年度は特定の種や目に限定しないパターンを抽出できるように手法の改良を行った。課題 3 に関して、2018 年度は汎用的な 2 クラス分類モデルを用いていたが、2019 年度はデータベースの特徴を考慮した本研究課題に特化した機械学習法を開発した。本研究におけるアレルゲン性判定・予測システムの概要を図 1 に示す。

A. 研究目的

ゲノム編集技術を用いた人工的な農産物の合成が行えるようになり、これまでにない食用タンパク質製品が登場する可能性がある。新たに合成された食用タンパク質は未知の特性を有しており、特定の人が摂取するとアレルギー反応が起こってしまうリスクがある。免疫反応においてタンパク質抗原のアミノ酸配列のうち、抗体が結合する部位をエピトープと呼び、エピトープを認識する抗体を人が持っている場合にアレルギー反応が引き起こされる。これまでの様々な研究から、いくつかのアレルゲンタンパク質において共通のエピトープ配列が見出されているが、アレルゲン性の単一因子は知られておらず、複数の因子が複雑に関連することでアレルゲン性を持つことが示唆されている。既存のアレルゲン性判定・予測ツールのうちもっとも基本的なアプローチはアレルゲン性を持つタンパク質とのアミノ酸配列の類似性（アミノ酸配列相同性）に基づくものである。しかし、このようなアプローチは偽陽性が高いことが指摘されており、ゲノム編集技術によって合成される新規タンパク質のアレルギー性判定には十分でない。また、別のアプローチとしては、タンパク質に含まれるアミノ酸の物理化学的な特徴の統計量

に基づいてアレルゲン性を判定する試みもなされている。このようなアプローチではアミノ酸の順序や位置関係を適切に考慮できないため、十分な精度ではないことが確認されている。アミノ酸配列パターンを用いたアプローチとして、Alledictor と呼ばれる方法が提案されたが、この方法では一定の長さのアミノ酸配列のみを抽出するものであり、すべてのエピトープを網羅できるようなものではない。このような背景のもと、本課題では人工知能や機械学習のアプローチを用い、食品のアレルゲン性を高精度で高信頼度で汎用性のあるアレルゲン性判定・予測が行えるシステムを開発することを目指す。本研究では、まず、食品タンパク質の大規模データベースを整備し、アレルゲン特異的な様々な長さのアミノ酸配列を抽出し、これらに基づいてアレルゲン性判定・予測システムを構築する。さまざまな数理技術、情報技術を活用することで高精度で信頼性が高く汎用性のあるアレルゲン性判定・予測システムを開発することを目的とする。2018 年度は、アレルゲン性判定・予測システムのプロトタイプを作成し、その高精度化、高信頼度化、汎用化に向けた問題抽出を行った。2019 年度は 2018 年度のプロトタイプの問題点を列挙し、それぞれを解決するための新たな数理技術、情報技術の開発を行った。

（課題１）人工知能や機械学習で判定・予測システムを構築するには訓練データベースが必要である。既存のアレルゲン性判定・予測システムで使われていたデータベースはアレルゲンタンパク質のみを用いたものであった。人工知能や機械学習では正例（positive example）だけでなく、負例（negative example）もあると有効なため、後者をデータベースに追加する必要がある。負例の追加では、アレルゲン性とは無関係のタンパク質データベースを取得し、そこからアレルゲン性のあるものを取り除く作業により行った。本データベースにおいて注意すべき問題は、アレルゲン性タンパク質数（正例数）と非アレルゲン性タンパク質数（負例数）に偏りがあることである。正例は生物学的な実験によって判定されたものであるため数が少なく、負例は通常のタンパク質データベースから大量に取得できる。一方、通常のタンパク質データベースから大量に取得した負例には誤陰性（False Negative）が多く含まれてしまうため、なんらかの対処が必要である。また、正例と負例の数が食物種目ごとにバラつきがある場合、特定の食物種目に特化したアミノ酸配列がアレルゲン性特異的なアミノ酸配列と誤って発見されてしまうリスクが生じる。2018 年度には 11 の食品種目のアレルゲンタンパク質と非アレルゲンタンパク質の訓練データベースを作成した。しかしながら、正例数が十分でないため、2019 年度はさらに非食品タンパク質においてアレルゲン性を持つことがわかっているタンパク質を正例として追加する。

（課題２）人工知能や機械学習でタンパク質の物性を判定・予測するにはタンパク質の特徴を機械学習が使える数値データとして抽出しなくてはならない。生物情報学で採用されているアプローチとして主に２通りのものがある。１つ目のアプローチは、タンパク質を構成するアミノ酸の物理化学的な特徴（疎水性、分子量など）を求め、その平均、分散、相関などを特徴として抽出することである。２つ目のアプローチは、アミノ酸の部分配列のうち、特定の物性を有するタンパク質に特化して頻出する部分配列を特徴として抽出することである。アプローチ１ではアミノ酸の順序や位置を考慮できないため、本研究ではアプローチ２を採用する。また、一般に、機械学習における特徴抽出は、教師なし学習と教師あり学習の２つのアプローチが存在する。本研究においては、前者はアレルゲン性タンパク質の情報のみから特徴抽出を行うことに相当し、既存のアレルゲン性判定・予測システムの多くではこのアプローチを採用されている。本研究では、より判定・予測に有用な特徴を抽出するため、教師あり特徴抽出のアプローチを採用する。2018 年度には食品タンパク質のみを扱っていたため、我々のグループが別の目的で既に確立した方法をそのまま適用することができた。2019 年度は非食品のアレルゲン性タンパク質を正例として追加したため、その対処が必要である。これは、既存の教師あり特徴抽出法を用いると、特定の非食品タンパク質に特化したアミノ酸部分配列がアレルゲン性特異的アミノ酸配列として誤って抽出されてしまうためである。

（課題３）正例と負例を含む訓練データベースを用いて、正負が未知の事例を判定・予測する問題は教師あり学習（supervised learning）と呼ばれている。アレルゲン性タンパク質を正例、非アレ

ルゲン性タンパク質を負例とみなせば、本研究課題は典型的な教師あり学習問題と解釈できるが、いくつか本研究課題特有の課題を解決する必要がある。まず、本課題の１つ目の特徴は訓練データベースに含まれるタンパク質が独立同一分布（ i.i.d.； independently, identically distributed）に従わない点である。この場合、通常の教師あり学習で多用されるクロスバリデーションなどのリサンプリング法をそのまま利用することができず様々な工夫が必要となる。また、正例数と負例数に偏りが生じてしまう点も本課題の特徴であり、注意深く対処する必要がある。本研究で用いるデータベースにおいて、食品タンパク質に関しては正例が負例に比べて極端に少なくなってしまっており、非食品タンパク質に関しては正例のみが存在する状況になってしまっている。また、アレルゲン性の原因となるエピトープはさまざまな長さであることが知られているため、さまざまな長さのアミノ酸部分系列特徴を抽出できるような工夫が必要である。さらに、アレルゲン性の判定は統計的信頼性が担保されたものである必要があるため、抽出された特徴の信頼性定量化を行う必要がある。加えて、特定の食品種目に特化したものでなく、一般的な特徴を抽出するための工夫が必要である。2018 年度では、訓練データベースが独立同一分布（IID）に従わない点と食品タンパク質における正例と負例の偏りを考慮したモデル作成法を構築した。2019 年度では、さらに非食品タンパク質を訓練データベースに追加した際の対処法を検討した。

B. 研究方法

課題１の訓練データベースの構築においては、アレルゲン性を持つ食品タンパク質の正例として COMPARE データベースのものを利用した。同じくアレルゲン性のない食品タンパク質の負例として UniProt データベースより取得した。UniProt データベースは汎用的なタンパク質データベースであるため、アレルゲン性を持つものも含まれている。そのため、既存のエピトープを含むもの、アレルゲンに関連するキーワードが付記されているものなどを削除した。またプロトタイプとして作成したアレルゲン性判定・予測システムにおいて偽陽性であったタンパク質に関して個別にデータベースを精査し、アレルゲン性を持つ可能性があるものは削除するなどの措置をとった。後述のように、課題２、３においては食品種目の情報を活用するため、食品種目分類の精査を行い、あいまい性のあるタンパク質はデータベースから削除するプロセスを行った。その他にもプロトタイプシステムや諸々のタンパク質データベースを活用することで訓練データベースの大規模化と高精度化を実現した。上述のように、本データベースに含まれる事例（タンパク質）は独立同一分布（IID）に従わないので、食品種目ごとにデータ分割を行う Leave-Food-Out クロスバリデーションと呼ぶ方法に基づいてデータ分析を実施した。2018 年度では、データベースが食品タンパク質のみから構成されていたが、2019 年度には非食品タンパク質も追加した。なお、非食品タンパク質でアレルゲン性のないものを網羅的に収集するのは困難であることが判明したため、本研究では、非食品タンパク質に関しては、アレルゲン性を有する正例のみを扱うこととした。

課題２の特徴抽出においては、本研究に特化したさまざまな工夫を行った。まず、異なる長さのアミノ酸部分配列を抽出できるようにするため、分担者の竹内らが開発したデータマイニング分野の技術を利用した。系列データから特定の性質を持つ部分系列を抽出する技術は系列マイニングと呼ばれ、さまざまな方法が提案されている。系列マイニングでは、系列を木構造と呼ばれるデータ構造で表現し、枝刈りと呼ばれる手順を導入することにより、膨大な部分系列から、特定の性質を満たすものを探索することができる。本研究の基本的な方針は、アレルゲン性タンパク質に高頻度で含まれ、非アレルゲン性タンパク質には低頻度でしか含まれない（あるいはまったく含まれない）ような部分配列を探索することである。頻度の違いを定量化する指標には様々なものがあるが、本研究ではフィッシャーの正確検定（Fisher Exact Test）に基づく指標を利用した。

例えば、20 種類のアミノ酸において長さ 10 までのアミノ酸の種類は 10 の 20 乗となり、その頻度を数えたデータテーブルを作ることは実質的に不可能である。

分担者の竹内らは、系列マイニングにおける木構造の枝刈りをフィッシャーの正確検定と統合する方法開発した（Sakuma et al., KDD2018）。詳細は割愛するが、この方法では、統計的に有意となり得ない部分配列を木構造の枝刈りによって排除できるため、膨大な数の候補から予測に最適な部

分配列を選択することができる。また、アレルゲン性予測モデルの信頼性を高めるため、統計的な有意性を持つ部分配列のみを用いることが望ましい。ある部分配列の出現頻度がアレルゲン性タンパク質と非アレルゲン性タンパク質で異なるかどうかの統計的検定を行う場合、フィッシャーの正確検定のｐ値(p-value)を利用することができる。しかしながら、膨大な部分系列の候補のなかから特に頻度の違いの大きなものを抽出してきた場合、選択バイアスが生じてしまい、所望の誤検出率を制御できなくなる。この選択バイアスの問題は多重検定問題（multiple hypothesis testing）と呼ばれており、その補正を行うためにはフィッシャーの正確検定によって得られたｐ値を適切に補正しなくてはならない。もっともよく使われている多重検定補正にボンフェローニ補正（Bonferroni correction）と呼ばれるものがあるが、選択における候補数が多い場合、補正が保守的になってしまう問題点が指摘されている。本研究ではこの問題に対処するため、Westfall Young 法と呼ばれるランダム化に基づく方法を採用した。これらの方法の開発と本データベースへの適用は主に 2018 年度に行ったが、2019 年度もアルゴリズムの改良や新たなデータへの適用などを行った。

2019 年度は、主に、非食品タンパク質においてはアレルゲン性を持つ正例のみがデータベースに含まれる点を考慮して特徴抽出を行った。この点を特に考慮せずに通常の機械学習アルゴリズムを適用すると、アレルゲン特異的でなく、非食品タンパク特異的なパターンが誤って検出されてしまう。この問題を回避するため、アレルゲン特異的パターンとして、条件 1） 食品タンパク質に含まれるか、条件 2） 非食品タンパク質のうち複数の目に含まれる、のどちらかの条件を満たすもののみを抽出することとした。2020 年度には、諸々のタンパク質データベースを活用し、非食品タンパク質でアレルゲン性を持たないものをデータベースに加えることができないか検討を進める。

課題３のアレルゲン性判定・予測システムの構築は上述の Leave-Food-Out クロスバリデーションを利用した教師あり学習によって行った。アミノ酸部分配列パターンを特徴として抽出したため、テスト対象のタンパク質がパターンを含むか否かをバイナリ表現した線形分類器をベース手法として採用した。パターン数が多いと解釈性が低く過学習のリスクがあるため、スパース正則化や二次正則化（Ridge Regression）を導入した。2018 年度は主にこのプロトタイプモデルに基づく考察を行った。2019 年度は、さらに、パターンが完全に含まれる（exact match）だけでなく、パターンが部分的に類似している場合（non-exact match）も考慮できるような工夫を導入した。20 種のアミノ酸の物理化学的な特徴に基づいてアミノ酸種間の類似度を定義し、タンパク質にパターンが含まれる程度を連続量として定量化した。さらに、2019 年度は、さらに、抽出されたパターンの生物学的な考察として、既存のエピトープとの一致度の確認や、結合性の確認なども行った。

C. 研究結果および考察

2019 年度は 2018 年度に構築したアレルゲン性判定・予測システムのプロトタイプの課題を抽出し、その精度、信頼性、汎用性を向上させるための様々な工夫を行った。

図 2 はアレルゲン性判定・予測システムを作成する際に利用する Leave-Food-Out クロスバリデーションの概要を示したものである。このような工夫をしないと、特定の食物に頻出するアミノ酸部分配列を誤ってアレルゲン特異的パターンとして抽出してしまうリスクが高まる。各食物種をまるごと削除した訓練データを作成して判定・予測システムを作成し、それを削除した食物種のタンパク質のアレルゲン性判定・予測に使うことで、偏りのない判定・予測精度を知ることができる。

図 3 はアレルゲン特異的パターンとして抽出されたパターンを示している。図の各行はアレルゲン性を持つタンパク質のアミノ酸配列を表しており、赤色の部分がアレルゲン特異的パターンとして抽出されたアミノ酸部分配列を表している。図より、アレルゲン性タンパク質が多くのアレルゲン特異的パターンを含んでいることがみてとれる。実際、これらのアレルゲン特異的パターンの生物学的特徴を調べたところ、既知のエピトープと類似していることが確認されている。2020 年度に、さらにこれらの抽出されたパターンの生物学的な分析を行う。

図 4 は 11 種の食物種それぞれに対してアレルゲン性判定・予測を行ったときの ROC 曲線を示している（それぞれのアレルゲン性予測・判定システムは、Leave-Food-Out クロスバリデーションにより、評価対象の食物を一切使わずに作成されて

いることに注意)。従来法を含む複数の判定・予測システムの結果が示されているが、本研究で構築した方法ではおおむねすべての場合において最もよい判定・予測性能を示している。2020 年度はさらにほかのアプローチとの比較も行うことで本システムの有効性の実証を行う予定である。

D. 結論と今後の展望

2019 年度は、2018 年度に構築したアレルゲン性判定・予測システムのプロトタイプにおいて問題点を抽出し、様々な改良を加えた。結果として、訓練データベースの大規模化と高精度化、アレルゲン特異的パターンの信頼性向上、判定・予測システムの精度向上が可能となった。2020 年度は、これまでの取り組みを論文としてまとめるとともに、予測・判定システムの実装を行う。

E. 業績

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F. 知的財産権の出願・登録状況

該当なし

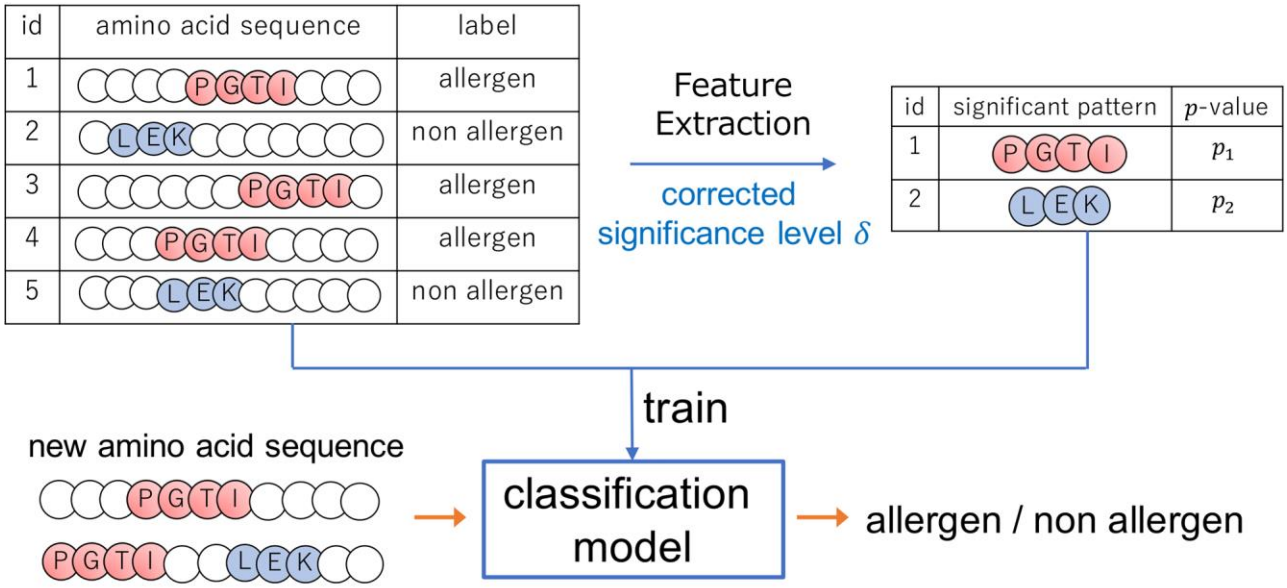

図１　アレルゲン性判定・予測システム構築の全体像

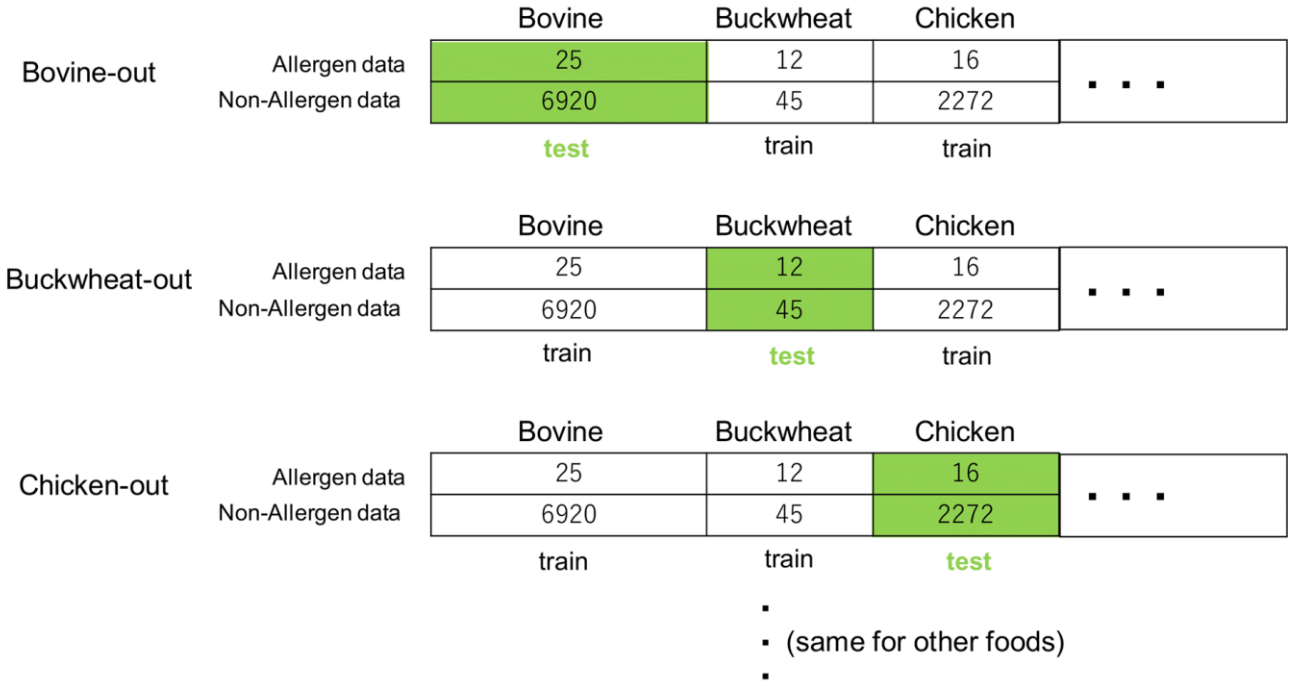

図２　訓練データの非独立同一分布性を考慮した評価方法の概略

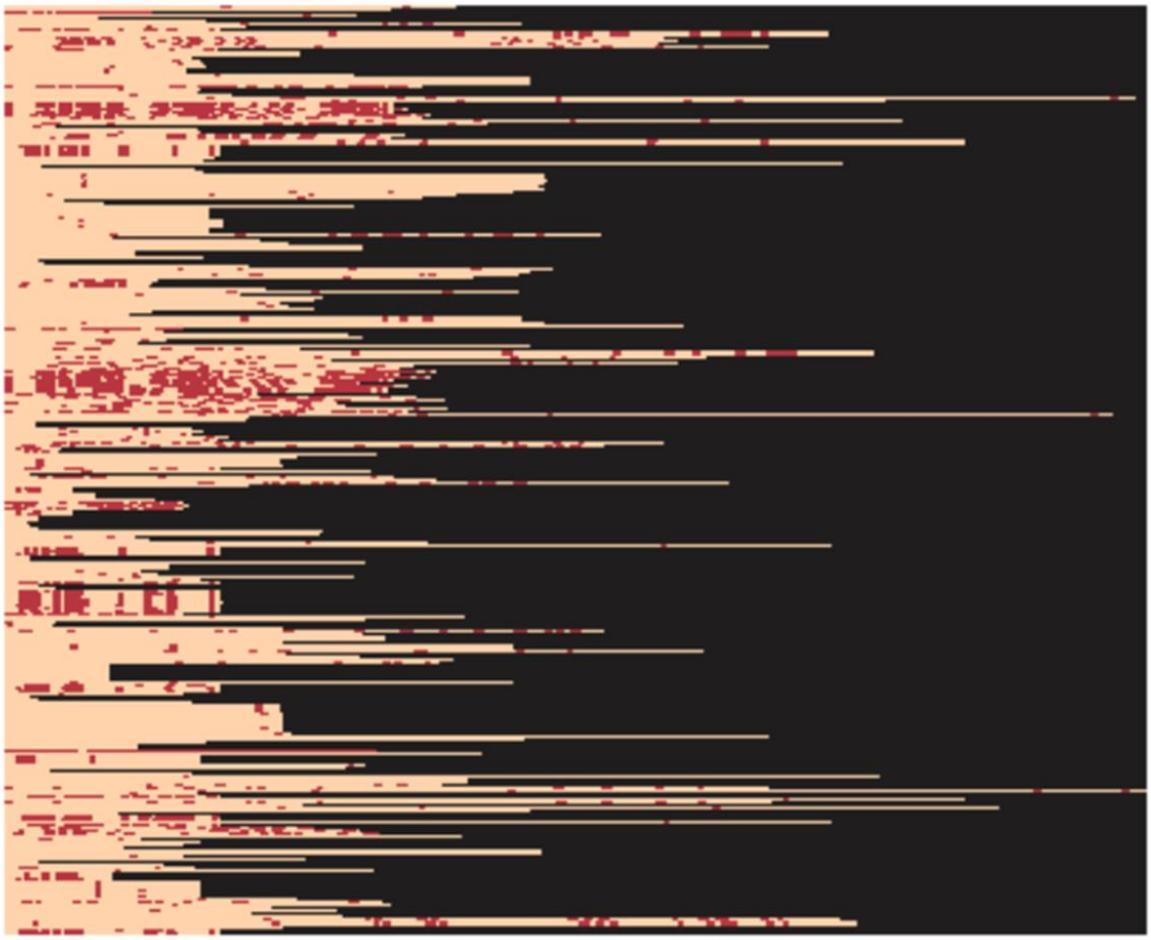

図３　抽出されたアレルゲン特異的パターン（アミノ酸部分配列）の例

各行がアレルゲン性タンパク質を表し、赤くハイライトされている部分がアレルゲン特異的パターンを表している。

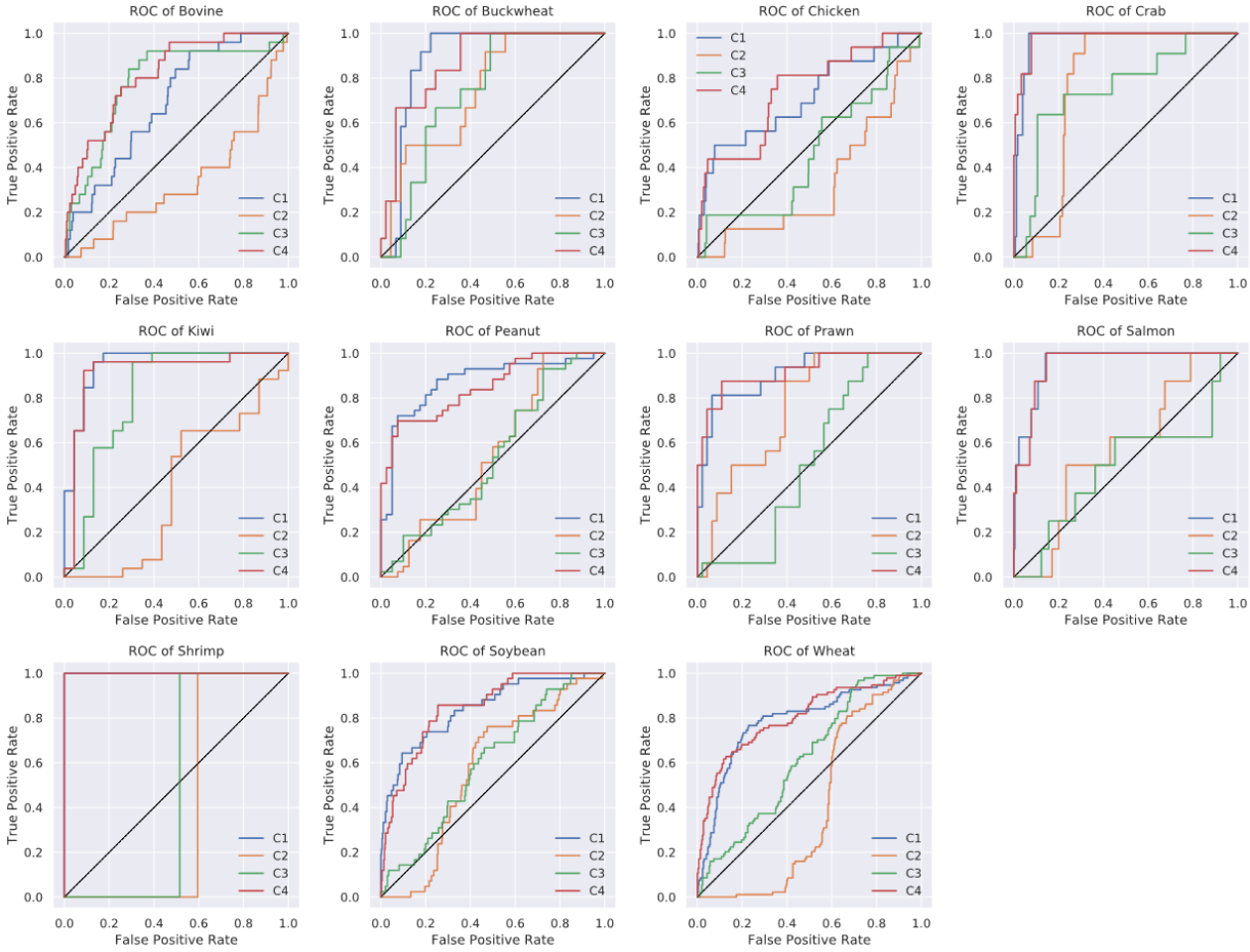

図４　11 種の食品種ごとのアレルゲン性判定・予測結果の AUC 曲線の例
（複数の線は比較した複数の手法に対応）

厚生労働科学研究費補助金（食品の安全確保推進研究事業）
「新たなバイオテクノロジーを用いて得られた食品の安全性確保と
リスクコミュニケーションのための研究」
分担研究報告書

ゲノム編集生物作製における現象解析と規制の進め方

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研究要旨：
我々は、これまでにゲノム編集技術（CRISPR/Cas9）を用いて、増肉形質を示すミオスタチン遺伝子破壊マダイ、高成長形質を示す食欲関連遺伝子破壊トラフグ系統の作製に成功している。本研究では、これらのゲノム編集養殖魚を用いて、非ゲノム編集個体との性状の相違を検討する。平成３１年度は、ミオスタチン遺伝子および、食欲関連遺伝子破壊トラフグで生産されると考えられる新規ペプチドについて、それらのアレルゲン性の追加検討を行った。その結果、昨年度と同様に既存のアレルゲンと相同性の高い配列は確認されなかった。また、高成長形質トラフグでのメタボロミックス解析およびゲノム解析を行なった結果、代謝産物およびゲノム配列に野生型と有意に異なる点は見つからなかった。これらのことから、高成長形質ゲノム編集トラフグの食品としての安全性は、通常トラフグと同等であると考えられた。加えて、フグ毒（TTX）体内分布検討実験を開始し、通常トラフグおよび高成長形質ゲノム編集トラフグへの TTX 投与および各組織の摘出を終えた。（結果は現在解析中である）。

A. 研究目的

近年急速に発展してきたゲノム編集技術は、「生物種を選ばず、ゲノム上の狙った配列を改変できる」という特性から、農林水産物の育種に応用され始めている。この技術では、「短期間で狙った形質を持つ品種」を作製することが可能であるため、本技術は品種改良方法・育種方法として、今後定着していくものと考えられる。

しかしながら、本技術は歴史が浅いため、この技術で作製された食品に対して、消費者が安全性に不安を持っているのが現状である。加えて、安全性を確認する機関においても安全性への具体的な評価基準の策定に至っていない。

水産物は、これまでの「とる漁業が中心」の時代から、「作る・育てる漁業」へと変わってきたものの、品種の作製や育種が、作物や畜産物に比べて大幅に遅れている。一方、世界的な人口増加と健康食志向の高まりから養殖業が発展してきており、消費者ニーズに合った水産物の作出が望まれるようになってきた。このような背景から、短期間で優良形質を固定化できるゲノム編集技術の導入が水産物育種に活用され始めた。今後、多様な水産物においてゲノム編集技術による新品種・新食品が作製されていくものと思われる。

そこで本研究分担者は、自身で作出したゲノム編集マダイおよびゲノム編集トラフグを用い、それらの特性を検討し、食品安全性評価法策定に提言を与えるのを目的とする。

具体的には、１）ゲノム編集により誘導される新規ペプチドのアレルゲン性を *in silico* 解析により評価する、２）第２世代または、第３世代でのオフターゲット候補配列の変異の有無を明らかにする、ターゲット配列の変異様式の伝達性を明らかにする、３）可食部（筋肉）のメタボローム解析を行い非編集魚との相違を明らかにする、ことを目的とする。

平成３１年度は、アレルゲン性の再評価、高成長形質ゲノム編集トラフグを用いた塩基配列解析によるオフターゲット影響とゲノム編集ツール残存性の評価、および、メタボロミックス解析を行い食品安全性を評価することと目的とした。また、ゲノム編集トラフグにおけるフグ毒分布検討実験を開始した。

B. 研究方法

１）新生ペプチドのアレルゲン性の検討：

ゲノム編集によりミオスタチン遺伝子（*mstn*）を破壊したマダイ３系統およびトラフグ３系統、レプチン受容体遺伝子（*lepr*）を破壊したトラフグ２系統、メラノコルチン４型受容体遺伝子（*mc4r*）

を破壊したトラフグ１系統について、予想される全アミノ酸配列、新生ペプチドとその直上１０アミノ酸部分、および、塩基欠失部位を挟んだ両側の終止コドン内で予想されるペプチドを用い、web上のアレルゲン検索サイト（後述）により、それらのアレルゲン性を検討した。３１年度は通常の読み枠（フレーム）に加えて、異なる読み枠および逆鎖でのアミノ酸配列においても評価した。アレルゲンとする基準は１）全長のアミノ酸配列において E.value ＜ 1 の相同性を示す、２）80アミノ酸のウインドウサイズで35%以上の相同性を示す、３）８アミノ酸配列が完全に一致する、ものとした(表１参照)。昨年度からの改良点として、「FAO での６アミノ酸の相同性評価」は過大評価をしているとの指摘があるため、Allergen Online の評価基準である「８アミノ酸の相同性」により評価した。また、E.value ＜ 1.0 となったものについては、より低値での解析を行った。
アレルゲン検索サイト：”Allergen Online”(University of Nebraska-Lincoln) および、”Allergen Database for Food Safety (ADFS)”（国立医薬品食品衛生研究所)。(表１参照)

２）オフターゲットと変異継代安定性の検討：
定)
lepr 破壊トラフグ第３世代の４個体、および、野生型４個体（雌雄２個体づつ）それぞれから、ゲノム DNA を抽出し全ゲノム配列を解読し、Integrative Genomics Viewer（IGV）による解析を行った。トラフグゲノム配列データベースから、*lepr* 中のターゲット配列（CCACTGTGTGCTGTTCCATCT）と欠失および挿入を含めミスマッチが２塩基以内の配列をオフターゲット候補とし、同領域におけるゲノム編集魚と野生型魚における塩基配列の変化を検討した。加えて PAM 配列近傍のシード配列が完全に一致している領域については､PCR で増幅後、塩基配列解析を行い、変異の有無を検討した。

３）可食部成分の検討：
上述のゲノム編集魚の全塩基配列データをゲノム編集時に使用した RNA の配列にマッピングを行い、そのリード数により評価した。この RNA 配列には、Cas9 RNA 合成に用いた pCS2+hspCas9 および guideRNA 合成に用いた pDR274+sgRNA の塩基配列を使用した。

３）ゲノム編集ツール残存性の評価：
上述のゲノム編集魚の全塩基配列データをゲノム編集時に使用した RNA の配列にマッピングを行い、そのリード数により評価した。この RNA 配列には、Cas9 RNA 合成に用いた pCS2+hspCas9 および guideRNA 合成に用いた pDR274+sgRNA の塩基配列を使用した。

４）可食部成分の検討（メタボロミクス)：
ゲノム編集により *mstn* 遺伝子を破壊したマダイ系統（１４塩基欠失）および *lepr* 遺伝子を破壊したトラフグ系統(４塩基欠失)の背部骨格筋を採取し、凍結乾燥後、粉末化した。この粉末 50mg にメタノール／クロロホルム／水 (2.5/1/1)を加えた後、水溶性画分を回収後、乾個・TMS 誘導化を行い、GC-MS(GSMS-QP2010 Ultra)にて分析を行った。分析により得られた水溶性一次代謝物を網羅的に検出し、多変量解析ソフト SIMA により解析した。

C. 研究結果および考察

１）新生ペプチドのアレルゲン性の検討：
野生型マダイとゲノム編集マダイのミオスタチン遺伝子から産生されるアミノ酸配列を図１に示す。マダイのミオスタチンタンパク質には、野生型においても、全長アミノ酸配列でアレルゲン性を示す配列（グリアジンと相同性を示す）が存在したが、それ以外にはアレルゲン性を示唆する配列は検出されなかった（図２)。また、新生アミノ酸とその上流１０アミノ酸配列中の８アミノ酸配列、および、別フレームではアレルゲン性が予測される配列は検出されなかった。
野生型トラフグとゲノム編集トラフグのミオスタチン遺伝子から産生されるアミノ酸配列を図３に示す。ミオスタチン遺伝子ゲノム編集トラフグの全長を解析した結果、8 塩基欠失（-8b）系統においてアオカビのタンパク質と相同性を示す配列が検出さてたが、その他の系統ではアレルゲン性を示す配列は検出されなかった（図４)。通常とは異なるフレームあるいは、相補鎖について検討した結果を図５に示す。その結果、各系統において、アレルゲン性を示す配列が検出されたが、いずれも E.value<0.05 ではアレルゲン性は示されなかった。
野生型トラフグとゲノム編集トラフグのレプチ

ン受容体遺伝子から産生されるアミノ酸配列を図６に示す。通常のフレームで全長アミノ酸を検討した場合、２塩基および４塩基欠失のいずれの系統においてもイネのαアミラーゼと相同性を示す配列が検出された（図７）。しかしながら、事実上アレルゲンとして有効であると考えられる E.value<0.05 で再評価した場合には相同性は検出されなかった。センス鎖の１塩基シフトしたフレームでは、いずれのゲノム編集系統でもオレオシンとの相同性が示されたが、E.value<0.05 ではアレルゲン性を示さなかった（図８）。他のフレームおよび相補鎖のすべてのフレームではアレルゲン性は検出されなかった（図８）。

野生型トラフグとゲノム編集トラフグのメラノコルチン４型受容体遺伝子から産生されるアミノ酸配列を図９に示す。図１０に示すように、センス鎖の通常のフレームではアレルゲン性は示されなかった。一方、通常ではない読枠ではアレルゲン性を示すものも有った（図１１）。中でもネッタイシマカの唾液腺中アレルゲンと E.value=0.0078 でアレルゲン性を示すものがあった。

２）レプチン受容体遺伝子破壊トラフグにおけるオフターゲットの検討：

全ゲノム配列解読は、各個体でゲノムサイズの３０倍以上のデータを取得した。また、ターゲット配列と２塩基以下のミスマッチ（欠失・挿入を含む）を有する配列は６１箇所存在した（図１２）。図１３に全塩基配列解析の結果を IGV で可視化した一例を示す。図１３内の拡大図に示すように、他個体と比較し塩基配列に違い（欠失）が存在すれば白抜きで示される。図１３ではターゲット配列領域を表示しているため、その中の同じ４塩基がゲノム編集魚でのみ欠失している。ゲノム編集魚に特異的にみられる欠失領域は、標的領域のみであった。つまり、オフターゲット影響は観察されなかった。また、異なる手法でのオフターゲットの検証として、候補領域を PCR により増幅後、電気泳動によるバンドシフトの観察と塩基配列解析を行った（図１４）。その結果、いずれの領域にも、変異は観察されなかった。

３）レプチン受容体遺伝子破壊トラフグにおけるゲノム編集ツール残存性の検討：

図１５に Cas9 RNA、図１６に guideRNA の残存性を示す。いずれにおいてもゲノム編集処理時に用いた RNA 配列は確認されなかった。一方、プラスミドのバックボーン配列（RNA に転写しておらず、ゲノム編集処理には用いていない配列）の一部に、ゲノム編集魚および野生型魚の両者で観察された。これは、トラフグゲノム内に一般的に侵入した細菌などの断片であると思われる。

４）可食部成分の検討：

野生型マダイと *mstn* 破壊マダイのメタボロミクスを比較した結果を図１７に示す。主成分分析(図１７-b)の信頼性を示す Q2 値が 0.5 以下であり（図１７-a)、主成分分析解析の信頼性は低いと判断される。つまり、比較した個体間で有意な差がないと判断された。

lepr 遺伝子破壊トラフグの結果を図１８に示す。Q2 値は 0.5 以下であったため、主成分分析の結果の信頼性は高くないが、*lepr* ゲノム編集トラフグと野生型トラフグ間で成分の差異があることが示唆された。そのため、最も差異を作り出す条件で解析を行った結果を図１９に示す。*lepr* ゲノム編集魚では、グリシンが増加し、リジンが減少していることが推察された。

D. 結論

mstn ゲノム編集マダイ、*mstn* ゲノム編集トラフグ、*lepr* ゲノム編集トラフグ、*mc4r* ゲノム編集トラフグにおいて、通常のフレームで産生されると予想されるタンパク質およびペプチドはアレルゲン性を示さないことが、アレルゲンデータベースとの比較により示された。通常のフレーム以外、あるいは相補鎖では、アレルゲン性が疑われるペプチド配列が存在するが、そのペプチドが各生物内で産生されている可能性は低いと思われる。そのため、今回解析したゲノム編集魚類では、アレルゲンの産生はないものと考えられる。

今年度新たに行った *lepr* ゲノム編集トラフグでは、オフターゲット変異はされず、また、ゲノム編集ツールの残存も確認されなかった。

メタボロミクス解析においてもゲノム編集魚と非編集魚（野生型）との有意な差は観察されなかった。

以上のことから、今回解析したゲノム編集魚の食品の安全性は、非編集魚とは同等であると判断できる。

E. 業績

１）市民向け説明会

・ ゲノム編集生物と社会について考える、2019 年 7 月 6 日、日本学術会議講堂、約 150 名、日本学術会議農学委員会・食料科学委員会合同遺伝子組換え作物分科会、講演者
・ 農林水産省アウトリーチ事業、2019 年 11 月 13 日、名古屋大学農学部、約 70 名、農林水産省、講師
・ 農林水産省アウトリーチ事業、2019 年 11 月 22 日、立命館高校、約 20 名、農林水産省、講師
・ 農林水産省アウトリーチ事業、2019 年 11 月 22 日、立命大学、約 20 名、農林水産省、講師
・ ステークホルダー会議、2019 年 12 月 5 日、京都テルサ、約 50 名、京都生活協同組合、講師

２）業界関係者向け説明会

・ 知的財産セミナー、2019 年 6 月 29 日、京都産業会館、約 30 名、日本弁理士会関西会京都地区会、招待講演者
・ ワークショップ:「ゲノム編集食品の安全・安心」、2019 年 7 月 17 日、大阪大学東京オフィス、約 20 名、一般社団法人 ゲノム編集学会、プレゼンター、話題提供者
・ アクションプラン 2019、2019 年 7 月 31 日、大阪帝国ホテル、約 300 名、三菱食品、招待講演者
・ 第６３回 滋賀県学校保健・安全研究大会、2019 年 10 月 10 日、近江八幡市 男女共同参画センター、約 60 名、滋賀県教育委員会
・ トランスフォーマティブ化学生命融合研究大学院プログラムセミナー、2019 年 10 月 15 日、名古屋大学農学部、約 70 名、名古屋大学、招待講演者
・ 養殖情報交換会、2019 年 12 月 10 日、シンフォニアテクノロジー響ホール伊勢、約５０名、国立研究開発法人水産研究・教育機構 増養殖研究所、招待講演者
・ JBA 発行と代謝講演会シンポジウム、2020 年 2 月 17 日、東京大学・中島董一郎記念ホール、80 名、バイオインダストリー協会、招待講演
・ ワークショップ「ゲノム編集食品に関する多様な意見をどう取り上げるか？」、2020 年 2 月 25 日、メルパルク京都、20 名、 ゲノム編集の未来を考える会（JST 来共創イノベーション活動支援)、招待講演

３）行政関係者向け説明会

・ ゲノム編集水産物に関する検討会（非公開)、2020 年 2 月 27 日、農林水産省、20 名、農林水産省消費安全局、有識者

４）学会（招待講演）

・ 第４回ゲノム編集学会、2019 年 6 月 3 日、タワーホール船堀
・ 第３１回日本比較免疫学会シンポジウム、2019 年 9 月 4 日、九州大学
・ Marine Biotechnology Conference 2019、2019 年 9 月 12 日、静岡県清水市清水文化会館
・ 第１７回食品安全フォーラム（日本薬学会)、2019 年 11 月 29 日、日本薬学会長井記念ホール
・ 第 31 回日本生命倫理学会年次大会 シンポジウム、2019 年 11 月 29 日、東北大学

６）総説など

・ 木下政人、「養殖業へのゲノム編集技術活用のために」、月刊養殖ビジネス（緑書房)、1 月号 pp61-64、2020
・ 木下政人、「ゲノム編集技術を使った肉厚マダイの作出と品種改良期間の短縮」、JATAFF ジャーナル（農林水産・食品産業技術振興協会)、No.2（２月号）pp8-12、2020

F. 知的財産権の出願と登録状況

該当なし

表１．アレルゲン検索データベース

サイト名	発行元	URL
Allergen Online	University of Nebraska-Lincoln	http://www.allergenonline.org
Allergen Database for Food Safety (ADFS)	国立医薬品食品衛生研究所	http://allergen.nihs.go.jp/ADFS/

以下の基準を満たすものをアレルゲンとした。
・全長のアミノ酸が高い相同性（E. value < 1 とした）
・80 aaのウインドウサイズで35％以上の相同性
・8 aaの完全一致

```
WT :          1 MHPSQIVLYL SLLIVLGPVV LSDQETQQQQ QQQQQQQPSA TSPEDTEQCA
mstn-14/-14 : 1 MHPSQIVLYL SLLIVLGPVV LSDQETQQQQ QQQQQQQPSA TSPEDTEQCA
mstn-8b/-8b : 1 MHPSQIVLYL SLLIVLGPVV LSDQETQQQQ QQQQQQQPSA TSPEDTEQCA
mstn-8a/-8a : 1 MHPSQIVLYL SLLIVLGPVV LSDQETQQQQ QQQQQQQPSA TSPEDTEQCA

WT :         51 TCEVRQQIKT MRLNAIKSQI LSKLRMKEAP NISRDIVKQL LPKAPPLQQL
mstn-14/-14 : 51 TCEVRQQIKT MRLNAIKSQI LSKLRMKEAP NISRDIVKQL LPKAPPLQQL
mstn-8b/-8b : 51 TCEVRQQIKT MRLNAIKSQI LSKLRMKEAP NISRDIVKQL LPKAPPLQQL
mstn-8a/-8a : 51 TCEVRQQIKT MRLNAIKSQI LSKLRMKEAP NISREAAPAQ SAAAAAASRP

                                                                  388
WT :        101 LDQYDVLGDD NRDVVMEEDD EHAITETIMM MATEPESVVQ ・・・・・・
mstn-14/-14 : 101 LDQYDVLGDD NRDVVMEEDD EHDYDDGH*
mstn-8b/-8b : 101 LDQYDVLGDD NRDVVMEEDD EHGDDYDDGH*
mstn-8a/-8a : 101 VRRAGRRQQG CGYGGGR*
```

図１．ミオスタチン遺伝子破壊マダイおよび野生型マダイのミオスタチン遺伝子から産生されるアミノ酸配列。

色字は新生ペプチドを示す。WT:野生型、 -14: 14 塩基欠失、-8a および -8b: 8 塩基欠失。

配列全長の比較

	-8a (Full)	-14 (Full)	-8b (Full)	WT
Full FASTA (E.value < 1)	グリアジン グルテニン	グリアジン グルテニン	グリアジン グルテニン	グリアジン グルテニン
FAO/WHO (>35% in 80 aa)	なし	なし	なし	なし

新生アミノ酸配列とその上流10アミノ酸をクエリとした比較

	-8a (10＋new aa)	-14 (10＋new aa)	-8b (10+new aa)
FAO/WHO (8 aa exact match)	なし	なし	なし

図２．ミオスタチン遺伝子破壊マダイのアレルゲン性の検討

別フレームおよび相補鎖でアレルゲン性は予測されなかった。
グリアジン：グルテニンは小麦のアレルゲン

```
WT :          1 MQLSPSMLHF SLMISLSLVV LSGQETHQQP PVGSPEDTEQ CVTCDVRQHI
mstn-8b/-8b :  1 MQLSPSMLHF SLMISLSLVV LSGQETHQQP PVGSPEDTEQ CVTCDVRQHI
mstn-41/-41 :  1 MQLSPSMLHF SLMISLSLVV LSGQETHQQP PVGSPEDTEQ CVTCDVRQHI
mstn-8a/-8a :  1 MQLSPSMLHF SLMISLSLVV LSGQETHQQP PVGSPEDTEQ CVTCDVRQHI

WT :          51 KTMRLNAIKS QILSKLRMKE APNISRDTVK QLLPKAPPLQ QLLDQYDVLG
mstn-8b/-8b :  51 KTMRLNAIKS QILSKLRMKE APNISRDTVK QLLPKAPPLQ QLLDQYDVLG
mstn-41/-41 :  51 KTMRLNAIKS QILSKLRMKE APNISRDTVK QLLPKAPPLQ QLLDQYDVLG
mstn-8a/-8a :  51 KTMRLNAIKS QILSKLRMKE APNISREAAP AQSAAAAAAP RPVRRAGR*

WT :          101 DDNRDVVTEE DDEHAITETI MM MATEPASVVQV ・・・ 376
mstn-8b/-8b :  101 DDNRDVVTEE DDEHGDHHDD GH*
mstn-41/-41 :  101 DDNRDHHDDGH*
```

図３．ミオスタチン遺伝子破壊トラフグおよび野生型トラフグのミオスタチン遺伝子から産生されるアミノ酸配列

色字は新生ペプチドを示す。WT:野生型、 -41: 41 塩基欠失、-8a および -8b: 8 塩基欠失。

配列全長の比較

	-8a (Full)	-8b (Full)	-41 (Full)	WT
Full FASTA (E.value < 1)	なし	ペルオキシソーム膜タンパク（アオカビ）	なし	グルテニン
FAO/WHO (>35% in 80 aa)	なし	なし	なし	なし

新生アミノ酸配列とその上流10アミノ酸をクエリとした比較

	-8a (10＋new aa)	-8b (10＋new aa)	-41 (10+new aa)
FAO/WHO (8 aa exact match)	なし	なし	なし

図4. ミオスタチン遺伝子破壊トラフグのアレルゲン性の検討

センス鎖の通常のフレームでの結果を示す。

センス鎖

フレーム	-8a	-41	-8b
1	グルテニン	なし	なし
2	2Sアルブミン（ピーナッツ） HLHタンパク（フザリウム属真菌） β-コングリシニン（大豆） ビシリン（マメ科植物）	なし	なし

相補鎖

フレーム	-8a	-41	-8b
0	なし	なし	脂質輸送タンパク （ナス科植物）
1	なし	なし	なし
2	なし	グリシニン （ピーナッツ）	グリシニン （ピーナッツ）

図５．ミオスタチン遺伝子破壊トラフグのアレルゲン性の検討

センス鎖の異なるフレームおよび相補鎖での結果を示す。

```
WT : 1MSSTMFGRVT LSVMVLGFLL SRGVLSLENS DAGGRHSGVL DLPWKDELCC
lepr-2/-2 : 1MSSTMFGRVT LSVMVLGFLL SRGVLSLENS DAGGRHSGVL DLPWKDELCC
lepr-4/-4 : 1MSSTMFGRVT LSVMVLGFLL SRGVLSLENS DAGGRHSGVL DLPWKDELCC
                                    .
                                    .
                                    .
WT : 351NQWVSQVTMR PSETGMYDLL QCTKKRMIAY SQVYVEGASI SISCETNGEI
lepr-2/-2 : 351NQWVSQVTMR PSETGMYDLL QCTKKRMIAY SQVYVEGASI SISCETNGEI
lepr-4/-4 : 351NQWVSQVTMR PSETGMYDLL QCTKKRMIAY SQVYVEGASI SISCETNGEI

WT : 401DAMDCRWNST QWLNPNFRTR WADLSCDVME ERERAGDNVG ・・・ 1117
lepr-2/-2 : 401DAMDCRWNST VAEPQLQNQV G*
lepr-4/-4 : 401DAMDCRWNSS G*
```

図６．レプチン受容体遺伝子破壊トラフグおよび野生型トラフグから産生されるアミノ酸配列

色字は新生ペプチドを示す。WT:野生型、 -2: 2 塩基欠失、-4 : 4 塩基欠失。

配列全長の比較

	-2　(Full)	-4　(Full)	WT
Full FASTA (E.value < 1)	αアミラーゼ インヒビター（イネ）＊	αアミラーゼ インヒビター（イネ）＊	なし
FAO/WHO (>35% in 80 aa)	なし	なし	なし

＊： E.value < 0.05 ではヒットしない

新生アミノ酸配列とその上流10アミノ酸をクエリとした比較

	-2 (10＋new aa)	-4 (10＋new aa)
FAO/WHO (8 aa exact match)	なし	なし

図７．レプチン受容体遺伝子破壊トラフグのアレルゲン性の検討

センス鎖の通常のフレームでの結果を示す。

センス鎖

フレーム	-2	-4
1	オレオシン（オリーブ花粉）	オレオシン（オリーブ花粉）
2	なし	なし

相補鎖

フレーム	-2	-4
0	なし	なし
1	なし	なし
2	なし	なし

図８. レプチン受容体遺伝子破壊トラフグの通常のフレーム以外でデータ上で翻訳されるペプチドのアレルゲン性の検討

E.value< 1での結果を示す。
E.value<0.05では、いずれもアレルゲン性は示されなかった。

WT：1 MNATDPPGRV QDFSNGSQTP ETDFPNEEKE SSTGCYEQML ISTEVFLTLG

mc4r$^{-13/-13}$：1 MNATDPPGRV QDFSNGRRT**F QTRRRNRLRD ATSRC***

WT：51 IISLLENILV VAAIVKNKNL HSPMYFFICS ・・・ 322

図９．メラノコルチン４型受容体遺伝子破壊トラフグおよび野生型トラフグから産生されるアミノ酸配列

色字は新生ペプチドを示す。WT:野生型、 -13: 13 塩基欠失。

配列全長の比較

	-13 (Full)	WT
Full FASTA (E.value < 1)	なし	なし
FAO/WHO (>35% in 80 aa)	なし	なし

新生アミノ酸配列とその上流10アミノ酸をクエリとした比較

	-13 (10＋new aa)
FAO/WHO (8 aa exact match)	なし

図１０.メラノコルチン４型受容体遺伝子破壊トラフグのアレルゲン性の検討

センス鎖の通常のフレームでの結果を示す。

センス鎖

フレーム	-13
1	β-コングリシニン（大豆）
2	グルテニン（小麦）

相補鎖

	フレーム		
解析方法	0	1	2
Full FASTA (E.value < 1)	花粉（ニガヨモギ）	グルテニン（コムギ） ※唾腺中アレルゲン（ネッタイシマカ）	なし
FAO/WHO (>35% in 80 aa)	なし	※唾腺中アレルゲン（ネッタイシマカ）	なし
FAO/WHO (8 aa exact match)	なし	なし	なし

図１１. レプチン受容体遺伝子破壊トラフグの通常のフレーム以外でデータ上で翻訳されるペプチドのアレルゲン性の検討

※E.value = 0.0078 と高い相同性が見られた
gi|2114497|gid|1024|Allergen 30 kDa salivary gland

#	results	strand	ensemble_search	gene	feature	abjct	mismatch	mis	del	ins	seed perfect match	
ont	-	-	chr20:11315793-11315813	ENSTRUG00000018553	exon	CCACTGTGTGCTGTTCCATCT		0	0	0	0	1
OT1	none	+	HE591747:303012-303031	ENSTRUG00000022625	intron	AGAT-GAACAACACACAGGAG		2	1	0	1	0
OT2	none	+	HE591855:167082-167102	none(Genescan_prediction+)		AGATGGAACAGCACACACAAG		1	1	0	0	0
OT3	none	+	HE591871:145815-145834	none(refseq+)	exon	TGA-GGAACAGCACACAGGAG		2	1	0	1	1
OT4	none	+	HE592464:18756-18776	ENSTRUG00000023752	3utr	AGATGGAACAG-ACGACAGCAG		2	0	1	1	0
OT5	none	+	HE592677:14859-14880	none		AGAGGGAATCAGCACACAGCAG		2	1	1	0	0
OT6	none	+	HE593051:5086-5107	none(Genescan_prediction+)		AGAGGGAATCAGCACACAGCAG		2	1	1	0	0
OT7	none	+	HE594516:1437-1457	none		AGCTGGAACAGCACAGAGAGG		2	2	0	0	0
OT8	none	+	HE594608:746-767	none		AGAGGGAATCAGCACACAGCAG		2	1	1	0	0
OT9	none	+	chr3:11677466-11677485	ENSTRUG00000013622	intron	AGATGGATCA-CACACAGAAG		2	1	0	1	0
OT10	none	+	chr5:283674-283694	ENSTRUG00000007413	intron	ACATGGAACAGCAAACAGAAG		2	2	0	0	0
OT11	none	+	chr5:4742359-4742380	ENSTRUG00000010563	exon	AGATGGACACAGCACACACAAG		2	1	1	0	0
OT12	none	+	chr5:4814740-4814761	ENSTRUG00000020691	intron	AGTATGGGACAGCACACAGAGG		2	1	1	0	0
OT13	none	+	chr6:4461642-4461664	none(Genescan_prediction+)		AGATGGAGAGCAGCACACAGAAG		2	0	2	0	0
OT14	none	+	chr8:8790955-8790975	none(Genescan_prediction+)		AGATGGAACAGGACACACCGG		2	2	0	0	0
OT15	none	+	chr9:7332440-7332459	ENSTRUG00000010186	intron	AAATGGAACAGCAC-CAGGAG		2	1	0	1	0
OT16	none	+	chr9:12360332-12360351	none(Genescan_prediction+)		AGA-GGAACACCACACAGCAG		2	1	0	1	0
OT17	none	+	chr10:3305528-3305547	none(Genescan_prediction+)		AGATGGAGCAGCACAC-GGAG		2	1	0	1	0
OT18	none	+	chr13:8706172-8706190	none(refseq+)	intron	AGATGG-A-AGCACACAGGAG		2	0	0	2	0
OT19	none	+	chr13:12693401-12693421	none		AGAGGGAACAGCACACAGCAG		1	1	0	0	1
OT20	none	+	chr15:3763545-3763565	ENSTRUG00000020956	3utr	AGATGGAAAAACACACAGCGG		2	2	0	0	0
OT21	none	+	chr15:4674220-4674238	none(refseq+)	exon	AGATGG-ACAGCACA-AGGAG		2	0	0	2	0
OT22	none	+	chr15:5006835-5006854	none(Genescan_prediction+)		ACAT-GAACAGCACACAGAAG		2	1	0	1	1
OT23	none	+	chr20:255241-255261	ENSTRUG00000024219	intron	AGACGAAACAGCACACAGAGG		2	2	0	0	1
OT24	none	+	chr21:4993307-4993326	ENSTRUG00000014047	intron	AGTTGGAACAGCACACA-AAG		2	1	0	1	0
OT25	none	+	chr22:8813489-8813508	none(refseq+)		AGATGGAACA-CACACAAAGG		2	1	0	1	0
OT26	none	+	chr22:9557216-9557235	ENSTRUG00000006539	3utr	AGA-GTAACAGCACACAGAGG		2	1	0	1	1
OT27	none	-	HE591784:27700-27720	none(Genescan_prediction+)		CTCCTGTCTGCAGTTCCATCT		2	2	0	0	0
OT28	none	-	HE591851:173713-173733	none(Genescan_prediction+)		CTTGTGTGTGCCGTTCCATCT		2	2	0	0	0
OT29	none	-	chr1:22100674-22100693	none(refseq+)	intron	CCCCTGTGTTCAGTTCCATCT		2	2	0	0	0
OT30	none	-	HE592491:20092-20112	none		CCCCTGTGTTCAGTTCCATCT		2	2	0	0	0
OT31	none	-	HE592521:4763-4784	none(refseq+)	intron	CCTCTGGAGTGCTGTTCCATCT		2	1	1	0	0
OT32	none	-	HE592751:4904-4924	none		CCTCTCTGTGCTGTTCCAGCT		2	2	0	0	0
OT33	none	-	HE593323:704-724	none		CCTCTCTGTGCTGTTCCAGCT		2	2	0	0	0
OT34	none	-	HE594615:2444-2465	none		CTGCTGTGTGCTGATTCCCTCT		2	1	1	0	0
OT35	none	-	HE594929:1098-1118	none(refseq+)	exon	CTTTTGTGTGCCGTTCCATCT		2	2	0	0	0
OT36	none	-	HE595267:768-788	none		CCTCTGTGTGCTGTTTCGTCT		2	2	0	0	1
OT37	none	-	chr1:22100674-22100693	none(refseq+)	3utr	CTACTGTG-GCTGTTTCATCT		2	1	0	1	0
OT38	none	-	chr2:9987523-9987542	none(Genescan_prediction+)		CTGCT-TGTGATGTTCCATCT		2	1	0	1	0
OT39	none	-	chr3:2687014-2687033	ENSTRUG00000014991	intron	CCAGTGTG-GCTGTTCCATCT		2	1	0	1	0
OT40	none	-	chr3:11354101-11354119	ENSTRUG00000012256	intron	CTG-TGTGTGCTGTT-CATCT		2	0	0	2	0
OT41	none	-	chr4:3738304-3738323	none(Genescan_prediction+)		CTACTGTA-GCTGTTCCATCT		2	1	0	1	0
OT42	none	-	chr7:7728697-7728716	ENSTRUG00000002103	exon	CCCCTGTGTGCTGTTGC-TCT		2	1	0	1	1
OT43	none	-	chr7:13468369-13468390	ENSTRUG00000004325	intron	CCCCTGTGTGCTGTTTCCATCT		1	0	1	0	0
OT44	none	-	chr8:12117618-12117636	ENSTRUG00000015511	intron	CTCCTGT-TGCT-TTCCATCT		2	0	0	2	0
OT45	none	-	chr13:11982850-11982868	none(Genescan_prediction+)		CTTCT-TGTGCT-TTCCATCT		2	0	0	2	0
OT46	none	-	chr13:12825026-12825045	none(Genescan_prediction+)		CCGCTGTGTGCTGCTCC-TCT		2	1	0	1	0
OT47	none	-	chr13:14225756-14225776	none(Genescan_prediction+)		CCAC-GTGTGCTGTTCCCATCT		2	0	1	1	0
OT48	none	-	chr14:4310825-4310845	ENSTRUG00000005874	exon	CTTCTGTGTGATCTTCCATCT		2	2	0	0	0
OT49	none	-	chr14:9383168-9383187	ENSTRUG00000013584	intron	CTT-TATGTGCTGTTCCATCT		2	1	0	1	0
OT50	none	-	chr15:5539820-5539839	ENSTRUG00000002628	intron	CCCCTGT-TGCTGTTCCATTT		2	1	0	1	0
OT51	none	-	chr15:9937138-9937157	ENSTRUG00000016772	intron	CTGC-GTGTGCTGTTCCATCC		2	1	0	1	0
OT52	none	-	chr17:429542-429560	none		CCTCTGT-T-CTGTTCCATCT		2	0	0	2	0
OT53	none	-	chr17:5091828-5091848	ENSTRUG00000025008	exon	CCACTGTCTGCTGTTCCCTCT		2	2	0	0	0
OT54	none	-	chr17:6271380-6271401	none(refseq+)	intron/exo	CCACTGTGTGTCTGTTCCAGCT		2	1	1	0	0
OT55	none	-	chr18:3986315-3986335	ENSTRUG00000013192	intron	CTTCTGTGTGTCTGTTCCAT-T		2	0	1	1	0
OT56	none	-	chr19:10079887-10079908	ENSTRUG00000006186	intron	CTGCTGTGGTGCTTTTCCATCT		2	1	1	0	0
OT57	none	-	chr20:9132257-9132277	none		CCGCTCTGTGCAGTTCCATCT		2	2	0	0	0
OT58	none	-	chr21:4886387-4886406	none		CCACTGTGT-CTGTTCCATCC		2	1	0	1	0
OT59	none	-	chr21:13673735-13673756	none(Genescan_prediction+)		CTGCCTGTGTGCTGTTACATCT		2	1	1	0	0
OT60	none	-	chr22:5805648-5805666	none		CTGCTGTGTGCTG-TCC-TCT		2	0	0	2	0
OT61	none	-	chr22:10306188-10306206	none(Genescan_prediction+)		CCCCTGTG-GCTGTTCC-TCT		2	0	0	2	0

図１２．レプチン受容体ゲノム編集ターゲット配列とオフターゲット候補配列

2 塩基ミスマッチまでの、候補配列(６１領域）を示す。黄色はターゲット配列。
濃青バック: シードシーケンス一致
薄青バック：1塩基ミスマッチ

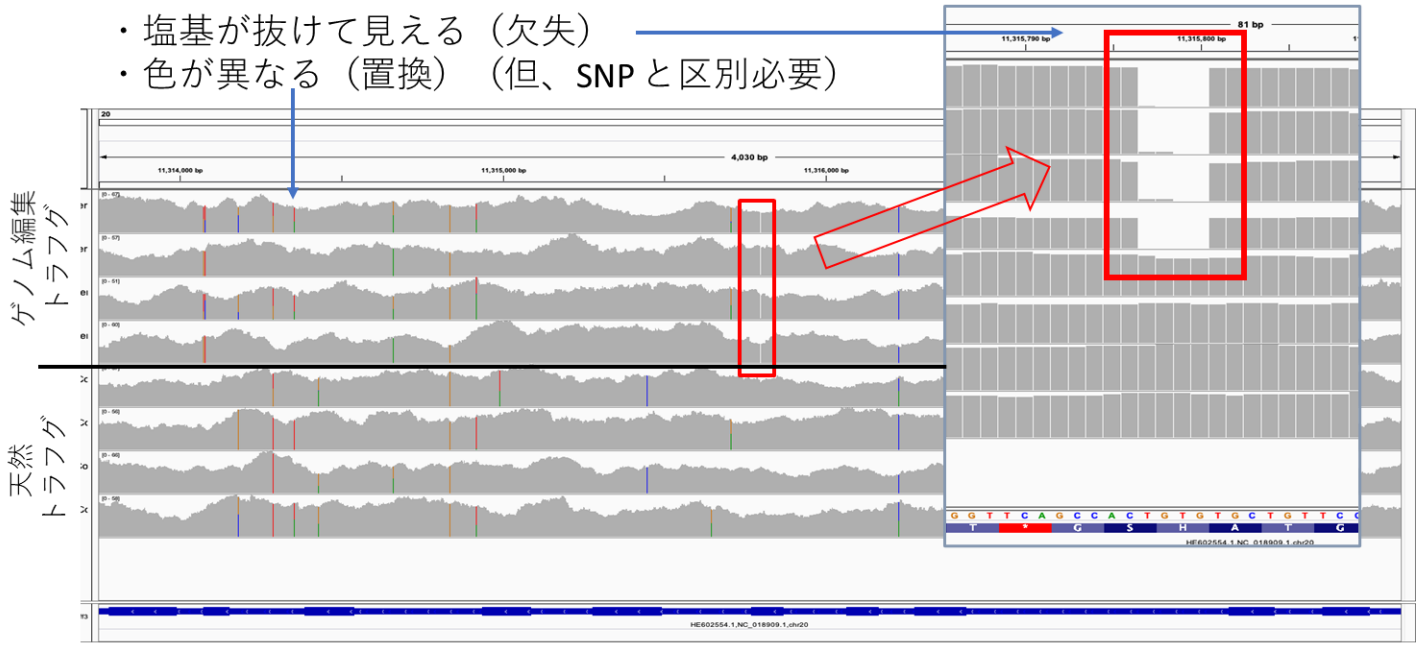


図１３．レプチン受容体遺伝子破壊および野生型トラフグの全塩基配列解析結果のIGV像

ターゲット入れる付近の結果を示す。縦軸はリード数を示す。横軸は塩基配列を示す。
拡大図に示されるように、塩基欠失箇所は白抜きで示される。

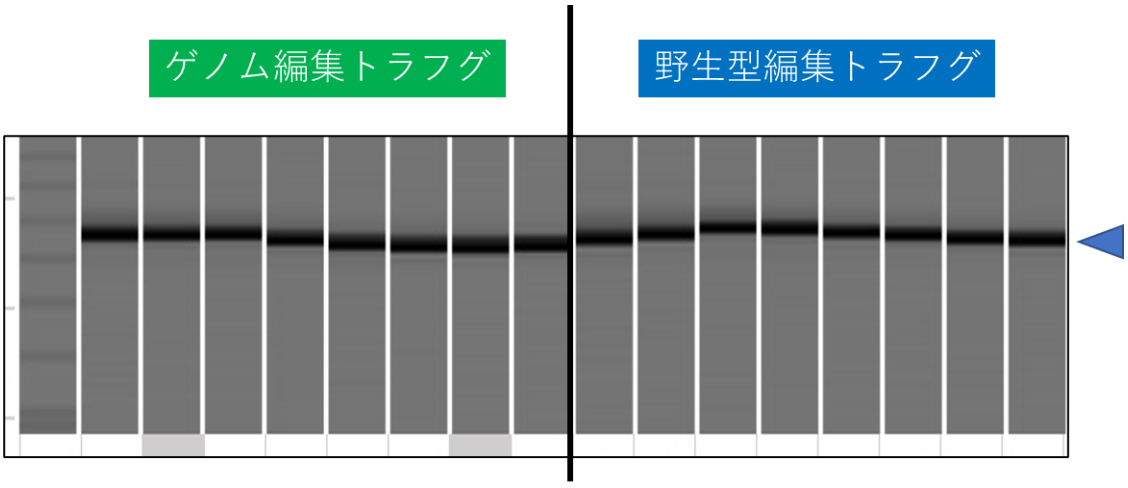


図１４．レプチン受容体遺伝子破壊および野生型トラフグのオフターゲット領域のPCR産物

一例として、オフターゲット候補配列:OT#2 を示す。
ゲノム編集および野生型個体それぞれ８個体の各 PCR 産物を自動電気泳動装置により解析した。続いて、これらの塩基配列をサンガーシーケンス法により解読した。その結果、いずれの領域にも変異は観察されなかった。

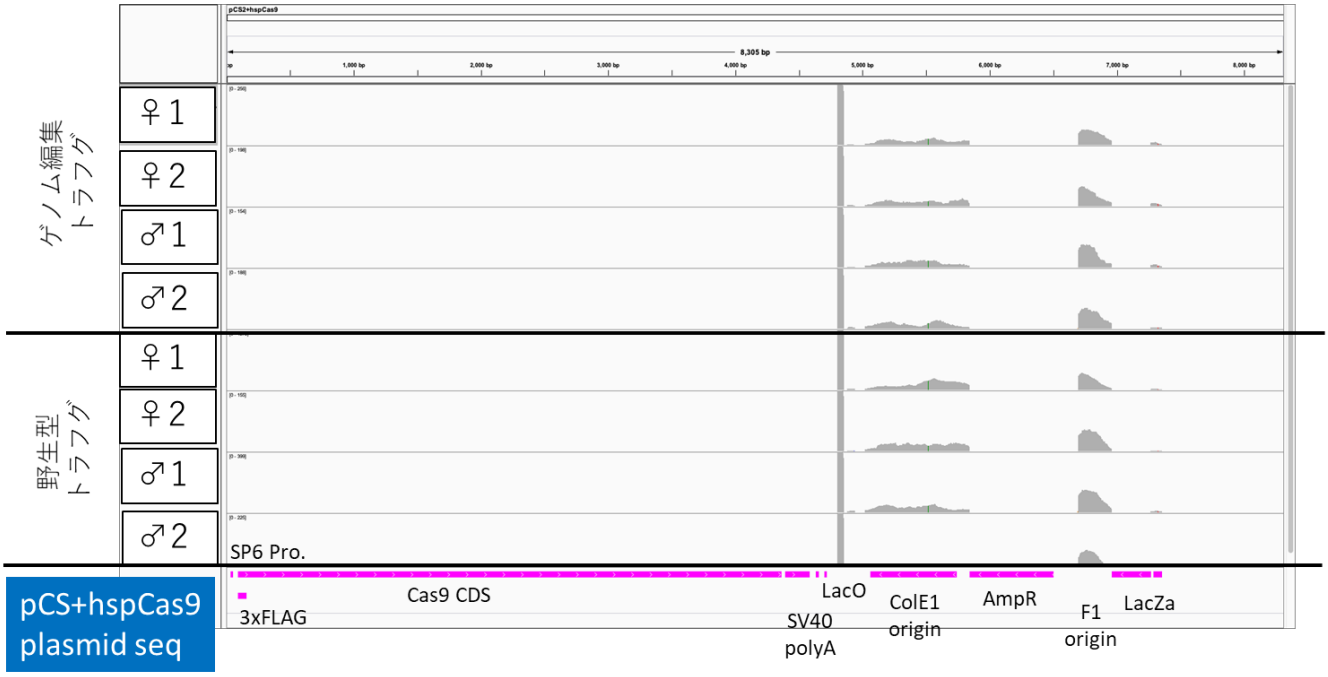


図１５．レプチン受容体遺伝子破壊トラフグ系統のゲノム編集ツール残存性の評価（pCS+hspCas9）

各個体の全塩基配列データをクエリーとしてCas9 RNA 合成時に用いたpCS+hspCas9にマッピングした。ゲノム編集個体および野生型個体ともに、プラスミドバックボーンの配列にマップされるものはあるが、Cas9 領域にはマップされなかった。

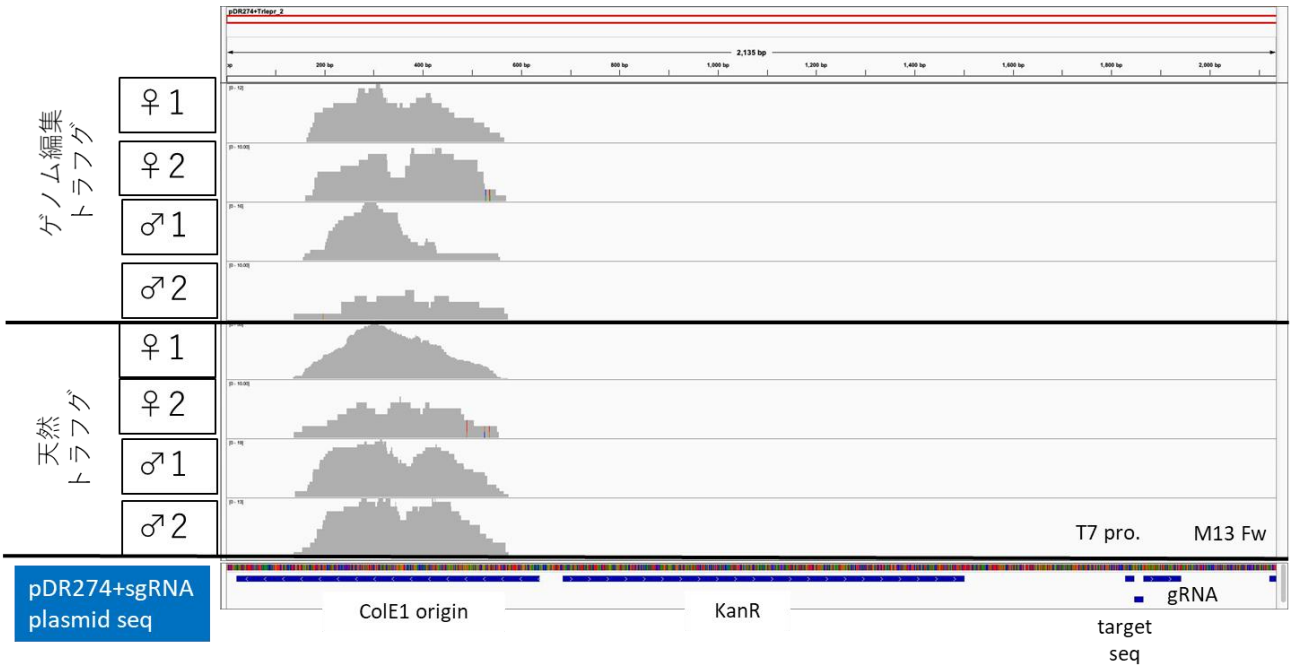


図１６．レプチン受容体遺伝子破壊トラフグ系統のゲノム編集ツール残存性の評価（pDR274+sgRNA）

各個体の全塩基配列データをクエリーとしてCas9 RNA 合成時に用いたpDR274+sgRNAにマッピングした。ゲノム編集個体および野生型個体ともに、プラスミドバックボーンの配列にマップされるものはあるが、標的配列を含む guide RNA 領域にはマップされなかった。

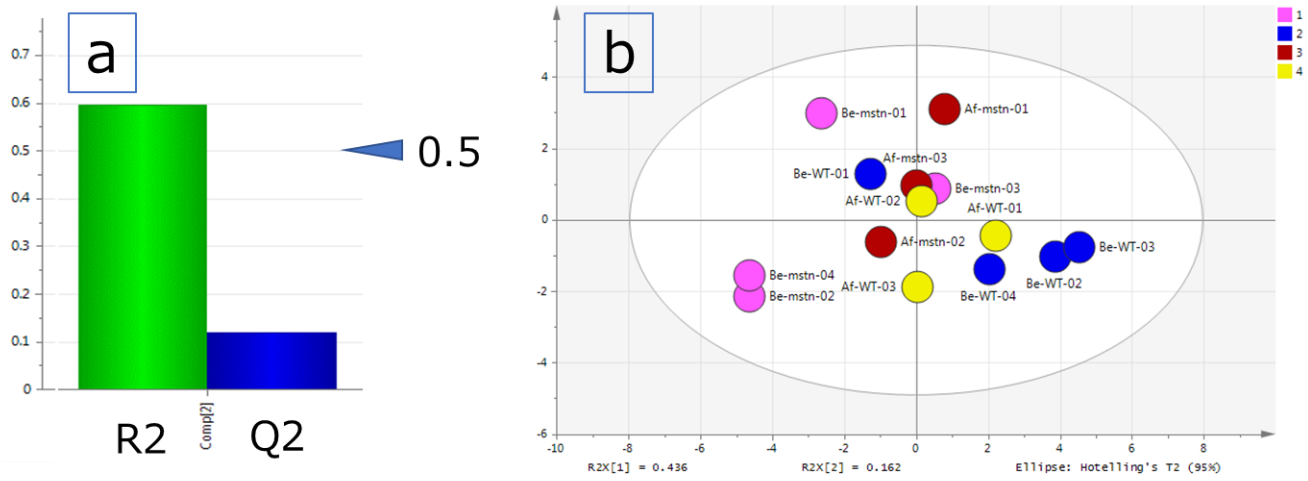
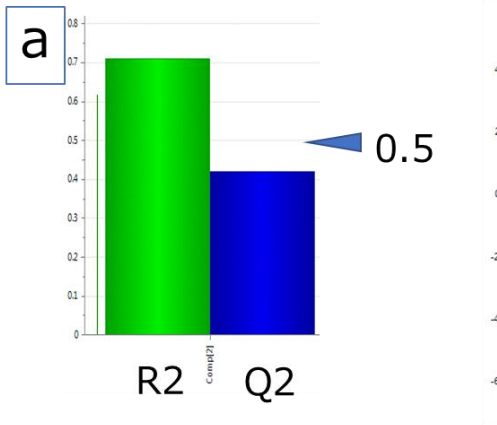
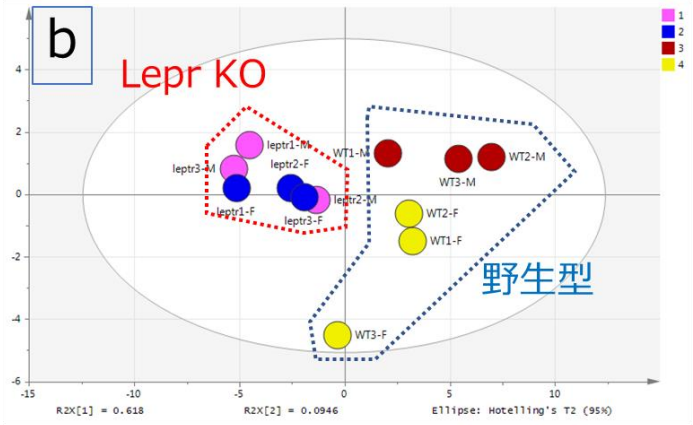


図１７．ミオスタチン遺伝子破壊マダイ系統メタボロミックス解析

a: データ解析の有意性を示す。 Q2値が 0.5 以上の場合、データ解析結果が信頼される。今回のQ2値は 0.5 以下であり b に示される解析の信頼性は低いと判断される。つまり、比較した個体間で有意な差がない。b：主成分分析の結果。mstn：mstn KO マダイ
WT： 野生型マダイ





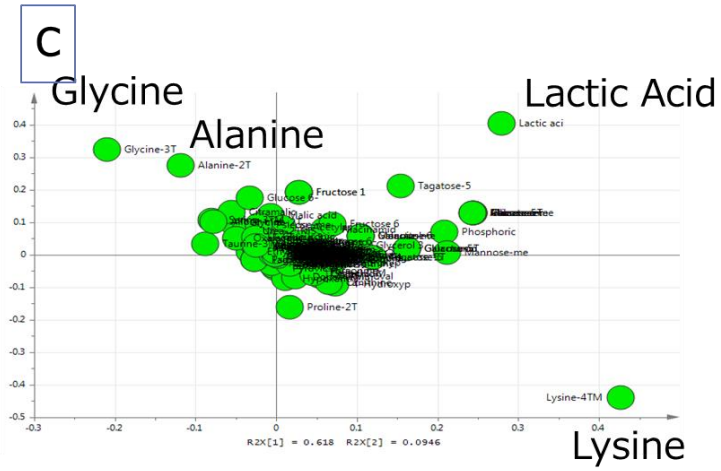
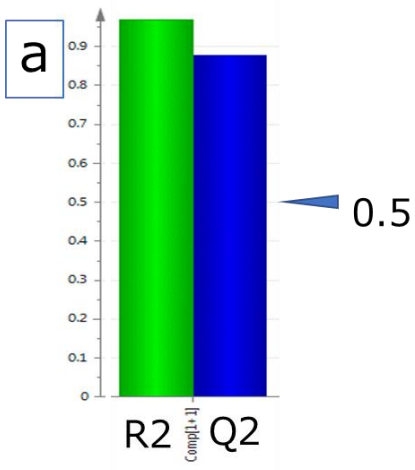
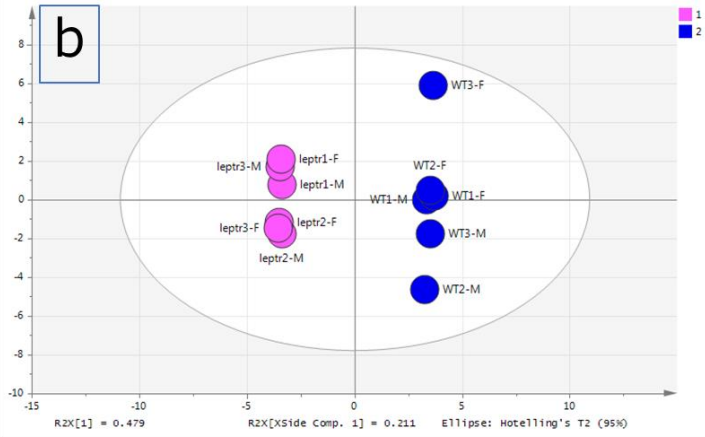


図１８．レプチン受容体遺伝子破壊トラフグ系統メタボロミックス解析

a: データ解析の有意性を示す。 Q2値が0.5 以上の場合、データ解析結果が信頼される。今回のQ2値は 0.5 以下であった。b : 主成分分析の結果。c : 主成分分析に寄与する因子の解析。 lepr KO トラフグ、WT： 野生型トラフグ





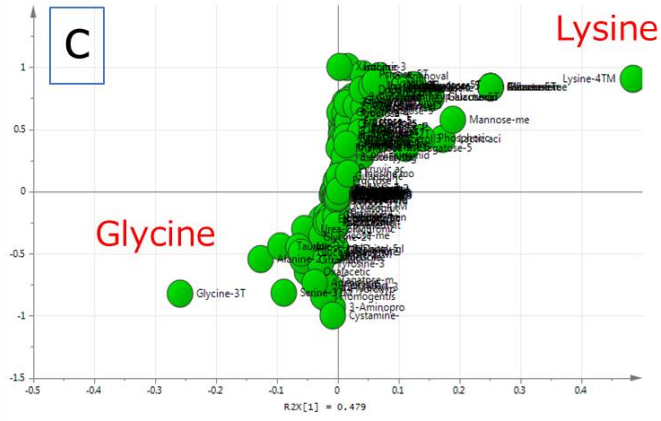


図１９．レプチン受容体遺伝子破壊トラフグ系統メタボロミックス解析に影響を与える因子

a: データ解析の有意性を示す。b : ゲノム編集魚と野生魚で最も差異が出るようにパラメータをせってした主成分分析の結果。c : 主成分分析に寄与する因子の解析。lepr : lepr KO トラフグ、WT： 野生型トラフグ

表３　諸外国でのゲノム編集技術を用いた研究動向調査

文献ID	生物種	種名	用いた技術	ターゲット遺伝子	雑誌名	TI	年	巻・号・ページ	著者	所属機関（国）	PMID	DOI	抄録	species2
1	animal	chicken	CRISPR;Cas9;	cadherin-like and PC-esterase domain containing 1	Bioscience reports	Cped1 promotes chicken SSCs formation with the aid of histone acetylation and transcription factor Sox2.	2018	38(5)	[Zhang C et al.]	Yangzhou University, Yangzhou, China.	30038055	10.1042/BSR20180707	Spermatogonial stem cells (SSCs) may apply to gene therapy, regenerative medicine in place of embryonic stem cells (ESCs). However, the application of SSCs was severely limited by the low induction efficiency and the lack of thorough analysis of the regulatory mechanisms of SSCs formation. Current evidences have demonstrated multiple marker genes of germ cells, while genes that specifically regulate the formation of SSCs have not been explored. In our study, cadherin-like and PC-esterase domain containing 1 (Cped1) expressed specifically in SSCs based on RNA-seq data analysis. To study the function of Cped1 in the formation of SSCs, we successfully established a CRISPR/Cas9 knockout system. The gene disruption frequency is 37% in DF1 and 25% in ESCs without off-target effects. Knockout of Cped1 could significantly inhibit the formation of SSCs in vivo and in vitro The fragment of -1050 to -1 bp had the activity as Cped1 gene promoter. Histone acetylation could regulate the expression of Cped1. We added 5-azaeytidi (DNA methylation inhibitors) and TSA (histone deacetylase inhibitors) respectively during the cultivation of SSCs. TSA was validated to promote the transcription of Cped1. Dual-luciferase reporter assay revealed that active control area of the chicken Cped1 gene is -296 to -1 bp. There are Cebpb, Sp1, and Sox2 transcription factor binding sites in this region. Point-mutation experiment results showed that Sox2 negatively regulates the transcription of Cped1. Above results demonstrated that Cped1 is a key gene that regulates the formation of SSCs. Histone	chicken
2	animal	chicken	CRISPR;Cas9;	transcription factors; enhancer	Development	Genome and epigenome engineering CRISPR toolkit for in vivo modulation of cis-regulatory interactions and gene expression in the chicken embryo.	2018	145(4)	[Williams RM et al.]	University of Oxford, Oxford, UK.	29386245	10.1242/dev.160333	CRISPR/Cas9 genome engineering has revolutionised all aspects of biological research, with epigenome engineering transforming gene regulation studies. Here, we present an optimised, adaptable toolkit enabling genome and epigenome engineering in the chicken embryo, and demonstrate its utility by probing gene regulatory interactions mediated by neural crest enhancers. First, we optimise novel efficient guide-RNA mini expression vectors utilising chick U6 promoters, provide a strategy for rapid somatic gene knockout and establish a protocol for evaluation of mutational penetrance by targeted next-generation sequencing. We show that CRISPR/Cas9-mediated disruption of transcription factors causes a reduction in their cognate enhancer-driven reporter activity. Next, we assess endogenous enhancer function using both enhancer deletion and nuclease-deficient Cas9 (dCas9) effector fusions to modulate enhancer chromatin landscape, thus providing the first report of epigenome engineering in a developing embryo. Finally, we use the synergistic activation mediator (SAM) system to activate an endogenous target promoter. The novel genome and epigenome engineering toolkit developed here enables manipulation of endogenous gene expression and enhancer activity in chicken embryos, facilitating high-resolution analysis of gene regulatory	chicken
3	animal	chicken	CRISPR;Cas9;	GAPDH	F1000Research	Successful CRISPR/Cas9 mediated homologous recombination in a chicken cell line.	2018	7:238	[Antonova E et al.]	Moscow Institute of Physics and Technology, Moscow Region, Russian Federation.	29946437	10.12688/f1000research.13457.2	Background: CRISPR/Cas9 system is becoming the dominant genome editing tool in a variety of organisms. CRISPR/Cas9 mediated knock out has been demonstrated both in chicken cell lines and in chicken germ cells that served to generate genetically modified birds. However, there is limited data about CRISPR/Cas9 dependent homology directed repair (HDR) for avian, even in cell culture. Few attempts have been made with integrations in safe harbor loci of chicken genome that induces constitutive expression of the inserted gene. Gene expression under an endogenous promoter would be more valuable than under a constitutive exogenous promoter, as it allows the gene expression to be tissue-specific. Methods: Three gRNAs were chosen to target chicken 3'-untranslated region of GAPDH gene. Cas9-mediated activity in the targeted locus for the gRNAs in DF-1 cells was estimated by T7E1 assay. To edit the locus, the HDR cassette was added along with CRISPR/Cas9. The inserted sequence contained eGFP in frame with a GAPDH coding sequence via P2A and Neomycin resistance gene (neoR) under cytomegalovirus promoter. Correct integration of the cassette was confirmed with fluorescent microscopy, PCR analysis and sequencing. Enrichment of modified cells was done by G418 selection. Efficiency of integration was assessed with fluorescence activated cell sorting (FACS). Results: We have established a CRISPR/Cas9 system to target an endogenous locus and precisely insert a gene under endogenous control. In our system, we used positive and negative selection to enrich modified cells and remove cells with undesirable insertions. The efficiency of CRISPR/Cas9-mediated HDR was increased up to 90% via G418 enrichment. We have successfully inserted eGFP under control of the chicken GAPDH promoter. Conclusions: The approach can be used further to insert genes of interest under control of tissue-specific promoters in primordial germ cells in order to produce	chicken
4	animal	chicken	CRISPR;Cas9;	TANK-binding kinase 1 (TBK-1)	Frontiers in immunology	CRISPR/Cas9-Mediated Chicken TBK1 Gene Knockout and Its Essential Role in STING-Mediated IFN-beta Induction in Chicken Cells.	2018	9:3010	[Cheng Y et al.]	Shanghai Jiao Tong University, Shanghai, China.	30662438	10.3389/fimmu.2018.03010	TANK-binding kinase 1 (TBK1) is involved in innate immunity, prompting transcriptional induction of type I interferons in response to pathogenic infection. Many studies have focused on mammals but the function of TBK1 in chickens remains poorly defined. CRISPR/Cas9 system has made gene-knockout easy to accomplish. Although CRISPR/Cas9 has been used in chicken cells, low mutation efficiency limits its wide application in chickens. In this study, an effective gene-knockout system was developed based on the CRISPR/Cas9 system in chicken embryonic fibroblast DF-1. Two CRISPR/Cas9 plasmids were constructed, TBK1-g1 and TBK1-g2, which express gRNAs targeting different sequences of the chicken TBK1 gene. After transfection and enrichment with puromycin screening, the mutation rates as assessed via T7E1 assay were 88.05 and 89.55%, respectively, and subsequent sequence analysis showed mutation efficiencies of 86.67 and 93.33%. With the limiting-dilution method, a chTBK1 gene-deficiency monoclonal cell line was obtained and was named DF-1-TBK1-C3. The DF-1-TBK1-C3 cells exhibited normal morphology and maintained stable proliferation ability compared to wild-type cells. The gene-overexpression system and luciferase reporter assay showed that IFN-beta induction induced by chSTING was almost completely blocked in DF-1-TBK1-C3 cells. With quantitative real-time PCR, we further confirmed the essential role of chTBK1 in the chSTING-mediated IFN-beta induction. At last, the study demonstrated that the chTBK1 knockout system is also applicable in primary chick embryo fibroblasts (CEFs). In this study, an effective gene-knockout system was applied in chickens, a TBK1 gene-deleted DF-1 cell line was successfully created using this system, and with the chTBK1 knockout cells, chTBK1 was revealed to be indispensable in STING-mediated IFN-beta activation in chicken	chicken

5	animal	chicken	Cas9	Nanos2	Journal of cellular biochemistry	Nanos2 promotes differentiation of chicken (Gallus gallus) embryonic stem cells to male germ cells.	2018	119(6):4435-4446	[Zhang W et al.]	Yangzhou University, Yangzhou, Jiangsu, China.	29143989	10.1002/jcb.26528	Nanos2 is an evolutionarily conserved RNA-binding protein containing 2 CCHC-type zinc finger motives. Here, we report that Nanos2 is strongly expressed in the testis compared to other tissues in chicken (Gallus gallus). Overexpression and knockout plasmid vectors were constructed, and in-vitro Cas9/gRNA digestion and T7 endonuclease I (T7E1) assay indicated that Nanos2-g1 possessed the highest knockout activity. In vitro and in vivo, Nanos2 overexpression accelerated the production of embryoid bodies (EBs) and SSC-like cells and promoted cvh, c-kit, and integrin alpha6 expression. Immunofluorescence staining, periodic acid schiff (PAS) and flow cytometry (FCM) assay showed that primordial germ cells (PGCs) and spermatogonial stem cells (SSCs) formation were significantly promoted. On the contrary, Nanos2 knockout delayed the production of EBs and SSC-like cells and correspondingly reduced cvh, c-kit, and integrin alpha6 expression. Simultaneously, the quantity of PGCs and SSCs was blocked. Collectively, these results uncovered a novel function of Nanos2 involved in chicken male germ cell differentiation, where it acts as a facilitator.	chicken
6	animal	chicken	CRISPR;Cas9;	transient receptor potential canonical channel (TRPC5)	Journal of neurophysiology	TRPC5 is required for the NO-dependent increase in dendritic Ca(2+) and GABA release from chick retinal amacrine cells.	2018	119(1):262-273	[Maddox JW et al.]	Louisiana State University , Baton Rouge, La, USA.	28978766	10.1152/jn.00500.2017	GABAergic signaling from amacrine cells (ACs) is a fundamental aspect of visual signal processing in the inner retina. We have previously shown that nitric oxide (NO) can elicit release of GABA independently from activation of voltage-gated Ca(2+) channels in cultured retinal ACs. This voltage-independent quantal GABA release relies on a Ca(2+) influx mechanism with pharmacological characteristics consistent with the involvement of the transient receptor potential canonical (TRPC) channels TRPC4 and/or TRPC5. To determine the identity of these channels, we evaluated the ability of NO to elevate dendritic Ca(2+) and to stimulate GABA release from cultured ACs under conditions known to alter the function of TRPC4 and 5. We found that these effects of NO are phospholipase C dependent, have a biphasic dependence on La(3+), and are unaffected by moderate concentrations of the TRPC4-selective antagonist ML204. Together, these results suggest that NO promotes GABA release by activating TRPC5 channels in AC dendrites. To confirm a role for TRPC5, we knocked down the expression of TRPC5 using CRISPR/Cas9-mediated gene knockdown and found that both the NO-dependent Ca(2+) elevations and increase in GABA release are dependent on the expression of TRPC5. These results demonstrate a novel NO-dependent mechanism for regulating neurotransmitter output from retinal ACs. NEW & NOTEWORTHY Elucidating the mechanisms regulating GABAergic synaptic transmission in the inner retina is key to understanding the flexibility of retinal ganglion cell output. Here, we demonstrate that nitric oxide (NO) can activate a transient receptor potential canonical 5 (TRPC5)-mediated Ca(2+) influx, which is sufficient to drive vesicular GABA release from retinal amacrine cells. This NO-dependent mechanism can bypass the need for depolarization and may have an important role in	chicken
7	animal	chicken	CRISPR;Cas9;		Scientific reports	High fidelity CRISPR/Cas9 increases precise monoallelic and biallelic editing events in primordial germ cells.	2018	8(1):15126	[Idoko-Akoh A et al.]	University of Edinburgh, Midlothian, UK.	30310080	10.1038/s41598-018-33244-x	Primordial germ cells (PGCs), the embryonic precursors of the sperm and egg, are used for the introduction of genetic modifications into avian genome. Introduction of small defined sequences using genome editing has not been demonstrated in bird species. Here, we compared oligonucleotide-mediated HDR using wild type SpCas9 (SpCas9-WT) and high fidelity SpCas9-HF1 in PGCs and show that many loci in chicken PGCs can be precise edited using donors containing CRISPR/Cas9-blocking mutations positioned in the protospacer adjacent motif (PAM). However, targeting was more efficient using SpCas9-HF1 when mutations were introduced only into the gRNA target sequence. We subsequently employed an eGFP-to-BFP conversion assay, to directly compare HDR mediated by SpCas9-WT and SpCas9-HF1 and discovered that SpCas9-HF1 increases HDR while reducing INDEL formation. Furthermore, SpCas9-HF1 increases the frequency of single allele editing in comparison to SpCas9-WT. We used SpCas9-HF1 to demonstrate the introduction of monoallelic and biallelic point mutations into the FGF20 gene and generate clonal populations of edited PGCs with defined homozygous and heterozygous genotypes. Our results demonstrate the use of oligonucleotide donors and high fidelity CRISPR/Cas9 variants to perform precise	chicken
8	animal	chicken	CRISPR;Cas9;	human interferon beta	Scientific reports	Efficient production of human interferon beta in the white of eggs from ovalbumin gene-targeted hens.	2018	8(1):10203	[Oishi I et al.]	National Institute of Advanced Industrial Science and Technology, Ikeda, Osaka, Japan.	29976933	10.1038/s41598-018-28438-2	Transgenic chickens could potentially serve as bioreactors for commercial production of recombinant proteins in egg white. Many transgenic chickens have been generated by randomly integrating viral vectors into their genomes, but transgene expression has proved insufficient and/or limited to the initial cohort. Herein, we demonstrate the feasibility of integrating human interferon beta (hIFN-beta) into the chicken ovalbumin locus and producing hIFN-beta in egg white. We knocked in hIFN-beta into primordial germ cells using a CRISPR/Cas9 protocol and then generated germline chimeric roosters by cell transplantation into recipient embryos. Two generation-zero founder roosters produced hIFN-beta knock-in offspring, and all knock-in female offspring produced abundant egg-white hIFN-beta (~3.5 mg/ml). Although female offspring of the first generation were sterile, their male counterparts were fertile and produced a second generation of knock-in hens, for which egg-white hIFN-beta production was comparable with that of the first generation. The hIFN-beta bioactivity represented only ~5% of total egg-white hIFN-beta, but unfolding and refolding of hIFN-beta in the egg white fully recovered the bioactivity. These results suggest that transgene insertion at the chicken ovalbumin locus can result in abundant and stable expression of an exogenous protein deposited into egg white and should be amenable to industrial	chicken
9	animal	chicken	CRISPR;Cas9;	IFN-induced proteins with tetratricopeptide repeats 5	Scientific reports	Chicken Interferon-induced Protein with Tetratricopeptide Repeats 5 Antagonizes Replication of RNA Viruses.	2018	8(1):6794	[Santhakumar D et al.]	Lancaster University, Lancaster, UK.	29717152	10.1038/s41598-018-24905-y	The intracellular actions of interferon (IFN)-regulated proteins, including IFN-induced proteins with tetratricopeptide repeats (IFITs), attribute a major component of the protective antiviral host defense. Here we applied genomics approaches to annotate the chicken IFIT locus and currently identified a single IFIT (chIFIT5) gene. The profound transcriptional level of this effector of innate immunity was mapped within its unique cis-acting elements. This highly virus- and IFN-responsive chIFIT5 protein interacted with negative sense viral RNA structures that carried a triphosphate group on its 5' terminus (ppp-RNA). This interaction reduced the replication of RNA viruses in lentivirus-mediated IFIT5-stable chicken fibroblasts whereas CRISPR/Cas9-edited chIFIT5 gene knockout fibroblasts supported the replication of RNA viruses. Finally, we generated mosaic transgenic chicken embryos stably expressing chIFIT5 protein or knocked-down for endogenous chIFIT5 gene. Replication kinetics of RNA viruses in these transgenic chicken embryos demonstrated the antiviral potential of chIFIT5 in ovo. Taken together, these findings propose that IFIT5 specifically antagonize RNA viruses by sequestering viral nucleic acids in chickens, which are unique in innate immune sensing and responses to viruses of both poultry and human health	chicken

10	animal	chicken	CRISPR;Cas9;	tva; tvc; tvj	Viruses	Genetic Resistance to Avian Leukosis Viruses Induced by CRISPR/Cas9 Editing of Specific Receptor Genes in Chicken Cells.	2018	10(11)	[Koslova A et al.]	Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic.	30400152	10.3390/v10110605	Avian leukosis viruses (ALVs), which are pathogens of concern in domestic poultry, utilize specific receptor proteins for cell entry that are both necessary and sufficient for host susceptibility to a given ALV subgroup. This unequivocal relationship offers receptors as suitable targets of selection and biotechnological manipulation with the aim of obtaining virus-resistant poultry. This approach is further supported by the existence of natural knock-outs of receptor genes that segregate in inbred lines of chickens. We used CRISPR/Cas9 genome editing tools to introduce frame-shifting indel mutations into tva, tvc, and tvj loci encoding receptors for the A, C, and J ALV subgroups, respectively. For all three loci, the homozygous frame-shifting indels generating premature stop codons induced phenotypes which were fully resistant to the virus of respective subgroup. In the tvj locus, we also obtained in-frame deletions corroborating the importance of W38 and the four amino-acids preceding it. We demonstrate that CRISPR/Cas9-mediated knock-out or the fine editing of ALV receptor genes might be the first step in the development of virus-resistant chickens.	chicken
11	animal	chicken	CRISPR;Cas9;	ovalbumin	3 Biotech	Efficient knock-in at the chicken ovalbumin locus using adenovirus as a CRISPR/Cas9 delivery system.	2019	9(12):454	[Qin X et al.]	Guangxi University, Guangxi, China.	31832301	10.1007/s13205-019-1966-3	In this study, efficient knock-in (KI) of human epidermal growth factor (hEGF) cDNA at the ovalbumin (OV) locus in cultured chicken cells was achieved using adenovirus as a delivery for CRISPR/Cas9 elements and optimizing donor vector construction. The strategy of recruiting donor DNA to the insertion site further improved the KI efficiency. The inserted hEGF cDNA can expressed in primary oviduct cells and secreted hEGF promoted proliferation of Hela cells. Moreover, we achieved efficient KI in blastoderm cells without altering their induction in vitro and obtained germline chimeric KI chicken embryos by transplanting KI blastoderm cells as well as injecting adenovirus directly, in vivo. Our results provided an efficient KI method for chicken cells and embryos, and lay the foundation for more convenient production of KI chicken at the OV locus, which will promote the development of oviduct-specific bioreactor.	chicken
12	animal	chicken	CRISPR;Cas9;	HMG-box protein 1	Biochemical and biophysical research communications	Transcription factor HBP1: A regulator of senescence and apoptosis of preadipocytes.	2019	517(2):216-220	[Chen H et al.]	Key Laboratory of Chicken Genetics and Breeding, Ministry of Agriculture and Rural Affairs, Harbin, China.	31331641	10.1016/j.bbrc.2019.07.048	BACKGROUND: /aim: HMG-box protein 1 (HBP1) plays an important role in the senescence and apoptosis of mammalian cells, but its role in chicken cells remains unclear. The aim of this study was to investigate the effects of HBP1 on senescence and apoptosis of chicken preadipocytes. METHODS: The immortalized chicken preadipocyte cell line (ICP2) was used as a cell model. Chicken HBP1 knockout and overexpressing preadipocyte cell lines were established using CRISPR/Cas9 gene editing technology and lentiviral infection. Western blotting was used to detect the protein expression of HBP1 and senescence markers p16 and p53. Cell senescence was measured by Sa-beta-Gal staining and apoptosis was detected by flow cytometry. RESULTS: HBP1 was highly expressed in senescent ICP2 cells compared with young ICP2 cells. After the deletion of HBP1, the degree of senescence, the apoptosis rate and the protein expression levels of p16 and p53 were significantly reduced. After the overexpression of HBP1, the degree of senescence, the apoptosis rate and the protein expression levels of p16 and p53 were significantly increased. CONCLUSION: HBP1 promotes the senescence and apoptosis of chicken preadipocytes.	chicken
13	animal	chicken	CRISPR;Cas9;	exogenous genes of donor plasmids into Z chromosomes	FASEB journal	Targeted gene insertion into Z chromosome of chicken primordial germ cells for avian sexing model development.	2019	33(7):8519-8529	[Lee HJ et al.]	Seoul National University, Seoul, South Korea.	30951374	10.1096/fj.201802671R	Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) have facilitated the production of genome-edited animals for use as models. Because of their unique developmental system, avian species offer many advantages as model vertebrates. Here, we report the development of novel chicken models using the CRISPR/Cas9-mediated nonhomologous end joining repair pathway in chicken primordial germ cells (PGCs). Through the introduction of a donor plasmid containing short guide RNA recognition sequences and CRISPR/Cas9 plasmids into chicken PGCs, exogenous genes of donor plasmids were precisely inserted into target loci, and production of transgenic chickens was accomplished through subsequent transplantation of the Z chromosome-targeted PGCs. Using this method, we successfully accomplished the targeted gene insertion to the chicken sex Z chromosome without detected off-target effects. The genome-modified chickens robustly expressed green fluorescent protein from the Z chromosome, which could then be used for easy sex identification during embryogenesis. Our results suggest that this powerful genome-editing method could be used to develop many chicken models and should significantly expand the application of genome-modified avians.-Lee, H. J., Yoon, J. W., Jung, K. M., Kim, Y. M., Park, J. S., Lee, K. Y., Park, K. J., Hwang, Y. S., Park, Y. H., Rengaraj, D., Han, J. Y. Targeted gene insertion into Z chromosome of chicken	chicken
14	animal	chicken	CRISPR;Cas9;	G0/G1 switch gene 2 (g0s2)	FASEB journal	Disruption of G0/G1 switch gene 2 (G0S2) reduced abdominal fat deposition and altered fatty acid composition in chicken.	2019	33(1):1188-1198	[Park TS et al.]	Seoul National University, Pyeongchang-gun, Gangwon-do, Korea.	30085885	10.1096/fj.201800784R	Chicken as a food source is one of the most widespread domestic animals, and it has been used extensively as a research model. The clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) system is the most efficient and reliable tool for precise genome-targeted modification and has generated considerable excitement for industrial applications, as well as biologic science. Unlike in mammals, germline-transmittable primordial germ cells (PGCs) in chicken were used as an alternative strategy for the production of genetically altered chickens. Here, by combining the CRISPR-Cas9 platform and germ cell-mediated germline transmission, we generated G0/G1 switch gene 2 (G0S2) knockout (KO) chickens, and G0S2 null KO chickens showed a dramatic reduction of abdominal fat deposition without affecting other economic traits. Additionally, G0S2 null KO chickens had altered fatty acid compositions in their blood and abdominal fat compared with wild-type chickens under normal dietary conditions. The global mRNA sequencing data showed that G0S2 disruption in chickens would activate the adipose tissue-specific peroxisomal oxidation pathway, and enoyl-coenzyme A (CoA), hydratase/3-hydroxyacyl CoA dehydrogenase might be a target molecule in metabolic homeostasis in the chicken adipose tissue. Our results demonstrate that the CRISPR-Cas9 system with chicken PGCs can facilitate the production of specific genome-edited chickens for practical applications, as well as basic research.-Park, T. S., Park, J., Lee, J. H., Park, J.-W., Park, B.-C. Disruption of G0/G1 switch gene 2 (G0S2) reduced abdominal fat deposition and altered fatty acid	chicken

15	animal	chicken	CRISPR;Cas9;	CXCR4	Frontiers in immunology	Blocking of the CXCR4-CXCL12 Interaction Inhibits the Migration of Chicken B Cells Into the Bursa of Fabricius.	2019	10:3057	[Laparidou M et al.]	Reproductive Biotechnology, School of Life Sciences Weihenstephan, Technical University of Munich, Freising, Germany.	31998323	10.3389/fimmu.2019.03057	B cells have first been described in chickens as antibody producing cells and were named after the Bursa of Fabricius, a unique organ supporting their development. Understanding different factors mediating the early migration of B cells into the bursa of Fabricius is crucial for the study of B cell biology. While CXCL12 (stromal derived factor 1) was found to play an important role in B lymphocyte trafficking in mammals, its role in the chicken is still unknown. Previous studies indicated that chicken CXCL12 and its receptor CXCR4 are simultaneously expressed during bursal development. In this study, we investigated whether the CXCR4/CXCL12 interaction mediates B cell migration in chicken embryo. We used the CRISPR/Cas9 system to induce a CXCR4 knockout in chicken B cells which led to chemotaxis inhibition toward CXCL12. This was confirmed by adoptive cell transfer and inhibition of the CXCR4/CXCL12 interaction by blocking with the small inhibitor AMD3100. In addition, we found that the chicken exhibits similarities to mice when it comes to CXCR4 being dependent on B cell receptor expression. B cells lacking the B cell receptor failed to migrate toward CXCL12 and showed no response upon CXCL12 stimulation. Overall, we demonstrated the significance of CXCR4/CXCL12 in chicken B cell development in vivo and the	chicken
16	animal	chicken	CRISPR;Cas9;	methyl binding domain protein 4 (mbd4)	Frontiers in immunology	Chicken MBD4 Regulates Immunoglobulin Diversification by Somatic Hypermutation.	2019	10:2540	[Costello R et al.]	University of Illinois at Chicago, Chicago, IL, USA.	31736964	10.3389/fimmu.2019.02540	Immunoglobulin (Ig) diversification occurs via somatic hypermutation (SHM) and class switch recombination (CSR), and is initiated by activation-induced deaminase (AID), which converts cytosine to uracil. Variable (V) region genes undergo SHM to create amino acid substitutions that produce antibodies with higher affinity for antigen. The conversion of cytosine to uracil in DNA promotes mutagenesis. Two distinct DNA repair mechanisms regulate uracil processing in Ig genes. The first involves base removal by the uracil DNA glycosylase (UNG), and the second detects uracil via the mismatch repair (MMR) complex. Methyl binding domain protein 4 (MBD4) is a uracil glycosylase and an intriguing candidate for involvement in somatic hypermutation because of its interaction with the MMR MutL homolog 1 (MLH1). We found that the DNA uracil glycosylase domain of MBD4 is highly conserved among mammals, birds, shark, and insects. Conservation of the human and chicken MBD4 uracil glycosylase domain structure is striking. Here we examined the function of MBD4 in chicken DT40 B cells which undergo constitutive SHM. We constructed structural variants of MBD4 DT40 cells using CRISPR/Cas9 genome editing. Disruption of the MBD4 uracil glycosylase catalytic region increased SHM frequency in IgM loss assays. We propose	chicken
17	animal	chicken	CRISPR;Cas9;	Atg5	In vitro cellular & developmental biology. Animal	Knockout of Atg5 inhibits proliferation and promotes apoptosis of DF-1 cells.	2019	55(5):341-348	[Liao Z et al.]	South China Agricultural University, Guangzhou, China.	31025250	10.1007/s11626-019-00342-7	Atg5, as a switch of cell autophagy and apoptosis, plays an important regulatory role in the occurrence and development of autophagy. Atg5 has been reported to involve the autophagy process but little in the apoptotic process. Here, we constructed an Atg5(-/-) DF-1 cell line using the CRISPR/Cas9 assay and confirmed the significant difference in growth kinetics between Atg5(-/-) DF-1 cells and wild-type DF-1 cells. Importantly, we found that Atg5 suppresses the cellular proliferation and induce the apoptosis in DF-1 cells by Hoechst's staining, flow cytometry, and caspase activity assay. All these findings indicated that Atg5 plays an important role in the proliferation of DF-1 cells. On the other hand, we compared the expression of autophagy key proteins LC3 and P62 in Atg5 knockout cells and wild-type cells, and detected the aggregation point distribution of LC3 protein in cells by laser confocal technique; our results showed that Atg5 knockout inhibited autophagy compared with wild-type cells. The present findings further help to resolve the molecular mechanisms regulating Atg5	chicken
18	animal	chicken	CRISPR;Cas9;	ALV subgroup A (tva)	Journal of animal science and biotechnology	Sequential disruption of ALV host receptor genes reveals no sharing of receptors between ALV subgroups A, B, and J.	2019	10:23	[Lee HJ et al.]	Seoul National University, Seoul, Korea.	30976416	10.1186/s40104-019-0333-x	Background: Previously, we showed that targeted disruption of viral receptor genes in avian leukosis virus (ALV) subgroups using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9))-based genome editing confers resistance to ALV subgroups B and J. Here, we used the same strategy to target the receptor expressed by ALV subgroup A (TVA) and generate chicken cells resistant to infection by this virus. Results: CRISPR/Cas9-based disruption of exon 2 within the tva gene of DF-1 fibroblasts conferred resistance to infection by ALV subgroup A regardless of whether frameshift mutations were introduced during editing. Conversely, overexpression of the wild-type TVA receptor (wtTVA) by tva-modified DF-1 clones restored susceptibility to ALV subgroup A. The results confirm that exon 2, which contains the low-density lipoprotein receptor class A domain of TVA, is critical for virus entry. Furthermore, we sequentially modified DF-1 cells by editing the tva, tvb, and Na(+)/H(+) exchange 1 (chNHE1) genes, which are the specific receptors for ALV subgroups A, B, and J, respectively. Conclusions: Simultaneous editing of multiple receptors to block infection by different subgroups of ALV confirmed that ALV subgroups A, B, and J do not share host receptors. This strategy could be used to generate cells resistant to multiple viral pathogens that use distinct receptors for cell	chicken
19	animal	chicken	CRISPR;Cas9;	dazl; pou5f3; ovalbumin	Journal of biological engineering	HMEJ-mediated efficient site-specific gene integration in chicken cells.	2019	13:90	[Xie L et al.]	Guangxi University, Nanning, Guangxi, China.	31832093	10.1186/s13036-019-0217-9	Background: The production of transgenic chicken cells holds great promise for several diverse areas, including developmental biology and biomedical research. To this end, site-specific gene integration has been an attractive strategy for generating transgenic chicken cell lines and has been successfully adopted for inserting desired genes and regulating specific gene expression patterns. However, optimization of this method is essential for improving the efficiency of genome modification in this species. Results: Here we compare gene knock-in methods based on homology-independent targeted integration (HITI), homology-directed repair (HDR) and homology mediated end joining (HMEJ) coupled with a clustered regularly interspaced short palindromic repeat associated protein 9 (CRISPR/Cas9) gene editing system in chicken DF-1 cells and primordial germ cells (PGCs). HMEJ was found to be a robust and efficient method for gene knock-in in chicken PGCs. Using this method, we successfully labeled the germ cell specific gene DAZL and the pluripotency-related gene Pou5f3 in chicken PGCs through the insertion of a fluorescent protein in the frame at the 3' end of the gene, allowing us to track cell migration in the embryonic gonad. HMEJ strategy was also successfully used in Ovalbumin, which accounts for more than 60% of proteins in chicken eggs, suggested its good promise for the mass production of protein with pharmaceutical importance using the chicken oviduct system. Conclusions: Taken together, these results demonstrate that HMEJ efficiently mediates site-specific gene integration in chicken PGCs, which holds great potential for the biopharmaceutical	chicken

20	animal	chicken	CRISPR;Cas9;	ovalbumin	Journal of bioscience and bioengineering	Targeted knock-in into the OVA locus of chicken cells using CRISPR/Cas9 system with homology-independent targeted integration.	2019		[Shi M et al.]	Kyushu University, Fukuoka, Japan.	31594694	10.1016/j.jbiosc.2019.09.011	It is anticipated that transgenic avian species will be used as living bioreactors for the production of biopharmaceutical proteins. Precise tissue-specific expression of exogenous genes is a major challenge for the development of avian bioreactors. No robust vector is currently available for highly efficient and specific expression. In recent years, genome-editing techniques such as the CRISPR/Cas9 system have emerged as efficient and user-friendly genetic modification tools. Here, to apply the CRISPR/Cas9 system for the development of transgenic chickens, guide RNA sequences (gRNAs) of the CRISPR/Cas9 system for the ovalbumin (OVA) locus were evaluated for the oviduct-specific expression of exogenous genes. An EGFP gene expression cassette was introduced into the OVA locus of chicken DF-1 and embryonic fibroblasts using the CRISPR/Cas9 system mediated by homology-independent targeted integration. For the knock-in cells, EGFP expression was successfully induced by activation of the endogenous OVA promoter using the dCas9-VPR transactivation system. The combination of gRNAs designed around the OVA TATA box was important to induce endogenous OVA gene expression with high efficiency. These methods provide a useful tool for studies on the creation of	chicken
21	animal	chicken	CRISPR;Cas9;	EAV-EP genome	Sheng wu gong cheng xue bao = Chinese journal of biotechnology	[CRISPR/Cas9-mediated foreign gene targeted knock-in into the chicken EAV-HP genome].	2019	35(2):236-243	[Guo M et al.]	Shaanxi University of Technology, Hanzhong, Shaanxi, China.	30806053	10.13345/j.cjb.180224	The study aims to use CRISPR/Cas9 introducing foreign gene targeted knock-in into chicken EAV-HP genome. First, specific primers were designed for amplification of EAV-HP left, right homologous arms and enhanced green fluorescent protein (eGFP) expression cassette. PCR products of homologous arms were ligated to both sides of eGFP by overlap extension PCR, resulting in full-length donor DNA fragment designated as LER. Then LER fragments were cloned into pMD19-T to obtain donor vector pMDT-LER. Subsequently, the donor vector pMDT-LER was transfected into HEK293T cells to verify the expression of eGFP gene. Furthermore, co-transfection of CRISPR/Cas9 expression vector and pMDT-LER into chicken DF-1 cells was performed to achieve eGFP transgenic cells. Meanwhile, eGFP expression was observed in cells, and the event of eGFP integration into EAV-HP genome was detectable by amplification of target DNA. Finally, the transgenic DF-1 cells were passaged seven times, and the stable integration and expression of eGFP was checked by PCR and Western blotting. These results demonstrated that eGFP gene was knocked into the EAV-HP genome successfully, which provides a new integration site	chicken
22	animal	chicken	CRISPR;Cas9;	activation-induced cytidine deaminase	Virologica Sinica	A Novel DT40 Antibody Library for the Generation of Monoclonal Antibodies.	2019	34(6):641-647	[Wang B et al.]	Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China.	31240617	10.1007/s12250-019-00142-z	Early etiological diagnosis is very important for the control of sudden viral infections, and requires antibodies with both high sensitivity and high specificity. Traditional antibody preparation methods have limitations, such as a long and arduous cycle, complicated operation, and high expenses. A chicken lymphoma cell line, DT40, is known to produce IgM-type antibodies and undergo gene conversion and somatic mutation in the variable region of the immunoglobulin gene during culture. Here, the DT40 cell line was developed to produce antibody libraries and prepare antibody rapidly in vitro. Since hypermutation in DT40 cells was regulated by the activation-induced cytidine deaminase (AID) gene, AID expression needs to be controlled to either fix the Ig sequence by stopping mutation or improve affinity by resuming mutation after the antibodies have been selected. In this study, we generated a novel AID-inducible DT40 cell line (DT40-H7), in which the endogenous AID gene was knocked out using the CRISPR/Cas9 genome editing system, and an inducible AID gene, based on the Tet-Off expression system, was stably transfected. AID expression was controlled in DT40-H7 cells in a simple and efficient manner; gene conversion and point mutations were observed only when AID was expressed. Using the antibody library generated from this cell line, we successfully obtained monoclonal antibodies against the NS1 protein of Zika virus. The DT40-H7 cell line represents a useful tool for the selection and evolution of antibodies and may also be a powerful tool for the rapid selection and	chicken
23	animal	cow	CRISPR;Cas9;	collagen type VIII alpha 1 chain (COL8A1)	Cell biology international	Effects of COL8A1 on the proliferation of muscle-derived satellite cells.	2018	42(9):1132-1140	[Li X et al.]	North-east Agricultural University, Harbin, China.	29696735	10.1002/cbin.10979	Collagen type VIII alpha 1 chain (COL8A1) is a component of the extracellular matrix. Our previous studies suggested that COL8A1 is associated with the proliferation of muscle-derived satellite cells (MDSCs). Additionally, it has been demonstrated that COL8A1 promotes the proliferation of smooth muscle cells and liver cancer cells. Therefore, we predicted that COL8A1 is associated with the proliferation of bovine MDSCs, which have potential applications in research. In this study, we constructed vectors to activate and repress COL8A1 in bovine MDSCs using the CRISPR/Cas9 technique and determined the effects of COL8A1 modulation by EdU labeling, Western blotting, and dual-luciferase reporter assays. The results showed that activation of COL8A1 increased the number of EdU-positive cells and expression of the proliferation markers cyclin B1 (CCNB1) and P-AKT. The expression of P-Akt was unchanged after addition of LY294002 (a protein kinase inhibitor capable of blocking the signal transduction pathway of the phosphoinositide 3-kinase). In contrast, repression of COL8A1 reduced the number of EdU-positive cells and expression of CCNB1 and P-AKT. We also observed upregulation and downregulation of COL8A1 following the overexpression and repression of EGR1, respectively. The dual-luciferase reporter assay revealed that EGR1 regulates the promoter activity of COL8A1. To our knowledge, this is the first study demonstrating that EGR1 positively regulates the expression of COL8A1, which in turn promotes the proliferation of bovine MDSCs via	cow
24	animal	cow	CRISPR;Cas9;	extracellular matrix protein 2	Cell biology international	Effect of ECM2 expression on bovine skeletal muscle-derived satellite cell differentiation.	2018	42(5):525-532	[Liu C et al.]	Northeast Agricultural University, Harbin, Heilongjiang, China.	29274297	10.1002/cbin.10927	Extracellular matrix components have important regulatory functions during cell proliferation and differentiation. In recent study, extracellular matrix were shown to have a strong effect on skeletal muscle differentiation. Here, we aimed to elucidate the effects of extracellular matrix protein 2 (ECM2), an extracellular matrix component, on the differentiation of bovine skeletal muscle-derived satellite cells (MDSCs). Western blot and immunofluorescence analyses were used to elucidate the ECM2 expression pattern in bovine MDSCs during differentiation in vitro. CRISPR/Cas9 technology was used to activate or inhibit ECM2 expression to study its effects on the in vitro differentiation of bovine MDSCs. ECM2 expression was shown to increase gradually during bovine MDSC differentiation, and the levels of this protein were higher in more highly differentiated myotubes. ECM2 activation promoted MDSC differentiation, whereas its suppression inhibited the differentiation of these cells. Here, for the first time, we demonstrated the importance of ECM2 expression during bovine MDSC differentiation; these results could lead to treatments that help to increase beef cattle	cow

25	animal	cow	CRISPR;	apoptosis-associated speck-like protein containing a caspase recruitment domain	Frontiers in microbiology	Lactobacillus rhamnosus GR-1 Ameliorates Escherichia coli-Induced Activation of NLRP3 and NLRC4 Inflammasomes With Differential Requirement for ASC.	2018	9:1661	[Wu Q et al.]	China Agricultural University, Beijing, China.	30087667	10.3389/fmicb.2018.01661	Escherichia coli is a common cause of mastitis in dairy cows. The adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) synergizes with caspase-1 to regulate inflammasome activation during pathogen infection. Here, the ASC gene was knocked out in bovine mammary epithelial (MAC-T) cells using clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated (Cas)-9 technology. MAC-T cells were pre-incubated with and without Lactobacillus rhamnosus GR-1 and then exposed to E. coli. Western blot analysis demonstrated increased expression of NLRP3 and NLRC4 following E. coli infection, but this increase was attenuated by pre-incubation with L. rhamnosus GR-1, regardless of ASC knockout. Western blot and immunofluorescence analyses revealed that pre-incubation with L. rhamnosus GR-1 decreased E. coli-induced caspase-1 activation at 6 h after E. coli infection, as also observed in ASC-knockout MAC-T cells. The E. coli-induced increase in caspase-4 mRNA expression was inhibited by pre-incubation with L. rhamnosus GR-1. ASC knockout diminished, but did not completely prevent, increased production of IL-1beta and IL-18 and cell pyroptosis associated with E. coli infection, whereas pre-incubation with L. rhamnosus GR-1 inhibited this increase. Our data indicate that L. rhamnosus GR-1 suppresses activation of ASC-dependent NLRP3 and NLRC4 inflammasomes and production of downstream IL-1beta and IL-18 during E. coli infection. L. rhamnosus GR-1 also inhibited E. coli-induced cell pyroptosis, in part through attenuation of NLRC4 and non-	cow
26	animal	cow	TALENs;	bta-miR-192	Genes & genomics	Selected microRNA-192 mutant indicates association with several function genes in bovine cells.	2018	40(4):361-371	[Zi C et al.]	Nanjing Agricultural University, Nanjing, China.	29892841	10.1007/s13258-017-0635-3	MicroRNAs are implicated in many cellular processes such as cell differentiation and development, tumorigenesis, and immune regulation. In this study, miR192 was detected using quantitative real-time polymerase chain reaction (qRT-PCR) when MDBK cells were exposed to Escherichia coli. Cells with malfunction of bta-miR-192 were established using transcription activator-like effector nuclease (TALEN) technology. Finally, bta-miR-192 mutant cells were screened for differentially expressed genes using RNA-sequencing (RNA-seq). The results showed that miR192 significantly decreased in cells exposed to E. coli F18ac and E. coli K88ac. The RNA-seq results showed that 1673 differentially expressed transcripts were identified; 890 genes were upregulated and 775 genes were downregulated. With the gene ontology enrichment analysis, 431 differentially expressed genes (DEGs) were classified into 937 gene ontology terms. The pathway enrichment analysis showed that 535 genes were involved in 254 pathway terms. Interestingly, most of these DEGs were associated with the pathways in cancers or infectious diseases. When the selected DEGs (n = 162) in these pathways were intersected with 120 differential transcripts, 11 DEGs were identified. Subsequently, several genes associated with regulation, cancers, or viral infections, such as LEF1, AXIN2, MX1, and FCGR2B, were identified among the DEGs using functional analysis. Furthermore, associations between bta-miR-192 and DEGs were detected by intersecting the bta-miR-192's target genes with the DEGs, indicating that three genes including CBL, DICER1 and TRERF1 were involved in this relationship. These findings provided useful guidance for investigating the role played	cow
27	animal	cow	CRISPR;	phosphodiesterase 2A	Journal of cellular physiology	MiR-139 promotes differentiation of bovine skeletal muscle-derived satellite cells by regulating DHFR gene expression.	2018	234(1):632-641	[Zhou S et al.]	Northeast Agricultural University, Harbin, Heilongjiang, China.	30078180	10.1002/jcp.26817	MicroRNAs play an important regulatory role in the proliferation and differentiation of skeletal muscle-derived satellite cells (MDSCs). In particular, miR-139 can inhibit tumor cell proliferation and invasion, and its expression is down-regulated during C2C12 myoblast differentiation. The aim of this study was thus to examine the effect and potential mechanism of miR-139 in bovine MDSCs. The expression of miR-139 was found to be significantly increased during bovine MDSC differentiation by stem-loop reverse transcription-polymerase chain reaction amplification. Statistical analysis of the myotube fusion rate was done through immunofluorescence detection of desmin, and western blotting was used to measure the change in protein expression of the muscle differentiation marker genes MYOG and MYH3. The results showed that the miR-139 mimic could enhance the differentiation of bovine MDSCs, whereas the inhibitor had the opposite effect. By using the dual-luciferase reporter system, miR-139 was found to target the 3'-untranslated region of the dihydrofolate reductase (DHFR) gene and regulate its expression. In addition, the expression of miR-139 was found to be regulated by its host gene phosphodiesterase 2A (PDE2A) via inhibition of the latter by CRISPR interference (CRISPRi). Overall, our findings indicate that miR-139 plays an important role in regulating the differentiation of bovine MDSCs.	cow
28	animal	cow	CRISPR;Cas9;	OCT4	Proceedings of the National Academy of Sciences of the United States of America	OCT4/POU5F1 is required for NANOG expression in bovine blastocysts.	2018	115(11):2770-2775	[Simmet K et al.]	Ludwig-Maximilians-Universitat Munchen, Munich, Germany.	29483258	10.1073/pnas.1718833115	Mammalian preimplantation development involves two lineage specifications: first, the CDX2-expressing trophectoderm (TE) and a pluripotent inner cell mass (ICM) are separated during blastocyst formation. Second, the pluripotent epiblast (EPI; expressing NANOG) and the differentiated primitive endoderm (PrE; expressing GATA6) diverge within the ICM. Studies in mice revealed that OCT4/POU5F1 is at the center of a pluripotency regulatory network. To study the role of OCT4 in bovine preimplantation development, we generated OCT4 knockout (KO) fibroblasts by CRISPR-Cas9 and produced embryos by somatic cell nuclear transfer (SCNT). SCNT embryos from nontransfected fibroblasts and embryos produced by in vitro fertilization served as controls. In OCT4 KO morulae (day 5), approximately 70% of the nuclei were OCT4 positive, indicating that maternal OCT4 mRNA partially maintains OCT4 protein expression during early development. In contrast, OCT4 KO blastocysts (day 7) lacked OCT4 protein entirely. CDX2 was detected only in TE cells; OCT4 is thus not required to suppress CDX2 in the ICM. Control blastocysts showed a typical salt-and-pepper distribution of NANOG- and GATA6-positive cells in the ICM. In contrast, NANOG was absent or very faint in the ICM of OCT4 KO blastocysts, and no cells expressing exclusively NANOG were observed. This mimics findings in OCT4-deficient human blastocysts but is in sharp contrast to Oct4-null mouse blastocysts, where NANOG persists and PrE development fails. Our study supports bovine embryogenesis as a model for early human development and exemplifies a general strategy for studying the	cow

29	animal	cow	ZFN;	beta-lactoglobulin	Scientific reports	Production of hypoallergenic milk from DNA-free beta-lactoglobulin (BLG) gene knockout cow using zinc-finger nucleases mRNA.	2018	8(1):15430	[Sun Z et al.]	China Agricultural University, Beijing, China.	30337546	10.1038/s41598-018-32024-x	The whey protein beta-lactoglobulin (BLG) is a major milk allergen which is absent in human milk. Here, we for the first time generated DNA-free BLG bi-allelic knockout cow by zinc-finger nuclease (ZFNs) mRNA and produced BLG-free milk. According to the allergenicity evaluation of BLG-free milk, we found it can trigger lower allergic reaction of Balb/c mice including the rectal temperature drop and the allergen-specific immunoglobulin IgE production; BLG free-milk was easily digested by pepsin at 2 min, while BLG in control milk was still not completely digested after 60 min, and the binding of IgE from cow's milk allergy (CMA) patients to BLG free-milk was significantly lower than that to the control milk. Meanwhile, the genome sequencing revealed that our animal is free of off-target events. Importantly, editing animal genomes without introducing foreign DNA into cells may alleviate regulatory concerns related to foods produced by genome edited animals. Finally, the ZFNs-mediated targeting in cow could be transmitted through the germline by breeding. These findings will open up unlimited possibilities of modifying milk composition to make it more suitable for human health	cow
30	animal	cow	TALENs;	Rosa26	Scientific reports	Efficient targeted integration into the bovine Rosa26 locus using TALENs.	2018	8(1):10385	[Wang M et al.]	China Agricultural University, Beijing, China.	29991797	10.1038/s41598-018-28502-x	The genetic modification of cattle has many agricultural and biomedical applications. However, random integration often results in the unstable expression of transgenes and unpredictable phenotypes. Targeting genes to the "safe locus" and stably expressing foreign genes at a high level are desirable methods for overcoming these hurdles. The Rosa26 locus has been widely used to produce genetically modified animals in some species expressing transgenes at high and consistent levels. For the first time, we identified a bovine orthologue of the mouse Rosa26 locus through a genomic sequence homology analysis. According to 5' rapid-amplification of cDNA ends (5'RACE), 3' rapid-amplification of cDNA ends (3'RACE), reverse transcription PCR (RT-PCR) and quantitative PCR (Q-PCR) experiments, this locus encodes a long noncoding RNA (lncRNA) comprising two exons that is expressed ubiquitously and stably in different tissues. The bovine Rosa26 (bRosa26) locus appears to be highly amenable to transcription activator-like effector nucleases (TALENs)-mediated knock-in, and ubiquitous expression of enhanced green fluorescent protein (EGFP) inserted in the bRosa26 locus was observed in various stages, including cells, embryos, fetus and cattle. Finally, we created a valuable master bRosa26-EGFP fetal fibroblast cell line in which any gene of interest can be efficiently introduced and stably expressed using recombinase-mediated cassette exchange (RMCE). The new tools described here will	cow
31	animal	cow	CRISPR;Cas9;	POU5F1	Scientific reports	Embryonic POU5F1 is Required for Expanded Bovine Blastocyst Formation.	2018	8(1):7753	[Daigneault BW et al.]	Michigan State University, East-Lansing, MI, USA.	29773834	10.1038/s41598-018-25964-x	POU5F1 is a transcription factor and master regulator of cell pluripotency with indispensable roles in early embryo development and cell lineage specification. The role of embryonic POU5F1 in blastocyst formation and cell lineage specification differs between mammalian species but remains completely unknown in cattle. The CRISPR/Cas9 system was utilized for targeted disruption of the POU5F1 gene by direct injection into zygotes. Disruption of the bovine POU5F1 locus prevented blastocyst formation and was associated with embryonic arrest at the morula stage. POU5F1 knockout morulas developed at a similar rate as control embryos and presented a similar number of blastomeres by day 5 of development. Initiation of SOX2 expression by day 5 of development was not affected by lack of POU5F1. On the other hand, CDX2 expression was aberrant in embryos lacking POU5F1. Notably, the phenotype observed in bovine POU5F1 knockout embryos reveals conserved functions associated with loss of human embryonic POU5F1 that differ from Pou5f1- null mice. The similarity observed in transcriptional regulation of early embryo development between cattle and humans combined with highly efficient gene editing techniques make the bovine a valuable model for human embryo biology with expanded applications	cow
32	animal	cow	TALENs;	beta-lactoglobulin	Scientific reports	Cattle with a precise, zygote-mediated deletion safely eliminate the major milk allergen beta-lactoglobulin.	2018	8(1):7661	[Wei J et al.]	AgResearch, Hamilton, New Zealand.	29769555	10.1038/s41598-018-25654-8	We applied precise zygote-mediated genome editing to eliminate beta-lactoglobulin (BLG), a major allergen in cows' milk. To efficiently generate LGB knockout cows, biopsied embryos were screened to transfer only appropriately modified embryos. Transfer of 13 pre-selected embryos into surrogate cows resulted in the birth of three calves, one dying shortly after birth. Deep sequencing results confirmed conversion of the genotype from wild type to the edited nine bp deletion by more than 97% in the two male calves. The third calf, a healthy female, had in addition to the expected nine bp deletion (81%), alleles with an in frame 21 bp deletion (<17%) at the target site. While her milk was free of any mature BLG, we detected low levels of a BLG variant derived from the minor deletion allele. This confirmed that the nine bp deletion genotype completely knocks out production of BLG. In addition, we showed that the LGB knockout animals are free of any TALEN-mediated off-target mutations or vector integration events using an unbiased whole genome analysis. Our study demonstrates the feasibility of generating precisely biallelically edited cattle by zygote-mediated editing for the safe	cow

33	animal	cow	TALENs	beta-glycosidase (LacS)	Theriogenology	Production of microhomologous-mediated site-specific integrated LacS gene cow using TALENs.	2018	119:282-288	[Su X et al.]	Sun Yat-sen University, Guangzhou, China.	30075414	10.1016/j.theriogenology.2018.07.011	Gene editing tools (Zinc-Finger Nucleases, ZFN; Transcription Activator-Like Effector Nucleases, TALEN; and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas)9, CRISPR-Cas9) provide us with a powerful means of performing genetic engineering procedures. A combinational approach that utilizes both somatic cell nuclear transfer (SCNT) and somatic cell gene editing facilitates the generation of genetically engineered animals. However, the associated research has utilized markers and/or selected genes, which constitute a potential threat to biosafety. Microhomologous-mediated end-joining (MMEJ) has showed the utilization of micro-homologous arms (5-25 bp) can mediate exogenous gene insertion. Dairy milk is a major source of nutrition worldwide. However, most people are not capable of optimally utilizing the nutrition in milk because of lactose intolerance. Sulfolobus solfataricus beta-glycosidase (LacS) is a lactase derived from the extreme thermophilic archaeon Sulfolobus solfataricus. Our finally aim was to site-specific integrated LacS gene into cow's genome through TALEN-mediated MMEJ and produce low-lactose cow. Firstly, we constructed TALENs vectors which target to the cow's beta-casein locus and LacS gene expression vector which contain TALEN reorganization sequence and micro-homologous arms. Then we co-transfected these vectors into fetal derived skin fibroblasts and cultured as monoclone. Positive cell clones were screened using 3' junction PCR amplification and sequencing analysis. The positive cells were used as donors for SCNT and embryo transfer (ET). Lastly, we detected the genotype through PCR of blood genomic DNA. This resulted in a LacS knock-in rate of 0.8% in TALEN-treated cattle fetal fibroblasts. The blastocyst rate of SCNT embryo was 27%. The 3 months pregnancy rate was 20%. Finally, we obtained 1 newborn cow (5%) and verified its genotype. We obtained 1 site-specific marker-free LacS transgenic cow. It provides a basis to solve lactose intolerance by gene engineering breeding. This study also provides us with a new strategy to facilitate gene	cow
34	animal	cow (Japanese Black cattle)	CRISPR;Cas9;	isoleucyl-tRNA synthetase	Biomedical research (Tokyo, Japan)	Establishment of protocol for preparation of gene-edited bovine ear-derived fibroblasts for somatic cell nuclear transplantation.	2018	39(2):95-104	[Ishino T et al.]	Rakuno Gakuen University, Japan.	29669988	10.2220/biomedres.39.95	Recently, gene-editing using the clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein 9 (Cas9) technique has attempted to utilize fibroblasts of livestock animals for somatic cell nuclear transfer. In this study, we establish the procedure for preparing skin fibroblast clones whose genes were edited by the CRISPR/Cas9 technique. After isolating fibroblasts from earlobes of Japanese Black cattle, subsequent collagenase-digestion and extensive wash procedures enabled us to avoid contamination of fungi. Electroporation using NEPA21, rather than lipofection using commercially available liposome reagents, allowed us to perform more efficient transfection of plasmid constructs. Although bovine ear-derived fibroblasts were not able to proliferate in single cell cultures in Dulbecco's modified Eagle medium containing 10% fetal calf serum, supplementation with insulin-transferrin-selenium mixture, human recombinant epidermal growth factor, or human recombinant basic fibroblast growth factor promoted proliferation of the cells, even in a single cell culture. Taking advantage of our established protocol, we eventually obtained eight ear-derived fibroblast clones with a recessive mutation in the isoleucyl-tRNA synthetase gene	cow
35	animal	cow	CRISPR;Cas9;	MSTN	In vitro cellular & developmental biology. Animal	Genome mutation after the introduction of the gene editing by electroporation of Cas9 protein (GEEP) system into bovine putative zygotes.	2019	55(8):598-603	[Namula Z et al.]	Guangdong Ocean University, Zhanjiang, China.	31297696	10.1007/s11626-019-00385-w	The present study was designed to investigate the effects of voltage strength on embryonic developmental rate and mutation efficiency in bovine putative zygotes during electroporation with the CRISPR/Cas9 system to target the MSTN gene at different time points after insemination. Results showed that there was no significant interaction between electroporation time and voltage strength on the embryonic cleavage and blastocyst formation rates. However, increasing the voltage strength to 20 V/mm to electroporate the zygotes at 10 h after the start of insemination yielded significantly lower blastocyst formation rates ($P < 0.05$) than those of the 10-V/mm electroporated zygotes. Mutation efficiency was then assessed in individual blastocysts by DNA sequence analysis of the target sites in the MSTN gene. A positive correlation between mutation rate and voltage strength was observed. The mutation efficiency in mutant blastocysts was significantly higher in the zygotes electroporated with 20 V/mm at 10 h after the start of insemination ($P < 0.05$) than in the zygotes electroporated at 15 h, irrespective of the voltage strength. We also noted that a certain number of blastocysts from zygotes that were electroporated with more than 15 V/mm at 10 h (4.8-16.7%) and 20 V/mm at 15 h (4.8%) were biallelic mutants. Our results suggest that the voltage strength during electroporation as well as electroporation time certainly have effects on the embryonic developmental rate and	cow
36	animal	cow	CRISPR;Cas9;	mitochondrial transcription factor A (TFAM)	PloS one	Edition of TFAM gene by CRISPR/Cas9 technology in bovine model.	2019	14(3):e0213376	[de Oliveira VC et al.]	University of Sao Paulo, Pirassununga, Sao Paulo, Brazil.	30845180	10.1371/journal.pone.0213376	The mitochondrial transcription factor A (TFAM) is a mitochondrial DNA (mtDNA) binding protein essential for the initiation of transcription and genome maintenance. Recently it was demonstrated that the primary role of TFAM is to maintain the integrity of mtDNA and that it is a key regulator of mtDNA copy number. It was also shown that TFAM plays a central role in the mtDNA stress-mediated inflammatory response. In our study, we proposed to evaluate the possibility of editing the TFAM gene by CRISPR/Cas9 technology in bovine fibroblasts, as TFAM regulates the replication specificity of mtDNA. We further attempted to maintain these cells in culture post edition in a medium supplemented with uridine and pyruvate to mimic Rho zero cells that are capable of surviving without mtDNA, because it is known that the TFAM gene is lethal in knockout mice and chicken. Moreover, we evaluated the effects of TFAM modification on mtDNA copy number. The CRISPR gRNA was designed to target exon 1 of the bovine TFAM gene and subsequently cloned. Fibroblasts were transfected with Cas9 and control plasmids. After 24 h of transfection, cells were analyzed by flow cytometry to evaluate the efficiency of transfection. The site directed-mutation frequency was assessed by T7 endonuclease assay, and cell clones were analyzed for mtDNA copy number by Sanger DNA sequencing. We achieved transfection efficiency of 51.3%. We selected 23 successfully transformed clones for further analysis, and seven of these exhibited directed mutations at the CRISPR/Cas9 targeted site. Moreover, we also found a decrease in mtDNA copy number in the gene edited clones compared to that in the controls. These TFAM gene mutant cells were viable in culture when supplemented with uridine and pyruvate. We conclude that this CRISPR/Cas9 design was efficient, resulting in seven heterozygous mutant clones and opening up the possibility to use these mutant cell lines as a model system to elucidate the role of	cow

37	animal	cow	CRISPR;Cas9;	ankyrin-repeat and SOCS-box protein 9	PloS one	Gonadotropin regulation of ankyrin-repeat and SOCS-box protein 9 (ASB9) in ovarian follicles and identification of binding partners.	2019	14(2):e0212571	[Benoit G et al.]	Universite de Montreal, St-Hyacinthe, Quebec, Canada.	30811458	10.1371/journal.pone.0212571	Ankyrin-repeat and SOCS-box protein 9 (ASB9) is a member of the large SOCS-box containing proteins family and acts as the specific substrate recognition component of E3 ubiquitin ligases in the process of ubiquitination and proteasomal degradation. We previously identified ASB9 as a differentially expressed gene in granulosa cells (GC) of bovine ovulatory follicles. This study aimed to further investigate ASB9 mRNA and protein regulation, identify binding partners in GC of bovine ovulatory follicles, and study its function. GC were obtained from small follicles (SF: 2-4 mm), dominant follicles at day 5 of the estrous cycle (DF), and ovulatory follicles, 24 hours following hCG injection (OF). Analyses by RT-PCR showed a 104-fold greater expression of ASB9 in GC of OF than in DF. Steady-state levels of ASB9 in follicular walls (granulosa and theca cells) analyzed at 0, 6, 12, 18 and 24 hours after hCG injection showed a significant induction of ASB9 expression at 12 and 18 hours, reaching a maximum induction of 10.2-fold at 24 hours post-hCG as compared to 0 hour. These results were confirmed in western blot analysis showing strongest ASB9 protein amounts in OF. Yeast two-hybrid screening of OF-cDNAs library resulted in the identification of 10 potential ASB9 binding partners in GC but no interaction was found between ASB9 and creatine kinase B (CKB) in these GC. Functional studies using CRISPR-Cas9 approach revealed that ASB9 inhibition led to increased GC proliferation and modulation of target genes expression. Overall, these results support a physiologically relevant role of ASB9 in the ovulatory follicle by targeting specific proteins likely for degradation, contributing to reduced GC proliferation, and could be involved in the final	cow
38	animal	cow	CRISPRi	EGR1	Zhongguo ying yong sheng li xue za zhi = Zhongguo yingyong shenglixue zazhi = Chinese journal of applied physiology	[Expression of EGR1 gene and location of EGR1 protein in differentiation of bovine skeletal muscle-derived satellite cells].	2019	35(1):5-8	[Zhang WW et al.]	College of Life Sciences, Agriculture and Forestry, China.	31245944	10.12047/j.cjap.5749.2019.002	OBJECTIVE: To investigate the expression of EGR1 gene and the localization of EGR1 protein in bovine skeletal muscle-derived satellite cells (MDSCs), as well as to investigate the mechanism that EGR1 protein enters the nucleus. METHODS: Bovine MDSCs were cultured in differentiation medium for 1 day, 3 days and 5 days, respectively, and each group was triplicate. The expression of EGR1 gene and the localization of EGR1 protein were studied at different differentiation period in MDSCs by qRT-PC and Western blot. Moreover, the changes on the expression of endogenous EGR1 gene and EGR1 proteins were explored by CRISPRi, site-directed mutagenesis and laser confocal method. RESULTS: The results from the qRT-PCR and Western blot showed that the expressions of EGR1 gene on transcription level and translation level were significantly higher in differentiated cells than those in undifferentiated cells. The highest expression was found on the third day after the differentiation, and then began to decline. Immunofluorescence assays showed that EGR1 proteins were preferentially expressed in differentiated MDSCs, and increased along with the increase of number of myotubes. Confocal observation revealed that some EGR1 proteins were transferred into the nucleus in the differentiation of cells, however, the EGR1 proteins would not be detected in the differentiated MDSCs nuclei if a site directed mutagenesis (serine533) on EGR1 protein occurred. CONCLUSION: During the differentiation of bovine skeletal muscle satellite cells, the transcriptional level of EGR1 gene is increased, and some EGR1 proteins are transferred into the nucleus. The serine phosphorylation at position 533 of the C terminal of EGR1 protein is necessary for the	cow
39	animal	Debao pig; swamp buffalo	CRISPR;Cas9;	growth differentiation factor 8 (GDF8)	In vitro cellular & developmental biology. Animal	Efficient genome editing in cultured cells and embryos of Debao pig and swamp buffalo using the CRISPR/Cas9 system.	2018	54(5):375-383	[Su X et al.]	Guangxi University, Nanning, China.	29556895	10.1007/s11626-018-0236-8	Myostatin (MSTN), a protein encoded by growth differentiation factor 8 (GDF8), is primarily expressed in skeletal muscle and negatively regulates the development and regeneration of muscle. Accordingly, myostatin-deficient animals exhibit a double-muscling phenotype. The CRISPR/Cas9 system has proven to be an efficient genome-editing tool and has been applied to gene modification in cells from many model organisms such as Drosophila melanogaster, zebrafish, mouse, rat, sheep, and human. Here, we edited the GDF8 gene in fibroblasts and embryos of Debao pig and swamp buffalo using the CRISPR/Cas9 system. The CRISPR/Cas9-mediated mutation efficiency in fibroblasts was as high as 87.5% in pig and 78.9% in buffalo. We then obtained single-cell clones with mutations at the specific sites of the GDF8 gene by screening with G418 in fibroblasts of pig and buffalo. In addition, the frequencies of Cas9/gRNA-mediated mutations were at 36 and 25% in the intracytoplasmic sperm injection embryos of pig and in vitro fertilization embryos of buffalo, respectively. Our work demonstrates that the Cas9/gRNA system is a highly efficient and fast tool for genome editing in cultured cells and embryos of Debao pig and swamp buffalo. These results can be helpful for the establishment of a new animal strain that can generate	cow; pig
40	animal	cow; pig	CRISPR;Cas9;	Nanos2	Biology of reproduction	Simplified pipelines for genetic engineering of mammalian embryos by CRISPR-Cas9 electroporationdagger.	2019	101(1):177-187	[Miao D et al.]	Washington State University, Pullman, WA, USA.	31095680	10.1093/biolre/ioz075	Gene editing technologies, such as CRISPR-Cas9, have important applications in mammalian embryos for generating novel animal models in biomedical research and lines of livestock with enhanced production traits. However, the lack of methods for efficient introduction of gene editing reagents into zygotes of various species and the need for surgical embryo transfer in mice have been technical barriers of widespread use. Here, we described methodologies that overcome these limitations for embryos of mice, cattle, and pigs. Using mutation of the Nanos2 gene as a readout, we refined electroporation parameters with preassembled sgRNA-Cas9 RNPs for zygotes of all three species without the need for zona pellucida dissolution that led to high-efficiency INDEL edits. In addition, we optimized culture conditions to support maturation from zygote to the multicellular stage for all three species that generates embryos ready for transfer to produce gene-edited animals. Moreover, for mice, we devised a nonsurgical embryo transfer method that yields offspring at an efficiency comparable to conventional surgical approaches. Collectively, outcomes of these studies provide simplified pipelines for CRISPR-Cas9-based gene editing that are applicable in a	cow; pig

41	animal	Cashmere Goat	CRISPR;Cas9;	EDAR	International journal of biological sciences	Generation of Cashmere Goats Carrying an EDAR Gene Mutant Using CRISPR-Cas9-Mediated Genome Editing.	2018	14(4):427-436	[Hao F et al.]	Inner Mongolia University, Hohhot, China.	29725264	10.7150/ijbs.23890	In recent years, while the use of the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 (Cas9) (CRISPR-Cas9) system for targeted genome editing has become a research hotspot, it has, to date, not proved adequate for genome editing in large mammals, such as goats. In this study, two opposite single-guide RNAs (sgRNAs) were designed for complete EDAR gene targeting in Cashmere goats, and co-transfected with a plasmid encoding Cas9 into goat fibroblasts. Among the 89 cell lines obtained through the cultivation of clonal cell lines, 62 were positive for EDAR gene targeting. Nine types of mutations were identified by sequencing analysis, and the mutation efficiency was 69.7%. Using one of these cell lines, EDAR gene-targeted Cashmere goat embryos were prepared by somatic cell cloning. Developed embryos were transferred to 79 Cashmere goat recipients, and, after a gestation period of five months six male EDAR gene-targeted Cashmere goats were born. Although only two of these goats survived, they had abnormal primary hair follicles and no hair on the top of their heads, which are the distinctive features of the EDAR gene-targeted Cashmere goats. Thus, this study provides a valuable animal model for future studies on EDAR gene-related phenotypes and hair follicle growth and development and shows that the CRISPR-Cas9 system can be used to edit genes in	goat
42	animal	goat	CRISPR;Cas9;	retinoic acid-inducible gene-1	Animal biotechnology	Generation of Genomic Deletions (of Rig-I GENE) in Goat Primary Cell Culture Using CRISPR/CAS9 Method.	2018	29(2):142-152	[Malpotra S et al.]	National Dairy Research Institute , Karnal , Haryana , India.	28662369	10.1080/10495398.2017.1331915	CRISPR/Cas9 system is a natural immune system in prokaryotes protecting them from infectious viral or plasmid DNA invading the cells. This RNA-guided system can act as powerful tool for introducing genomic alterations in eukaryotic cells with high efficiency. In the present study, Rig-Igene is taken as model gene to study the efficiency of CRISPR/Cas9 system induced gene deletion in primary fibroblast cell culture. Rig-I(retinoic acid-inducible gene-1) is involved in regulating immune response in mammals. In this study, we optimized the CRISPR/Cas9 method for knocking out Rig-Igene in Goat primary fibroblasts by using a NHEJ pathway. Cells were screened for inactivation of the Rig-Igene and two positive clones were found out of thirty colonies screened. Thus, cells containing Rig-Igene inactivation could be achieved by	goat
43	animal	goat	CRISPR;Cas9;	MSTN	Animal genetics	CRISPR/Cas9-mediated MSTN disruption and heritable mutagenesis in goats causes increased body mass.	2018	49(1):43-51	[Wang X et al.]	Northwest A&F University, Yangling, China.	29446146	10.1111/age.12626	Genetic engineering in livestock has been greatly enhanced through the use of artificial programmed nucleases such as the recently emerged clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) system. We recently reported our successful application of the CRISPR/Cas9 system to engineer the goat genome through micro-injection of Cas9 mRNA and sgRNAs targeting MSTN and FGF5 in goat embryos. The phenotypes induced by edited loss-of-function mutations of MSTN remain to be evaluated extensively. We demonstrate the utility of this approach by disrupting MSTN, resulting in enhanced body weight and larger muscle fiber size in Cas9-mediated gene-modified goats. The effects of genome modifications were further characterized by H&E staining, quantitative PCR, Western blotting and immunofluorescence staining. Morphological and genetic analyses indicated the occurrence of phenotypic and genotypic modifications. We further provide sufficient evidence, including breeding data, to demonstrate the transmission of the knockout alleles through the germline. By phenotypic and genotypic characterization, we demonstrated the merit of using the CRISPR/Cas9 approach for establishing	goat
44	animal	goat	CRISPR;Cas9;	myostatin	Bioscience reports	Use of CRISPR/Cas9 technology efficiently targetted goat myostatin through zygotes microinjection resulting in double-muscled phenotype in goats.	2018	38(6)	[He Z et al.]	Yangzhou University, Yangzhou, Jiangsu, China.	30201688	10.1042/BSR20180742	Myostatin gene (MSTN) can inhibit the proliferation of myoblast, which in turn promotes muscle growth and inhibits adipocyte differentiation in livestock. MSTN mutation may lead to muscle hypertrophy or double-muscled (DM) phenotype. MSTN mutation animal, such as sheep, dog, and rabbit have been generated through CRISPR/Cas9 technology. However, goats with promising MSTN mutation have not been generated. We designed two sgRNAs loci targetting exon3 of MSTN gene to destroy the MSTN cysteines knots. We got seven goats from seven recipients, in which six were MSTN knocked-out (KO) goats, with a mutation rate of 85.7%. Destroyed cysteine knots caused MSTN structure inactivation. The average body weight gain (BWG) per day of MSTN KO goats was significantly higher than that of wild-type (WT) goats. MSTN KO goats showed abnormal sugar, fat, and protein metabolism compared with wild-type controls (MSTN(+/+)). Inheritance of mutations was observed in offspring of MSTN KO goats by PCR analysis.	goat
45	animal	goat	CRISPR;Cas9;	myostatin; fat-1	FEBS journal	CRISPR/Cas9-mediated specific integration of fat-1 at the goat MSTN locus.	2018	285(15):2828-2839	[Zhang J et al.]	Inner Mongolia University, Hohhot, China.	29802684	10.1111/febs.14520	Recent advances in understanding the CRISPR/Cas9 system have provided a precise and versatile approach for genome editing in various species. However, no study has reported simultaneous knockout of endogenous genes and site-specific knockin of exogenous genes in large animal models. Using the CRISPR/Cas9 system, this study specifically inserted the fat-1 gene into the goat MSTN locus, thereby achieving simultaneous fat-1 insertion and MSTN mutation. We introduced the Cas9, MSTN knockout small guide RNA and fat-1 knockin vectors into goat fetal fibroblasts by electroporation, and obtained a total of 156 positive clonal cell lines. PCR and sequencing were performed for identification. Of the 156 clonal strains, 40 (25.6%) had simultaneous MSTN knockout and fat-1 insertion at the MSTN locus without drug selection, and 55 (35.25%) and 101 (67.3%) had MSTN mutations and fat-1 insertions, respectively. We generated a site-specific knockin Arbas cashmere goat model using a combination of CRISPR/Cas9 and somatic cell nuclear transfer for the first time. For biosafety, we mainly focused on unmarked and non-resistant gene screening, and point-specific gene editing. The results showed that simultaneous editing of the two genes (simultaneous knockout and knockin) was achieved in large animals, demonstrating that the CRISPR/Cas9 system has the potential to become an	goat

46	animal	goat	CRISPR;Cas9;	MSTN; FGF5	Frontiers in genetics	Trio-Based Deep Sequencing Reveals a Low Incidence of Off-Target Mutations in the Offspring of Genetically Edited Goats.	2018	9:449	[Li C et al.]	Northwest A&F University, Yangling, China.	30356875	10.3389/fgene.2018.00449	Unintended off-target mutations induced by CRISPR/Cas9 nucleases may result in unwanted consequences, which will impede the efficient applicability of this technology for genetic improvement. We have recently edited the goat genome through CRISPR/Cas9 by targeting MSTN and FGF5, which increased muscle fiber diameter and hair fiber length, respectively. Using family trio-based sequencing that allow better discrimination of variant origins, we herein generated offspring from edited goats, and sequenced the members of four family trios (gene-edited goats and their offspring) to an average of approximately 36.8x coverage. This data was to systematically examined for mutation profiles using a stringent pipeline that comprehensively analyzed the sequence data for de novo single nucleotide variants, indels, and structural variants from the genome. Our results revealed that the incidence of de novo mutations in the offspring was equivalent to normal populations. We further conducted RNA sequencing using muscle and skin tissues from the offspring and control animals, the differentially expressed genes (DEGs) were related to muscle fiber development in muscles, skin development, and immune responses in skin tissues. Furthermore, in contrast to recently reports of Cas9 triggered p53 expression alterations in cultured cells, we provide primary evidence to show that Cas9-mediated genetic modification does not induce apparent p53 expression changes in animal tissues. This work provides adequate molecular evidence to support the reliability of conducting Cas9-mediated	goat
47	animal	goat	CRISPR;Cas9;	stearoyl-CoA desaturase 1	Journal of agricultural and food chemistry	CRISPR/Cas9-mediated Stearoyl-CoA Desaturase 1 (SCD1) Deficiency Affects Fatty Acid Metabolism in Goat Mammary Epithelial Cells.	2018	66(38):10041-10052	[Tian H et al.]	Northwest A&F University , Yangling , China.	30180552	10.1021/acs.jafc.8b03545	Stearoyl-CoA desaturase 1 (SCD1) is a fatty acid desaturase catalyzing cis-double-bond formation in the Delta9 position to produce monounsaturated fatty acids essential for the synthesis of milk fat. Previous studies using RNAi methods have provided support for a role of SCD1 in goat mammary epithelial cells (GMEC); however, RNAi presents several limitations that might preclude a truthful understanding of the biological function of SCD1. To explore the function of SCD1 on fatty acid metabolism in GMEC, we used CRISPR-Cas9-mediated SCD1 knockout through non-homologous end-joining (NHEJ) and homology-directed repair (HDR) pathways in GMEC. We successfully introduced nucleotide deletions and mutations in the SCD1 gene locus through the NHEJ pathway and disrupted its second exon via insertion of an EGFP-PuroR segment using the HDR pathway. In clones derived from the latter, gene- and protein-expression data indicated that we obtained a monoallelic SCD1 knockout. A T7EN1-mediated assay revealed no off-targets in the surveyed sites. The contents of triacylglycerol and cholesterol and the desaturase index were significantly decreased as a consequence of SCD1 knockout. The deletion of SCD1 decreased the expression of other genes involved in de novo fatty acid synthesis, including SREBF1 and FASN, as well the fatty acid transporters FABP3 and FABP4. The downregulation of these genes partly explains the decrease of intracellular triacylglycerols. Our results indicate a successful SCD1 knockout in goat mammary cells using CRISPR-Cas9. The demonstration of the successful use of CRISPR-Cas9 in GMEC is an important step to	goat
48	animal	goat	CRISPR;Cas9;	growth differentiation factor 9 (GDF9)	Reproduction, fertility, and development	Efficient generation of goats with defined point mutation (I397V) in GDF9 through CRISPR/Cas9.	2018	30(2):307-312	[Niu Y et al.]	Northwest A&F University, Yangling, China.	28692815	10.1071/RD17068	The recent emergence of the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) 9 system has attracted significant attention for its potential to improve traits of agricultural importance. However, most applications in livestock species to date have depended on aberrant DNA repair to generate frameshifting indels. Whether this genomic engineering technique involving homology-dependent repair (HDR) can be used to introduce defined point mutations has been less explored. Previously, we reported a G-->A point mutation (g.231A>G, p.Val397Ile) in the growth differentiation factor 9 (GDF9) gene that has a large effect on the litter size of cashmere goats. In the present study we report that by co-injecting synthesised RNAs and single-stranded oligo deoxynucleotide (ssODN) donor sequences into goat zygotes, we successfully introduced defined point mutations resulting in single amino acid substitutions in the proteins as expected. The efficiency of this precise single-nucleotide substitution in newborn kids was as high as 24% (4/17), indicating that ssODN-directed HDR via zygote injection is efficient at introducing point mutations in the goat genome. The findings of the present study further highlight the complex genome modifications facilitated by the CRISPR/Cas9 system, which is able to introduce defined point mutations. This represents a significant development for the improvement of reproduction traits in goats, as well as for validating the roles of	goat
49	animal	Alpas cashmere goat	CRISPR;Cas9;TALENs;	myostatin	Theriogenology	Comparison of gene editing efficiencies of CRISPR/Cas9 and TALEN for generation of MSTN knock-out cashmere goats.	2019	132:1-11	[Zhang J et al.]	Inner Mongolia University, Hohhot, China.	30981084	10.1016/j.theriogenology.2019.03.029	The genome editors CRISPR/Cas9 (clustered regularly interspaced short palindromicrepeats/Cas9 nuclease-null) and TALENs (transcription activator-like effector nuclease) are popularly used for targeted modification of the mammalian genome. To date, few comparative studies have been carried out to investigate the differences between the use of CRISPR/Cas9 and TALENs in genome editing for goat breeding. Here, we compared CRISPR/Cas9 and TALEN technologies at multiple levels for generating a knock out (KO) of the Alpas cashmere goat myostatin (MSTN) gene, which negatively regulates the proliferation and differentiation of skeletal muscle cells. The electrotransfection efficiency observed using CRISPR/Cas9 was 8.1% more than that observed using TALEN for generating MSTN KO cells. In addition, the cutting efficiency of CRISPR/Cas9 for editing exon 1 of the MSTN gene was higher than that of TALENs. However, the off-target effects of the CRISPR/Cas9 system were also higher than those of TALENs. Further, we found that the frequency of obtaining MSTN(-/-) mutations by CRISPR/Cas9 was 8.5 times higher than that by TALEN. The CRISPR/Cas9-edited colonies involved longer deletions (up to 117 bp) than the TALEN-edited colonies (up to 13 bp). Remarkably, when embryos used to generate cloned goat via somatic cell nuclear transfer were compared, we found that the TALEN MSTN KO embryos easily developed to 8cells and their cleavage rate was significantly higher than that of CRISPR/Cas9-edited embryos. Finally, we produced a MSTN KO lamb using CRISPR/Cas9, which suggested that a high level of targeted gene modification could be achieved in goat using CRISPR/Cas9. Taken together, our study indicates that although TALEN enables a variety of genome modifications and may have some advantages over CRISPR/Cas9, the latter provides a significant advantage by permitting precise and efficient gene editing. Thus, CRISPR/Cas9 has more	goat

50	animal	goat	CRISPR;Cas9;	Tbeta4; CCR5	International journal of biological sciences	Generation of Tbeta4 knock-in Cashmere goat using CRISPR/Cas9.	2019	15(8):1743-1754	[Li X et al.]	Inner Mongolia University, Hohhot, China.	31360116	10.7150/ijbs.34820	The cashmere goat breed is known to provide excellent quality cashmere. Here, we attempted to breed high-yielding cashmere goats by specifically inserting the Tbeta4 gene into the goat CCR5 locus and provided an animal model for future research. We successfully obtained Tbeta4 knock-in goat without any screening and fluorescent markers using CRISPR/Cas9 technology. A series of experiments were performed to examine physical conditions and characteristics of the Tbeta4 knock-in goat. The goat exhibited an increase in cashmere yield by 74.5% without affecting the fineness and quality. Additionally, RNA-seq analysis indicated that Tbeta4 may promote hair growth by affecting processes such as vasoconstriction, angiogenesis, and vascular permeability around secondary hair follicles. Together, our study can significantly improve the breeding of cashmere goat and thereby increase economic efficiency.	goat
51	animal	goat	CRISPR;Cas9;		Reproduction, fertility, and development	Optimisation of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9:single-guide RNA (sgRNA) delivery system in a goat model.	2019		[Huang Y et al.]	Northwest A&F University, Yangling, China	31079595	10.1071/RD18485	The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is an efficient method for the production of gene-edited animals. We have successfully generated gene-modified goats and sheep via zygote injection of Cas9 mRNA and single-guide RNA (sgRNA) mixtures. However, the delivery system for microinjection largely refers to methods established for mice; optimised injection conditions are urgently required for the generation of large animals. Here, we designed a study to optimise the Cas9 mRNA and sgRNA delivery system for goats. By comparing four computational tools for sgRNA design and validating the targeting efficiency in goat fibroblasts, we suggest a protocol for the selection of desirable sgRNAs with higher targeting efficiency and negligible off-target mutations. We further evaluated the editing efficiency in goat zygotes injected with Cas9:sgRNA (sg8) and found that injection with 50ngmuL-1 Cas9 mRNA and 25ngmuL-1 sgRNA yielded an increased editing efficiency. Our results provide a reference protocol for the optimisation of the injection conditions for the efficient editing of large animal genomes via the zygote injection approach.	goat
52	animal	Shaanbei white cashmere goat	CRISPR;Cas9;	vitamin D receptor	PeerJ	CRISPR/Cas9-mediated VDR knockout plays an essential role in the growth of dermal papilla cells through enhanced relative genes.	2019	7:e7230	[Gao Y et al.]	Shanxi Datong University, Datong, China.	31309000	10.7717/peerj.7230	Background: Hair follicles in cashmere goats are divided into primary and secondary hair follicles (HFs). HF development, which determines the morphological structure, is regulated by a large number of vital genes; however, the key functional genes and their interaction networks are still unclear. Although the vitamin D receptor (VDR) is related to cashmere goat HF formation, its precise effects are largely unknown. In the present study, we verified the functions of key genes identified in previous studies using hair dermal papilla (DP) cells as an experimental model. Furthermore, we used CRISPR/Cas9 technology to modify the VDR in DP cells to dissect the molecular mechanism underlying HF formation in cashmere goats. Results: The VDR expression levels in nine tissues of Shaanbei white cashmere goats differed significantly between embryonic day 60 (E60) and embryonic day 120 (E120). At E120, VDR expression was highest in the skin. At the newborn and E120 stages, the VDR protein was highly expressed in the root sheath and hair ball region of Shaanbei cashmere goats. We cloned the complete CDS of VDR in the Shaanbei white cashmere goat and constructed a VDR-deficient DP cell model by CRISPR/Cas9. Heterozygous and homozygous mutant DP cells were produced. The growth rate of mutant DP cells was significantly lower than that of wild-type DP cells ($P < 0.05$) and VDR mRNA levels in DP cells decreased significantly after VDR knockdown ($P < 0.05$). Further, the expression levels of VGF, Noggin, Lef1, and beta-catenin were significantly downregulated ($P < 0.05$). Conclusions: Our results indicated that VDR has a vital role	goat
53	animal	Bama miniature pig	CRISPR;Cas9;	Apolipoprotein E (ApoE)	Disease models & mechanisms	Apolipoprotein E deficiency accelerates atherosclerosis development in miniature pigs.	2018	11(10)	[Fang B et al.]	Nanjing Medical University, Nanjing, China.	30305304	10.1242/dmm.036632	Miniature pigs have advantages over rodents in modeling atherosclerosis because their cardiovascular system and physiology are similar to that of humans. Apolipoprotein E (ApoE) deficiency has long been implicated in cardiovascular disease in humans. To establish an improved large animal model of familial hypercholesterolemia and atherosclerosis, the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 system (CRISPR/Cas9) was used to disrupt the ApoE gene in Bama miniature pigs. Biallelic-modified ApoE pigs with in-frame mutations (ApoE(m/m)) and frameshift mutations (ApoE(-/-)) were simultaneously produced. ApoE(-/-) pigs exhibited moderately increased plasma cholesterol levels when fed with a regular chow diet, but displayed severe hypercholesterolemia and spontaneously developed human-like atherosclerotic lesions in the aorta and coronary arteries after feeding on a high-fat and high-cholesterol (HFHC) diet for 6 months. Thus, these ApoE(-/-) pigs could be valuable large animal models for providing further insight into	pig
54	animal	Guangxi Bama minipig	CRISPR;Cas9;	SCNA	Scientific reports	CRISPR/Cas9-Mediated Generation of Guangxi Bama Minipigs Harboring Three Mutations in alpha-Synuclein Causing Parkinson's Disease.	2018	8(1):12420	[Zhu XX et al.]	Guangxi University, Nanning, China.	30127453	10.1038/s41598-018-30436-3	Parkinson's disease (PD) is a common, progressive neurodegenerative disorder characterized by classical motor dysfunction and is associated with alpha-synuclein-immunopositive pathology and the loss of dopaminergic neurons in the substantia nigra (SN). Several missense mutations in the alpha-synuclein gene SCNA have been identified as cause of inherited PD, providing a practical strategy to generate genetically modified animal models for PD research. Since minipigs share many physiological and anatomical similarities to humans, we proposed that genetically modified minipigs carrying PD-causing mutations can serve as an ideal model for PD research. In the present study, we attempted to model PD by generating Guangxi Bama minipigs with three PD-causing missense mutations (E46K, H50Q and G51D) in SCNA using CRISPR/Cas9-mediated gene editing combining with somatic cell nuclear transfer (SCNT) technique. We successfully generated a total of eight SCNT-derived Guangxi Bama minipigs with the desired heterozygous SCNA mutations integrated into genome, and we also confirmed by DNA sequencing that these minipigs expressed mutant alpha-synuclein at the transcription level. However, immunohistochemical analysis was not able to detect PD-specific pathological changes such as alpha-synuclein-immunopositive pathology and loss of SN dopaminergic neurons in the gene-edited minipigs at 3 months of age. In summary, we successfully generated Guangxi Bama minipigs harboring three PD-casusing mutations (E46K, H50Q and G51D) in SCNA. As they continue to develop, these gene editing minipigs need to be regularly teseted for the presence of PD-like pathological features in order to validate the use	pig

55	animal	pig	CRISPR;Cas9;	UL16-binding protein 1	3 Biotech	The production of UL16-binding protein 1 targeted pigs using CRISPR technology.	2018	8(1):70	[Joanna Z et al.]	Poznan University of Life Sciences, Poznan, Poland.	29354381	10.1007/s13205-018-1107-4	Two sgRNAs were designed to target the region of exon 2 of the pULBP1 gene by microinjection. The co-injection of modified Cas9-D10A nickase with a pair of sgRNAs into the zygote's cytoplasm easily and efficiently generated biallelic modification of the pULBP1 gene in one step. Five out of nine F0 generation piglets showed insertions or deletions in the targeting site of the pULBP1 gene, indicating that pULBP1 mutation efficiency reached about 56% (5/9). Quantitative determination of pULBP1 showed approximately a 1.53-fold reduction in the amount of protein ULBP1 on the cell surface (ELISA). A human NK-cell cytotoxicity test leads to the conclusion that higher cell viability is observed for -/- ULBP1 (survival rate 85.36%) compared to +/+ ULBP1 (69.58%). ULBP1-KO pigs will provide a more progressive xenograft source for further research studies, especially those measuring the effects of abolishing the gene function in terms of the complexity of the immunological interactions.	pig
56	animal	pig	CRISPR;Cas9;	alpha(1,3) galactosyltransferase (GGTA1 gene); CMP-Neu5Ac hydroxylase (CMAH gene); beta-1,4-N-acetyl-galactosaminyl transferase 2 (beta4GalNT2)	Acta biomaterialia	Reducing immunoreactivity of porcine bioprosthetic heart valves by genetically-deleting three major glycan antigens, GGTA1/beta4GalNT2/CMAH.	2018	72:196-205	[Zhang R et al.]	Nanjing Medical University, Nanjing, China.	29631050	10.1016/j.actbio.2018.03.055	Bioprosthetic heart valves (BHVs) originating from pigs are extensively used for heart valve replacement in clinics. However, recipient immune responses associated with chronic calcification lead to structural valve deterioration (SVD) of BHVs. Two well-characterized epitopes on porcine BHVs have been implicated in SVD, including galactose-alpha1,3-galactose (alphaGal) and N-glycolylneuraminic acid (Neu5Gc) whose synthesis are catalyzed by alpha(1,3) galactosyltransferase (encoded by the GGTA1 gene) and CMP-Neu5Ac hydroxylase (encoded by the CMAH gene), respectively. It has been reported that BHV from alphaGal-knockout pigs are associated with a significantly reduced immune response by human serum. Moreover, valves from alphaGal/Neu5Gc-deficient pigs could further reduce human IgM/IgG binding when compared to BHV from alphaGal-knockout pigs. Recently, another swine xenoantigen, Sd(a), produced by beta-1,4-N-acetyl-galactosaminyl transferase 2 (beta4GalNT2), has been identified. To explore whether tissue from GGTA1, CMAH, and beta4GalNT2 triple gene-knockout (TKO) pigs would further minimize human antibody binding to porcine pericardium, TKO pigs were successfully produced by CRISPR/Cas9 mediated gene targeting. Our results showed that the expression of alphaGal, Neu5G and Sd(a) on TKO pigs was negative, and that human IgG/IgM binding to pericardium was minimal. Moreover, the analysis of collagen composition and physical characteristics of porcine pericardium from the TKO pigs indicated that elimination of the three xenoantigens had no significant impact on the physical proprieties of porcine pericardium. Our results demonstrated that TKO pigs would be an ideal source of BHVs. STATEMENT OF SIGNIFICANCE: Surgical heart valve replacement is an established lifesaving treatment for diseased heart valve. Bioprosthetic heart valves (BHVs) made from glutaraldehyde-fixed porcine or bovine tissues are widely used in clinics but exhibit age-dependent structural valve degeneration (SVD) which is associated with the immune response against BHVs. Three major xenoantigens present on commercial BHVs, Galactosea alpha1,3 galactose (alphaGal), N-glycolylneuraminic acid (Neu5Gc) and glycan products of beta-1,4-N-acetyl-galactosaminyl transferase 2 (beta4GalNT2) are eliminated through CRISPR/Cas9 mediated gene targeting in the present study. The genetically modified porcine pericardium showed reduced immunogenicity but comparable collagen composition and physical characteristics of	pig
57	animal	pig	CRISPR;Cas	Gal; Sda	Annals of surgery	Xenoantigen Deletion and Chemical Immunosuppression Can Prolong Renal Xenograft Survival.	2018	268(4):564-573	[Adams AB et al.]	Emory School of Medicine, Atlanta, GA, USA.	30048323	10.1097/SLA.0000000000002977	OBJECTIVE: Xenotransplantation using pig organs could end the donor organ shortage for transplantation, but humans have xenoreactive antibodies that cause early graft rejection. Genome editing can eliminate xenoantigens in donor pigs to minimize the impact of these xenoantibodies. Here we determine whether an improved cross-match and chemical immunosuppression could result in prolonged kidney xenograft survival in a pig-to-rhesus preclinical model. METHODS: Double xenoantigen (Gal and Sda) knockout (DKO) pigs were created using CRISPR/Cas. Serum from rhesus monkeys (n = 43) was cross-matched with cells from the DKO pigs. Kidneys from the DKO pigs were transplanted into rhesus monkeys (n = 6) that had the least reactive cross-matches. The rhesus recipients were immunosuppressed with anti-CD4 and anti-CD8 T-cell depletion, anti-CD154, mycophenolic acid, and steroids. RESULTS: Rhesus antibody binding to DKO cells is reduced, but all still have positive CDC and flow cross-match. Three grafts were rejected early at 5, 6, and 6 days. Longer survival was achieved in recipients with survival to 35, 100, and 435 days. Each of the 3 early graft losses was secondary to IgM antibody-mediated rejection. The 435-day graft loss occurred secondary to IgG antibody-mediated rejection. CONCLUSIONS: Reducing xenoantigens in donor pigs and chemical immunosuppression can be used to achieve prolonged renal xenograft survival in a preclinical model, suggesting that if a negative cross-match can be obtained for humans then prolonged survival could be achieved.	pig
58	animal	pig	CRISPR;Cas9;	CD163	Antiviral research	CD163 knockout pigs are fully resistant to highly pathogenic porcine reproductive and respiratory syndrome virus.	2018	151:63-70	[Yang H et al.]	South China Agricultural University, Guangzhou, China.	29337166	10.1016/j.antiviral.2018.01.004	Porcine reproductive and respiratory syndrome virus (PRRSV) causes severe economic losses to current swine production worldwide. Highly pathogenic PRRSV (HP-PRRSV), originated from a genotype 2 PRRSV, is more virulent than classical PRRSV and further exacerbates the economic impact. HP-PRRSV has become the predominant circulating field strain in China since 2006. CD163 is a cellular receptor for PRRSV. The depletion of CD163 whole protein or SRCR5 region (interaction site for the virus) confers resistance to infection of several PRRSV isolates in pigs or cultured host cells. In this study, we described the generation of a CD163 knockout (KO) pig in which the CD163 protein was ablated by using CRISPR/Cas9 gene targeting and somatic cell nuclear transfer (SCNT) technologies. Challenge with HP-PRRSV TP strain showed that CD163 KO pigs are completely resistant to viral infection manifested by the absence of viremia, antibody response, high fever or any other PRRS-associated clinical signs. By comparison, wild-type (WT) controls displayed typical signs of PRRSV infection and died within 2 weeks after infection. Deletion of CD163 showed no adverse effects to the macrophages on immunophenotyping and biological activity as hemoglobin-haptoglobin scavenger. The results demonstrated that CD163 knockout confers full resistance to HP-PRRSV infection to pigs without impairing the biological	pig

59	animal	pig	CRISPR;Cas9;	cluster of differentiation 163 (CD163)	Asian-Australasian journal of animal sciences	Multi-resistance strategy for viral diseases and in vitro short hairpin RNA verification method in pigs.	2018	31(4):489-498	[Oh JN et al.]	Seoul National University, Seoul, Korea.	29268580	10.5713/ajas.17.0749	OBJECTIVE: Foot and mouth disease (FMD) and porcine reproductive and respiratory syndrome (PRRS) are major diseases that interrupt porcine production. Because they are viral diseases, vaccinations are of only limited effectiveness in preventing outbreaks. To establish an alternative multi-resistant strategy against FMD virus (FMDV) and PRRS virus (PRRSV), the present study introduced two genetic modification techniques to porcine cells. METHODS: First, cluster of differentiation 163 (CD163), the PRRSV viral receptor, was edited with the clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 technique. The CD163 gene sequences of edited cells and control cells differed. Second, short hairpin RNA (shRNAs) were integrated into the cells. The shRNAs, targeting the 3D gene of FMDV and the open reading frame 7 (ORF7) gene of PRRSV, were transferred into fibroblasts. We also developed an in vitro shRNA verification method with a target gene expression vector. RESULTS: shRNA activity was confirmed in vitro with vectors that expressed the 3D and ORF7 genes in the cells. Cells containing shRNAs showed lower transcript levels than cells with only the expression vectors. The shRNAs were integrated into CD163-edited cells to combine the two techniques, and the viral genes were suppressed in these cells. CONCLUSION: We established a multi-resistant strategy	pig
60	animal	pig	CRISPR;Cas9;	FBXO40	Biochemical and biophysical research communications	An FBXO40 knockout generated by CRISPR/Cas9 causes muscle hypertrophy in pigs without detectable pathological effects.	2018	498(4):940-945	[Zou Y et al.]	China Agricultural University, Beijing, China.	29545179	10.1016/j.bbrc.2018.03.085	The regulatory function of Fbxo40 has been well characterized in mice. As a key component of the SCF-E3 ubiquitin ligase complex, Fbxo40 induces IRS1 ubiquitination, thus inactivating the IGF1/Akt pathway. The expression of Fbxo40 is restricted to muscle, and mice with an Fbxo40 null mutation exhibit muscle hypertrophy. However, the function of FBXO40 has not been elucidated in pigs, and it is not known whether FBXO40 mutations affect their health. We therefore generated FBXO40 knockout pigs using somatic cell nuclear transfer (SCNT) technology. CRISPR/Cas9 technology was combined with G418 selection, making it possible to generate donor cells at an efficiency of 75.86%. In muscle from FBXO40 knockout pigs, IRS1 levels were higher, and the IGF1/Akt pathway was stimulated. Mutant animals also had approximately 4% more muscle mass compared to WT controls. The knockout pigs developed normally and no pathological changes were found in major organs. These results demonstrate that FBXO40 is a promising candidate gene for improving production traits in agricultural livestock and for developing therapeutic interventions for muscle diseases.	pig
61	animal	pig	CRISPR;Cas9;TALENs;	SURF1	Biochimica et biophysica acta. Molecular basis of disease	SURF1 knockout cloned pigs: Early onset of a severe lethal phenotype.	2018	1864(6 Pt A):2131-2142	[Quadalti C et al.]	Avantea, Cremona, Italy.	29601977	10.1016/j.bbadis.2018.03.021	Leigh syndrome (LS) associated with cytochrome c oxidase (COX) deficiency is an early onset, fatal mitochondrial encephalopathy, leading to multiple neurological failure and eventually death, usually in the first decade of life. Mutations in SURF1, a nuclear gene encoding a mitochondrial protein involved in COX assembly, are among the most common causes of LS. LS(SURF1) patients display severe, isolated COX deficiency in all tissues, including cultured fibroblasts and skeletal muscle. Recombinant, constitutive SURF1(-/-) mice show diffuse COX deficiency, but fail to recapitulate the severity of the human clinical phenotype. Pigs are an attractive alternative model for human diseases, because of their size, as well as metabolic, physiological and genetic similarity to humans. Here, we determined the complete sequence of the swine SURF1 gene, disrupted it in pig primary fibroblast cell lines using both TALENs and CRISPR/Cas9 genome editing systems, before finally generating SURF1(-/-) and SURF1(-/+) pigs by Somatic Cell Nuclear Transfer (SCNT). SURF1(-/-) pigs were characterized by failure to thrive, muscle weakness and highly reduced life span with elevated perinatal mortality, compared to heterozygous SURF1(-/+) and wild type littermates. Surprisingly, no obvious COX deficiency was detected in SURF1(-/-) tissues, although histochemical analysis revealed the presence of COX deficiency in jejunum villi and total mRNA sequencing (RNAseq) showed that several COX subunit-encoding genes were significantly down-regulated in SURF1(-/-) skeletal muscles. In addition, neuropathological findings, indicated a delay in central nervous system development of newborn SURF1(-/-) piglets. Our results suggest a broader role of sSURF1 in	pig
62	animal	pig	TALENs;	miR-192	Bioscience reports	Insight into the molecular mechanism of miR-192 regulating Escherichia coli resistance in piglets.	2018	38(1)	[Sun L et al.]	Yangzhou University, Yangzhou, China.	29363554	10.1042/BSR20171160	MicroRNAs (miRNAs) have important roles in many cellular processes, including cell proliferation, growth and development, and disease control. Previous study demonstrated that the expression of two highly homologous miRNAs (miR-192 and miR-215) was up-regulated in weaned piglets with Escherichia coli F18 infection. However, the potential molecular mechanism of miR-192 in regulating E. coli infection remains unclear in pigs. In the present study, we analyzed the relationship between level of miR-192 and degree of E. coli resistance using transcription activator-like effector nuclease (TALEN), in vitro bacterial adhesion assays, and target genes research. A TALEN expression vector that specifically recognizes the pig miR-192 was constructed and then monoclonal epithelial cells defective in miR-192 were established. We found that miR-192 knockout led to enhance the adhesion ability of the E. coli strains F18ab, F18ac and K88ac, meanwhile increase the expression of target genes (DLG5 and ALCAM) by qPCR and Western blotting analysis. The results suggested that miR-192 and its key target genes (DLG5 and ALCAM) could have a key role in E. coli infection. Based on our findings, we propose that further investigation of miR-192 function is likely to lead to insights into the molecular mechanisms of E. coli	pig
63	animal	pig	Cas9;	A sequence from the mMalat1 gene	BioTechniques	Single step production of Cas9 mRNA for zygote injection.	2018	64(3):118-124	[Redel BK et al.]	University of Missouri, Columbia, MO, USA.	29570443	10.2144/btn-2017-0116	Production of Cas9 mRNA in vitro typically requires the addition of a 5 cap and 3 polyadenylation. A plasmid was constructed that harbored the T7 promoter followed by the EMCV IRES and a Cas9 coding region. We hypothesized that the use of the metastasis associated lung adenocarcinoma transcript 1 (Malat1) triplex structure downstream of an IRES/Cas9 expression cassette would make polyadenylation of in vitro produced mRNA unnecessary. A sequence from the mMalat1 gene was cloned downstream of the IRES/Cas9 cassette described above. An mRNA concentration curve was constructed with either commercially available Cas9 mRNA or the IRES/Cas9/triplex, by injection into porcine zygotes. Blastocysts were genotyped to determine if differences existed in the percent of embryos modified. The concentration curve identified differences due to concentration and RNA type injected. Single step production of Cas9 mRNA provides an alternative source of Cas9 for use in zygote	pig

64	animal	pig	CRISPR;Cas9;	huntingtin	Cell	A Huntingtin Knockin Pig Model Recapitulates Features of Selective Neurodegeneration in Huntington's Disease.	2018	173(4):989-1002.e13	[Yan S et al.]	Jinan University, Guangzhou, China.	29606351	10.1016/j.cell.2018.03.005	Huntington's disease (HD) is characterized by preferential loss of the medium spiny neurons in the striatum. Using CRISPR/Cas9 and somatic nuclear transfer technology, we established a knockin (KI) pig model of HD that endogenously expresses full-length mutant huntingtin (HTT). By breeding this HD pig model, we have successfully obtained F1 and F2 generation KI pigs. Characterization of founder and F1 KI pigs shows consistent movement, behavioral abnormalities, and early death, which are germline transmittable. More importantly, brains of HD KI pig display striking and selective degeneration of striatal medium spiny neurons. Thus, using a large animal model of HD, we demonstrate for the first time that overt and selective neurodegeneration seen in HD patients can be recapitulated by endogenously expressed mutant proteins in large mammals, a finding that also underscores the importance of using large mammals to investigate the pathogenesis of neurodegenerative diseases and their therapeutics.	pig
65	animal	pig	CRISPR;Cas9;	insulin-like growth factor 2	Cellular and molecular life sciences	Editing porcine IGF2 regulatory element improved meat production in Chinese Bama pigs.	2018	75(24):4619-4628	[Xiang G et al.]	Institute of Zoology, Chinese Academy of Sciences, Beijing, China.	30259067	10.1007/s00018-018-2917-6	Insulin-like growth factor 2 (IGF2) is an important growth factor, which promotes growth and development in mammals during fetal and postnatal stages. Using CRISPR-Cas9 system, we generated multiple founder pigs containing 12 different mutant alleles around a regulatory element within the intron 3 of IGF2 gene. Crossing two male founders passed four mutant alleles onto F1 generation, and these mutations abolished repressor ZBED6 binding and rendered this regulatory element nonfunctional. Both founders and F1 animals showed significantly faster growth, without affecting meat quality. These results indicated that editing IGF2 intron 3-3072 site using CRISPR-Cas9 technology improved meat production in Bama pigs. This is the first demonstration that editing non-coding region can improve economic traits in livestock.	pig
66	animal	pig	CRISPR;Cpf1;		Cellular and molecular life sciences	Engineering CRISPR/Cpf1 with tRNA promotes genome editing capability in mammalian systems.	2018	75(19):3593-3607	[Wu H et al.]	Joint School of Life Sciences, Guangzhou Institutes of Biomedicine and Health, Guangzhou Medical University, Chinese Academy of Sciences, Guangzhou, China.	29637228	10.1007/s00018-018-2810-3	CRISPR/Cpf1 features a number of properties that are distinct from CRISPR/Cas9 and provides an excellent alternative to Cas9 for genome editing. To date, genome engineering by CRISPR/Cpf1 has been reported only in human cells and mouse embryos of mammalian systems and its efficiency is ultimately lower than that of Cas9 proteins from Streptococcus pyogenes. The application of CRISPR/Cpf1 for targeted mutagenesis in other animal models has not been successfully verified. In this study, we designed and optimized a guide RNA (gRNA) transcription system by inserting a transfer RNA precursor (pre-tRNA) sequence downstream of the gRNA for Cpf1, protecting gRNA from immediate digestion by 3'-to-5' exonucleases. Using this new gRNA(tRNA) system, genome editing, including indels, large fragment deletion and precise point mutation, was induced in mammalian systems, showing significantly higher efficiency than the original Cpf1-gRNA system. With this system, gene-modified rabbits and pigs were generated by embryo injection or somatic cell nuclear transfer (SCNT) with an efficiency comparable to that of the Cas9 gRNA system. These results demonstrated that this refined gRNA(tRNA) system can boost the targeting capability	pig
67	animal	pig	CRISPR;Cas9;	fat-1 (inserted);Rosa26	G3	Site-Specific Fat-1 Knock-In Enables Significant Decrease of n-6PUFAs/n-3PUFAs Ratio in Pigs.	2018	8(5):1747-1754	[Li M et al.]	Jilin University, Changchun, Jilin, China.	29563188	10.1534/g3.118.200114	The fat-1 gene from Caenorhabditis elegans encodes a fatty acid desaturase which was widely studied due to its beneficial function of converting n-6 polyunsaturated fatty acids (n-6PUFAs) to n-3 polyunsaturated fatty acids (n-3PUFAs). To date, many fat-1 transgenic animals have been generated to study disease pathogenesis or improve meat quality. However, all of them were generated using a random integration method with variable transgene expression levels and the introduction of selectable marker genes often raise biosafety concern. To this end, we aimed to generate marker-free fat-1 transgenic pigs in a site-specific manner. The Rosa26 locus, first found in mouse embryonic stem cells, has become one of the most common sites for inserting transgenes due to its safe and ubiquitous expression. In our study, the fat-1 gene was inserted into porcine Rosa 26 (pRosa26) locus via Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9) system. The Southern blot analysis of our knock-in pigs indicated a single copy of the fat-1 gene at the pRosa26 locus. Furthermore, this single-copy fat-1 gene supported satisfactory expression in a variety of tissues in F1 generation pigs. Importantly, the gas chromatography analysis indicated that these fat-1 knock-in pigs exhibited a significant increase in the level of n-3PUFAs, leading to an obvious decrease in the n-6PUFAs/n-3PUFAs ratio from 9.36 to 2.12 (***P < 0.0001). Altogether, our fat-1 knock-in pigs hold great promise for improving the nutritional value of pork and serving as an animal model to investigate therapeutic effects of n-3PUFAs on various	pig
68	animal	pig	CRISPR;Cas9;	MYH-7	Genes	Efficient Knock-in of a Point Mutation in Porcine Fibroblasts Using the CRISPR/Cas9-GMNN Fusion Gene.	2018	9(6)	[Gerlach M et al.]	Hannover Medical School, Hannover, Germany.	29899280	10.3390/genes9060296	During CRISPR/Cas9 mediated genome editing, site-specific double strand breaks are introduced and repaired either unspecific by non-homologous end joining (NHEJ) or sequence dependent by homology directed repair (HDR). Whereas NHEJ-based generation of gene knock-out is widely performed, the HDR-based knock-in of specific mutations remains a bottleneck. Especially in primary cell lines that are essential for the generation of cell culture and animal models of inherited human diseases, knock-in efficacy is insufficient and needs significant improvement. Here, we tested two different approaches to increase the knock-in frequency of a specific point mutation into the MYH7-gene in porcine fetal fibroblasts. We added a small molecule inhibitor of NHEJ, SCR7 (5,6-bis((E)-benzylideneamino)-2-mercaptopyrimidin-4-ol), during genome editing and screened cell cultures for the point mutation. However, this approach did not yield increased knock-in rates. In an alternative approach, we fused humanized Cas9 (hCas9) to the N-terminal peptide of the Geminin gene (GMNN). The fusion protein is degraded in NHEJ-dominated cell cycle phases, which should increase HDR-rates. Using hCas9-GMNN and point mutation-specific real time PCR screening, we found a two-fold increase in genome edited cell cultures. This increase of HDR by hCas9-GMNN provides a promising way to enrich specific knock-in in porcine fibroblast	pig

69	animal	pig	CRISPR;Cas9;	GGTA1; CMAH; beta 4GalNT2	Journal of biomedical research	Antigenicity of tissues and organs from GGTA1/CMAH/beta4GalNT2 triple gene knockout pigs.	2018		[Wang RG et al.]	Nanjing Medical University, Nanjing, Jiangsu, China.	30007952	10.7555/JBR.32.20180018	Clinical xenotransplantations have been hampered by human preformed antibody-mediated damage of the xenografts. To overcome biological incompatibility between pigs and humans, one strategy is to remove the major antigens [Gal, Neu5Gc, and Sd(a)] present on pig cells and tissues. Triple gene (GGTA1, CMAH, and beta 4GalNT2) knockout (TKO) pigs were produced in our laboratory by CRISPR-Cas9 targeting. To investigate the antigenicity reduction in the TKO pigs, the expression levels of these three xenoantigens in the cornea, heart, liver, spleen, lung, kidney, and pancreas tissues were examined. The level of human IgG/IgM binding to those tissues was also investigated, with wildtype pig tissues as control. The results showed that alphaGal, Neu5Gc, and Sd(a) were markedly positive in all the examined tissues in wildtype pigs but barely detected in TKO pigs. Compared to wildtype pigs, the liver, spleen, and pancreas of TKO pigs showed comparable levels of human IgG and IgM binding, whereas corneas, heart, lung, and kidney of TKO pigs exhibited significantly reduced human IgG and IgM binding. These results indicate that the antigenicity of TKO pig is significantly reduced and the remaining xenoantigens on porcine tissues can be	pig
70	animal	pig	CRISPR;Cas9;	fatty acid synthase	Journal of cellular physiology	Fatty acid synthase knockout impairs early embryonic development via induction of endoplasmic reticulum stress in pigs.	2018	233(5):4225-4234	[Guo J et al.]	Chungbuk National University, Cheongju, Chungbuk, Korea.	29058795	10.1002/jcp.26241	Fatty acid synthase (FAS) is an important enzyme involved in the de novo synthesis of long-chain fatty acids. During development, the function of FAS in growth is greater than that in energy storage pathways; therefore, we hypothesized that knockout of FAS would affect early embryonic development owing to the induction of endoplasmic reticulum (ER) stress. In the present study, the function of FAS was studied using the CRISPR (clustered regularly interspaced short palindromic repeats)/ CRISPR-associated protein 9 (Cas9) system. Cas9 and single-guide RNA (sgRNA) were injected into parthenotes to decrease the number of FAS-positive embryos. The efficiency of knockout was assayed by DNA sequencing. We found that FAS knockout caused excessive production of reactive oxygen species (ROS). Excess ROS induced ER stress, resulting in activation of the adaptive unfolded protein response (UPR). FAS knockout caused splicing of the X-box binding protein 1 gene (XBP1) and expression of spliced XBP1 mRNA. In addition, FAS knockout caused phosphorylation of PKR-like ER kinase (PERK), and an increase in the mRNA expression of the ER stress-regulated genes, activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP). Finally, Ca(2+) was released from the ER and taken up by the mitochondria. As the ER stress became intolerable, apoptosis was initiated. These results demonstrate that FAS knockout induced ROS generation, which mediated the activation of UPR via	pig
71	animal	pig	Cas9;	thrombomodulin	Journal of surgical research	CRISPR/Cas and recombinase-based human-to-pig orthotopic gene exchange for xenotransplantation.	2018	229:28-40	[Nunes Dos Santos RM et al.]	Indiana University School of Medicine, Indianapolis, IN, USA.	29937002	10.1016/j.jss.2018.03.051	BACKGROUND: Tools for genome editing in pigs are improving rapidly so that making precise cuts in DNA for the purposes of deleting genes is straightforward. Development of means to replace pig genes with human genes with precision is very desirable for the future development of donor pigs for xenotransplantation. MATERIALS AND METHODS: We used Cas9 to cut pig thrombomodulin (pTHBD) and replace it with a plasmid containing a promoterless antibiotic selection marker and the exon for human thrombomodulin. PhiC31 recombinase was used to remove the antibiotic selection marker to create porcine aortic endothelial cells expressing human instead of pTHBD, driven by the endogenous pig promoter. RESULTS: The promoterless selection cassette permitted efficient enrichment of cells containing correctly inserted transgene. Recombinase treatment of selected cells excised the resistance marker permitting expression of the human transgene by the endogenous pTHBD promoter. Gene regulation was maintained after gene replacement because pig endogenous promoter was kept intact in the correct position. CONCLUSIONS: Cas9 and recombinase technology make orthotopic human for pig gene exchange feasible and pave the way for creation of pigs with human genes that can be expressed in the	pig

72	animal	pig	Cas9;	growth hormone receptor (GHR)	Journal of translational medicine	Generation of GHR-modified pigs as Laron syndrome models via a dual-sgRNAs/Cas9 system and somatic cell nuclear transfer.	2018	16(1):41	[Yu H et al.]	ShanghaiTech University, Shanghai, China.	29482569	10.1186/s12967-018-1409-7	BACKGROUND: Laron syndrome is an autosomal disease resulting from mutations in the growth hormone receptor (GHR) gene. The only therapeutic treatment for Laron syndrome is recombinant insulin-like growth factor I (IGF-I), which has been shown to have various side effects. The improved Laron syndrome models are important for better understanding the pathogenesis of the disease and developing corresponding therapeutics. Pigs have become attractive biomedical models for human condition due to similarities in anatomy, physiology, and metabolism relative to humans, which could serve as an appropriate model for Laron syndrome. METHODS: To further improve the GHR knockout (GHRKO) efficiency and explore the feasibility of precise DNA deletion at targeted sites, the dual-sgRNAs/Cas9 system was designed to target GHR exon 3 in pig fetal fibroblasts (PFFs). The vectors encoding sgRNAs and Cas9 were co-transfected into PFFs by electroporation and GHRKO cell lines were established by single cell cloning culture. Two biallelic knockout cell lines were selected as the donor cell line for somatic cell nuclear transfer for the generation of GHRKO pigs. The genotype of colonies, cloned fetuses and piglets were identified by T7 endonuclease I (T7ENI) assay and sequencing. The GHR expression in the fibroblasts and piglets was analyzed by confocal microscopy, quantitative polymerase chain reaction (q-PCR), western blotting (WB) and immunohistochemical (IHC) staining. The phenotype of GHRKO pigs was recapitulated through level detection of IGF-I and glucose, and measurement of body weight and body size. GHRKO F1 generation were generated by crossing with wild-type pigs, and their genotype was detected by T7ENI assay and sequencing. GHRKO F2 generation was obtained via self-cross of GHRKO F1 pigs. Their genotypes of GHRKO F2 generation was also detected by Sanger sequencing. RESULTS: In total, 19 of 20 single-cell colonies exhibited biallelic modified GHR (95%), and the efficiency of DNA deletion mediated by dual-sgRNAs/Cas9 was as high as 90% in 40 GHR alleles of 20 single-cell colonies. Two types of GHR allelic single-cell colonies (GHR(-47/-1), GHR(-47/-46)) were selected as donor cells for the generation of GHRKO pigs. The reconstructed embryos were transferred into 15 recipient gilts, resulting in 15 GHRKO newborn piglets and 2 fetuses. The GHRKO pigs exhibited slow growth rates and small body sizes. From birth to 13 months old, the average body weight of wild-type pigs varied from 0.6 to 89.5 kg, but that of GHRKO pigs varied from only 0.9 to 37.0 kg. Biochemically, the knockout pigs exhibited decreased serum levels of IGF-I and glucose. Furthermore, the GHRKO pigs had normal reproduction ability, as eighteen GHRKO F1 piglets were obtained via mating a GHRKO pig with wild-type pigs and five GHRKO F2 piglets were obtained by self-cross of F1 generation, indicating that modified GHR alleles can pass to the next generation via germline transmission. CONCLUSION: The dual-sgRNAs/Cas9 is a reliable system for DNA deletion and that GHRKO pigs conform to typical phenotypes of those observed in Laron patients.	pig
73	animal	pig	CRISPR;Cas9;	immunoglobulin heavy chain JH	Journal of virology	Infection Dynamics of Hepatitis E Virus in Wild-Type and Immunoglobulin Heavy Chain Knockout JH (-/-) Gnotobiotic Piglets.	2018	92(21)	[Yugo DM et al.]	Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA.	30111571	10.1128/JVI.01208-18	Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important but incompletely understood pathogen causing high mortality during pregnancy and leading to chronic hepatitis in immunocompromised individuals. The underlying mechanisms leading to hepatic damage remain unknown; however, the humoral immune response is implicated. In this study, immunoglobulin (Ig) heavy chain JH (-/-) knockout gnotobiotic pigs were generated using CRISPR/Cas9 technology to deplete the B-lymphocyte population, resulting in an inability to generate a humoral immune response to genotype 3 HEV infection. Compared to wild-type gnotobiotic piglets, the frequencies of B lymphocytes in the Ig heavy chain JH (-/-) knockouts were significantly lower, despite similar levels of other innate and adaptive T-lymphocyte cell populations. The dynamic of acute HEV infection was subsequently determined in heavy chain JH (-/-) knockout and wild-type gnotobiotic pigs. The data showed that wild-type piglets had higher viral RNA loads in feces and sera compared to the JH (-/-) knockout pigs, suggesting that the Ig heavy chain JH (-/-) knockout in pigs actually decreased the level of HEV replication. Both HEV-infected wild-type and JH (-/-) knockout gnotobiotic piglets developed more pronounced lymphoplasmacytic hepatitis and hepatocellular necrosis lesions than other studies with conventional pigs. The HEV-infected JH (-/-) knockout pigs also had significantly enlarged livers both grossly and as a ratio of liver/body weight compared to phosphate-buffered saline-inoculated groups. This novel gnotobiotic pig model will aid in future studies into HEV pathogenicity, an aspect which has thus far been difficult to reproduce in the available animal model systems.IMPORTANCE According to the World Health Organization, approximately 20 million HEV infections occur annually, resulting in 3.3 million cases of hepatitis E and >44,000 deaths. The lack of an efficient animal model that can mimic the full-spectrum of infection outcomes hinders our ability to delineate the mechanism of HEV pathogenesis. Here, we successfully generated immunoglobulin heavy chain JH (-/-) knockout gnotobiotic pigs using CRISPR/Cas9 technology, established a novel JH (-/-) knockout and wild-type gnotobiotic pig model for HEV, and systematically determined the dynamic of acute HEV infection in gnotobiotic pigs. It was demonstrated that knockout of the Ig heavy chain in pigs decreased the level of HEV replication. Infected wild-type and JH (-/-) knockout gnotobiotic piglets developed more pronounced HEV-specific lesions than other studies using conventional pigs, and the infected JH (-/-)	pig

74	animal	pig	CRISPR;Cas9;	CD163	Journal of virology	Pigs Lacking the Scavenger Receptor Cysteine-Rich Domain 5 of CD163 Are Resistant to Porcine Reproductive and Respiratory Syndrome Virus 1 Infection.	2018	92(16)	[Burkard C et al.]	University of Edinburgh, Midlothian, UK.	29925651	10.1128/JVI.00415-18	Porcine reproductive and respiratory syndrome virus (PRRSV) has a narrow host cell tropism, limited to cells of the monocyte/macrophage lineage. CD163 protein is expressed at high levels on the surface of specific macrophage types, and a soluble form is circulating in blood. CD163 has been described as a fusion receptor for PRRSV, with the scavenger receptor cysteine-rich domain 5 (SRCR5) region having been shown to be the interaction site for the virus. As reported previously, we have generated pigs in which exon 7 of the CD163 gene has been deleted using CRISPR/Cas9 editing in pig zygotes. These pigs express CD163 protein lacking SRCR5 (DeltaSRCR5 CD163) and show no adverse effects when maintained under standard husbandry conditions. Not only was DeltaSRCR5 CD163 detected on the surface of macrophage subsets, but the secreted, soluble protein can also be detected in the serum of the edited pigs, as shown here by a porcine soluble CD163-specific enzyme-linked immunosorbent assay (ELISA). Previous results showed that primary macrophage cells from DeltaSRCR5 CD163 animals are resistant to PRRSV-1 subtype 1, 2, and 3 as well as PRRSV-2 infection in vitro Here, DeltaSRCR5 pigs were challenged with a highly virulent PRRSV-1 subtype 2 strain. In contrast to the wild-type control group, DeltaSRCR5 pigs showed no signs of infection and no viremia or antibody response indicative of a productive infection. Histopathological analysis of lung and lymph node tissue showed no presence of virus-replicating cells in either tissue. This shows that DeltaSRCR5 pigs are fully resistant to infection by the virus.IMPORTANCE Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) is the etiological agent of PRRS, causing late-term abortions, stillbirths, and respiratory disease in pigs, incurring major economic losses to the worldwide pig industry. The virus is highly mutagenic and can be divided into two species, PRRSV-1 and PRRSV-2, each containing several subtypes. Current control strategies mainly involve biosecurity measures, depopulation, and vaccination. Vaccines are at best only partially protective against infection with heterologous subtypes and sublineages, and modified live vaccines have frequently been reported to revert to virulence. Here, we demonstrate that a genetic-control approach results in complete resistance to PRRSV infection in vivo CD163 is edited so as to remove the viral interaction domain while maintaining protein expression and biological function, averting any potential adverse effect	pig
75	animal	pig	CRISPR;Cas9;	growth hormone receptor	Molecular metabolism	Growth hormone receptor-deficient pigs resemble the pathophysiology of human Laron syndrome and reveal altered activation of signaling cascades in the liver.	2018	11:113-128	[Hinrichs A et al.]	LMU Munich, Munich, Germany.	29678421	10.1016/j.molmet.2018.03.006	OBJECTIVE: Laron syndrome (LS) is a rare, autosomal recessive disorder in humans caused by loss-of-function mutations of the growth hormone receptor (GHR) gene. To establish a large animal model for LS, pigs with GHR knockout (KO) mutations were generated and characterized. METHODS: CRISPR/Cas9 technology was applied to mutate exon 3 of the GHR gene in porcine zygotes. Two heterozygous founder sows with a 1-bp or 7-bp insertion in GHR exon 3 were obtained, and their heterozygous F1 offspring were intercrossed to produce GHR-KO, heterozygous GHR mutant, and wild-type pigs. Since the latter two groups were not significantly different in any parameter investigated, they were pooled as the GHR expressing control group. The characterization program included body and organ growth, body composition, endocrine and clinical-chemical parameters, as well as signaling studies in liver tissue. RESULTS: GHR-KO pigs lacked GHR and had markedly reduced serum insulin-like growth factor 1 (IGF1) levels and reduced IGF-binding protein 3 (IGFBP3) activity but increased IGFBP2 levels. Serum GH concentrations were significantly elevated compared with control pigs. GHR-KO pigs had a normal birth weight. Growth retardation became significant at the age of five weeks. At the age of six months, the body weight of GHR-KO pigs was reduced by 60% compared with controls. Most organ weights of GHR-KO pigs were reduced proportionally to body weight. However, the weights of liver, kidneys, and heart were disproportionately reduced, while the relative brain weight was almost doubled. GHR-KO pigs had a markedly increased percentage of total body fat relative to body weight and displayed transient juvenile hypoglycemia along with decreased serum triglyceride and cholesterol levels. Analysis of insulin receptor related signaling in the liver of adult fasted pigs revealed increased phosphorylation of IRS1 and PI3K. In agreement with the loss of GHR, phosphorylation of STAT5 was significantly reduced. In contrast, phosphorylation of JAK2 was significantly increased, possibly due to the increased serum leptin levels and increased hepatic leptin receptor expression and activation in GHR-KO pigs. In addition, increased mTOR phosphorylation was observed in GHR-KO liver samples, and phosphorylation studies of downstream substrates suggested the activation of mainly mTOR complex 2. CONCLUSION: GHR-KO pigs	pig
76	animal	pig	TALENs;	DMD	Molecular reproduction and development	TALEN-mediated gene targeting in porcine spermatogonia.	2018	85(3):250-261	[Tang L et al.]	University of Calgary, Calgary, Canada.	29393557	10.1002/mrd.22961	Spermatogonia represent a diploid germ cell population that includes spermatogonial stem cells. In this report, we describe new methods for isolation of highly enriched porcine spermatogonia based on light scatter properties, and for targeted mutagenesis in porcine spermatogonia using nucleofection and TALENs. We optimized a nucleofection protocol to deliver TALENs specifically targeting the DMD locus in porcine spermatogonia. We also validated specific sorting of porcine spermatogonia based on light scatter properties. We were able to obtain a highly enriched germ cell population with over 90% of cells being UCH-L1 positive undifferentiated spermatogonia. After gene targeting in porcine spermatogonia, indel (insertion or deletion) mutations as a result of non-homologous end joining (NHEJ) were detected in up to 18% of transfected cells. Our report demonstrates for the first time an approach to obtain a live cell population highly enriched in undifferentiated spermatogonia from immature porcine testes, and that gene targeting can be achieved in porcine	pig

77	animal	pig	CRISPR;Cas9;	Synaptogyrin-2 (SYNGR2)	PLoS genetics	Synaptogyrin-2 influences replication of Porcine circovirus 2.	2018	14(10):e1007750	[Walker LR et al.]	University of Nebraska, Lincoln, NE, USA.	30379811	10.1371/journal.pgen.1007750	Porcine circovirus 2 (PCV2) is a circular single-stranded DNA virus responsible for a group of diseases collectively known as PCV2 Associated Diseases (PCVAD). Variation in the incidence and severity of PCVAD exists between pigs suggesting a host genetic component involved in pathogenesis. A large-scale genome-wide association study of experimentally infected pigs (n = 974), provided evidence of a host genetic role in PCV2 viremia, immune response and growth during challenge. Host genotype explained 64% of the phenotypic variation for overall viral load, with two major Quantitative Trait Loci (QTL) identified on chromosome 7 (SSC7) near the swine leukocyte antigen complex class II locus and on the proximal end of chromosome 12 (SSC12). The SNP having the strongest association, ALGA0110477 (SSC12), explained 9.3% of the genetic and 6.2% of the phenotypic variance for viral load. Dissection of the SSC12 QTL based on gene annotation, genomic and RNA-sequencing, suggested that a missense mutation in the SYNGR2 (SYNGR2 p.Arg63Cys) gene is potentially responsible for the variation in viremia. This polymorphism, located within a protein domain conserved across mammals, results in an amino acid variant SYNGR2 p.63Cys only observed in swine. PCV2 titer in PK15 cells decreased when the expression of SYNGR2 was silenced by specific-siRNA, indicating a role of SYNGR2 in viral replication. Additionally, a PK15 edited clone generated by CRISPR-Cas9, carrying a partial deletion of the second exon that harbors a key domain and the SYNGR2 p.Arg63Cys, was associated with a lower viral titer compared to wildtype PK15 cells (>24 hpi) and supernatant (>48hpi)($P < 0.05$). Identification of a non-conservative substitution in this key domain of SYNGR2 suggests that the SYNGR2 p.Arg63Cys variant may underlie the observed genetic	pig
78	animal	pig	CRISPR;Cas9	TP53	PloS one	Generation of a TP53-modified porcine cancer model by CRISPR/Cas9-mediated gene modification in porcine zygotes via electroporation.	2018	13(10):e0206360	[Tanihara F et al.]	Tokushima University, Tokushima, Japan.	30352075	10.1371/journal.pone.0206360	TP53 (which encodes p53) is one of the most frequently mutated genes in cancers. In this study, we generated TP53-mutant pigs by gene editing via electroporation of the Cas9 protein (GEEP), a process that involves introducing the Cas9 protein and single-guide RNA (sgRNA) targeting exon 3 and intron 4 of TP53 into in vitro-fertilized zygotes. Zygotes modified by the sgRNAs were transferred to recipients, two of which gave birth to a total of 11 piglets. Of those 11 piglets, 9 survived. Molecular genetic analysis confirmed that 6 of 9 live piglets carried mutations in TP53, including 2 piglets with no wild-type (WT) sequences and 4 genetically mosaic piglets with WT sequences. One mosaic piglet had 142 and 151 bp deletions caused by a combination of the two sgRNAs. These piglets were continually monitored for 16 months and three of the genome-edited pigs (50%) exhibited various tumor phenotypes that we presumed were caused by TP53 mutations. Two mutant pigs with no WT sequences developed mandibular osteosarcoma and nephroblastoma. The mosaic pig with a deletion between targeting sites of two sgRNAs exhibited malignant fibrous histiocytoma. Tumor phenotypes of TP53 mosaic mutant pigs have not been previously reported. Our results indicated that the mutations caused by gene editing successfully induced tumor	pig
79	animal	pig	CRISPR;Cas9;	Rosa26	PLoS pathogens	Genetically modified pigs are protected from classical swine fever virus.	2018	14(12):e1007193	[Xie Z et al.]	Jilin University, Changchun, Jilin, China.	30543715	10.1371/journal.ppat.1007193	Classical swine fever (CSF) caused by classical swine fever virus (CSFV) is one of the most detrimental diseases, and leads to significant economic losses in the swine industry. Despite efforts by many government authorities to stamp out the disease from national pig populations, the disease remains widespread. Here, antiviral small hairpin RNAs (shRNAs) were selected and then inserted at the porcine Rosa26 (pRosa26) locus via a CRISPR/Cas9-mediated knock-in strategy. Finally, anti-CSFV transgenic (TG) pigs were produced by somatic nuclear transfer (SCNT). Notably, in vitro and in vivo viral challenge assays further demonstrated that these TG pigs could effectively limit the replication of CSFV and reduce CSFV-associated clinical signs and mortality, and disease resistance could be stably transmitted to the F1-generation. Altogether, our work demonstrated that RNA interference (RNAi) technology combining CRISPR/Cas9 technology offered the possibility to produce TG animal with improved resistance to viral infection. The use of these TG pigs can reduce CSF-related economic losses and this antiviral strategy may be useful for future antiviral research.	pig
80	animal	pig	CRISPR;Cas9;	IL1B2	Proceedings of the National Academy of Sciences of the United States of America	Inactivation of porcine interleukin-1beta results in failure of rapid conceptus elongation.	2018	115(2):307-312	[Whyte JJ et al.]	University of Missouri, Columbia, MO, USA.	29279391	10.1073/pnas.1718004115	Conceptus expansion throughout the uterus of mammalian species with a noninvasive epitheliochorial type of placentation is critical establishing an adequate uterine surface area for nutrient support during gestation. Pig conceptuses undergo a unique rapid morphological transformation to elongate into filamentous threads within 1 h, which provides the uterine surface to support development and maintain functional corpora lutea through the production of estrogen. Conceptus production of a unique interleukin 1beta, IL1B2, temporally increases during the period of trophoblast remodeling during elongation. CRISPR/Cas9 gene editing was used to knock out pig conceptus IL1B2 expression and the secretion of IL1B2 during the time of conceptus elongation. Trophoblast elongation occurred on day 14 in wild-type (IL1B2(+/+)) conceptuses but did not occur in ILB2-null (IL1B2(-/-)) conceptuses. Although the morphological transition of IL1B2(-/-) conceptuses was inhibited, expression of a number of conceptus developmental genes was not altered. However, conceptus aromatase expression and estrogen secretion were decreased, indicating that IL1B2 may be involved in the spatiotemporal increase in conceptus estrogen synthesis needed for the establishment of pregnancy in the pig and may serve to regulate the proinflammatory response of endometrium to IL1B2 during conceptus elongation and attachment to the	pig

81	animal	pig	CRISPR;Cas9;	FGF10	Reproduction in domestic animals = Zuchthygiene	Effects of voltage strength during electroporation on the development and quality of in vitro-produced porcine embryos.	2018	53(2):313-318	[Nishio K et al.]	Tokushima University, Tokushima, Japan.	29135047	10.1111/rda.13106	This study was conducted to determine suitable conditions for an experimental method in which the CRISPR/Cas9 system is introduced into in vitro-produced porcine zygotes by electroporation. In the first experiment, when putative zygotes derived from in vitro fertilization (IVF) were electroporated by either unipolar or bipolar pulses, keeping the voltage, pulse duration and pulse number fixed at 30 V/mm, 1 msec and five repeats, respectively, the rate of blastocyst formation from zygotes electroporated by bipolar pulses decreased compared to zygotes electroporated by unipolar pulses. In the second experiment, the putative zygotes were electroporated by electroporation voltages ranging from 20 V/mm-40 V/mm with five 1-msec unipolar pulses. The rate of cleavage and blastocyst formation of zygotes electroporated at 40 V/mm was significantly lower ($p < .05$) than that of zygotes electroporated at less than 30 V/mm. Moreover, the apoptotic nuclei indices of blastocysts derived from zygotes electroporated by voltages greater than 30 V/mm significantly increased compared with those from zygotes electroporated by voltages less than 25 V/mm ($p < .05$). When zygotes were electroporated with Cas9 mRNA and single-guide RNA (sgRNA) targeting site in the FGF10 exon 3, the proportions of blastocysts with targeted genomic sequences were 7.7% (2/26) and 3.6% (1/28) in the embryos derived from zygotes electroporated at 25 V/mm and 30 V/mm, respectively. Our results indicate that electroporation at 25 V/mm may be an acceptable condition for introducing Cas9 mRNA and sgRNA into pig IVF zygotes under which the viability of the embryos is not	pig
82	animal	pig	CRISPR;Cas9;	Pifs501	Scientific reports	Screen and Verification for Transgene Integration Sites in Pigs.	2018	8(1):7433	[Ma L et al.]	China Agricultural University, Beijing, China.	29743638	10.1038/s41598-018-24481-1	Efficient transgene expression in recipient cells constitutes the primary step in gene therapy. However, random integration in host genome comprises too many uncertainties. Our study presents a strategy combining bioinformatics and functional verification to find transgene integration sites in pig genome. Using an in silico approach, we screen out two candidate sites, namely, Pifs302 and Pifs501, located in actively transcribed intergenic regions with low nucleosome formation potential and without potential non-coding RNAs. After CRISPR/Cas9-mediated site-specific integration on Pifs501, we detected high EGFP expression in different pig cell types and ubiquitous EGFP expression in diverse tissues of transgenic pigs without adversely affecting 600 kb neighboring gene expression. Promoters integrated on Pifs501 exhibit hypomethylated modification, which suggest a permissive epigenetic status of this locus. We establish a versatile master cell line on Pifs501, which allows us to achieve site-specific exchange of EGFP to Follistatin with Cre/loxP system conveniently. Through in vitro and in vivo functional assays, we demonstrate the effectiveness of this screening method, and take Pifs501 as a potential site for transgene insertion in pigs. We anticipate that Pifs501 will have useful applications in pig genome engineering, though the identification of genomic safe harbor should over	pig
83	animal	pig	TALENs;	MYH7	Scientific reports	Successful knock-in of Hypertrophic Cardiomyopathy-mutation R723G into the MYH7 gene mimics HCM pathology in pigs.	2018	8(1):4786	[Montag J et al.]	Hannover Medical School, Hannover, Germany.	29555974	10.1038/s41598-018-22936-z	Familial Hypertrophic Cardiomyopathy (HCM) is the most common inherited cardiac disease. About 30% of the patients are heterozygous for mutations in the MYH7 gene encoding the ss-myosin heavy chain (MyHC). Hallmarks of HCM are cardiomyocyte disarray and hypertrophy of the left ventricle, the symptoms range from slight arrhythmias to sudden cardiac death or heart failure. To gain insight into the underlying mechanisms of the diseases' etiology we aimed to generate genome edited pigs with an HCM-mutation. We used TALEN-mediated genome editing and successfully introduced the HCM-point mutation R723G into the MYH7 gene of porcine fibroblasts and subsequently cloned pigs that were heterozygous for the HCM-mutation R723G. No off-target effects were determined in the R723G-pigs. Surprisingly, the animals died within 24 h post partem, probably due to heart failure as indicated by a shift in the a/ss-MyHC ratio in the left ventricle. Most interestingly, the neonatal pigs displayed features of HCM, including mild myocyte disarray, malformed nuclei, and MYH7-overexpression. The finding of HCM-specific pathology in neonatal R723G-piglets suggests a very early onset of the disease and highlights the importance of novel large animal models for studying causative mechanisms and long-term progression of human	pig
84	animal	pig	CRISPR;Cas9;	neurogenin 3	Scientific reports	Targeted Mutation of NGN3 Gene Disrupts Pancreatic Endocrine Cell Development in Pigs.	2018	8(1):3582	[Sheets TP et al.]	University of Maryland, College Park, MD, USA.	29483633	10.1038/s41598-018-22050-0	The domestic pig is an attractive model for biomedical research because of similarities in anatomy and physiology to humans. However, key gaps remain in our understanding of the role of developmental genes in pig, limiting its full potential. In this publication, the role of NEUROGENIN 3 (NGN3), a transcription factor involved in endocrine pancreas development has been investigated by CRISPR/Cas9 gene ablation. Precomplexed Cas9 ribonucleoproteins targeting NGN3 were injected into in vivo derived porcine embryos, and transferred into surrogate females. On day 60 of pregnancy, nine fetuses were collected for genotypic and phenotypic analysis. One of the piglets was identified as an in-frame biallelic knockout (Delta2/Delta2), which showed a loss of putative NGN3-downstream target genes: NEUROD1 and PAX4, as well as insulin, glucagon, somatostatin and pancreatic polypeptide-Y. Fibroblasts from this fetus were used in somatic cell nuclear transfer to generate clonal animals to qualify the effect of mutation on embryonic lethality. Three live piglets were born, received colostrum and suckled normally, but experienced extreme weight loss over a 24 to 36-hour period requiring humane euthanasia. Expression of pancreatic endocrine hormones: insulin, glucagon, and somatostatin were lost. The data support a critical role	pig

85	animal	pig	CRISPR;Cas9;	alpha-1,3-galactosyltransferase	Theriogenology	Timing of CRISPR/Cas9-related mRNA microinjection after activation as an important factor affecting genome editing efficiency in porcine oocytes.	2018	108:29-38	[Sato M et al.]	Kagoshima University, Kagoshima, Japan.	29195121	10.1016/j.theriogenology.2017.11.030	Recently, successful one-step genome editing by microinjection of CRISPR/Cas9-related mRNA components into the porcine zygote has been described. Given the relatively long gestational period and the high cost of housing swine, the establishment of an effective microinjection-based porcine genome editing method is urgently required. Previously, we have attempted to disrupt a gene encoding alpha-1,3-galactosyltransferase (GGTA1), which synthesizes the alpha-Gal epitope, by microinjecting CRISPR/Cas9-related nucleic acids and enhanced green fluorescent protein (EGFP) mRNA into porcine oocytes immediately after electrical activation. We found that genome editing was indeed induced, although the resulting blastocysts were mosaic and the frequency of modified cells appeared to be low (50%). To improve genome editing efficiency in porcine oocytes, cytoplasmic injection was performed 6 h after electrical activation, a stage wherein the pronucleus is formed. The developing blastocysts exhibited higher levels of EGFP. Furthermore, the T7 endonuclease 1 assay and subsequent sequencing demonstrated that these embryos exhibited increased genome editing efficiencies (69%), although a high degree of mosaicism for the induced mutation was still observed. Single blastocyst-based cytochemical staining with fluorescently labeled isolectin BS-I-B4 also confirmed this mosaicism. Thus, the development of a technique that avoids or reduces such mosaicism would be a key	pig
86	animal	pig	CRISPR;Cas9;	INS	Transgenic research	Generation of insulin-deficient piglets by disrupting INS gene using CRISPR/Cas9 system.	2018	27(3):289-300	[Cho B et al.]	Mgenplus Co., Ltd., Seoul, Korea.	29691708	10.1007/s11248-018-0074-1	Diabetes mellitus is a chronic disease with accompanying severe complications. Various animal models, mostly rodents due to availability of genetically modified lines, have been used to investigate the pathophysiology of diabetes. Using pigs for diabetic research can be beneficial because of their similarity in size, pathogenesis pathway, physiology, and metabolism with human. However, the use of pigs for diabetes research has been hampered due to only few pig models presenting diabetes symptoms. In this study, we have successfully generated insulin-deficient pigs by generating the indels of the porcine INS gene in somatic cells using CRISPR/Cas9 system followed by somatic cell nuclear transfer. First, somatic cells carrying a modified INS gene were generated using CRISPR/Cas9 system and their genotypes were confirmed by T7E1 assay; targeting efficiency was 40.4% (21/52). After embryo transfer, three live and five stillborn piglets were born. As expected, INS knockout piglets presented high blood glucose levels and glucose was detected in the urine. The level of insulin and c-peptide in the blood serum of INS knockout piglets were constant after feeding and the expression of insulin in the pancreas was absent in those piglets. This study demonstrates effectiveness of CRISPR/Cas9 system in generating novel pig models. We expect that these insulin-deficient pigs can be used in diabetes research to test the efficacy and safety of new drugs and the recipient of islet transplantation to	pig
87	animal	pig	CRISPR;Cas9;	retinoic acid-inducible gene I (RIG-I)	Virology journal	RIG-I is responsible for activation of type I interferon pathway in Seneca Valley virus-infected porcine cells to suppress viral replication.	2018	15(1):162	[Li P et al.]	Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China.	30352599	10.1186/s12985-018-1080-x	BACKGROUND: Retinoic acid-inducible gene I (RIG-I) is a key cytosolic receptor of the innate immune system. Seneca valley virus (SVV) is a newly emerging RNA virus that infects pigs causing significant economic losses in pig industry. RIG-I plays different roles during different viruses infections. The role of RIG-I in SVV-infected cells remains unknown. Understanding of the role of RIG-I during SVV infection will help to clarify the infection process of SVV in the infected cells. METHODS: In this study, we generated a RIG-I knockout (KO) porcine kidney PK-15 cell line using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9) genome editing tool. The RIG-I gene sequence of RIG-I KO cells were determined by Sanger sequencing method, and the expression of RIG-I protein in the RIG-I KO cells were detected by Western bloting. The activation status of type I interferon pathway in Sendai virus (SeV)- or SVV-infected RIG-I KO cells was investigated by measuring the mRNA expression levels of interferon (IFN)-beta and IFN-stimulated genes (ISGs). The replicative state of SVV in the RIG-I KO cells was evaluated by qPCR, Western bloting, TCID50 assay and indirect immunofluorescence assay. RESULTS: Gene editing of RIG-I in PK-15 cells successfully resulted in the destruction of RIG-I expression. RIG-I KO PK-15 cells had a lower expression of IFN-beta and ISGs compared with wildtype (WT) PK-15 cells when stimulated by the model RNA virus SeV. The amounts of viral RNA and viral protein as well as viral yields in SVV-infected RIG-I WT and KO cells were determined and compared, which showed that knockout of RIG-I significantly increased SVV replication and propagation. Meanwhile, the expression of IFN-beta and ISGs were considerably decreased in RIG-I KO cells compared with that in RIG-I WT cells during SVV infection. CONCLUSION: Altogether, this study indicated that RIG-I showed an antiviral role against SVV and was essential for activation of type I IFN signaling during SVV infection. In addition, this study suggested that the CRISPR/Cas9 system can be	pig
88	animal	pig	CRISPR;Cas9;	miR-302/367	Yi chuan = Hereditas	[Assessing abundance and specificity of different types of sgRNA targeting miRNA precursors].	2018	40(7):561-571	[Liu HL et al.]	Huazhong Agricultural University, Wuhan, China.	30021719	10.16288/j.yczz.17-417	CRISPR/Cas technology enables efficient and specific editing the genome. Since different bacterial sources or artificially modified Cas9, as well as Cpf1 and other nucleases, recognize different PAMs (protospacer adjacent motifs), different gene editing nucleases may use different types of sgRNAs (small guide RNA). MicroRNAs (miRNAs) are a class of regulatory small non-coding RNAs. To determine whether specific targets for sgRNAs in miRNA precursor exit, the abundance and specificity of 11 different types of sgRNA targeting 28 645 miRNA precursors were analyzed in the present study using the CRISPR-offinder, a bioinformatics software developed in our own laboratory. The CRISPR/Cas9 lentivirus technology was used to target the miR-302/367 cluster in a porcine cell line, and its knockout efficiency for the miRNA target was evaluated. The results show that there are about 8 different types of sgRNAs that can target individual miRNA precursors. By assessing the off-target effect, only 18.2% of the sgRNAs showed high specificity for targeting the porcine miRNA precursors. Lastly, using the miR-302/367 cluster target as an example, we showed that the CRISPR/Cas9 lentivirus technology was 40% efficient in successfully establishing correct knockout of the target miRNA in the porcine cell line. This present study provides an important resource for the use of CRISPR/Cas technology to target	pig

89	animal	Bama miniature pig	CRISPR;Cas9;	GHR	In vitro cellular & developmental biology. Animal	Efficient generation of GHR knockout Bama minipig fibroblast cells using CRISPR/Cas9-mediated gene editing.	2019	55(10):784-792	[Wang R et al.]	Guangxi University, Nanning, China.	31456163	10.1007/s11626-019-00397-6	Dwarfism, also known as growth hormone deficiency (GHD), is a disease caused by genetic mutations that result in either a lack of growth hormone or insufficient secretion of growth hormone, resulting in a person's inability to grow normally. In the past, many studies focusing on GHD have made use of models of other diseases such as metabolic or infectious diseases. A viable GHD specific model system has not been used previously, thus limiting the interpretation of GHD results. The Bama minipig is unique to Guangxi province and has strong adaptability and disease resistance, and an incredibly short stature, which is especially important for the study of GHD. In addition, studies of GHR knockout Bama minipigs and GHR knockout Bama minipig fibroblast cells generated using CRISPR/Cas9 have not been previously reported. Therefore, the Bama minipig was selected as an animal model and as a tool for the study of GHD in this work. In this study, a Cas9 plasmid with sgRNA targeting the first exon of the GHR gene was transfected into Bama minipig kidney fibroblast cells to generate 22 GHR knockout Bama minipig kidney fibroblast cell lines (12 male monoclonal cells and 10 female monoclonal cells). After culture and identification, 11 of the 12 male clone cell lines showed double allele mutations, and the rate of positive alteration of GHR was 91.67%. Diallelic mutation of the target sequence occurred in 10 female clonal cell lines, with an effective positive mutation rate of 100%. Our experimental results not only showed that CRISPR/Cas9 could efficiently be used for gene editing in Bama minipig cells but also identified a highly efficient target site for the generation of a GHR knockout in other porcine models. Thus, the generation of GHR knockout male and female Bama fibroblast cells could lay a foundation for the birth of a future dwarfism model pig. We anticipate that the "mini" Bama minipig will be of improved use for biomedical and agricultural scientific research and for furthering our understanding of	pig
90	animal	Bama miniature pig	CRISPR;Cas9;	oxysterol binding protein like 2	Journal of genetics and genomics = Yi chuan xue bao	OSBPL2-disrupted pigs recapitulate dual features of human hearing loss and hypercholesterolaemia.	2019	46(8):379-387	[Yao J et al.]	Nanjing Medical University, Nanjing, China.	31451425	10.1016/j.jgg.2019.06.006	Oxysterol binding protein like 2 (OSBPL2), an important regulator in cellular lipid metabolism and transport, was identified as a novel deafness-causal gene in our previous work. To resemble the phenotypic features of OSBPL2 mutation in animal models and elucidate the potential genotype-phenotype associations, the OSBPL2-disrupted Bama miniature (BM) pig model was constructed using CRISPR/Cas9-mediated gene editing, somatic cell nuclear transfer (SCNT) and embryo transplantation approaches, and then subjected to phenotypic characterization of auditory function and serum lipid profiles. The OSBPL2-disrupted pigs displayed progressive hearing loss (HL) with degeneration/apoptosis of cochlea hair cells (HCs) and morphological abnormalities in HC stereocilia, as well as hypercholesterolaemia. High-fat diet (HFD) feeding aggravated the development of HL and led to more severe hypercholesterolaemia. The dual phenotypes of progressive HL and hypercholesterolaemia resembled in OSBPL2-disrupted pigs confirmed the implication of OSBPL2 mutation in nonsyndromic hearing loss (NSHL) and contributed to the	pig
91	animal	Liang Guang Small Spotted pig	CRISPR;Cas9;	insulin-like growth factor 2	Transgenic research	Disruption of the ZBED6 binding site in intron 3 of IGF2 by CRISPR/Cas9 leads to enhanced muscle development in Liang Guang Small Spotted pigs.	2019	28(1):141-150	[Liu X et al.]	Sun Yat-sen University, Guangzhou, China.	30488155	10.1007/s11248-018-0107-9	Insulin-like growth factor 2 (IGF2) plays an important role in the development of the foetus and in post-natal growth and development. A SNP within intron 3 of porcine IGF2 disrupts a binding site for the repressor, zinc finger BED-type containing 6 (ZBED6), leading to up-regulation of IGF2 in skeletal muscle and major effects on muscle growth, heart size, and fat deposition. This favourable mutation is common in Western commercial pig populations, but is not present in most indigenous Chinese pig breeds. Here, we described the efficient disruption of the ZBED6 binding site motif in intron 3 of IGF2 by CRISPR/Cas9 in porcine embryonic fibroblasts (PEFs) from the indigenous Chinese pig breed, Liang Guang Small Spotted pig. Disruption of the binding motif led to a drastic up-regulation of IGF2 expression in PEFs and enhanced myogenic potential and cell proliferation of PEFs. IGF2-edited pigs were then generated using somatic cell nuclear transfer. Enhanced muscle development was evident in one pig with biallelic deletion of the ZBED6 binding site motif, implying that the release of ZBED6 repression has a major effect on porcine muscle development. Our study confirmed the important effect of a mutation in the ZBED6 binding site motif on IGF2 expression and myogenesis, thus providing the basis for breeding a new line of Liang Guang Small Spotted pigs with improved lean meat percentage, a trait of great	pig
92	animal	meishan pig	ZFN;	myostatin	Genome	An integrated analysis of mRNA and miRNA in skeletal muscle from myostatin-edited Meishan pigs.	2019	62(5):305-315	[Xie S et al.]	Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China.	30913397	10.1139/gen-2018-0110	Myostatin (MSTN) is a key muscle factor that negatively regulates skeletal muscle growth and development. Our laboratory recently produced genetically engineered Meishan pigs containing a ZFN-edited MSTN loss-of-function mutation (MSTN(-/-), MKO) that led to the hypertrophy of skeletal muscles. In this study, we performed transcriptome sequencing and miRNA sequencing in skeletal muscle samples from MKO and wildtype Meishan (MWT) pigs to investigate the effect of MSTN(-/-) on expression of mRNA and miRNA. Our results indicated that, compared to MWT pigs, there were 200 genes and 4 miRNAs being significantly up-regulated, and 238 genes and 5 miRNAs being significantly down-regulated in MKO pigs. Analysis by GO and KEGG pathways revealed that differentially expressed miRNAs and their target genes of those differentially expressed miRNAs were involved in the signal pathways of skeletal muscle growth and development such as AMPK, mTOR, and TGF-beta. An integrated analysis of the correlation between miRNA-mRNA and transcriptome predicated that XK and METTL8 were target genes for miR-499-5p, while LRP4 was a target gene for miR-490-3p. Our results provide important clues to help us further investigate MSTN's regulatory mechanisms during skeletal muscle growth and development.	pig

93	animal	Meishan pig	ZFN;	myostatin	PloS one	Effect of ZFN-edited myostatin loss-of-function mutation on gut microbiota in Meishan pigs.	2019	14(1):e0210619	[Cui WT et al.]	Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China.	30645618	10.1371/journal.pone.0210619	Intestine contains the body's second largest genetic information, so a relatively stable microbiota ecosystems and interactions between intestinal micro-organisms play a pivotal role in the normal growth and development in animals. The establishment of intestinal microflora is affected by a variety of factors such as species, environmental factors, developmental stage, organizational structure and physiological characteristics of various parts of the digestive tract. Gene editing technology such as ZFN has recently been used as a new approach to replace the traditional transgenic technology and to make genetic modifications in animals. However, it is not known if genetic modification by gene editing technology will have any impact on gut microbiota. In this study, by sequencing 16S rRNA collected from rectum, we investigated the effects of ZFN-mediated myostatin (MSTN) loss-of-function mutation (MSTN-/-) on gut microbiota in Meishan pigs. Our results showed that the fecal microbial composition is very similar between MSTN-/- Meishan pigs and wild type Meishan pigs. Although significant differences in certain individual strains were observed, all the dominant microorganism species are basically the same between MSTN-/- and wild type pigs. However, these differences do not adversely affect MSTN-/- Meishan pigs. Thus, it is concluded that ZFN-mediated MSTN loss-of-function mutation did not have any	pig
94	animal	pig	CRISPR;Cas9;	beta2-microglobulin	American journal of transplantation	Triple (GGTA1, CMAH, B2M) modified pigs expressing an SLA class I(low) phenotype-Effects on immune status and susceptibility to human immune responses.	2019		[Hein R et al.]	Hannover Medical School, Hannover, Germany.	31733031	10.1111/ajt.15710	Porcine xenografts lacking swine leukocyte antigen (SLA) class I are thought to be protected from human T cell responses. We have previously shown that SLA class I deficiency can be achieved in pigs by CRISPR/Cas9-mediated deletion of beta2 -microglobulin (B2M). Here, we characterized another line of genetically modified pigs in which targeting of the B2M locus did not result in complete absence of B2M and SLA class I but rather in significantly reduced expression levels of both molecules. Residual SLA class I was functionally inert, because no proper differentiation of the CD8(+) T cell subset was observed in B2M(low) pigs. Cells from B2M(low) pigs were less capable in triggering proliferation of human peripheral blood mononuclear cells in vitro, which was mainly due to the nonresponsiveness of CD8(+) T cells. Nevertheless, cytotoxic effector cells developing from unaffected cell populations (eg, CD4(+) T cells, natural killer cells) lysed targets from both SLA class I(+) wildtype and SLA class I(low) pigs with similar efficiency. These data indicate that the absence of SLA class I is an effective approach to prevent the activation of human CD8(+) T cells during the induction phase of an anti-xenograft response. However, cytotoxic activity of cells during the effector phase cannot be controlled by this approach.	pig
95	animal	pig	CRISPR;Cas9;	cd163	Animal biotechnology	Generation of CD163-edited pig via electroporation of the CRISPR/Cas9 system into porcine in vitro-fertilized zygotes.	2019	1-8	[Tanihara F et al.]	Tokushima University, Tokushima, Japan.	31558095	10.1080/10495398.2019.1668801	CD163 is a putative fusion receptor for virus of porcine reproductive and respiratory syndrome (PRRS). In this study, we introduced a CRISPR/Cas9 system [guide RNAs (gRNAs) with Cas9 protein] targeting the CD163 gene into in vitro-fertilized porcine zygotes by electroporation to generate CD163-modified pigs. First, we designed four types of gRNAs that targeted distinct sites in exon 7 of the CD163 gene. Cas9 protein with different gRNAs was introduced into in vitro-fertilized zygotes by electroporation. When the electroporated zygotes were allowed to develop to blastocysts in vitro and the genome editing efficiency was evaluated using these blastocysts, three (gRNA1, 2, and 4) of the four gRNAs tested successfully edited the CD163 gene. To generate CD163-knockout pigs, a total of 200 electroporated zygotes using these three gRNAs were transferred into the oviducts of oestrous-synchronized surrogate and the surrogate gave birth to eight piglets. Subsequent sequence analysis revealed that one of the piglets carried no wild-type sequence in CD163 gene. The other seven piglets carried only wild-type sequence. Thus, we successfully generated a CD163-edited pig by electroporation of the CRISPR/Cas9 system into in vitro-fertilized zygotes, although further improvement is required to generate genetically modified pigs with high	pig
96	animal	pig	CRISPR;Cas9;	pancreas duodenum homeobox 1 (PDX-1)	Animal science journal = Nihon chikusan Gakkaiho	Generation of PDX-1 mutant porcine blastocysts by introducing CRISPR/Cas9-system into porcine zygotes via electroporation.	2019	90(1):55-61	[Tanihara F et al.]	Tokushima University, Ishii-cho, Tokushima, Japan.	30368976	10.1111/asj.13129	Recently, we established the GEEP ("gene editing by electroporation of Cas9 protein") method, in which the CRISPR/Cas9 system, consisting of a Cas9 protein and single guide RNA (sgRNA), is introduced into pig zygotes by electroporation and thus induces highly efficient targeted gene disruption. In this study, we examined the effects of sgRNA on the blastocyst formation of porcine embryos and evaluated their genome-editing efficiency. To produce an animal model for diabetes, we targeted PDX-1 (pancreas duodenum homeobox 1), a gene that is crucial for pancreas development during the fetal period and whose monoallelic disruption impairs insulin secretion. First, Cas9 protein with different sgRNAs that targeted distinct sites in the PDX-1 exon 1 was introduced into in vitro-fertilized zygotes by the GEEP method. Of the six sgRNAs tested, three sgRNAs (sgRNA1, 2, and 3) successfully modified PDX-1 gene. The blastocyst formation rate of zygotes edited with sgRNA3 was significantly ($p < 0.05$) lower than that of control zygotes without the electroporation treatment. Our study indicates that the GEEP method can be successfully used to generate PDX-1 mutant blastocysts, but the development and the efficiency of editing the genome of zygotes may be affected by the sgRNA used for CRISPR/Cas9 system.	pig
97	animal	pig	Cas9;	Porcine endogenous retrovirus (PERV) pol	Animals	The Relationship between Embryonic Development and the Efficiency of Target Mutations in Porcine Endogenous Retroviruses (PERVs) Pol Genes in Porcine Embryos.	2019	9(9)	[Hirata M et al.]	Tokushima University, Myozai-gun, Tokushima, Japan.	31443357	10.3390/ani9090593	Porcine endogenous retrovirus (PERV) is a provirus found in the pig genome that may act as an infectious pathogen in humans who receive pig organ xenotransplantation. Inactivation of the PERV pol gene in porcine cells reportedly affects cell growth. Therefore, the mutation of PERV pol gene in porcine embryos using genome editing may affect the embryonic development. The present study was carried out to investigate the relationship between the mutation of the PERV pol gene in porcine embryos and their development. We introduced, either alone or in combination, three different gRNAs (gRNA1, 2, and 3) into porcine zygotes by genome editing using electroporation of the Cas9 protein (GEEP) system. All three gRNAs targeted the PERV pol gene, and we assessed their effects on porcine embryonic development. Our results showed that the blastocyst formation rates of zygotes electroporated with gRNA3-alone and in combination-were significantly lower ($p < 0.05$) than those of zygotes electroporated with gRNA1. The mutation rates assessed by the PERV pol gene target site sequencing in individual blastocysts and pooled embryos at the 2-to-8-cell stage did not differ among the three gRNAs. However, the frequency of indel mutations in mutant embryos at the 2-to-8-cell stage trended higher in the embryos electroporated with gRNA3 alone and in combination. Embryonic development may be	pig

98	animal	pig	CRISPR;Cas9;	rsad2	Antiviral research	Generation of pRSAD2 gene knock-in pig via CRISPR/Cas9 technology.	2019	174:104696	[Xie Z et al.]	Jilin University, Changchun, Jilin, China	31862502	10.1016/j.antiviral.2019.104696	A wide range of endemic and epidemic viruses, including classic swine fever virus (CSFV), pseudorabies virus (PRV) and others, are among the most economically important pathogens in pigs and have severely affected the national economy, human health and animal welfare and productivity. The RSAD2 exhibits antiviral activity against various DNA and RNA viruses. In this study, we successfully accomplished site-specific insertion of the porcine RSAD2 gene (pRSAD2) at the porcine ROSA26 (pROSA26) locus, generating pRSAD2 gene knock-in (pRSAD2-KI) PK-15 cells and porcine foetal fibroblasts (PFFs) via CRISPR/Cas9 technology. Gene expression analysis confirmed that pRSAD2-KI cells stably and efficiently overexpressed the pRSAD2 gene. Furthermore, viral challenge studies in vitro indicated that site-specific integration of the pRSAD2 gene not only effectively reduced CSFV infection but also PRV infection. More importantly, we ultimately successfully produced a pRSAD2-KI pig that constitutively overexpressed the pRSAD2, viral challenge results indicated that fibroblasts isolated from the pRSAD2-KI pig reduced CSFV infection. Taken together, these results suggest that CRISPR/Cas9-mediated knock-in strategy can be used for	pig
99	animal	pig	CRISPR;Cas9;	Rosa26	Applied biochemistry and biotechnology	Establishment of CRISPR/Cas9-Mediated Knock-in System for Porcine Cells with High Efficiency.	2019	189(1):26-36	[Zhang J et al.]	Northwest A&F University, Yangling, Shaanxi, China.	30859452	10.1007/s12010-019-02984-5	Since the birth of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, the new genome engineering technology has become a hot topic in the scientific community. However, for swine, the system of pig cells' homology directed repair (HDR) is generally unstable and costly. Here, we aim to make knock-in of porcine cells more realizable. The Rosa26 locus was chosen for gene editing. Through the optimization of strategy, an efficient sgRNA was selected by TIDE analysis. Correspondingly, a vector system was constructed for gene insertion in pRosa26 locus by homologous recombination. A large percentage of cells whose gene is edited easily result in apoptosis. To improve the positive rate, culturing systems have been optimized. Sequence alignment and nuclear transfer confirmed that we got two knock-in cell lines and transgene primary porcine fetal fibroblasts (PFFs) successfully. Results showed that the gene editing platform we used can obtain genetically modified pig cells stably and efficiently. This system can contribute to pig gene research and production	pig
100	animal	pig	CRISPR;Cas9;	prostaglandin-endoperoxide synthase 2 (ptgs2)	Biology of reproduction	Ablation of conceptus PTGS2 expression does not alter early conceptus development and establishment of pregnancy in the pigdagger.	2019		[Pfeiffer CA et al.]	University of Missouri, Columbia, MO, USA.	31616930	10.1093/biolre/ioz192	Pig conceptuses secrete estrogens (E2), interleukin 1 beta 2 (IL1B2), and prostaglandins (PG) during the period of rapid trophoblast elongation and establishment of pregnancy. Previous studies established that IL1B2 is essential for rapid conceptus elongation, whereas E2 is not essential for conceptus elongation or early maintenance of the corpora lutea. The objective of the present study was to determine if conceptus expression of prostaglandin-endoperoxide synthase 2 (PTGS2) and release of PG are important for early development and establishment of pregnancy. To understand the role of PTGS2 in conceptus elongation and pregnancy establishment, a loss-of-function study was conducted by editing PTGS2 using CRISPR/Cas9 technology. Wild-type (PTGS2+/+) and null (PTGS2-/-) fibroblast cells were used to create embryos through somatic cell nuclear transfer. Immunolocalization of PTGS2 and PG production was absent in cultured PTGS2-/- blastocysts on day 7. PTGS2+/+ and PTGS2-/- blastocysts were transferred into surrogate gilts, and the reproductive tracts were collected on either day 14, 17 or 35 of pregnancy. After flushing the uterus on day 14 and 17, filamentous conceptuses were cultured for 3 h to determine PG production. Conceptus release of total PG, PGF2alpha and PGE in culture media was lower with PTGS2-/- conceptuses compared to PTGS2+/+ conceptuses. However, the total PG, PGF2alpha and PGE content in the uterine flushings was not different. PTGS2-/- conceptus surrogates allowed to continue pregnancy were maintained beyond 30 days of gestation. These results indicate that pig conceptus PTGS2 is not essential for early	pig
101	animal	pig	CRISPR;Cas9;	CYP19A1	Biology of reproduction	New perspective on conceptus estrogens in maternal recognition and pregnancy establishment in the pigdagger.	2019	101(1):148-161	[Meyer AE et al.]	University of Missouri, Columbia, MO, USA.	31066888	10.1093/biolre/ioz058	The proposed signal for maternal recognition of pregnancy in pigs is estrogen (E2), produced by the elongating conceptuses between days 11 to 12 of pregnancy with a more sustained increase during conceptus attachment and placental development on days 15 to 30. To understand the role of E2 in porcine conceptus elongation and pregnancy establishment, a loss-of-function study was conducted by editing aromatase (CYP19A1) using CRISPR/Cas9 technology. Wild-type (CYP19A1+/+) and (CYP19A1-/-) fibroblast cells were used to create embryos through somatic cell nuclear transfer, which were transferred into recipient gilts. Elongated and attaching conceptuses were recovered from gilts containing CYP19A1+/+ or CYP19A1-/- embryos on day 14 and 17 of pregnancy. Total E2 in the uterine flushings of gilts with CYP19A1-/- embryos was lower than recipients containing CYP19A1+/+ embryos with no difference in testosterone, PGF2alpha, or PGE2 on either day 14 or 17. Despite the loss of conceptus E2 production, CYP19A1-/- conceptuses were capable of maintaining the corpora lutea. However, gilts gestating CYP19A1-/- embryos aborted between days 27 and 31 of gestation. Attempts to rescue the pregnancy of CYP19A1-/- gestating gilts with exogenous E2 failed to maintain pregnancy. However, CYP19A1-/- embryos could be rescued when co-transferred with embryos derived by in vitro fertilization. Endometrial transcriptome analysis revealed that ablation of conceptus E2 resulted in disruption of a number biological pathways. Results demonstrate that intrinsic E2 conceptus production is not essential for pre-implantation development, conceptus elongation, and early CL maintenance, but is essential for maintenance of pregnancy	pig
102	animal	pig	CRISPR;Cas9;	ribonuclease L	BioMed research international	CRISPR-Cas9 Mediated RNase L Knockout Regulates Cellular Function of PK-15 Cells and Increases PRV Replication.	2019	2019:7398208	[Sui C et al.]	Shandong Agricultural University, Tai'an, China.	30941371	10.1155/2019/7398208	Ribonuclease L (RNase L) is an important antiviral endoribonuclease regulated by type I IFN. RNase L is activated by viral infection and dsRNA. Because the role of swine RNase L (sRNase L) is not fully understood, in this study, we generated a sRNase L knockout PK-15 (KO-PK) cell line through the CRISPR/Cas9 gene editing system to evaluate the function of sRNase L. After transfection with CRISPR-Cas9 followed by selection using puromycin, sRNase L knockout in PK-15 cells was further validated by agarose gel electrophoresis, DNA sequencing, and Western blotting. The sRNase L KO-PK cells failed to trigger RNA degradation and induced less apoptosis than the parental PK-15 cells after transfected with poly (I: C). Furthermore, the levels of ISGs mRNA in sRNase L KO-PK cells were higher than those in the parental PK-15 cells after treated with poly (I: C). Finally, both wild type and attenuated pseudorabies viruses (PRV) replicated more efficiently in sRNase L KO-PK cells than the parental PK-15 cells. Taken together, these findings suggest that sRNase L has multiple biological functions including cellular single-stranded RNA degradation, induction of apoptosis, downregulation of transcript levels of ISGs, and antiviral activity against PRV. The sRNase L KO-PK cell line will be a valuable tool for studying functions of sRNase L as	pig

103	animal	pig	CRISPR;Cas9;	Mx2; beta 1,4 N-acetylgalactosaminyltransferase	Bioscience reports	Porcine antiviral activity is increased by CRISPRa-SAM system.	2019	39(8)	[Jiang J et al.]	Huazhong Agricultural University, Wuhan, Hubei, China.	31371630	10.1042/BSR20191496	Clustered Regularly Interspaced Short Palindromic Repeat activation-synergistic activation mediator system (CRISPRa-SAM) has been efficiently used to up-regulate the targeted genes in human and mouse. But it is not known whether the CRISPRa-SAM system can be used against porcine disease because its two important transcriptional activation domains (P65 and heat shock transcription factor 1 (HSF1)) are from mouse and human, respectively. Pig is one of the most important meat sources, porcine viral infectious diseases cause massive economic losses to the swine industry and threaten the public health. We aimed to investigate whether the CRISPRa-SAM system could increase porcine antiviral activity by mediating two pig-specific target genes (Mx2 and beta1,4 N-acetylgalactosaminyltransferase (B4galnt2)). First, we constructed PK-15 and IPEC-J2 cell lines expressing nuclease-deficient Cas9 (dCas9)-vp64 and MS2-P65-HSF1 stably. Next, in these two cell models, we activated Mx2 and B4galnt2 expression through CRISPRa-SAM system. Antiviral activity to PRV or H9N2 was improved in PK-15 cells where Mx2 or B4galnt2 was activated. Altogether, our results demonstrated the potential of CRISPRa-SAM system as a powerful tool for activating pig genes and improving porcine antiviral activity.	pig
104	animal	pig	CRISPR;Cas9;	RAG2; IL2RG; SCD5; Ig Heavy chain	BMC biotechnology	Frequency of off-targeting in genome edited pigs produced via direct injection of the CRISPR/Cas9 system into developing embryos.	2019	19(1):25	[Carey K et al.]	Virginia Tech, Blacksburg, VA, USA.	31060546	10.1186/s12896-019-0517-7	BACKGROUND: The CRISPR/Cas9 system can effectively introduce site-specific modifications to the genome. The efficiency is high enough to induce targeted genome modifications during embryogenesis, thus increasing the efficiency of producing genetically modified animal models and having potential clinical applications as an assisted reproductive technology. Because most of the CRISPR/Cas9 systems introduce site-specific double-stranded breaks (DSBs) to induce site-specific modifications, a major concern is its potential off-targeting activity, which may hinder the application of the technology in clinics. In this study, we investigated off-targeting events in genome edited pigs/fetuses that were generated through direct injection of the CRISPR/Cas9 system into developing embryos; off-targeting activity of four different sgRNAs targeting RAG2, IL2RG, SCD5, and Ig Heavy chain were examined. RESULTS: First, bioinformatics analysis was applied to identify 27 potential off-targeting genes from the sgRNAs. Then, PCR amplification followed by sequencing analysis was used to verify the presence of off-targeting events. Off-targeting events were only identified from the sgRNA used to disrupt Ig Heavy chain in pigs; frequency of off-targeting was 80 and 70% on AR and RBFOX1 locus respectively. A potential PAM sequence was present in both of the off-targeting genes adjacent to probable sgRNA binding sites. Mismatches against sgRNA were present only on the 5' side of AR, suggesting that off-targeting activities are systematic events. However, the mismatches on RBFOX1 were not limited to the 5' side, indicating unpredictability of the events. CONCLUSIONS: The prevalence of off-targeting is low via direct injection of CRISPR/Cas9 system into developing embryos, but the events cannot be accurately predicted. Off-targeting frequency of each CRISPR/Cas9 system should be	pig
105	animal	pig	CRISPR;Cas9;	islet amyloid polypeptide	Cell death & disease	Preparation of a new type 2 diabetic miniature pig model via the CRISPR/Cas9 system.	2019	10(11):823	[Zou X et al.]	Jilin University, Changchun, Jilin, China.	31659151	10.1038/s41419-019-2056-5	Diabetes has become one of the major noninfectious diseases that seriously endanger public health. The formation of islet amyloid polypeptide (IAPP) affects the normal physiological functions of the body, such as glucose metabolism and lipid metabolism. The mature human IAPP protein (hIAPP) has a strong tendency to misfold and is considered to be one of the major causes of amyloid changes in islets. Deposition of hIAPP is considered to be one of the leading causes of type 2 diabetes mellitus (T2DM). Miniature pigs are experimental animal models that are well suited for research on gene function and human diabetes. In our study, we obtained IAPP gene-humanized miniature pigs via the CRISPR/Cas9 system and somatic cell nuclear transfer (SCNT) technology. The hIAPP pigs can be used to further study the pathogenesis and related complications of T2DM and to lay a solid foundation for the prevention and treatment	pig
106	animal	pig	CRISPR;Cas9;	miR-17-92 cluster	Cells	CRISPR/Cas9-Mediated Hitchhike Expression of Functional shRNAs at the Porcine miR-17-92 Cluster.	2019	8(2)	[Lu C et al.]	Jilin University, Changchun, China.	30717310	10.3390/cells8020113	Successful RNAi applications depend on strategies allowing stable and persistent expression of minimal gene silencing triggers without perturbing endogenous gene expression. In this study, we proposed an endogenous microRNA (miRNA) cluster as a novel integration site for small hairpin RNAs (shRNAs). We successfully integrated exogenous shRNAs at the porcine miRNA-17-92 (pmiR-17-92) cluster via a CRISPR/Cas9-mediated knock-in strategy. The anti-EGFP or anti-CSFV shRNAs could be stably and effectively expressed at the control of the endogenous promoter of the pmiR-17-92 cluster. Importantly, we confirmed that hitchhike expression of anti-classical swine fever (CSFV) shRNA had no effect on cell growth, blastocyst development and endogenous pmiR-17-92 expression in selected transgene (TG) porcine fetal fibroblasts (PFFs) clones. Moreover, these TG PFFs could inhibit the replication of CSFV by half and could be further used for generation of transgenic pigs. Taken together, these results show that our RNA interference (RNAi) expression strategy benefits numerous applications, from miRNA, genome and transgenic research.	pig
107	animal	pig	CRISPR;Cas9	fumarylacetoacetate hydrolase (Fah); Recombinant activation gene 2 (Rag2)	DNA and cell biology	Efficient Generation of an Fah/Rag2 Dual-Gene Knockout Porcine Cell Line Using CRISPR/Cas9 and Adenovirus.	2019	38(4):314-321	[Gao M et al.]	Sichuan University, Chengdu, China.	30762444	10.1089/dna.2018.4493	The shortage of human hepatocytes continues to be a significant limitation for the widespread application of hepatocyte transplantation and bioartificial liver (BAL) support therapy. Recombinant activation gene 2 (Rag2) and fumarylacetoacetate hydrolase (Fah)-deficient mice could be highly repopulated with human hepatocytes. However, Fah/Rag2-deficient mice can only produce up to 1 x 10(8) human hepatocytes per mouse. We hypothesized that 2-10 x 10(10) human hepatocytes can be produced per Fah/Rag2-deficient pig, which is an adequate supply for hepatocyte transplantation and BAL therapy. In a novel approach, we used stably transfected Cas9 cells and single-guide RNA adenoviruses containing fluorescent reporters to enrich porcine cells with Fah/Rag2 dual gene mutations. This resulted in the construction of Fah/Rag2 double knockout porcine iliac artery endothelial cells, which were subsequently used for generating Fah/Rag2-deficient pigs.	pig

108	animal	pig	CRISPR;Cas9;	hairless	Experimental animals	Hairless-knockout piglets generated using the clustered regularly interspaced short palindromic repeat/CRISPR-associated-9 exhibit abnormalities in the skin and thymus.	2019	68(4):519-529	[Gao QS et al.]	Yanbian University, Yanji, Jilin, China.	31308290	10.1538/expanim.19-0018	The nuclear receptor corepressor Hairless (HR) interacts with nuclear receptors and controls expression of specific target genes involved in hair morphogenesis and hair follicle cycling. Patients with HR gene mutations exhibit atrichia, and in rare cases, immunodeficiency. Pigs with HR gene mutations may provide a useful model for developing therapeutic strategies because pigs are highly similar to humans in terms of anatomy, genetics, and physiology. The present study aimed to knockout the HR gene in pigs using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated-9 (Cas9) system and to investigate the molecular and structural alterations in the skin and thymus. We introduced a biallelic mutation into the HR gene in porcine fetal fibroblasts and generated nine piglets via somatic cell nuclear transfer. These piglets exhibited a lack of hair on the eyelids, abnormalities in the thymus and peripheral blood, and altered expression of several signaling factors regulated by HR. Our results indicate that introduction of the biallelic mutation successfully knocked out the HR gene, resulting in several molecular and structural changes in the skin and thymus. These pigs will provide a useful model for studying human hair disorders associated with HR gene mutations and the underlying molecular	pig
109	animal	pig	Cas9;	IGF2	Frontiers in genetics	sgRNA-shRNA Structure Mediated SNP Site Editing on Porcine IGF2 Gene by CRISPR/StCas9.	2019	10:347	[Sun Y et al.]	Northwest A&F University, Yangling, China.	31057603	10.3389/fgene.2019.00347	The SNP within intron 3 of the porcine IGF2 gene (G3072A) plays an important role for muscle growth and fat deposition in pigs. In this study, the StCas9 derived from Streptococcus thermophilus together with the Drosha-mediated sgRNA-shRNA structure were combined to boost the G to A base editing on the IGF2 SNP site, which we called "SNP editing." The codon-humanized StCas9 as we previously reported was firstly compared with the prevalently used SpCas9 derived from Streptococcus pyogenes using our idiomatic surrogate report assay, and the StCas9 demonstrated a comparable targeting activity. On the other hand, by combining shRNA with sgRNA, simultaneous gene silencing and genome targeting can be achieved. Thus, the novel IGF2.sgRNA-LIG4.shRNA-IGF2.sgRNA structure was constructed to enhance the sgRNA/Cas9-mediated HDR-based IGF2 SNP editing by silencing the LIG4 gene, which is a key molecule of the HDR's competitive NHEJ pathway. The sgRNA-shRNA/StCas9 all-in-one expression vector and the IGF2.sgRNA/StCas9 as control were separately used to transfect porcine PK15 cells together with an ssODNs donor for the IGF2 SNP editing. The editing events were detected by the RFLP assay, Sanger sequencing as well as Deep-sequencing, and the Deep-sequencing results finally demonstrated a significant higher HDR-based editing efficiency (16.38%) for our sgRNA-shRNA/StCas9 strategy. In short, we achieved effective IGF2 SNP editing by using the combined sgRNA-shRNA/StCas9 strategy, which will facilitate the further production of base-edited animals and perhaps extend for the gene therapy for the base correction of	pig
110	animal	pig	CRISPR;Cas9;	integrin beta5 subunit (ITGB5)	Frontiers in immunology	ITGB5 Plays a Key Role in Escherichia coli F4ac-Induced Diarrhea in Piglets.	2019	10:2834	[Wang W et al.]	Shandong Agricultural University, Tai'an, China.	31921118	10.3389/fimmu.2019.02834	Enterotoxigenic Escherichia coli (ETEC) that expresses F4ac fimbriae is the major pathogenic microorganism responsible for bacterial diarrhea in neonatal piglets. The susceptibility of piglets to ETEC F4ac is determined by a specific receptor on the small intestinal epithelium surface. We performed an iTRAQ-labeled quantitative proteome analysis using a case-control design in which susceptible and resistant full-sib piglets were compared for the protein expression levels. Two thousand two hundred forty-nine proteins were identified, of which 245 were differentially expressed (fold change > 1.5, FDR-adjusted $P < 0.05$). The differentially expressed proteins fell into four functional classes: (I) cellular adhesion and binding, (II) metabolic process, (III) apoptosis and proliferation, and (IV) immune response. The integrin signaling pathway merited particular interest based on a pathway analysis using statistical overexpression and enrichment tests. Genomic locations of the integrin family genes were determined based on the most recent porcine genome sequence assembly (Sscrofa11.1). Only one gene, ITGB5, which encodes the integrin beta5 subunit that assorts with the alphav subunit to generate integrin alphavbeta5, was located within the SSC13q41 region between 13:133161078 and 13:139609422, where strong associations of markers with the ETEC F4ac susceptibility were found in our previous GWAS results. To identify whether integrin alphavbeta5 is the ETEC F4acR, we established an experimental model for bacterial adhesion using IPEC-J2 cells. Then, the ITGB5 gene was knocked out in IPEC-J2 cell lines using CRISPR/Cas9, resulting in a biallelic deletion cell line (ITGB5 (-/-)). Disruption of ITGB5 significantly reduced ETEC F4ac adhesion to porcine intestinal epithelial cells. In contrast, overexpression of ITGB5 significantly enhanced the adhesion. A GST pull-down assay with purified FaeG and ITGB5 also showed that FaeG binds directly to ITGB5. Together, the results suggested that ITGB5	pig
111	animal	pig	CRISPR;Cas9;	cep112	G3	CRISPR/Cas9-Mediated Integration of Large Transgene into Pig CEP112 Locus.	2019		[Li G et al.]	South China Agricultural University, China.	31818875	10.1534/g3.119.400810	Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) is a precise genome manipulating tool that can produce targeted gene mutations in various cells and organisms. Although CRISPR/Cas9 can efficiently generate gene knockout, the gene knock-in (KI) efficiency mediated by homology-directed repair remains low, especially for large fragment integration. In this study, we established an efficient method for the CRISPR/Cas9-mediated integration of large transgene cassette, which carries salivary gland-expressed multiple digestion enzymes (approximately 20 kbp) in CEP112 locus in pig fetal fibroblasts (PFFs). Our results showed that using an optimal homology donor with a short and a long arm yielded the best CRISPR/Cas9-mediated KI efficiency in CEP112 locus, and the targeting efficiency in CEP112 locus was higher than in ROSA26 locus. The CEP112 KI cell lines were used as nuclear donors for somatic cell nuclear transfer to create genetically modified pigs. We found that KI pig (705) successfully expressed three microbial enzymes (beta-glucanase, xylanase, and phytase) in salivary gland. This finding suggested that the CEP112 locus supports exogenous gene expression by a tissue-specific promoter. In summary, we successfully targeted CEP112 locus in pigs by using our optimal homology arm system and established a modified pig model for foreign	pig

112	animal	pig	CRISPR;Cas9;	Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	Genes	Identification of Glyceraldehyde-3-Phosphate Dehydrogenase Gene as an Alternative Safe Harbor Locus in Pig Genome.	2019	10(9)	[Han X et al.]	Breeding and Reproduction (Huazhong Agricultural University), Ministry of Education, Wuhan, China.	31470649	10.3390/genes10090660	The ectopic overexpression of foreign genes in animal genomes is an important strategy for gain-of-function study and establishment of transgenic animal models. Previous studies showed that two loci (Rosa26 and pH11) were identified as safe harbor locus in pig genomes, which means foreign genes can be integrated into this locus for stable expression. Moreover, integration of a transgene may interfere with the endogenous gene expression of the target locus after the foreign fragments are inserted. Here, we provide a new strategy for efficient transgene knock-in in the endogenous GAPDH gene via CRISPR/Cas9 mediated homologous recombination. This strategy has no influence on the expression of the endogenous GAPDH gene. Thus, the GAPDH locus is a new alternative safe harbor locus in the pig genome for foreign gene knock-ins. This strategy is promising for agricultural breeding and biomedical model	pig
113	animal	pig	CRISPR;Cas9;	cystic fibrosis transmembrane conductance regulator	Human gene therapy	In Vitro Validation of a CRISPR-Mediated CFTR Correction Strategy for Preclinical Translation in Pigs.	2019	30(9):1101-1116	[Zhou ZP et al.]	University of Toronto, Toronto, Canada.	31099266	10.1089/hum.2019.074	Early efforts in cystic fibrosis (CF) gene therapy faced major challenges in delivery efficiency and sustained therapeutic gene expression. Recent advancements in engineered site-specific endonucleases such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 make permanent CF transmembrane conductance regulator (CFTR) gene correction possible. However, because of safety concerns of the CRISPR/Cas9 system and challenges in in vivo delivery to inflamed CF airway, CRISPR-based gene correction strategies need to be tested in proper animal models. In this study, we aimed at creating vectors for testing CFTR gene correction in pig models. We constructed helper-dependent adenoviral (HD-Ad) vectors to deliver CRISPR/Cas9 and a donor template (a 6 kb LacZ or 8.7 kb human CFTR expression cassette) into cultured pig cells. We demonstrated precise integration of each donor into the GGTA1 safe harbor through Cas9-induced homology directed repair with 3 kb homology arms. In addition, we showed that both LacZ and hCFTR were persistently expressed in transduced cells. Furthermore, we created a CFTR-deficient cell line for testing CFTR correction. We detected hCFTR mRNA and protein expression in cells transduced with the hCFTR vector. We also demonstrated CFTR function in the CF cells transduced with the HD-Ad delivering the CRISPR-Cas9 system and hCFTR donor at late cellular passages using the membrane potential sensitive dye-based assay (FLIPR((R))). Combined with our previous report on gene delivery to pig airway basal cells, these data provide the feasibility of testing CRISPR/Cas9-mediated	pig
114	animal	pig	CRISPR;Cas9;	MSTN; FGF10	In vitro cellular & developmental biology. Animal	Genome mutation after introduction of the gene editing by electroporation of Cas9 protein (GEEP) system in matured oocytes and putative zygotes.	2019	55(4):237-242	[Hirata M et al.]	Tokushima University, Tokushima, Japan.	30820813	10.1007/s11626-019-00338-3	The application of CRISPR/Cas9 strategy promises to rapidly increase the production of genetically engineered animals since it yields stably integrated transgenes. In the present study, we investigated the efficiency of target mutations after electroporation with the CRISPR/Cas9 system using sgRNAs to target the MSTN or FGF10 genes in porcine-matured oocytes and putative zygotes. Effects of pulse number (3-7 pulse repetitions) during electroporation on the embryonic development and mutation efficiency were also investigated. Our results showed that the cleavage rate of matured oocytes with electroporation treatment significantly decreased as compared with electroporated putative zygotes ($p < 0.05$). Moreover, the rates of blastocyst formation from oocytes/zygotes electroporated with more than 5 pulses decreased. Mutation efficiency was then assessed after sequencing the target sites in individual blastocysts derived from oocytes/zygotes electroporated by 3 and 5 pulses. No bi-allelic mutations in all examined blastocysts were observed in this study. There were no differences in the mutation rates (50-60%) between blastocysts derived from matured oocytes electroporated by 3 and 5 pulses, irrespective of targeting gene. In the targeting MSTN gene, however, the mutation rate (12.5%) of blastocysts derived from putative zygotes electroporated by 3 pulses tended to be lower than that (60%) from 5-pulsed electroporated putative zygotes. These data indicate that the type of eggs may influence not only their development after electroporation treatment but also the	pig
115	animal	pig	CRISPR;Cas9;	CD163	International journal of biological sciences	Deletion of CD163 Exon 7 Confers Resistance to Highly Pathogenic Porcine Reproductive and Respiratory Viruses on Pigs.	2019	15(9):1993-2005	[Wang H et al.]	China Agricultural University, Beijing, China.	31523199	10.7150/ijbs.34269	Porcine reproductive and respiratory syndrome (PRRS) caused by PRRS virus (PRRSV) is a severe infectious disease in the swine industry. PRRSV infection is mediated by porcine CD163 (pCD163). Scavenger receptor cysteine-rich domain 5 coded by exon 7 of pCD163 is essential for PRRSV infection. In this study, we generated CD163 exon 7 deleted (CD163E7D) pigs using CRISPR/Cas9 mediated homologous recombination and somatic cell nuclear transfer (SCNT). The deletion of exon 7 had no adverse effects on CD163-associated functions. Pigs were further challenged with a highly pathogenic PRRSV (HP-PRRSV) strain. The CD163E7D pigs exhibited mild clinical symptoms and had decreased viral loads in blood. All CD163E7D pigs survived the viral challenge, while all the WT pigs displayed severe symptoms, and 2 out of 6 WT pigs died during the challenge. Our results demonstrated that CD163 exon 7 deletion confers resistance to HP-PRRSV infection without impairing the biological functions of CD163.	pig
116	animal	pig	CRISPR;Cas9;	CD163	International journal of biological sciences	Generation of Pigs Resistant to Highly Pathogenic-Porcine Reproductive and Respiratory Syndrome Virus through Gene Editing of CD163.	2019	15(2):481-492	[Chen J et al.]	China Agricultural University, Beijing, China.	30745836	10.7150/ijbs.25862	Porcine reproductive and respiratory syndrome (PRRS) is a highly contagious disease and the most economically important disease of the swine industry worldwide. Highly pathogenic-PRRS virus (HP-PRRSV) is a variant of PRRSV, which caused high morbidity and mortality. Scavenger receptor CD163, which contains nine scavenger receptor cysteine-rich (SRCR) domains, is a key entry mediator for PRRSV. A previous study demonstrated that SRCR domain 5 (SRCR5), encoded by exon 7, was essential for PRRSV infection in vitro. Here, we substituted exon 7 of porcine CD163 with the corresponding exon of human CD163-like 1 (hCD163L1) using a CRISPR/Cas9 system combined with a donor vector. In CD163(Mut/Mut) pigs, modifying CD163 gene had no adverse effects on hemoglobin-haptoglobin (Hb-Hp) complex clearance or erythroblast growth. In vitro infection experiments showed that the CD163 mutant strongly inhibited HP-PRRSV replication by inhibiting virus uncoating and genome release. Compared to wild-type (WT) pigs in vivo, HP-PRRSV-infected CD163(Mut/Mut) pigs showed a substantially decreased viral load in blood and relief from PRRSV-induced fever. While all WT pigs were dead, there of four CD163(Mut/Mut) pigs survived and recovered at the termination of the experiment. Our data demonstrated that modifying CD163 remarkably inhibited PRRSV replication and protected pigs from HP-PRRSV infection, thus establishing a good foundation for breeding PRRSV-resistant pigs via gene editing	pig

117	animal	pig	CRISPR;Cas9;	triphosphohydrolase (SAMHD1)	Journal of general virology	Human SAMHD1 restricts the xenotransplantation relevant porcine endogenous retrovirus (PERV) in non-dividing cells.	2019	100(4):656-661	[Al-Shehabi H et al.]	Robert Koch Institute, Berlin, Germany.	30767852	10.1099/jgv.0.001232	The release of porcine endogenous retrovirus (PERV) particles from pig cells is a potential risk factor during xenotransplantation by way of productively infecting the human transplant recipient. Potential countermeasures against PERV replication are restriction factors that block retroviral replication. SAMHD1 is a triphosphohydrolase that depletes the cellular pool of dNTPs in non-cycling cells starving retroviral reverse transcription. We investigated the antiviral activity of human SAMHD1 against PERV and found that SAMHD1 potently restricts its reverse transcription in human monocytes, monocyte-derived dendritic cells (MDDC), or macrophages (MDM) and in monocytic THP-1 cells. Degradation of SAMHD1 by SIVmac Vpx or CRISPR/Cas9 knock-out of SAMHD1 allowed for PERV reverse transcription. Addition of deoxynucleosides alleviated the SAMHD1-mediated restriction suggesting that SAMHD1-mediated degradation of dNTPs restricts PERV replication in these human immune cells. In conclusion, our findings highlight SAMHD1 as a potential barrier to	pig
118	animal	pig	Cas9;	X-linked Dmd	Journal of genetics and genomics = Yi chuan xue bao	Production of non-mosaic genome edited porcine embryos by injection of CRISPR/Cas9 into germinal vesicle oocytes.	2019	46(7):335-342	[Su X et al.]	Sun Yat-sen University, Guangzhou, China.	31378649	10.1016/j.jgg.2019.07.002	Genetically modified pigs represent a great promise for generating models of human diseases and producing new breeds. Generation of genetically edited pigs using somatic cell nuclear transfer (SCNT) or zygote cytoplasmic microinjection is a tedious process due to the low developmental rate or mosaicism of the founder (F0). Herein, we developed a method termed germinal vesicle oocyte gene editing (GVGE) to produce non-mosaic porcine embryos by editing maternal alleles during the GV to M transition. Injection of Cas9 mRNA and X-linked Dmd gene-specific gRNA into GV oocytes did not affect their developmental potential. The M oocytes edited during in vitro maturation (IVM) could develop into blastocysts after parthenogenetic activation (PA) or in vitro fertilization (IVF). Genotyping results indicated that the maternal gene X-linked Dmd could be efficiently edited during oocyte maturation. Up to 81.3% of the edited IVF embryos were non-mosaic Dmd gene mutant embryos. In conclusion, GVGE might be a valuable method for the generation of non-mosaic maternal allele edited F0	pig
119	animal	pig	CRISPR;Cas9;	alpha-1,3-galactosyltranferase	Journal of reproduction and development	Effects of concentration of CRISPR/Cas9 components on genetic mosaicism in cytoplasmic microinjected porcine embryos.	2019	65(3):209-214	[Tanihara F et al.]	Tokushima University, Tokushima, Japan.	30726783	10.1262/jrd.2018-116	Cytoplasmic microinjection (CI) of the CRISPR/Cas9 system enabled the induction of site-specific mutations in porcine zygotes and resulting pigs. However, mosaicism is a serious problem for genetically modified pigs. In the present study, we investigated suitable timing and concentration of CRISPR/Cas9 components for introduction into oocytes/zygotes by CI, to reduce mosaicism in the resulting blastocysts. First, we introduced 20 ng/mul of Cas9 protein and guide RNA (gRNA), targeting the alpha-1,3-galactosyltransferase (GalT) gene in oocytes before in vitro fertilization (IVF), in zygotes after IVF, or in oocytes/zygotes before and after IVF, twice. CI treatment had no detrimental effects on blastocyst formation rates. The highest value of the rate of mutant blastocysts was observed in zygotes injected after IVF. Next, we injected Cas9 protein and gRNA into zygotes after IVF at a concentration of 20 ng/mul each (20 ng/mul group) or 100 ng/mul each (100 ng/mul group). The ratio of the number of blastocysts that carried mutations to the total number of blastocysts examined in the 100 ng/mul group was significantly higher ($P < 0.05$) than that in the 20 ng/mul group. Although no blastocysts from the 20 ng/mul group carried a biallelic mutation, 16.7% of blastocysts from the 100 ng/mul group carried a biallelic mutation. In conclusion, increasing the concentration of Cas9 protein and gRNA is effective in generating biallelic mutant blastocysts. To reduce mosaicism, however, further optimization of the timing of CI, and the concentration of CRISPR/Cas9 components, is needed.	pig
120	animal	pig	CRISPR;Cas9;		Journal of veterinary science	The length of guide RNA and target DNA heteroduplex effects on CRISPR/Cas9 mediated genome editing efficiency in porcine cells.	2019	20(3):e23	[Lv J et al.]	Northeast Agricultural University, Harbin, China.	31161741	10.4142/jvs.2019.20.e23	The clustered regularly interspaced short palindrome repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a versatile genome editing tool with high efficiency. A guide sequence of 20 nucleotides (nt) is commonly used in application of CRISPR/Cas9; however, the relationship between the length of the guide sequence and the efficiency of CRISPR/Cas9 in porcine cells is still not clear. To illustrate this issue, guide RNAs of different lengths targeting the EGFP gene were designed. Specifically, guide RNAs of 17 nt or longer were sufficient to direct the Cas9 protein to cleave target DNA sequences, while 15 nt or shorter guide RNAs had loss-of-function. Full-length guide RNAs complemented with mismatches also showed loss-of-function. When the shortened guide RNA and target DNA heteroduplex (gRNA:DNA heteroduplex) was blocked by mismatch, the CRISPR/Cas9 would be interfered with. These results suggested the length of the gRNA:DNA heteroduplex was a key factor for maintaining high efficiency of the CRISPR/Cas9 system rather than weak bonding between	pig
121	animal	pig	CRISPR;Cas9;	RING finger protein 114	Journal of virology	Porcine RING Finger Protein 114 Inhibits Classical Swine Fever Virus Replication via K27-Linked Polyubiquitination of Viral NS4B.	2019	93(21)	[Zhang Y et al.]	Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China.	31413123	10.1128/JVI.01248-19	In the host, many RING domain E3 ligases have been reported to inhibit viral replication through various mechanisms. In a previous screen, we found that porcine RING finger protein 114 (pRNF114), a RING domain E3 ubiquitin ligase, inhibits classical swine fever virus (CSFV) replication. This study aimed to clarify the underlying antiviral mechanism of pRNF114 against CSFV. Upon CSFV infection, pRNF114 mRNA was upregulated both in vitro and in vivo CSFV replication was significantly suppressed in PK-pRNF114 cells stably expressing pRNF114 by the lentivirus-delivered system, whereas CSFV growth was enhanced in PK-15 cells with RNF114 knockout by the CRISPR/Cas9 system. The RING domain of pRNF114, which has E3 ubiquitin ligase activity, is crucial for its antiviral activity. Mechanistically, pRNF114 interacted with the CSFV NS4B protein through their C-terminal domains, which led to the K27-linked polyubiquitination and degradation of NS4B through a proteasome-dependent pathway. Collectively, these findings indicate that pRNF114 as a critical regulator of CSFV replication and uncover a mechanism by which pRNF114 employs its E3 ubiquitin ligase activity to inhibit CSFV replication.IMPORTANCE Porcine RING finger protein 114 (pRNF114) is a member of the RING domain E3 ligases. In this study, it was shown that pRNF114 is a potential anti-CSFV factor and the anti-CSFV effect of pRNF114 depends on its E3 ligase activity. Notably, pRNF114 targets and catalyzes the K27-linked polyubiquitination of the NS4B protein and then promotes proteasome-dependent degradation of NS4B, inhibiting the replication of CSFV. To our knowledge, pRNF114 is the first E3 ligase to be identified as being involved in anti-CSFV activity.	pig

122	animal	pig	Cas9;	Melanocortin 3 receptor (MC3R)	Lipids in health and disease	Generation of an MC3R knock-out pig by CRSPR/Cas9 combined with somatic cell nuclear transfer (SCNT) technology.	2019	18(1):122	[Yin Y et al.]	Jiaxing University, Jiaxing, China.	31138220	10.1186/s12944-019-1073-9	BACKGROUND: Melanocortin 3 receptor (MC3R), a rhodopsin-like G protein-coupled receptor, is an important regulator of metabolism. Although MC3R knock-out (KO) mice and rats were generated in earlier studies, the function of MC3R remains elusive. Since pig models have many advantages over rodents in metabolism research, we generated an MC3R-KO pig using a CRSPR/Cas9-based system combined with somatic cell nuclear transfer (SCNT) technology. METHOD: Four CRSPR/Cas9 target vectors were constructed and then their cleavage efficiency was tested in porcine fetal fibroblasts (PFFs). The pX330-sgRNA1 and pX330-sgRNA4 vectors were used to co-transfect PFFs to obtain positive colonies. PCR screening and sequencing were conducted to identify the genotype of the colonies. The biallelically modified colonies and wild-type control colonies were used simultaneously as donor cells for SCNT. A total of 1203 reconstructed embryos were transferred into 6 surrogates, of which one became pregnant. The genotypes of the resulting piglets were determined by PCR and sequencing, and off-target effects in the MC3R KO piglets were detected by sequencing. Then, offspring were obtained through breeding and six male KO pigs were used for the growth performance analysis. RESULTS: Four vectors were constructed successfully, and their cleavage efficiencies were 27.96, 44.89, 32.72 and 38.86%, respectively. A total of 21 mutant colonies, including 11 MC3R(-/-) and 10 MC3R(+/-) clones, were obtained, corresponding to a gene targeting efficiency of 29.17%, with 15.28% biallelic mutations. A total of 6 piglets were born, and only two MC3R KO piglets were generated, one with malformations and a healthy one. No off-target effects were detected by sequencing in the healthy mutant. Six male MC3R KO pigs were obtained in the F2 generation and their body weight and body fat were both increased compared to wild-type full siblings. CONCLUSION: A MC3R KO pig strain was generated using the CRSIPR/Cas9-based system, which makes it possible to study the biological function	pig
123	animal	pig	CRISPR;Cas9;	H11	Molecular biotechnology	Improved Delivery of CRISPR/Cas9 System Using Magnetic Nanoparticles into Porcine Fibroblast.	2019	61(3):173-180	[Hryhorowicz M et al.]	Poznan University of Life Sciences, Poznan, Poland.	30560399	10.1007/s12033-018-0145-9	Genetically modified pigs play an important role in agriculture and biomedical research; hence, new efficient methods are needed to obtain genetically engineered cells and animals. The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas (CRISPR-associated) system represents an effective genome editing tool. It consists of two key molecules: single guide RNA (sgRNA) and the Cas9 endonuclease that can be introduced into the cells as one plasmid. Typical delivery methods for CRISPR/Cas9 components are limited by low transfection efficiency or toxic effects on cells. Here, we describe the use of magnetic nanoparticles and gradient magnetic field to improve delivery of CRISPR/Cas9 constructs into porcine fetal fibroblasts. Polyethylenimine-coated nanoparticles with magnetic iron oxide core were used to form magnetic plasmid DNA lipoplexes. CRISPR/Cas9 construct was prepared to induce site-specific cutting at the porcine H11 locus. Quantitative assessment of genomic cleavage by sequence trace decomposition demonstrated that the magnetofection efficiency was more than 3.5 times higher compared to the classic lipofection method. The Tracking of Indels by Decomposition web tool precisely determined the spectrum of indels that occurred. Simultaneously, no additional cytotoxicity associated with the utilization of magnetic nanoparticles was observed. Our results indicate that magnetofection enables effective delivery of the CRISPR/Cas9 construct into porcine fetal fibroblasts with low	pig
124	animal	pig	CRISPR;Cas9;	CMP-N-glycolylneuraminic acid hydroxylase	PloS one	Lessening of porcine epidemic diarrhoea virus susceptibility in piglets after editing of the CMP-N-glycolylneuraminic acid hydroxylase gene with CRISPR/Cas9 to nullify N-glycolylneuraminic acid expression.	2019	14(5):e0217236	[Tu CF et al.]	Agricultural Technology Research Institute, Xiangshan, Hsinchu, Taiwan	31141512	10.1371/journal.pone.0217236	The porcine epidemic diarrhoea virus (PEDV) devastates the health of piglets but may not infect piglets whose CMP-N-glycolylneuraminic acid hydroxylase (CMAH) gene is mutated (knockouts, KO) by using CRISPR/Cas9 gene editing techniques. This hypothesis was tested by using KO piglets that were challenged with PEDV. Two single-guide RNAs targeting the CMAH gene and Cas9 mRNA were microinjected into the cytoplasm of newly fertilized eggs. Four live founders generated and proven to be biallelic KO, lacking detectable N-glycolylneuraminic acid (NGNA). The founders were bred, and homozygous offspring were obtained. Two-day-old (in exps. I, n = 6, and III, n = 15) and 3-day-old (in exp. II, n = 9) KO and wild-type (WT, same ages in respective exps.) piglets were inoculated with TCID50 1x103 PEDV and then fed 20 mL of infant formula (in exps. I and II) or sow's colostrum (in exp. III) every 4 hours. In exp. III, the colostrum was offered 6 times and was then replaced with Ringer/5% glucose solution. At 72 hours post-PEDV inoculation (hpi), the animals either deceased or euthanized were necropsied and intestines were sampled. In all 3 experiments, the piglets showed apparent outward clinical manifestations suggesting that infection occurred despite the CMAH KO. In exp. I, all 6 WT piglets and only 1 of 6 KO piglets died at 72 hpi. Histopathology and immunofluorescence staining showed that the villus epithelial cells of WT piglets were severely exfoliated, but only moderate exfoliation and enterocyte vacuolization was observed in KO piglets. In exp. II, delayed clinical symptoms appeared, yet the immunofluorescence staining/histopathologic inspection (I/H) scores of the two groups differed little. In exp. III, the animals exhibited clinical and pathological signs after inoculation similar to those in exp. II. These results suggest that porcine CMAH KO with nullified NGNA expression are not immune to PEDV but that this KO	pig
125	animal	pig	CRISPR;Cas9;	insulin receptor substrate 2	Polish journal of veterinary sciences	Porcine insulin receptor substrate 2: molecular cloning, tissues distribution, and functions in hepatocyte and aortic endothelial cells.	2019	22(3):589-598	[Yin Z et al.]	Northeast Agricultural University, Harbin, China.	31560477	10.24425/pjvs.2019.129968	Insulin receptor substrate 2 (IRS-2) modulates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which controls the suppression of gluconeogenic genes; IRS-2 is also a critical node of insulin signaling. Because of the high homology between pig and human IRS-2, we investigated the expression pattern and function of porcine IRS-2. QPCR and immunoblotting were used to detect the IRS-2 expression level in different tissues. There were high IRS-2 levels in the cerebral cortex, hypothalamus, and cerebellum in the central nervous system. In peripheral tissues, IRS-2 was expressed at relatively high levels in the liver. Immunohistochemistry analysis revealed that IRS-2 was mainly distributed in the hypothalamus and cerebral cortex. Furthermore, IRS-2 knockdown porcine hepatocytes and porcine aortic endothelial cells (PAECs) were generated. The IRS-2 knockdown induced abnormal expression of genes involved in glycolipid metabolism in hepatocytes and reduced the antiatherosclerosis ability in PAECs. In addition, we disrupted IRS-2 in porcine embryonic fibroblasts (PEFs) using the CRISPR/Cas9 genome editing system, before finally generating IRS-2 knockout embryos by somatic cell nuclear transfer (SCNT). Taken together, our results indicate that IRS-2 might be a valuable target to establish diabetes and vascular disease	pig

126	animal	pig	ZFN;	GGTA1	Polish journal of veterinary sciences	Production of ZFN-mediated GGTA1 knock-out pigs by microinjection of gene constructs into pronuclei of zygotes.	2019	22(1):91-100	[Lipinski D et al.]	Poznan University of Life Sciences, Poznan, Poland.	30997769	10.24425/pjvs.2018.125611	Animals as a source of organs and tissues for xenotransplantation could become a backup solution for the growing shortage of human donors. The presence of human xenoreactive anti- bodies directed against Galalpha1,3Gal antigens on the cell surface of a pig donor triggers the activa- tion of the complement leading to a hyperacute reaction. The development of genetic engineer- ing techniques has enabled the modification of genomes by knocking in and/or knocking out genes. In this paper, we report the generation of modified pigs with ZFN mediated disruption of the GGTA1 gene encoding the enzyme responsible for synthesis of Galalpha1,3Gal antigens. ZFN plasmids designed to target the exon 9 region of the pig GGTA1 gene encoding the catalytic domain were injected into the pronuclei of fertilized egg cells. Among 107 piglets of the F0 gene- ration analyzed, one female with 9-nt deletion in exon 9 of the GGTA1 gene was found. 13 of 33 piglets of the F1 generation represented the +/- GGTA1 genotype and 2 of 13 F2 piglets repre- sented the -/- GGTA1 genotype. No changes in the animals' behavior, phenotype or karyotype were observed. Analysis confirmed heredity of the trait in all animals. A complex functional analysis of the modified animals, including flow cytometry, human serum cytotoxicity test and immunohistochemical detection, was performed to estimate the phenotype effect of	pig
127	animal	pig	CRISPR;Cas9;	aminopeptidase-N	Scientific reports	Aminopeptidase N-null neonatal piglets are protected from transmissible gastroenteritis virus but not porcine epidemic diarrhea virus.	2019	9(1):13186	[Luo L et al.]	Zhejiang University, Hangzhou, Zhejiang, China.	31515498	10.1038/s41598-019-49838-y	Swine enteric diseases have caused significant economic loss and have been considered as the major threat to the global swine industry. Several coronaviruses, including transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV), have been identified as the causative agents of these diseases. Effective measures to control these diseases are lacking. The major host cells of transmissible gastroenteritis virus and porcine epidemic diarrhea virus have thought to be epithelial cells on small intestine villi. Aminopeptidase-N (APN) has been described as the putative receptor for entry of transmissible gastroenteritis virus and porcine epidemic diarrhea virus into cells in vitro. Recently, Whitworth et al. have reported that APN knockout pigs are resistant to TGEV but not PEDV after weaning. However, it remains unclear if APN-null neonatal pigs are protected from TGEV. Here we report the generation of APN-null pigs by using CRISPR/Cas9 technology followed by somatic cell nuclear transfer. APN-null pigs are produced with normal pregnancy rate and viability, indicating lack of APN is not embryonic lethal. After viral challenge, APN-null neonatal piglets are resistant to highly virulent transmissible gastroenteritis virus. Histopathological analyses indicate APN-null pigs exhibit normal small intestine villi, while wildtype pigs show typical lesions in small intestines. Immunochemistry analyses confirm that no transmissible gastroenteritis virus antigen is detected in target tissues in APN-null piglets. However, upon porcine epidemic diarrhea virus challenge, APN-null pigs are still susceptible with 100% mortality. Collectively, this report provides a viable tool for producing animals with enhanced resistance to TGEV and clarifies that	pig
128	animal	pig	CRISPR;Cas9;	trex2	The Journal of reproduction and development	Suppression of mosaic mutation by co-delivery of CRISPR associated protein 9 and three-prime repair exonuclease 2 into porcine zygotes via electroporation.	2019		[Yamashita S et al.]	Central Research Institute for Feed and Livestock of Zen-noh, Ibaraki, Japan.	31761839	10.1262/jrd.2019-088	Gene-modified animals, including pigs, can be generated efficiently by introducing CRISPR associated protein 9 (CRISPR/Cas9) into zygotes. However, in many cases, these zygotes tend to become mosaic mutants with various different mutant cell types, making it difficult to analyze the phenotype of gene-modified founder animals. To reduce the mosaic mutations, we introduced three-prime repair exonuclease 2 (Trex2), an exonuclease that improves gene editing efficiency, into porcine zygotes along with CRISPR/Cas9 via electroporation. Although the rate of porcine blastocyst formation decreased due to electroporation (25.9 +/- 4.6% vs. 41.2 +/- 2.0%), co-delivery of murine Trex2 (mTrex2) mRNA with CRISPR/Cas9 did not affect it any further (25.9 +/- 4.6% vs. 31.0 +/- 4.6%). In addition, there was no significant difference in the diameter of blastocysts carrying CRISPR/Cas9 (164.7 +/- 10.2 mum), and those with CRISPR/Cas9 + mTrex2 (151.9 +/- 5.1 mum) as compared to those from the control group (178.9 +/- 9.0 mum). These results revealed that mTrex2 did not affect the development of pre-implantation embryo. We also found bi-allelic, as well as mono-allelic, non-mosaic homozygous mutations in the blastocysts. Most importantly, co-delivery of mTrex2 with CRISPR/Cas9 increased non-mosaic mutant blastocysts (29.3 +/- 4.5%) and reduced mosaic mutant blastocysts (70.7 +/- 4.5%) as compared to CRISPR/Cas9 alone (5.6 +/- 6.4% and92.6 +/- 8.6%, respectively). These data suggest that the co-delivery of CRISPR/Cas9 and mTrex2 is a useful method to suppress	pig
129	animal	pig	CRISPR;Cas9;	aminopeptidase N	Transgenic research	Resistance to coronavirus infection in amino peptidase N-deficient pigs.	2019	28(1):21-32	[Whitworth KM et al.]	University of Missouri, Columbia, MO, USA.	30315482	10.1007/s11248-018-0100-3	The alphacoronaviruses, transmissible gastroenteritis virus (TGEV) and Porcine epidemic diarrhea virus (PEDV) are sources of high morbidity and mortality in neonatal pigs, a consequence of dehydration caused by the infection and necrosis of enterocytes. The biological relevance of amino peptidase N (ANPEP) as a putative receptor for TGEV and PEDV in pigs was evaluated by using CRISPR/Cas9 to edit exon 2 of ANPEP resulting in a premature stop codon. Knockout pigs possessing the null ANPEP phenotype and age matched wild type pigs were challenged with either PEDV or TGEV. Fecal swabs were collected daily from each animal beginning 1 day prior to challenge with PEDV until the termination of the study. The presence of virus nucleic acid was determined by PCR. ANPEP null pigs did not support infection with TGEV, but retained susceptibility to infection with PEDV. Immunohistochemistry confirmed the presence of PEDV reactivity and absence of TGEV reactivity in the enterocytes lining the ileum in ANPEP null pigs. The different receptor requirements for TGEV and PEDV have important implications in the development of new genetic tools	pig

130	animal	pig	CRISPR;Cas9;	endogenous class I and class II HLA; B-cell receptor; Fc receptor genes	Transplantation	HLA Class I-sensitized Renal Transplant Patients Have Antibody Binding to SLA Class I Epitopes.	2019	103(8):1620-1629	[Martens GR et al.]	University of Alabama at Birmingham, Birmingham, AL, USA.	30951017	10.1097/TP.0000000000002739	BACKGROUND: Highly sensitized patients are difficult to match with suitable renal allograft donors and may benefit from xenotransplant trials. We evaluate antibody binding from sensitized patients to pig cells and engineered single allele cells to identify anti-human leukocyte antigen (HLA) antibody cross-species reactivity with swine leukocyte antigen (SLA). These novel testing strategies assess HLA/SLA epitopes and antibody-binding patterns and introduce genetic engineering of SLA epitopes. METHODS: Sensitized patient sera were grouped by calculated panel reactive antibody and luminex single antigen reactivity profile and were tested with cloned GGTA1/CMAH/B4GalNT2 glycan knockout porcine cells. Pig reactivity was assessed by direct flow cytometric crossmatch and studied following elution from pig cells. To study the antigenicity of individual class I HLA and SLA alleles in cells, irrelevant sera binding to lymphoblastoid cells were minimized by CRISPR/Cas9 elimination of endogenous class I and class II HLA, B-cell receptor, and Fc receptor genes. Native HLA, SLA, and mutants of these proteins after mutating 144K to Q were assessed for antibody binding. RESULTS: Those with predominately anti-HLA-B&C antibodies, including Bw6 and Bw4 sensitization, frequently have low pig reactivity. Conversely, antibodies eluted from porcine cells are more commonly anti-HLA-A. Single HLA/SLA expressing engineered cells shows variable antigenicity and mutation of 144K to Q reduces antibody binding for some sensitized patients. CONCLUSIONS: Anti-HLA antibodies cross-react with SLA class I in predictable patterns, which can be identified	pig
131	animal	pig	CRISPR;Cas9;	beta2 microglobulin	Xenotransplantation	Possible detrimental effects of beta-2-microglobulin knockout in pigs.	2019	26(6):e12525	[Sake HJ et al.]	Friedrich-Loeffler-Institut, Neustadt, Germany.	31119817	10.1111/xen.12525	BACKGROUND: Despite major improvements in pig-to-primate xenotransplantation, long-term survival of xenografts is still challenging. The major histocompatibility complex (MHC) class I, which is crucial in cellular immune response, is an important xenoantigen. Abrogating MHC class I expression on xenografts might be beneficial for extending graft survival beyond current limits. METHODS: In this study, we employed the CRISPR/Cas9 system to target exon 2 of the porcine beta-2-microglobulin (B2M) gene to abrogate SLA class I expression on porcine cells. B2M-KO cells served as donor cells for somatic cell nuclear transfer, and cloned embryos were transferred to three recipient sows. The offspring were genotyped for mutations at the B2M locus, and blood samples were analyzed via flow cytometry for the absence of SLA class I molecules. RESULTS: Pregnancies were successfully established and led to the birth of seven viable piglets. Genomic sequencing proved that all piglets carried biallelic modifications at the B2M locus leading to a frameshift, a premature stop codon, and ultimately a functional knockout. However, survival times of these animals did not exceed 4 weeks due to unexpected disease processes. CONCLUSION: Here, we demonstrate the feasibility of generating SLA class I knockout pigs by targeting the porcine beta-2-microglobulin gene using the CRISPR/Cas9 system. Additionally, our findings indicate for the first time that this genetic modification might have a negative impact on the viability of the animals. These issues need to be solved to unveil the real	pig
132	animal	pig	CRISPR;Cas9;	porcine endogenous retrovirus reverse transcriptase	Xenotransplantation	Characterization of porcine endogenous retrovirus particles released by the CRISPR/Cas9 inactivated cell line PK15 clone 15.	2019	e12563	[Godehardt AW et al.]	Paul-Ehrlich-Institut, Langen, Germany.	31667881	10.1111/xen.12563	The infection of human transplant recipients by porcine endogenous retrovirus (PERV) is a safety issue for xenotransplantation (XTx). CRISPR/Cas9 technology has enabled the generation of pigs free of functional PERVs, and the susceptibility of these animals to reinfection by PERVs remains unclear. To assess virological safety, we characterized a cell line in which PERVs have been inactivated by CRISPR/Cas9 (PK15 clone 15) for its susceptibility to infectious PERV. First, basal expression of PERV pol, the porcine PERV-A receptor (POPAR), and reverse transcriptase (RT) activity of PERV were determined. PK15 clone 15 cells were inoculated with PERV and monitored post infection for virus expression and RT activity. Particles were visualized by electron microscopy. Our data show that PK15 clone 15 cells still produce viral proteins that assemble to produce impaired viral particles. These virions have an irregular morphology that diverges from that of mature wild type. The particles are no longer infectious when tested in a downstream infection assay using supernatants of PK15 clone 15 cells to infect susceptible swine testis-IOWA (ST-IOWA) cells. The expression of POPAR was quantified to exclude the possibility that lack of susceptibility to reinfection, for PERV-A, is caused by absence of viral host receptor(s). PK15 and PK15 clone 15 cells do, in fact, express POPAR equally. PERV RT inactivation mediated by CRISPR/Cas9 does not compromise virus assembly but affects virion structure and proviral integration. The constitutive virion production seems to maintain cellular resistance to superinfection and possibly indicates a	pig
133	animal	pig	CRISPR;Cas9;	GGTA1; CMAH; B4GALNT2; SLA class I	Xenotransplantation	Viable pigs after simultaneous inactivation of porcine MHC class I and three xenoreactive antigen genes GGTA1, CMAH and B4GALNT2.	2019	e12560	[Fischer K et al.]	Technische Universitat Munchen, Freising, Germany.	31591751	10.1111/xen.12560	BACKGROUND: Cell surface carbohydrate antigens play a major role in the rejection of porcine xenografts. The most important for human recipients are alpha-1,3 Gal (Galactose-alpha-1,3-galactose) causing hyperacute rejection, also Neu5Gc (N-glycolylneuraminic acid) and Sd(a) blood group antigens both of which are likely to elicit acute vascular rejection given the known human immune status. Porcine cells with knockouts of the three genes responsible, GGTA1, CMAH and B4GALNT2, revealed minimal xenoreactive antibody binding after incubation with human serum. However, human leucocyte antigen (HLA) antibodies cross-reacted with swine leucocyte antigen class I (SLA-I). We previously demonstrated efficient generation of pigs with multiple xeno-transgenes placed at a single genomic locus. Here we wished to assess whether key xenoreactive antigen genes can be simultaneously inactivated and if combination with the multi-transgenic background further reduces antibody deposition and complement activation. METHODS: Multiplex CRISPR/Cas9 gene editing and somatic cell nuclear transfer were used to generate pigs carrying functional knockouts of GGTA1, CMAH, B4GALNT2 and SLA class I. Fibroblasts derived from one- to four-fold knockout animals, and from multi-transgenic cells (human CD46, CD55, CD59, HO1 and A20) with the four-fold knockout were used to examine the effects on human IgG and IgM binding or complement activation in vitro. RESULTS: Pigs were generated carrying four-fold knockouts of important xenoreactive genes. In vitro assays revealed that combination of all four gene knockouts reduced human IgG and IgM binding to porcine kidney cells more effectively than single or double knockouts. The multi-transgenic background combined with GGTA1 knockout alone reduced C3b/c and C4b/c complement activation to such an extent that further knockouts had no significant additional effect. CONCLUSION: We showed that pigs carrying several xenoprotective transgenes and knockouts of xenoreactive antigens can be readily generated and these	pig

134	animal	pig	CRISPR;Cas9;	SIX1; SIX4	Xenotransplantation	Disabling of nephrogenesis in porcine embryos via CRISPR/Cas9-mediated SIX1 and SIX4 gene targeting.	2019	26(3):e12484	[Wang J et al.]	Nanjing Medical University, Nanjing, China.	30623494	10.1111/xen.12484	SIX1 and SIX4 genes play critical roles in kidney development. We evaluated the effect of these genes on pig kidney development by generating SIX1(-/-) and SIX1(-/-)/SIX4(-/-) pig foetuses using CRISPR/Cas9 and somatic cell nuclear transfer. We obtained 3 SIX1(-/-) foetuses and 16 SIX1(-/-)/SIX4(-/-) foetuses at different developmental stages. The SIX1(-/-) foetuses showed a migration block of the left kidney and a smaller size for both kidneys. The ureteric bud failed to form the normal branching and collecting system. Abnormal expressions of kidney development-related genes (downregulation of PAX2, PAX8, and BMP4 and upregulation of EYA1 and SALL1) were also observed in SIX1(-/-) foetal kidneys and confirmed in vitro in porcine kidney epithelial cells (PK15) following SIX1 gene deletion. The SIX1(-/-)/SIX4(-/-) foetuses exhibited more severe phenotypes, with most foetuses showing retarded development at early stages of gestation. The kidney developed only to the initial stage of metanephros formation. These results demonstrated that SIX1 and SIX4 are key genes for porcine metanephros development. The creation of kidney-deficient porcine foetuses provides a platform for generating human kidneys inside pigs using	pig
135	animal	tibet minipig	Cas9;	tyr; il2rg; rag1	International journal of biological sciences	Optimization Strategy for Generating Gene-edited Tibet Minipigs by Synchronized Oestrus and Cytoplasmic Microinjection.	2019	15(12):2719-2732	[Chen B et al.]	Southern Medical University, Guangzhou, China.	31754342	10.7150/ijbs.35930	The Tibet minipig is a rare highland pig breed worldwide and has many applications in biomedical and agricultural research. However, Tibet minipigs are not like domesticated pigs in that their ovulation number is low, which is unfavourable for the collection of zygotes. Partly for this reason, few studies have reported the successful generation of genetically modified Tibet minipigs by zygote injection. To address this issue, we described an efficient way to generate gene-edited Tibet minipigs, the major elements of which include the utilization of synchronized oestrus instead of superovulation to obtain zygotes, optimization of the preparation strategy, and co-injection of clustered regularly interspaced short palindromic repeat sequences associated protein 9 (Cas9) mRNA and single-guide RNAs (sgRNAs) into the cytoplasm of zygotes. We successfully obtained allelic TYR gene knockout (TYR (-/-)) Tibet minipigs with a typical albino phenotype (i.e., red-coloured eyes with light pink-tinted irises and no pigmentation in the skin and hair) as well as TYR (-/-) IL2RG (-/-) and TYR (-/-) RAG1 (-/-) Tibet minipigs with typical phenotypes of albinism and immunodeficiency, which was characterized by thymic atrophy and abnormal immunocyte proportions. The overall gene editing efficiency was 75% for the TYR single gene knockout, while for TYR-IL2RG and TYR-RAG1 dual gene editing, the values were 25% and 75%, respectively. No detectable off-target mutations were observed. By intercrossing F0 generation minipigs, targeted genetic mutations can also be transmitted to gene-edited minipigs' offspring through germ line transmission. This study is a valuable exploration for the efficient generation of gene-edited Tibet minipigs with medical research value in the	pig
136	animal	yorkshire pig	CRISPR;Cas9	KIT	BMC molecular and cell biology	Highly efficient correction of structural mutations of 450 kb KIT locus in kidney cells of Yorkshire pig by CRISPR/Cas9.	2019	20(1):4	[Qin K et al.]	Sun Yat-sen University, Guangzhou, China.	31041890	10.1186/s12860-019-0184-5	The white coat colour of Yorkshire and Landrace pig breeds is caused by the dominant white I allele of KIT, associated with 450-kb duplications and a splice mutation (G > A) at the first base in intron 17. To test whether genome editing can be employed to correct this structural mutation, and to investigate the role of KIT in the control of porcine coat colour, we designed sgRNAs targeting either intron 16 or intron 17 of KIT, and transfected Cas9/sgRNA co-expression plasmids into the kidney cells of Yorkshire pigs. The copy number of KIT was reduced by about 13%, suggesting the possibility of obtaining cells with corrected structural mutations of the KIT locus. Using single cell cloning, from 24 successfully expanded single cell clones derived from cells transfected with sgRNA targeting at intron 17, we obtained 3 clones with a single copy of KIT without the splice mutation. Taken together, the 12.5% (3/24) efficiency of correction of structural mutations of 450 kb fragments is highly efficient, providing a solid basis for the generation of genome edited Yorkshire pigs with a normal KIT locus. This provides an insight into the underlying genetic mechanisms of porcine coat colour.	pig
137	animal	Yucatan minipig	CRISPR;Cas9;	LMNA	Cell discovery	Generation and characterization of a novel knockin minipig model of Hutchinson-Gilford progeria syndrome.	2019	5:16	[Dorado B et al.]	Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain.	30911407	10.1038/s41421-019-0084-z	Hutchinson-Gilford progeria syndrome (HGPS) is an extremely rare genetic disorder for which no cure exists. The disease is characterized by premature aging and inevitable death in adolescence due to cardiovascular complications. Most HGPS patients carry a heterozygous de novo LMNA c.1824C > T mutation, which provokes the expression of a dominant-negative mutant protein called progerin. Therapies proven effective in HGPS-like mouse models have yielded only modest benefit in HGPS clinical trials. To overcome the gap between HGPS mouse models and patients, we have generated by CRISPR-Cas9 gene editing the first large animal model for HGPS, a knockin heterozygous LMNA c.1824C > T Yucatan minipig. Like HGPS patients, HGPS minipigs endogenously co-express progerin and normal lamin A/C, and exhibit severe growth retardation, lipodystrophy, skin and bone alterations, cardiovascular disease, and die around puberty. Remarkably, the HGPS minipigs recapitulate critical cardiovascular alterations seen in patients, such as left ventricular diastolic dysfunction, altered cardiac electrical activity, and loss of vascular smooth muscle cells. Our analysis also revealed reduced myocardial perfusion due to microvascular damage and myocardial interstitial fibrosis, previously undescribed readouts potentially useful for monitoring disease progression in patients. The HGPS minipigs provide an appropriate preclinical model in which to test human-size interventional devices and optimize candidate therapies before advancing to clinical trials, thus accelerating the development of	pig

138	animal	sheep	CRISPR;Cas9;		BMC genomics	Low incidence of SNVs and indels in trio genomes of Cas9-mediated multiplex edited sheep.	2018	19(1):397	[Wang X et al.]	College of Animal Science and Technology, Yangling, China.	29801435	10.1186/s12864-018-4712-z	BACKGROUND: The simplicity of the CRISPR/Cas9 system has enabled its widespread applications in generating animal models, functional genomic screening and in treating genetic and infectious diseases. However, unintended mutations produced by off-target CRISPR/Cas9 nuclease activity may lead to negative consequences. Especially, a very recent study found that gene editing can introduce hundreds of unintended mutations into the genome, and have attracted wide attention. RESULTS: To address the off-target concerns, urgent characterization of the CRISPR/Cas9-mediated off-target mutagenesis is highly anticipated. Here we took advantage of our previously generated gene-edited sheep and performed family trio-based whole genome sequencing which is capable of discriminating variants in the edited progenies that are inherited, naturally generated, or induced by genetic modification. Three family trios were re-sequenced at a high average depth of genomic coverage (~ 25.8x). After developing a pipeline to comprehensively analyze the sequence data for de novo single nucleotide variants, indels and structural variations from the genome; we only found a single unintended event in the form of a 2.4 kb inversion induced by site-specific double-strand breaks between two sgRNA targeting sites at the MSTN locus with a low incidence. CONCLUSIONS: We provide the first report on the fidelity of CRISPR-based modification for sheep genomes targeted simultaneously for gene breaks at three coding sequence locations. The trio-based sequencing approach revealed almost negligible off-target modifications, providing timely evidences of the safe application of	sheep
139	animal	sheep	CRISPR;Cas9;	Cystic fibrosis transmembrane conductance regulator (CFTR)	JCI insight	A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption of the CFTR gene.	2018	3(19)	[Fan Z et al.]	Utah State University, Logan, UT, USA.	30282831	10.1172/jci.insight.123529	Cystic fibrosis (CF) is a genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. The major cause of limited life span in CF patients is progressive lung disease. CF models have been generated in 4 species (mice, rats, ferrets, and pigs) to enhance our understanding of the CF pathogenesis. Sheep may be a particularly relevant animal to model CF in humans due to the similarities in lung anatomy and development in the two species. Here, we describe the generation of a sheep model for CF using CRISPR/Cas9 genome editing and somatic cell nuclear transfer (SCNT) techniques. We generated cells with CFTR gene disruption and used them for production of CFTR-/- and CFTR+/- lambs. The newborn CFTR-/- sheep developed severe disease consistent with CF pathology in humans. Of particular relevance were pancreatic fibrosis, intestinal obstruction, and absence of the vas deferens. Also, substantial liver and gallbladder disease may reflect CF liver disease that is evident in humans. The phenotype of CFTR-/- sheep suggests this large animal model will be a useful resource to advance the development of new CF therapeutics. Moreover, the generation of specific human CF disease-associated mutations in sheep may advance personalized medicine for this common genetic disorder.	sheep
140	animal	sheep	CRISPR;	acetyl-coenzyme A acyltranferase 2	Journal of cellular biochemistry	Acetyl-coenzyme A acyltransferase 2 promote the differentiation of sheep precursor adipocytes into adipocytes.	2018		[Zhang Y et al.]	Yangzhou University, Yangzhou, Jiangsu, China.	30485515	10.1002/jcb.28080	The acetyl CoA acyltransferase 2 (ACAA2) is a key enzyme of the fatty acid oxidation pathway, catalyzing the last step of the mitochondrial beta oxidation, thus playing an important role in the fatty acid metabolism. The purpose of this study was to investigate the effect of knocking out ACAA2 on the expression of genes lipoprteinlipase (LPL), peroxisome proliferator-activated receptor-gamma (PPAR-gamma), fatty acid synthase, fat mass and obesity-associated gene, adipocyte fatty acid-binding protein (AP2) in precursor adipocytes and their differentiation into adipocytes. The knockout vector was constructed using CRISPR-Cas RNA-guided nuclease technology with an efficiency of 23.80%, and the vector was transfected into precursor adipocyte cells, while an overexpression vector of the ACAA2 gene was also transfected in another group of preadipocytes. Quantitative polymerase chain reaction showed that the expression of the PPAR-gamma, LPL, and AP2 was significantly lower in the knockout compared with the overexpression group, while there was no difference in cell growth. After induction of adipocyte precursor cells into adipocytes using dexamethasone, insulin, and IBMX, oil red staining showed a significantly different number of lipid droplets in the knockout group. These results provide a preliminary	sheep
141	animal	sheep	CRISPR;Cas9;	myostatin	Journal of cellular biochemistry	CRISPR/Cas9-mediated sheep MSTN gene knockout and promote sSMSCs differentiation.	2018		[Zhang Y et al.]	Yangzhou University, Yangzhou, China.	30242885	10.1002/jcb.27474	Myostatin (MSTN) is an important gene involved in the regulation of embryonic muscle cells and adult muscle development; it has a good application prospect in transgenic animal production by improving the yield of muscle. The purpose of this study is to construct MSTN gene knockout vector using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). The knockout efficiency was evaluated in sheep ear fibroblasts (SEFs) by cleavage activity of transcription of guide RNA (gRNA), luciferase-single-strand annealing assay, T7 endonuclease I assay (T7E1), and TA clone sequence (10/38); and above all, detection showed that the cleavage activity of CRISPR/Cas9-mediated MSTN reached 29%. MSTN-Cas9/gRNA4 was transfected into sheep skeletal muscle satellite cell (sSMSC) to confirm the function of MSTN in myotomes formation induced by starvation in low-serum medium. The results showed that myotubes formation efficiency were 11.2 +/- 1.3% and 19.5 +/- 2.1% in the control group and knockout group, respectively. The average length of myotomes was 22 +/- 5.3 and 47 +/- 3.6 mum, displaying that MSTN knockout can promote sSMSC differentiation in number and length. The unlabeled MSTN-Cas9/gRNA4 was transfected into SEFs and monoclonal positive cells was obtained after 48 hours transfection. The MSTN-positive cells were used as donor cells to perform somatic cell nuclear transplantation to produce transgenic sheep. A total of 20 embryos were transplanted into surrogate mothers, four of them normally produce offspring. The genomic DNA of surviving lambs were used as a template, three positive individuals were identified by T7E1 digestion. All the results demonstrated that the CRISPR/Cas9 system has the potential to become an important and applicable	sheep

142	animal	sheep	CRISPR;Cas9;	bone morphogenetic protein receptor type 1b	Reproduction, fertility, and development	Generation of gene-edited sheep with a defined Booroola fecundity gene (FecB(B)) mutation in bone morphogenetic protein receptor type 1B (BMPR1B) via clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) 9.	2018	30(12):1616-1621	[Zhou S et al.]	Northwest A&F University, Yangling, China.	31039970	10.1071/RD18086	Since its emergence, the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated (Cas) 9 system has been increasingly used to generate animals for economically important traits. However, most CRISPR/Cas9 applications have been focused on non-homologous end joining, which results in base deletions and insertions, leading to a functional knockout of the targeted gene. The Booroola fecundity gene (FecBB) mutation (p.Q249R) in bone morphogenetic protein receptor type 1B (BMPR1B) has been demonstrated to exert a profound effect on fecundity in many breeds of sheep. In the present study, we successfully obtained lambs with defined point mutations resulting in a p.249Q>R substitution through the coinjection of Cas9 mRNA, a single guide RNA and single-stranded DNA oligonucleotides into Tan sheep zygotes. In the newborn lambs, the observed efficiency of the single nucleotide exchange was as high as 23.8%. We believe that our findings will contribute to improved reproduction traits in sheep, as well as to the generation of defined point mutations in	sheep
143	animal	sheep	CRISPR;Cas9;	tissue nonspecific alkaline phosphatase	Scientific reports	Genetic engineering a large animal model of human hypophosphatasia in sheep.	2018	8(1):16945	[Williams DK et al.]	Texas A&M University, College Station, TX, USA.	30446691	10.1038/s41598-018-35079-y	The availability of tools to accurately replicate the clinical phenotype of rare human diseases is a key step toward improved understanding of disease progression and the development of more effective therapeutics. We successfully generated the first large animal model of a rare human bone disease, hypophosphatasia (HPP) using CRISPR/Cas9 to introduce a single point mutation in the tissue nonspecific alkaline phosphatase (TNSALP) gene (ALPL) (1077 C > G) in sheep. HPP is a rare inherited disorder of mineral metabolism that affects bone and tooth development, and is associated with muscle weakness. Compared to wild-type (WT) controls, HPP sheep have reduced serum alkaline phosphatase activity, decreased tail vertebral bone size, and metaphyseal flaring, consistent with the mineralization deficits observed in human HPP patients. Computed tomography revealed short roots and thin dentin in incisors, and reduced mandibular bone in HPP vs. WT sheep, accurately replicating odonto-HPP. Skeletal muscle biopsies revealed aberrant fiber size and disorganized mitochondrial cristae structure in HPP vs. WT sheep. These genetically engineered sheep accurately phenocopy human HPP and provide a novel large animal platform for the longitudinal study of HPP progression, as well as other rare human bone diseases.	sheep
144	animal	sheep	CRISPR;Cas9;	PDX1	Transgenic research	Mosaicism diminishes the value of pre-implantation embryo biopsies for detecting CRISPR/Cas9 induced mutations in sheep.	2018	27(6):525-537	[Vilarino M et al.]	University of California Davis, Davis, CA, USA.	30284144	10.1007/s11248-018-0094-x	The production of knock-out (KO) livestock models is both expensive and time consuming due to their long gestational interval and low number of offspring. One alternative to increase efficiency is performing a genetic screening to select pre-implantation embryos that have incorporated the desired mutation. Here we report the use of sheep embryo biopsies for detecting CRISPR/Cas9-induced mutations targeting the gene PDX1 prior to embryo transfer. PDX1 is a critical gene for pancreas development and the target gene required for the creation of pancreatogenesis-disabled sheep. We evaluated the viability of biopsied embryos in vitro and in vivo, and we determined the mutation efficiency using PCR combined with gel electrophoresis and digital droplet PCR (ddPCR). Next, we determined the presence of mosaicism in ~ 50% of the recovered fetuses employing a clonal sequencing methodology. While the use of biopsies did not compromise embryo viability, the presence of mosaicism diminished the diagnostic value of the technique. If mosaicism could be overcome, pre-implantation embryo biopsies for mutation screening represents a powerful approach	sheep
145	animal	sheep	CRISPR;Cas9;	suppresor cytokine signaling 2	Frontiers in genetics	Programmable Base Editing of the Sheep Genome Revealed No Genome-Wide Off-Target Mutations.	2019	10:215	[Zhou S et al.]	Northwest A&F University, Yangling, China.	30930940	10.3389/fgene.2019.00215	Since its emergence, CRISPR/Cas9-mediated base editors (BEs) with cytosine deaminase activity have been used to precisely and efficiently introduce single-base mutations in genomes, including those of human cells, mice, and crop species. Most production traits in livestock are induced by point mutations, and genome editing using BEs without homology-directed repair of double-strand breaks can directly alter single nucleotides. The p.96R > C variant of Suppressor cytokine signaling 2 (SOCS2) has profound effects on body weight, body size, and milk production in sheep. In the present study, we successfully obtained lambs with defined point mutations resulting in a p.96R > C substitution in SOCS2 by the co-injection of BE3 mRNA and a single guide RNA (sgRNA) into sheep zygotes. The observed efficiency of the single nucleotide exchange in newborn animals was as high as 25%. Observations of body size and body weight in the edited group showed that gene modification contributes to enhanced growth traits in sheep. Moreover, targeted deep sequencing and unbiased family trio-based whole genome sequencing revealed undetectable off-target mutations in the edited animals. This study demonstrates the potential for the application of BE-mediated point mutations in large animals for the improvement of production traits in	sheep
146	animal	sheep	CRISPR;Cas9;	Fibroblast growth factor 5	Gene	Bioinformatics analysis of evolutionary characteristics and biochemical structure of FGF5 Gene in sheep.	2019	702:123-132	[Zhang R et al.]	China Agricultural University, Beijing, China.	30926307	10.1016/j.gene.2019.03.040	Fibroblast growth factor (FGF) 5 regulates the development and periodicity of hair follicles, which can affect hair traits. Loss-of-function mutations associated with long-hair phenotypes have been described in several mammalian species. Sheep is an important economic animal, however, the evolution characterizations and biological mechanism of oFGF5 (Ovis aries FGF5) gene are still poorly understood. In this study, oFGF5 gene was obtained by resequencing the whole genome of three Dorper sheep and RACE of two Kazakh sheep FGF5. We proposed FGF5 was phylogenetically related to FGF4 family and oFGF5 clearly orthologed to goat FGF5. Six loci were found from the positive selection results of FGF5 and half of them located on signal peptide. The basically similar rates of function-altering substitutions in sheep and goat lineage and the rest of the mammalian lineage of 365 SNPs indicated that the FGF5 gene was quite conservative during evolution. Homology modeling of the oFGF5 suggested that it has a highly conserved FGF superfamily domain containing 10 beta-strands. Furthermore, the protein-protein docking analysis revealed that oFGF5 have the potential to form heterodimers with oFGFR1, the predicted interaction interface of FGF5-FGFR1 heterodimer was formed mainly by residues from FGF superfamily domain. Our observations suggested the evolutionary and structural biology features of oFGF5 might be relevant to its function about hair follicle development and modulating hair growth, and we confirmed our speculation by using the FGF5 gene editing sheep	sheep

147	animal	sheep	CRISPR;Cas9;	palmitoyl-protein thioesterase 1	Scientific reports	CRISPR/Cas9 mediated generation of an ovine model for infantile neuronal ceroid lipofuscinosis (CLN1 disease).	2019	9(1):9891	[Eaton SL et al.]	University of Edinburgh, Edinburgh, UK.	31289301	10.1038/s41598-019-45859-9	The neuronal ceroid lipofuscinoses (NCLs) are a group of devastating monogenetic lysosomal disorders that affect children and young adults with no cure or effective treatment currently available. One of the more severe infantile forms of the disease (INCL or CLN1 disease) is due to mutations in the palmitoyl-protein thioesterase 1 (PPT1) gene and severely reduces the child's lifespan to approximately 9 years of age. In order to better translate the human condition than is possible in mice, we sought to produce a large animal model employing CRISPR/Cas9 gene editing technology. Three PPT1 homozygote sheep were generated by insertion of a disease-causing PPT1 (R151X) human mutation into the orthologous sheep locus. This resulted in a morphological, anatomical and biochemical disease phenotype that closely resembles the human condition. The homozygous sheep were found to have significantly reduced PPT1 enzyme activity and accumulate autofluorescent storage material, as is observed in CLN1 patients. Clinical signs included pronounced behavioral deficits as well as motor deficits and complete loss of vision, with a reduced lifespan of 17 +/- 1 months at a humanely defined terminal endpoint. Magnetic resonance imaging (MRI) confirmed a significant decrease in motor cortical volume as well as increased ventricular volume corresponding with observed brain atrophy and a profound reduction in brain mass of 30% at necropsy, similar to alterations observed in human patients. In summary, we have generated the first CRISPR/Cas9 gene edited NCL model. This novel sheep model of CLN1 disease develops biochemical, gross morphological and in vivo brain alterations confirming the efficacy of the targeted modification and potential relevance	sheep
148	animal (fish)	african cichlid	CRISPR;Cas9;	agouti-related peptide 2	Science	Agouti-related peptide 2 facilitates convergent evolution of stripe patterns across cichlid fish radiations.	2018	362(6413):457-460	[Kratochwil CF et al.]	University of Konstanz, Konstanz, Germany.	30361373	10.1126/science.aao6809	The color patterns of African cichlid fishes provide notable examples of phenotypic convergence. Across the more than 1200 East African rift lake species, melanic horizontal stripes have evolved numerous times. We discovered that regulatory changes of the gene agouti-related peptide 2 (agrp2) act as molecular switches controlling this evolutionarily labile phenotype. Reduced agrp2 expression is convergently associated with the presence of stripe patterns across species flocks. However, cis-regulatory mutations are not predictive of stripes across radiations, suggesting independent regulatory mechanisms. Genetic mapping confirms the link between the agrp2 locus and stripe patterns. The crucial role of agrp2 is further supported by a CRISPR-Cas9 knockout that reconstitutes stripes in a nonstriped cichlid. Thus, we unveil how a single gene affects the convergent evolution of a	fish
149	animal (fish)	Astyanax mexicanus	CRISPR;Cas9;	oculocutaneous albinism II (oca2)	Developmental biology	CRISPR mutagenesis confirms the role of oca2 in melanin pigmentation in Astyanax mexicanus.	2018	441(2):313-318	[Klaassen H et al.]	Iowa State University, Ames, IA, USA.	29555241	10.1016/j.ydbio.2018.03.014	Understanding the genetic basis of trait evolution is critical to identifying the mechanisms that generated the immense amount of diversity observable in the living world. However, genetically manipulating organisms from natural populations with evolutionary adaptations remains a significant challenge. Astyanax mexicanus exists in two interfertile forms, a surface-dwelling form and multiple independently evolved cave-dwelling forms. Cavefish have evolved a number of morphological and behavioral traits and multiple quantitative trait loci (QTL) analyses have been performed to identify loci underlying these traits. These studies provide a unique opportunity to identify and test candidate genes for these cave-specific traits. We have leveraged the CRISPR/Cas9 genome editing techniques to characterize the effects of mutations in oculocutaneous albinism II (oca2), a candidate gene hypothesized to be responsible for the evolution of albinism in A. mexicanus cave populations. We generated oca2 mutant surface A. mexicanus. Surface fish with oca2 mutations are albino due to a disruption in the first step of the melanin synthesis pathway, the same step that is disrupted in albino cavefish. Hybrid offspring from crosses between oca2 mutant surface and cavefish are albino, definitively demonstrating the role of this gene in the evolution of albinism in this species. This research elucidates the role oca2 plays in pigmentation in fish, and establishes that this gene is solely responsible for the evolution of albinism in multiple cavefish populations. Finally, it demonstrates the utility of using genome	fish
150	animal (fish)	channel catfish	CRISPR;Cas9;	toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1); rhamnose binding lectin (RBL)	Scientific reports	Effects of CRISPR/Cas9 dosage on TICAM1 and RBL gene mutation rate, embryonic development, hatchability and fry survival in channel catfish.	2018	8(1):16499	[Elaswad A et al.]	Auburn University, AL, USA.	30405210	10.1038/s41598-018-34738-4	The current study was conducted to assess the effects of microinjection of different dosages of guide RNA (gRNA)/Cas9 protein on the mutation rate, embryo survival, embryonic development, hatchability and early fry survival in channel catfish, Ictalurus punctatus. Guide RNAs targeting two of the channel catfish immune-related genes, toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) and rhamnose binding lectin (RBL) genes, were designed and prepared. Three dosages of gRNA/Cas9 protein (low, 2.5 ng gRNA/7.5 ng Cas9, medium, 5 ng gRNA/15 ng Cas9 and high, 7.5 ng gRNA/22.5 ng Cas9) were microinjected into the yolk of one-cell embryos. Mutation rate increased with higher dosages ($p < 0.05$). Higher dosages increased the mutation frequency in individual embryos where biallelic mutations were detected. For both genes, microinjection procedures increased the embryo mortality ($p < 0.05$). Increasing the dosage of gRNA/Cas9 protein increased the embryo mortality and reduced the hatching percent ($p < 0.05$). Embryonic development was delayed when gRNAs targeting RBL gene were injected. Means of fry survival time were similar for different dosages ($p > 0.05$). The current results lay the foundations for designing gene editing experiments in channel catfish and can be used as a guide for other fish	fish
151	animal (fish)	channel catfish (Ictalurus punctatus)	CRISPR;Cas9;	toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1); rhamnose binding lectin (RBL)	Journal of visualized experiments	Microinjection of CRISPR/Cas9 Protein into Channel Catfish, Ictalurus punctatus, Embryos for Gene Editing.	2018	(131)	[Elaswad A et al.]	Auburn University, USA.	29443028	10.3791/56275	The complete genome of the channel catfish, Ictalurus punctatus, has been sequenced, leading to greater opportunities for studying channel catfish gene function. Gene knockout has been used to study these gene functions in vivo. The clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) system is a powerful tool used to edit genomic DNA sequences to alter gene function. While the traditional approach has been to introduce CRISPR/Cas9 mRNA into the single cell embryos through microinjection, this can be a slow and inefficient process in catfish. Here, a detailed protocol for microinjection of channel catfish embryos with CRISPR/Cas9 protein is described. Briefly, eggs and sperm were collected and then artificial fertilization performed. Fertilized eggs were transferred to a Petri dish containing Holtfreter's solution. Injection volume was calibrated and then guide RNAs/Cas9 targeting the toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) gene and rhamnose binding lectin (RBL) gene were microinjected into the yolk of one-cell embryos. The gene knockout was successful as indels were confirmed by DNA sequencing. The predicted protein sequence alterations due to these mutations included frameshift and truncated protein due to premature stop	fish

152	animal (fish)	common carp	CRISPR;Cas9;	HIF-1 alpha	Archives of virology	CRISPR/Cas9-mediated knockout of HIF-1alpha gene in epithelioma papulosum cyprini (EPC) cells inhibited apoptosis and viral hemorrhagic septicemia virus (VHSV) growth.	2018	163(12):3395-3402	[Kim MS et al.]	Sejong University, Seoul, Korea.	30220031	10.1007/s00705-018-4018-0	Hypoxia-inducible factor-1 (HIF-1) is a heterodimer of HIF-1alpha and HIF-1beta, and its key role in the regulation of cellular responses to hypoxia has been well-demonstrated. The participation of HIF-1alpha in apoptosis has been reported in mammals, however, a little information is available on the role of HIF-1alpha in the progression of apoptosis in fish. In this study, to know the role of HIF-1alpha in the apoptosis of fish cells, we produced HIF-1alpha knockout Epithelioma papulosum cyprini (EPC) cells using a CRISPR/Cas9 vector, and a single cell clone showing a heterozygous insertion/deletion (indel) mutation (one nucleotide insertion and one nucleotide deletion in HIF-1alpha gene) was chosen for further experiments. To confirm the knockout of HIF-1alpha, cells were transfected with a hypoxia reporting vector containing hypoxic response elements (HREs). EPC cells transfected with the reporting plasmids showed significantly increased luminescence by exposure to cobalt chloride, a prolyl hydroxylases inhibitor. On the other hand, HIF-1alpha knockout EPC cells showed a non-responsiveness to a cobalt chloride exposure, suggesting that functional HIF-1alpha protein was not produced in the HIF-1alpha knockout EPC cells. Apoptosis progression induced by camptothecin and viral hemorrhagic septicemia virus (VHSV) infection was severely inhibited by HIF-1alpha knockout, and the replication of VHSV was significantly retarded in HIF-1alpha knockout EPC cells. These results suggest that HIF-1alpha in EPC cells acts as a pro-apoptotic factor in the progression	fish
153	animal (fish)	common carp	CRISPR;Cas9;	IFN regulatory factor 9 (IRF9)	Fish & shellfish immunology	Increase of viral hemorrhagic septicemia virus growth by knockout of IRF9 gene in Epithelioma papulosum cyprini cells.	2018	83:443-448	[Kim MS et al.]	Sejong University, Seoul, Korea.	30244086	10.1016/j.fsi.2018.09.025	Viral hemorrhagic septicemia virus (VHSV) has been a notorious pathogen in freshwater and marine fish. Due to the lack of effective treatment measures against VHSV disease, the development of prophylactic vaccines has been required, and methods that can produce high-titered viruses would be advantageous in producing cost-effective vaccines. Type I interferon (IFN) responses are the key elements of vertebrates' antiviral activities, and IFN-stimulated gene factor 3 (ISGF3) complex formed through type I IFNs up-regulates the expression of IFN-stimulated genes (ISGs). IFN regulatory factor 9 (IRF9) is a key component of ISGF3, so the inhibition of IRF9 would compromise host's type I IFN responses, which would weaken host antiviral activity. In this study, to increase the replication of VHSV, we generated IRF9 knockout Epithelioma papulosum cyprini (EPC) cells using a CRISPR/Cas9 vector that contains an EPC cell's U6 promoter-driven guide RNA cassette (targeting IRF9 gene) and a Cas9 expressing cassette. In the clones of IRF9 knockout EPC cells, there were no increase in ISG15 gene by poly I:C, and in Mx1 gene by both poly I:C and VHSV. Interestingly, although the increased folds were conspicuously lower than control EPC cells, the expression of ISG 15 gene in all the IRF9 knockout clones was significantly increased by VHSV infection. Control EPC cells pre-treated with poly I:C did not show any CPE when infected with VHSV, however, IRF9 knockout EPC cells showed CPE by VHSV infection in spite of being pretreated with poly I:C. The replication of VHSV in IRF9 knockout EPC cells was significantly faster and higher than that in control EPC cells indicating that the IRF9 knockout-mediated decrease of type I IFN responses allowed VHSV to replicate efficiently. Considering an economical aspect for the production of fish vaccines, the present IRF9 knockout EPC cells can be used to get	fish
154	animal (fish)	Danionella translucida	CRISPR;Cas9;		Nature methods	Transparent Danionella translucida as a genetically tractable vertebrate brain model.	2018	15(11):977-983	[Schulze L et al.]	Charite-Universitatsmedizin Berlin, Berlin, Germany.	30323353	10.1038/s41592-018-0144-6	Understanding how distributed neuronal circuits integrate sensory information and generate behavior is a central goal of neuroscience. However, it has been difficult to study neuronal networks at single-cell resolution across the entire adult brain in vertebrates because of their size and opacity. We address this challenge here by introducing the fish Danionella translucida to neuroscience as a potential model organism. This teleost remains small and transparent even in adulthood, when neural circuits and behavior have matured. Despite having the smallest known adult vertebrate brain, D. translucida displays a rich set of complex behaviors, including courtship, shoaling, schooling, and acoustic communication. In order to carry out optical measurements and perturbations of neural activity with genetically encoded tools, we established CRISPR-Cas9 genome editing and Tol2 transgenesis techniques. These features make D. translucida a promising model organism for the study of adult	fish
155	animal (fish)	grass carp (Ctenopharyngodon idellus)	CRISPR;Cas9;	Junctional Adhesion Molecule-A (gcJAM-A)	Fish & shellfish immunology	Efficient resistance to grass carp reovirus infection in JAM-A knockout cells using CRISPR/Cas9.	2018	76:206-215	[Ma J et al.]	Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, Hubei, China.	29477498	10.1016/j.fsi.2018.02.039	The hemorrhagic disease of grass carp (Ctenopharyngodon idellus) induced by grass carp reovirus (GCRV) leads to huge economic losses in China and currently, there are no effective methods available for prevention and treatment. The various GCRV genotypes may be one of the major obstacles in the pursuit of an effective antiviral treatment. In this study, we exploited CRISPR/Cas9 gene editing to specifically knockout the DNA sequence of the grass carp Junctional Adhesion Molecule-A (gcJAM-A) and evaluated in vitro resistance against various GCRV genotypes. Our results show that CRISPR/Cas9 effectively knocked out gcJAM-A and reduced GCRV infection for two different genotypes in permissive grass carp kidney cells (CIK), as evidenced by suppressed cytopathic effect (CPE) and GCRV progeny production in infected cells. In addition, with ectopic expression of gcJAM-A in cells, non-permissive cells derived from Chinese giant salamander (Andrias davidianus) muscle (GSM) could be highly infected by both GCRV-JX0901 and Hubei grass carp disease reovirus (HGDRV) strains that have different genotypes. Taken together, the results demonstrate that gcJAM-A is necessary for GCRV infection, implying a potential	fish

156	animal (fish)	medaka	CRISPR;Cas9;		Biology open	Efficient genome editing using CRISPR/Cas9 ribonucleoprotein approach in cultured Medaka fish cells.	2018	7(8)	[Liu Q et al.]	National University of Singapore, Singapore.	30072445	10.1242/bio.035170	Gene editing with CRISPR/Cas9 is a powerful tool to study the function of target genes. Although this technology has demonstrated wide efficiency in many species, including fertilized zebrafish and medaka fish embryos when microinjected, its application to achieve efficient gene editing in cultured fish cells have met some difficulty. Here, we report an efficient and reliable approach to edit genes in cultured medaka (Oryzias latipes) fish cells using pre-formed gRNA-Cas9 ribonucleoprotein (RNP) complex. Both medaka fish haploid and diploid cells were transfected with the RNP complex by electroporation. Efficient gene editing was demonstrated by polymerase chain reaction (PCR) amplification of the target gene from genomic DNA and heteroduplex mobility assay carried out with polyacrylamide gel electrophoresis (PAGE). The heteroduplex bands caused by RNP cleavage and non-homologous end joining could be readily detected by PAGE. DNA sequencing confirmed that these heteroduplex bands contains the mutated target gene sequence. The average gene editing efficiency in haploid cells reached 50%, enabling us to generate a clonal cell line with ntrk3b gene mutation for further study. This RNP transfection method also works efficiently in diploid medaka cells, with the highest mutation efficiency of 61.5%. The specificity of this synthetic RNP CRISPR/Cas9 approach was verified by candidate off-target gene sequencing. Our result indicated that transfection of pre-formed gRNA-Cas9 RNP into fish cells is efficient and reliable to edit target genes in cultured	fish
157	animal (fish)	medaka	CRISPR;Cas9;	tyr	Journal of experimental zoology. Part B, Molecular and developmental evolution	Generation of albino medaka (Oryzias latipes) by CRISPR/Cas9.	2018	330(4):242-246	[Fang J et al.]	Huazhong Agricultural University, Wuhan, Hubei, China.	29873175	10.1002/jez.b.22808	CRISPR/Cas9 system is a powerful tool to produce the genetic modification in plants and animals such as mouse and zebrafish. However, this technique was less reported in fish model medaka (Oryzias latipes). Here, we describe an efficient and rapid procedure for genome editing in medaka tyr and generate a stable albino strain. The Cas9 mRNA and gRNA for tyr gene were injected into the embryos of orange-red medaka, and the tyr gene was disrupted in more than 90% of embryos in F0 and F1, which were validated by observation and sequencing of targeted locus. The pigment cells were largely decreased in the mutant medaka because open reading frames of tyr were shifted near the targeted locus, generating albino medaka. Taken together, this method provides a detailed procedure to generate the genetic modification medaka by using an optimized CRISPR/Cas9 system, and the new albino medaka provides an	fish
158	animal (fish)	medaka	CRISPR;Cas9;	ZRS	Nature genetics	A conserved Shh cis-regulatory module highlights a common developmental origin of unpaired and paired fins.	2018	50(4):504-509	[Letelier J et al.]	Centro Andaluz de Biologia del Desarrollo (CABD), Consejo Superior de Investigaciones Cientificas/Universidad Pablo de Olavide/Junta de Andalucia, Sevilla, Spain.	29556077	10.1038/s41588-018-0080-5	Despite their evolutionary, developmental and functional importance, the origin of vertebrate paired appendages remains uncertain. In mice, a single enhancer termed ZRS is solely responsible for Shh expression in limbs. Here, zebrafish and mouse transgenic assays trace the functional equivalence of ZRS across the gnathostome phylogeny. CRISPR/Cas9-mediated deletion of the medaka (Oryzias latipes) ZRS and enhancer assays identify the existence of ZRS shadow enhancers in both teleost and human genomes. Deletion of both ZRS and shadow ZRS abolishes shh expression and completely truncates pectoral fin formation. Strikingly, deletion of ZRS results in an almost complete ablation of the dorsal fin. This finding indicates that a ZRS-Shh regulatory module is shared by paired and median fins and that paired fins likely emerged by the co-option of developmental programs established in the median fins of stem gnathostomes. Shh function was later reinforced in pectoral fin development with	fish
159	animal (fish)	medaka	CRISPR;Cas9;	miR-202	PLoS genetics	MiR-202 controls female fecundity by regulating medaka oogenesis.	2018	14(9):e1007593	[Gay S et al.]	INRA, Rennes, France.	30199527	10.1371/journal.pgen.1007593	Female gamete production relies on coordinated molecular and cellular processes that occur in the ovary throughout oogenesis. In fish, as in other vertebrates, these processes have been extensively studied both in terms of endocrine/paracrine regulation and protein expression and activity. The role of small non-coding RNAs in the regulation of animal reproduction remains however largely unknown and poorly investigated, despite a growing interest for the importance of miRNAs in a wide variety of biological processes. Here, we analyzed the role of miR-202, a miRNA predominantly expressed in male and female gonads in several vertebrate species. We studied its expression in the medaka ovary and generated a mutant line (using CRISPR/Cas9 genome editing) to determine its importance for reproductive success with special interest for egg production. Our results show that miR-202-5p is the most abundant mature form of the miRNA and that it is expressed in granulosa cells and in the unfertilized egg. The knock out (KO) of mir-202 gene resulted in a strong phenotype both in terms of number and quality of eggs produced. Mutant females exhibited either no egg production or produced a dramatically reduced number of eggs that could not be fertilized, ultimately leading to no reproductive success. We quantified the size distribution of the oocytes in the ovary of KO females and performed a large-scale transcriptomic analysis approach to identified dysregulated molecular pathways. Together, cellular and molecular analyses indicate that the lack of miR-202 impairs the early steps of oogenesis/folliculogenesis and decreases the number of large (i.e. vitellogenic) follicles, ultimately leading to dramatically reduced female fecundity. This study sheds new light on the regulatory mechanisms that control the early steps of follicular development, including possible targets of miR-202-5p, and provides the first	fish
160	animal (fish)	medaka	CRISPR;Cas9;	5 genetic loci	Zoological letters	Highly efficient generation of knock-in transgenic medaka by CRISPR/Cas9-mediated genome engineering.	2018	4:3	[Watakabe I et al.]	National Institute for Basic Biology, Okazaki, Aichi, Japan.	29445519	10.1186/s40851-017-0086-3	Background: Medaka (Oryzias latipes) is a popular animal model used in vertebrate genetic analysis. Recently, an efficient (~ 30%) knock-in system via non-homologous end joining (NHEJ) was established in zebrafish using the CRISPR/Cas9 system. If the same technique were applicable in medaka, it would greatly expand the usefulness of this model organism. The question of the applicability of CRISPR/Cas9 in medaka, however, has yet to be addressed. Results: We report the highly efficient generation of knock-in transgenic medaka via non-homologous end joining (NHEJ). Donor plasmid containing a heat-shock promoter and a reporter gene was co-injected with a short guide RNA (sgRNA) targeted for genome digestion, an sgRNA targeted for donor plasmid digestion, and Cas9 mRNA. Broad transgene expression in the expression domain of a target gene was observed in approximately 25% of injected embryos. By raising these animals, we established stable knock-in transgenic fish with several different constructs for five genetic loci, obtaining transgenic founders at efficiencies of > 50% for all five loci. Further, we show that the method is useful for obtaining mutant alleles. In the experiments where transgene integrations were targeted between the transcription start site and the initiation methionine, the resultant transgenic fish became mutant alleles. Conclusion: With its simplicity, design flexibility, and high efficiency, we propose that CRISPR/Cas9-mediated knock-in via NHEJ will become a	fish

161	animal (fish)	medaka; zebrafish	CRISPR;Cas9;TALENs;	sox5; sox10	PLoS genetics	Distinct interactions of Sox5 and Sox10 in fate specification of pigment cells in medaka and zebrafish.	2018	14(4):e1007260	[Nagao Y et al.]	Nagoya University, Nagoya, Aichi, Japan.	29621239	10.1371/journal.pgen.1007260	Mechanisms generating diverse cell types from multipotent progenitors are fundamental for normal development. Pigment cells are derived from multipotent neural crest cells and their diversity in teleosts provides an excellent model for studying mechanisms controlling fate specification of distinct cell types. Zebrafish have three types of pigment cells (melanocytes, iridophores and xanthophores) while medaka have four (three shared with zebrafish, plus leucophores), raising questions about how conserved mechanisms of fate specification of each pigment cell type are in these fish. We have previously shown that the Sry-related transcription factor Sox10 is crucial for fate specification of pigment cells in zebrafish, and that Sox5 promotes xanthophores and represses leucophores in a shared xanthophore/leucophore progenitor in medaka. Employing TILLING, TALEN and CRISPR/Cas9 technologies, we generated medaka and zebrafish sox5 and sox10 mutants and conducted comparative analyses of their compound mutant phenotypes. We show that specification of all pigment cells, except leucophores, is dependent on Sox10. Loss of Sox5 in Sox10-defective fish partially rescued the formation of all pigment cells in zebrafish, and melanocytes and iridophores in medaka, suggesting that Sox5 represses Sox10-dependent formation of these pigment cells, similar to their interaction in mammalian melanocyte specification. In contrast, in medaka, loss of Sox10 acts cooperatively with Sox5, enhancing both xanthophore reduction and leucophore increase in sox5 mutants. Misexpression of Sox5 in the xanthophore/leucophore progenitors increased xanthophores and reduced leucophores in medaka. Thus, the mode of Sox5 function in xanthophore specification differs between medaka (promoting) and zebrafish (repressing), which is also the case in adult fish. Our findings reveal surprising diversity in even the mode of the interactions between Sox5 and Sox10 governing specification of pigment cell types in	fish
162	animal (fish)	paddlefish (Acipenseriformes)	CRISPR;Cas9;	GLP1R; GIPR	Peptides	Glucagon-related peptides from phylogenetically ancient fish reveal new approaches to the development of dual GCGR and GLP1R agonists for type 2 diabetes therapy.	2018	110:19-29	[Graham GV et al.]	Ulster University, Coleraine, Northern Ireland, UK.	30391422	10.1016/j.peptides.2018.10.013	The insulinotropic and antihyperglycaemic properties of glucagons from the sea lamprey (Petromyzontiformes), paddlefish (Acipenseriformes) and trout (Teleostei) and oxyntomodulin from dogfish (Elasmobranchii) and ratfish (Holocephali) were compared with those of human glucagon and GLP-1 in mammalian test systems. All fish peptides produced concentration-dependent stimulation of insulin release from BRIN-BD11 rat and 1.1 B4 human clonal beta-cells and isolated mouse islets. Paddlefish glucagon was the most potent and effective peptide. The insulinotropic activity of paddlefish glucagon was significantly ($P < 0.01$) decreased after incubating BRIN-BD11 cells with the GLP1R antagonist, exendin-4(9-39) and the GCGR antagonist [des-His(1),Pro(4), Glu(9)] glucagon amide but GIPR antagonist, GIP(6-30)Cex-K(40)[palmitate] was without effect. Paddlefish and lamprey glucagons and dogfish oxyntomodulin (10 nmol $L(-1)$) produced significant ($P < 0.01$) increases in cAMP concentration in Chinese hamster lung (CHL) cells transfected with GLP1R and human embryonic kidney (HEK293) cells transfected with GCGR. The insulinotropic activity of paddlefish glucagon was attenuated in CRISPR/Cas9-engineered GLP1R knock-out INS-1 cells but not in GIPR knock-out cells. Intraperitoneal administration of all fish peptides, except ratfish oxyntomodulin, to mice together with a glucose load produced significant ($P < 0.05$) decreases in plasma glucose concentrations and paddlefish glucagon produced a greater release of insulin compared with GLP-1. Paddlefish glucagon shares the sequences Glu(15)-Glu(16) and Glu(24)-Trp(25)-Leu(26)-Lys(27)-Asn(28)-Gly(29) with the potent GLP1R agonist, exendin-4 so may be regarded as a naturally occurring, dual-agonist hybrid peptide that may serve as a template design of new drugs for type	fish
163	animal (fish)	pufferfish (Takifugu rubripes)	CRISPR;	pufferfish saxitoxin- and tetrodotoxin-binding protein 2	Toxicon	Genome editing of pufferfish saxitoxin- and tetrodotoxin-binding protein type 2 in Takifugu rubripes.	2018	153:58-61	[Kato-Unoki Y et al.]	Kyushu University, Fukuoka, Japan.	30170168	10.1016/j.toxicon.2018.08.001	The pufferfish saxitoxin- and tetrodotoxin-binding protein 2 (PSTBP2), which is involved in toxin accumulation, was knocked out in Takifugu rubripes embryos by using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 genome-editing technology. Treating the embryos with one of two single-guide RNA (sgRNA) resulted in mutation rates of 57.1% and 62.5%, respectively, as estimated using a heteroduplex mobility assay at 3 days postfertilization. Both sgRNAs might induced frameshift mutations that knocked out the T. rubripes PSTBP2.	fish
164	animal (fish)	rainbow trout (Oncorhynchus mykiss)	CRISPR;Cas9;	insulin-like growth factor binding protein-2b	Scientific reports	Editing the duplicated insulin-like growth factor binding protein-2b gene in rainbow trout (Oncorhynchus mykiss).	2018	8(1):16054	[Cleveland BM et al.]	Agricultural Research Service, United States Department of Agriculture, Kearneysville, WV, USA.	30375441	10.1038/s41598-018-34326-6	In salmonids, the majority of circulating insulin-like growth factor-I (IGF-I) is bound to IGF binding proteins (IGFBP), with IGFBP-2b being the most abundant in circulation. We used CRISPR/Cas9 methodology to disrupt expression of a functional IGFBP-2b protein by co-targeting for gene editing IGFBP-2b1 and IGFBP-2b2 subtypes, which represent salmonid-specific gene duplicates. Twenty-four rainbow trout were produced with mutations in the IGFBP-2b1 and IGFBP-2b2 genes. Mutant fish exhibited between 8-100% and 2-83% gene disruption for IGFBP-2b1 and IGFBP-2b2, respectively, with a positive correlation ($P < 0.001$) in gene mutation rate between individual fish. Analysis of IGFBP-2b protein indicated reductions in plasma IGFBP-2b abundance to between 0.04-0.96-fold of control levels. Plasma IGF-I, body weight, and fork length were reduced in mutants at 8 and 10 months post-hatch, which supports that IGFBP-2b is significant for carrying IGF-I. Despite reduced plasma IGF-I and IGFBP-2b in mutants, growth retardation in mutants was less severe between 10 and 12 months post-hatch ($P < 0.05$), suggesting a compensatory growth response occurred. These findings indicate that gene editing using CRISPR/Cas9 and ligand blotting is a feasible approach for characterizing protein-level functions of duplicated IGFBP genes in salmonids and	fish
165	animal (fish)	rare minnow (Gobiocypris rarus)	CRISPR;Cas9;	integrin beta-1	International journal of molecular sciences	ITGB1b-Deficient Rare Minnows Delay Grass Carp Reovirus (GCRV) Entry and Attenuate GCRV-Triggered Apoptosis.	2018	19(10)	[Chen G et al.]	Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.	30326628	10.3390/ijms19103175	Integrin beta-1 (ITGB1) is a transmembrane protein belonging to the integrin family and it plays an important role in viral entry. In this study, the itgb1b gene of the rare minnow, Gobiocypris rarus, was cloned and analyzed. To investigate the possible role of itgb1b on grass carp reovirus (GCRV) infection, we generated an ITGB1b-deficient rare minnow (ITGB1b(-/-)) using the CRISPR/Cas9 system. Following stimulation with GCRV, the survival time of the -ITGB1b(-/-) rare minnows was extended in comparison to the wild-type minnows. Moreover, the relative copy number of GCRV and the level of clathrin-mediated endocytosis-associated and apoptosis-related gene expression in the ITGB1b(-/-) rare minnows was significantly lower than that of the wild-type minnows. These results suggested that the absence of itgb1b reduced viral entry efficiency and the expression of apoptosis-related genes. Moreover, the data suggested that itgb1b played an important role in mediating the entry of viruses into the cells via clathrin. Therefore, these findings provide novel insight into the function	fish

166	animal (fish)	sea lamprey	CRISPR;Cas9;	Semaphorin3F (Sema3F)	Development	An ancestral role for Semaphorin3F–Neuropilin signaling in patterning neural crest within the new vertebrate head.	2018	145(14)	[York JR et al.]	University of Oklahoma, Norman, OK, USA.	29980564	10.1242/dev.164780	The origin of the vertebrate head is one of the great unresolved issues in vertebrate evolutionary developmental biology. Although many of the novelties in the vertebrate head and pharynx derive from the neural crest, it is still unknown how early vertebrates patterned the neural crest within the ancestral body plan they inherited from invertebrate chordates. Here, using a basal vertebrate, the sea lamprey, we show that homologs of Semaphorin3F (Sema3F) ligand and its Neuropilin (Nrp) receptors show complementary and dynamic patterns of expression that correlate with key periods of neural crest development (migration and patterning of cranial neural crest–derived structures). Using CRISPR/Cas9–mediated mutagenesis, we demonstrate that lamprey Sema3F is essential for patterning of neural crest–derived melanocytes, cranial ganglia and the head skeleton, but is not required for neural crest migration or patterning of trunk neural crest derivatives. Based on comparisons with jawed vertebrates, our results suggest that the deployment of Nrp–Sema3F signaling, along with other intercellular guidance cues, was pivotal in allowing early vertebrates to organize and pattern cranial neural crest cells into many of the hallmark structures that define the	fish
167	animal (fish)	sea lamprey	CRISPR;Cas9;	key genes required for the differentiation of oligodendrocytes in gnathostomes, including Nkx2.2, SoxE genes, and PDGFR	Developmental biology	Gliogenesis in lampreys shares gene regulatory interactions with oligodendrocyte development in jawed vertebrates.	2018	441(1):176–190	[Yuan T et al.]	University of Oklahoma, Norman, OK, USA.	29981309	10.1016/j.ydbio.2018.07.002	Glial cells in the nervous system regulate and support many functions related to neuronal activity. Understanding how the vertebrate nervous system has evolved demands a greater understanding of the mechanisms controlling evolution and development of glial cells in basal vertebrates. Among vertebrate glia, oligodendrocytes form an insulating myelin layer surrounding axons of the central nervous system (CNS) in jawed vertebrates. Jawless vertebrates lack myelinated axons but it is unclear when oligodendrocytes or the regulatory mechanisms controlling their development evolved. To begin to investigate the evolution of mechanisms controlling glial development, we identified key genes required for the differentiation of oligodendrocytes in gnathostomes, including Nkx2.2, SoxE genes, and PDGFR, analyzed their expression, and used CRISPR/Cas9 genome editing to perturb their functions in a primitively jawless vertebrate, the sea lamprey. We show in lamprey that orthologs required for oligodendrocyte development in jawed vertebrates are expressed in the lamprey ventral neural tube, in similar locations where gnathostome oligodendrocyte precursor cells (OPC) originate. In addition, they appear to be under the control of conserved mechanisms that regulate OPC development in jawed vertebrates and may also function in gliogenesis. Our results suggest that although oligodendrocytes first emerged in jawed vertebrates, regulatory mechanisms required for their development	fish
168	animal (fish)	shrimp	CRISPR;Cas9;	EcgC1qR	Fish & shellfish immunology	Biological function of a gC1qR homolog (EcgC1qR) of Exopalaemon carinicauda in defending bacteria challenge.	2018	82:378–385	[Zhang J et al.]	Hebei University, Baoding, Hebei, China.	30144564	10.1016/j.fsi.2018.08.046	The gC1qR is a ubiquitously expressed cell protein that interacts with the globular heads of C1q (gC1q) and many other ligands. In this study, one gC1qR homolog gene was obtained from Exopalaemon carinicauda and named EcgC1qR. The complete nucleotide sequence of EcgC1qR contained a 774 bp open reading frame (ORF) encoding EcgC1qR precursor of 257amino acids. The deduced amino acid sequence of EcgC1qR revealed a 55–amino–acid–long mitochondrial targeting sequence at the N–terminal and a mitochondrial acidic matrix protein of 33kDa (MAM33) domain. The genomic organization of EcgC1qR gene showed that EcgC1qR gene contained five exons and four introns. EcgC1qR could express in all of the detected tissues and its expression was much higher in hepatopancreas and hemocytes. The expression of EcgC1qR in the hepatopancreas of prawns challenged with Vibrio parahaemolyticus and Aeromonas hydrophila changed in a time–dependent manner. The expression of EcgC1qR in prawns challenged with V. parahaemolyticus was up–regulated at 6h ($p<0.05$), and significantly up–regulated at 12h and 24h ($p<0.01$), and then returned to the control levels at 48h post–challenge ($p>0.05$). At the same time, the expression in Aeromonas–challenged group was significantly up–regulated at 6, 12 and 24h. The recombinant EcgC1qR could inhibit the growth of two tested bacteria. In addition, we successfully deleted EcgC1qR gene through CRISPR/Cas9 technology and it was the first time to obtain the mutant of gC1qR homolog gene in crustacean. It's a great	fish
169	animal (fish)	shrimp (ridgetail white prawn Exopalaemon carinicauda)	CRISPR;Cas9;	EcMIH	Fish & shellfish immunology	CRISPR/Cas9–mediated deletion of EcMIH shortens metamorphosis time from mysis larva to postlarva of Exopalaemon carinicauda.	2018	77:244–251	[Zhang J et al.]	Hebei University, Baoding, Hebei, China.	29621632	10.1016/j.fsi.2018.04.002	The recently emerged CRISPR/Cas9 technology is the most flexible means to produce targeted mutations at the genomic loci in a variety of organisms. In Crustaceans, molt–inhibiting hormone (MIH) is an important negative–regulatory factor and plays a key role in suppressing the molting process. However, whether precise disruption of MIH in crustacean can be achieved and successfully used to improve the development and growth has not been proved. In this research, the complementary DNA (cDNA) and genomic DNA, including flanking regions of the MIH gene (EcMIH) of ridgetail white prawn Exopalaemon carinicauda, were cloned and sequenced. Sequence analysis revealed that EcMIH was composed of three exons and two introns. Analysis by RT–PCR showed that EcMIH mainly expressed in eyestalks. During different development periods, EcMIH was highest in juvenile stage and extremely low in others but adult prawns eyestalks. In addition, we applied CRISPR/Cas9 technology to generate EcMIH knock–out (KO) prawns and then analyzed the changes in their phenotypes. We efficiently generated 12 EcMIH–KO prawns out of 250 injected one–cell stage embryos and the mutant rate reached 4.8% after embryo injection with one sgRNA targeting the second exon of EcMIH. The EcMIH–KO prawns exhibited increased the body length and shortened the metamorphosis time of larvae from mysis larva to postlarva. Meanwhile, EcMIH–KO did not cause the health problems such as early stage death or deformity. In conclusion, we successfully obtained EcMIH gene and generated EcMIH–KO prawns using CRISPR/Cas9 technology. This study will certainly lead to a wide	fish

170	animal (fish)	zebrafish	CRISPR;Cas9;	histamine receptor H3	Acta physiologica	Knockout of histamine receptor H3 alters adaptation to sudden darkness and monoamine levels in the zebrafish.	2018	222(3)	[Puttonen HAJ et al.]	University of Helsinki, Helsinki, Finland.	29044927	10.1111/apha.12981	AIM: Histamine receptor H3 (HRH3) has substantial neuropharmacological potential. Currently, knockout models of this receptor have been investigated only in mice. We characterized the expression of this receptor in the zebrafish and generated a zebrafish HRH3 knockout line. Using this model, we studied the role of HRH3 in important behaviours. We also analysed the effect of HRH3 knockout on monoaminergic systems, which has not been thoroughly studied in any animal model. METHODS: Generation of a mutant zebrafish line using the CRISPR-Cas9 system. Analysis of locomotor and social behaviour. Expression of HRH3 was characterized using in situ hybridization. Analysis of monoamine networks using HPLC, immunohistochemistry and quantitative PCR. RESULTS: We found that HRH3 knockout zebrafish larvae showed a shorter period of increased locomotion after a sudden onset of darkness, while the knockout larvae had a wild-type-like acute response to sudden darkness. Adult knockout fish showed decreased swimming velocity, although locomotor activity of knockout larvae was unaltered. Additionally, levels of dopamine and serotonin were significantly decreased in the knockout fish, while monoamine-related gene expression and immunohistochemistry patterns were unchanged. CONCLUSIONS: Our results show that HRH3 knockout larvae adapt faster to sudden darkness, suggesting a role for this receptor in regulating responses to changes in the environment. The decreased levels of dopamine and serotonin provide the first direct evidence that knockout of	fish
171	animal (fish)	zebrafish	CRISPR;Cas9;	exosc9	American journal of human genetics	Variants in EXOSC9 Disrupt the RNA Exosome and Result in Cerebellar Atrophy with Spinal Motor Neuronopathy.	2018	102(5):858-873	[Burns DT et al.]	Newcastle University, Newcastle upon Tyne, UK.	29727687	10.1016/j.ajhg.2018.03.011	The exosome is a conserved multi-protein complex that is essential for correct RNA processing. Recessive variants in exosome components EXOSC3, EXOSC8, and RBM7 cause various constellations of pontocerebellar hypoplasia (PCH), spinal muscular atrophy (SMA), and central nervous system demyelination. Here, we report on four unrelated affected individuals with recessive variants in EXOSC9 and the effect of the variants on the function of the RNA exosome in vitro in affected individuals' fibroblasts and skeletal muscle and in vivo in zebrafish. The clinical presentation was severe, early-onset, progressive SMA-like motor neuronopathy, cerebellar atrophy, and in one affected individual, congenital fractures of the long bones. Three affected individuals of different ethnicity carried the homozygous c.41T>C (p.Leu14Pro) variant, whereas one affected individual was compound heterozygous for c.41T>C (p.Leu14Pro) and c.481C>T (p.Arg161(*)). We detected reduced EXOSC9 in fibroblasts and skeletal muscle and observed a reduction of the whole multi-subunit exosome complex on blue-native polyacrylamide gel electrophoresis. RNA sequencing of fibroblasts and skeletal muscle detected significant >2-fold changes in genes involved in neuronal development and cerebellar and motor neuron degeneration, demonstrating the widespread effect of the variants. Morpholino oligonucleotide knockdown and CRISPR/Cas9-mediated mutagenesis of exosc9 in zebrafish recapitulated aspects of the human phenotype, as they have in other zebrafish models of exosomal disease. Specifically, portions of the cerebellum and hindbrain were absent, and motor neurons failed to develop and migrate properly. In summary, we show that variants in EXOSC9 result in a neurological syndrome combining cerebellar atrophy and spinal	fish
172	animal (fish)	zebrafish	CRISPR ;	tyrosine kinase containing immunoglobulin and epidermal growth factor homology 1 antisense	Arteriosclerosis, thrombosis, and vascular biology	Temporal and Spatial Post-Transcriptional Regulation of Zebrafish tie1 mRNA by Long Noncoding RNA During Brain Vascular Assembly.	2018	38(7):1562-1575	[Chowdhury TA et al.]	Massachusetts General Hospital, Charlestown. MA, USA.	29724820	10.1161/ATVBAHA.118.310848	OBJECTIVE: Tie1 (tyrosine kinase containing immunoglobulin and epidermal growth factor homology 1), an endothelial and hematopoietic cell-specific receptor tyrosine kinase, is an important regulator of angiogenesis and critical for maintaining vascular integrity. The post-transcriptional regulation of tie1 mRNA expression is not understood, but it might partly explain Tie1's differential expression pattern in endothelium. Following up on our previous work that identified natural antisense transcripts from the tie1 locus-tie1 antisense (tie1AS), which regulates tie1 mRNA levels in zebrafish-we attempted to identify the mechanism of this regulation. APPROACH AND RESULTS: Through in vitro and in vivo ribonucleoprotein binding studies, we demonstrated that tie1AS long noncoding RNA interacts with an RNA binding protein-embryonic lethal and abnormal vision Drosophila-like 1 (Elavl1)-that regulates tie1 mRNA levels. When we disrupted the interaction between tie1AS and Elavl1 by using constitutively active antisense morpholino oligonucleotides or photoactivatable morpholino oligonucleotides, tie1 mRNA levels increased between 26 and 31 hours post-fertilization, particularly in the head. This increase correlated with dilation of primordial midbrain channels, smaller eyes, and reduced ventricular space. We also observed these phenotypes when we used CRISPR (clustered regularly interspaced short palindromic repeats)-mediated CRISPRi (CRISPR-mediated interference) to knock down tie1AS. Treatment of the morpholino oligonucleotide-injected embryos with a small molecule that decreased tie1 mRNA levels rescued all 3 abnormal phenotypes. CONCLUSIONS: We identified a novel mode of temporal and	fish
173	animal (fish)	zebrafish	CRISPR;Cas9;	leucyl-tRNA synthetase (larsb)	Biochemical and biophysical research communications	Loss of Leucyl-tRNA synthetase b leads to ILFS1-like symptoms in zebrafish.	2018	505(2):378-384	[Wang Z et al.]	Southwest University, Beibei, Chongqing, China.	30262142	10.1016/j.bbrc.2018.09.133	Leucyl-tRNA synthetase (LARS) is a kind of aminoacyl-tRNA synthetases (aaRSs), which is important for protein synthesis. Following the discovery of three clinical cases which carry LARS mutations, it has been designated as the infantile liver failure syndrome type 1 (ILFS1) gene. ILFS1 is a kind of infantile hepatopathy, which is difficult to diagnose and manage. As the mechanism underlying this disease is poorly understood and LARS is conserved among vertebrates, we obtained zebrafish larsb(cq68) mutant via CRISPR/Cas9 technology to investigate the role of larsb in vivo. In mutant, the proliferation ability of liver was drastically decreased at later stages accompanied with severe DNA damage. Further studies demonstrated that the mTORC1 signaling was hyperactivated in larsb(cq68) mutant. Inhibition of mTORC1 signaling pathway by Rapamycin or mTORC1 morpholino can partially rescue the liver failure of the mutants. These data revealed that larsb mutation caused ILFS1-like phenotype in zebrafish, and indicated this mutant may serve as a potential model for ILFS1. Furthermore, we demonstrated that rapamycin treatment can partially rescue	fish

174	animal (fish)	zebrafish	CRISPR;Cas9;	ras related	Biochemical and biophysical research communications	Small GTPase R-Ras participates in neural tube formation in zebrafish embryonic spinal cord.	2018	501(3):786-790	[Ohata S et al.]	Musashino University, Tokyo, Japan.	29772239	10.1016/j.bbrc.2018.05.074	Ras related (R-Ras), a small GTPase, is involved in the maintenance of apico-basal polarity in neuroepithelial cells of the zebrafish hindbrain, axonal collapse in cultured murine hippocampal neurons, and maturation of blood vessels in adult mice. However, the role of R-Ras in neural tube formation remains unknown. Using antisense morpholino oligonucleotides (AMOs), we found that in the spinal cord of zebrafish embryos, the lumen was formed bilaterally in rras morphants, whereas it was formed at the midline in control embryos. As AMO can cause off-target effects, we generated rras mutant zebrafish lines using CRISPR/Cas9 technology. Although these rras mutant embryos did not have a bilateral lumen in the spinal cord, the following findings suggest that the phenotype is unlikely due to an off-target effect of rras AMO: 1) The rras morphant phenotype was rescued by an injection of AMO-resistant rras mRNA, and 2) a bilaterally segregated spinal cord was not observed in rras mutant embryos injected with rras AMO. The results suggest that the function of other ras family genes may be redundant in rras mutants. Previous research reported a bilaterally formed lumen in the spinal cord of zebrafish embryos with a mutation in a planar cell polarity (PCP) gene, van gogh-like 2 (vangl2). In the present study, in cultured cells, R-Ras was co-immunoprecipitated with Vangl2 but not with another PCP regulator, Pricke1. Interestingly, the interaction between R-Ras and Vangl2 was stronger in guanine-nucleotide free point mutants of R-Ras than in wild-type or constitutively active (GTP-bound) forms of R-Ras. R-Ras may regulate neural tube formation in cooperation	fish
175	animal (fish)	zebrafish	CRISPR;Cas9;	SET- and MYND domain containing protein 1 (smyd1a)	Biochemical and biophysical research communications	Loss of zebrafish Smyd1a interferes with myofibrillar integrity without triggering the misfolded myosin response.	2018	496(2):339-345	[Paone C et al.]	University of Ulm, Ulm, Germany.	29331378	10.1016/j.bbrc.2018.01.060	Sarcomeric protein turnover needs to be tightly balanced to assure proper assembly and renewal of sarcomeric units within muscle tissues. The mechanisms regulating these fundamental processes are only poorly understood, but of great clinical importance since many cardiac and skeletal muscle diseases are associated with defective sarcomeric organization. The SET- and MYND domain containing protein 1b (Smyd1b) is known to play a crucial role in myofibrillogenesis by functionally interacting with the myosin chaperones Unc45b and Hsp90alpha1. In zebrafish, Smyd1b, Unc45b and Hsp90alpha1 are part of the misfolded myosin response (MMR), a regulatory transcriptional response that is activated by disturbed myosin homeostasis. Genome duplication in zebrafish led to a second smyd1 gene, termed smyd1a. Morpholino- and CRISPR/Cas9-mediated knockdown of smyd1a led to significant perturbations in sarcomere structure resulting in decreased cardiac as well as skeletal muscle function. Similar to Smyd1b, we found Smyd1a to localize to the sarcomeric M-band in skeletal and cardiac muscles. Overexpression of smyd1a efficiently compensated for the loss of Smyd1b in flatline (fla) mutant zebrafish embryos, rescued the myopathic phenotype and suppressed the MMR in Smyd1b-deficient embryos, suggesting overlapping functions of both Smyd1 paralogs. Interestingly, Smyd1a is not transcriptionally activated in Smyd1b-deficient fla mutants, demonstrating lack of genetic compensation	fish
176	animal (fish)	zebrafish	CRISPR;	Niemann-Pick type C1	Biological chemistry	Model construction of Niemann-Pick type C disease in zebrafish.	2018	399(8):903-910	[Lin Y et al.]	Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.	29897878	10.1515/hsz-2018-0118	Niemann-Pick type C disease (NPC) is a rare human disease, with limited effective treatment options. Most cases of NPC disease are associated with inactivating mutations of the NPC1 gene. However, cellular and molecular mechanisms responsible for the NPC1 pathogenesis remain poorly defined. This is partly due to the lack of a suitable animal model to monitor the disease progression. In this study, we used CRISPR to construct an NPC1-/- zebrafish model, which faithfully reproduced the cardinal pathological features of this disease. In contrast to the wild type (WT), the deletion of NPC1 alone caused significant hepatosplenomegaly, ataxia, Purkinje cell death, increased lipid storage, infertility and reduced body length and life span. Most of the NPC1-/- zebrafish died within the first month post fertilization, while the remaining specimens developed slower than the WT and died before reaching 8 months of age. Filipin-stained hepatocytes of the NPC1-/- zebrafish were clear, indicating abnormal accumulation of unesterified cholesterol. Lipid profiling showed a significant difference between NPC1-/- and WT zebrafish. An obvious accumulation of seven sphingolipids was detected in livers of NPC1-/- zebrafish. In summary, our results provide a valuable model system that could identify promising therapeutic targets and	fish
177	animal (fish)	zebrafish	Cas9;	ERV	Biotechnology letters	Generation of Cas9 transgenic zebrafish and their application in establishing an ERV-deficient animal model.	2018	40(11-12):1507-1518	[Yang Z et al.]	Yangzhou University, Yangzhou, Jiangsu, China.	30244429	10.1007/s10529-018-2605-5	OBJECTIVES: To investigate the effect of endogenous Cas9 on genome editing efficiency in transgenic zebrafish. RESULTS: Here we have constructed a transgenic zebrafish strain that can be screened by pigment deficiency. Compared with the traditional CRISPR injection method, the transgenic zebrafish can improve the efficiency of genome editing significantly. At the same time, we first observed that the phenotype of vertebral malformation in early embryonic development of zebrafish after ZFERV knockout. CONCLUSIONS: The transgenic zebrafish with expressed Cas9, is more efficient in genome editing. And the results of ZFERV knockout indicated that ERV may affect the vertebral development by Notch1/Delta D signal pathway.	fish

178	animal (fish)	zebrafish	CRISPR;Cas9;	dual-specificity phosphatase (Dusp) family (dusp6, dusp2)	BMC developmental biology	A parental requirement for dual-specificity phosphatase 6 in zebrafish.	2018	18(1):6	[Maurer JM et al.]	University of Massachusetts Medical School, Worcester, MA, USA.	29544468	10.1186/s12861-018-0164-6	BACKGROUND: Signaling cascades, such as the extracellular signal-regulated kinase (ERK) pathway, play vital roles in early vertebrate development. Signals through these pathways are initiated by a growth factor or hormone, are transduced through a kinase cascade, and result in the expression of specific downstream genes that promote cellular proliferation, growth, or differentiation. Tight regulation of these signals is provided by positive or negative modulators at varying levels in the pathway, and is required for proper development and function. Two members of the dual-specificity phosphatase (Dusp) family, dusp6 and dusp2, are believed to be negative regulators of the ERK pathway and are expressed in both embryonic and adult zebrafish, but their specific roles in embryogenesis remain to be fully understood. RESULTS: Using CRISPR/Cas9 genome editing technology, we generated zebrafish lines harboring germ line deletions in dusp6 and dusp2. We do not detect any overt defects in dusp2 mutants, but we find that approximately 50% of offspring from homozygous dusp6 mutants do not proceed through embryonic development. These embryos are fertilized, but are unable to proceed past the first zygotic mitosis and stall at the 1-cell stage for several hours before dying by 10 h post fertilization. We demonstrate that dusp6 is expressed in gonads of both male and female zebrafish, suggesting that loss of dusp6 causes defects in germ cell production. Notably, the 50% of homozygous dusp6 mutants that complete the first cell division appear to progress through embryogenesis normally and give rise to fertile adults. CONCLUSIONS: The fact that offspring of homozygous dusp6 mutants stall prior to activation of the zygotic genome, suggests that loss of dusp6 affects gametogenesis and/or parentally-directed early development. Further, since only approximately 50% of homozygous dusp6 mutants are affected, we postulate that ERK signaling is tightly regulated and that dusp6 is required to keep ERK signaling within a range that is permissive for proper embryogenesis. Lastly, since dusp6 is expressed throughout zebrafish embryogenesis, but dusp6 mutants do not exhibit defects after the first cell division, it is possible that other regulators of the ERK	fish
179	animal (fish)	zebrafish	CRISPR;Cas9;	CreER(T2) (inserted);at otx2 locus	Cell and tissue research	Targeted knock-in of CreER (T2) in zebrafish using CRISPR/Cas9.	2018	372(1):41-50	[Kesavan G et al.]	Technische Universitat Dresden, Dresden, Germany.	29435650	10.1007/s00441-018-2798-x	New genome-editing approaches, such as the CRISPR/Cas system, have opened up great opportunities to insert or delete genes at targeted loci and have revolutionized genetics in model organisms like the zebrafish. The Cre-loxp recombination system is widely used to activate or inactivate genes with high spatial and temporal specificity. Using a CRISPR/Cas9-mediated knock-in strategy, we inserted a zebrafish codon-optimized CreER (T2) transgene at the otx2 gene locus to generate a conditional Cre-driver line. We chose otx2 as it is a patterning gene of the anterior neural plate that is expressed during early development. By knocking in CreER (T2) upstream of the endogenous ATG of otx2, we utilized this gene's native promoter and enhancer elements to perfectly match CreER (T2) and endogenous otx2 expression patterns. Next, by combining this novel driver line with a Cre-dependent reporter line, we show that only in the presence of tamoxifen can efficient Cre-loxp-mediated recombination be achieved in the anterior neural plate-derived tissues like the telencephalon, the eye and the optic tectum. Our results imply that the otx2:CreER (T2) transgenic fish will be a valuable tool for lineage tracing and conditional mutant studies in larval and adult	fish
180	animal (fish)	zebrafish	CRISPR;Cas9;	heat shock factor 5	Cell reports	Heat Shock Factor 5 Is Essential for Spermatogenesis in Zebrafish.	2018	25(12):3252-3261.e4	[Saju JM et al.]	Temasek Life Sciences Laboratory, Singapore, Singapore.	30566854	10.1016/j.celrep.2018.11.090	Heat shock factors (Hsfs) are transcription factors that regulate responses to heat shock and other environmental stimuli. Four heat shock factors (Hsf1-4) have been characterized from vertebrates to date. In addition to stress response, they also play important roles in development and gametogenesis. Here, we study the fifth member of heat shock factor family, Hsf5, using zebrafish as a model organism. Mutant hsf5(-/-) males, generated by CRISPR/Cas9 technique, were infertile with drastically reduced sperm count, increased sperm head size, and abnormal tail architecture, whereas females remained fertile. We show that Hsf5 is required for progression through meiotic prophase 1 during spermatogenesis as suggested by the accumulation of cells in the leptotene and zygotene-pachytene stages and increased apoptosis in post-meiotic cells. hsf5(-/-) mutants show gonadal misregulation of a substantial number of genes with roles in cell cycle, apoptosis, protein modifications, and signal transduction, indicating an important role of Hsf5 in early stages of spermatogenesis.	fish
181	animal (fish)	zebrafish	CRISPR;Cas9;	dopamine-beta-hydrolase	Cell reports	The Locus Coeruleus Modulates Intravenous General Anesthesia of Zebrafish via a Cooperative Mechanism.	2018	24(12):3146-3155.e3	[Du WJ et al.]	Zunyi Medical College, Zunyi, China.	30231998	10.1016/j.celrep.2018.08.046	How general anesthesia causes loss of consciousness has been a mystery for decades. It is generally thought that arousal-related brain nuclei, including the locus coeruleus (LC), are involved. Here, by monitoring locomotion behaviors and neural activities, we developed a larval zebrafish model for studying general anesthesia induced by propofol and etomidate, two commonly used intravenous anesthetics. Local lesion of LC neurons via two-photon laser-based ablation or genetic depletion of norepinephrine (NE; a neuromodulator released by LC neurons) via CRISPR/Cas9-based mutation of dopamine-beta-hydroxylase (dbh) accelerates induction into and retards emergence from general anesthesia. Mechanistically, in vivo whole-cell recording revealed that both anesthetics suppress LC neurons' activity through a cooperative mechanism, inhibiting presynaptic excitatory inputs and inducing GABAA receptor-mediated hyperpolarization of these neurons. Thus, our study indicates that the LC-NE system plays a modulatory role in both induction of and emergence from intravenous general	fish
182	animal (fish)	zebrafish	CRISPR;Cas9;	class II phosphatidylinositol transfer protein (PITP) family member (pitpnc1a)	Cell reports	Pitpnc1a Regulates Zebrafish Sleep and Wake Behavior through Modulation of Insulin-like Growth Factor Signaling.	2018	24(6):1389-1396	[Ashlin TG et al.]	University College London, London, UK.	30089250	10.1016/j.celrep.2018.07.012	The lipid transporters of the phosphatidylinositol transfer protein (PITP) family dictate phosphoinositide compartmentalization, and specific phosphoinositides play crucial roles in signaling cascades, membrane traffic, ion channel regulation, and actin dynamics. Although PITPs are enriched in the brain, their physiological functions in neuronal signaling pathways in vivo remain ill defined. We describe a CRISPR/Cas9-generated zebrafish mutant in a brain-specific, conserved class II PITP member, pitpnc1a. Zebrafish pitpnc1a mutants are healthy but display widespread aberrant neuronal activity and increased wakefulness across the day-night cycle. The loss of Pitpnc1a increases insulin-like growth factor (IGF) signaling in the brain, and inhibition of IGF pathways is sufficient to rescue both neuronal and behavioral hyperactivity in pitpnc1a mutants. We propose that Pitpnc1a-expressing neurons alter behavior via modification of neuro-modulatory IGF that acts on downstream wake-promoting	fish

183	animal (fish)	zebrafish	CRISPR;Cas9;	tbx5a; downstream elements	Cell reports	Genomic Knockout of Two Presumed Forelimb Tbx5 Enhancers Reveals They Are Nonessential for Limb Development.	2018	23(11):3146-3151	[Cunningham TJ et al.]	Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA.	29898387	10.1016/j.celrep.2018.05.052	A standard approach in the identification of transcriptional enhancers is the use of transgenic animals carrying DNA elements joined to reporter genes inserted randomly in the genome. We examined elements near Tbx5, a gene required for forelimb development in humans and other vertebrates. Previous transgenic studies reported a mammalian Tbx5 forelimb enhancer located in intron 2 containing a putative retinoic acid response element and a zebrafish tbx5a forelimb (pectoral fin) enhancer located downstream that is conserved from fish to mammals. We used CRISPR/Cas9 gene editing to knockout the endogenous elements and unexpectedly found that deletion of the intron 2 and downstream elements, either singly or together in double knockouts, resulted in no effect on forelimb development. Our findings show that reporter transgenes may not identify endogenous enhancers and that in vivo genetic loss-of-function studies are required, such as CRISPR/Cas9, which is similar in effort to production of animals carrying reporter transgenes.	fish
184	animal (fish)	zebrafish	CRISPR;Cas9;	runx2	Cells	New Insights into the Runt Domain of RUNX2 in Melanoma Cell Proliferation and Migration.	2018	7(11)	[Deiana M et al.]	University of Verona, Verona, Italy.	30463392	10.3390/cells7110220	The mortality rate for malignant melanoma (MM) is very high, since it is highly invasive and resistant to chemotherapeutic treatments. The modulation of some transcription factors affects cellular processes in MM. In particular, a higher expression of the osteogenic master gene RUNX2 has been reported in melanoma cells, compared to normal melanocytes. By analyzing public databases for recurrent RUNX2 genetic and epigenetic modifications in melanoma, we found that the most common RUNX2 genetic alteration that exists in transcription upregulation is, followed by genomic amplification, nucleotide substitution and multiple changes. Additionally, altered RUNX2 is involved in unchecked pathways promoting tumor progression, Epithelial Mesenchymal Transition (EMT), and metastasis. In order to investigate further the role of RUNX2 in melanoma development and to identify a therapeutic target, we applied the CRISPR/Cas9 technique to explore the role of the RUNT domain of RUNX2 in a melanoma cell line. RUNT-deleted cells showed reduced proliferation, increased apoptosis, and reduced EMT features, suggesting the involvement of the RUNT domain in different pathways. In addition, del-RUNT cells showed a downregulation of genes involved in migration ability. In an in vivo zebrafish model, we observed that wild-type melanoma cells migrated in 81% of transplanted fishes, while del-RUNT cells migrated in 58%. All these findings strongly suggest the involvement of the RUNT domain in melanoma metastasis.	fish
185	animal (fish)	zebrafish	CRISPR;Cas9;		Chemical communications	A G-quadruplex motif at the 3' end of sgRNAs improves CRISPR-Cas9 based genome editing efficiency.	2018	54(19):2377-2380	[Nahar S et al.]	Academy of Scientific & Innovative Research, New Delhi, India.	29450416	10.1039/c7cc08893k	Originating as a component of prokaryotic adaptive immunity, the type II CRISPR/Cas9 system has been repurposed for targeted genome editing in various organisms. Although Cas9 can bind and cleave DNA efficiently under in vitro conditions, its activity inside a cell can vary dramatically between targets owing to the differences between genomic loci and the availability of enough Cas9/sgRNA (single guide RNA) complex molecules for cleavage. Most methods have so far relied on Cas9 protein engineering or base modifications in the sgRNA sequence to improve CRISPR/Cas9 activity. Here we demonstrate that a structure based rational design of sgRNAs can enhance the efficiency of Cas9 cleavage in vivo. By appending a naturally forming RNA G-quadruplex motif to the 3' end of sgRNAs we can improve its stability and target cleavage efficiency in zebrafish embryos without inducing off-target activity, thereby underscoring its value in the design of better and optimized genome editing triggers.	fish
186	animal (fish)	zebrafish	CRISPR;Cas9;	tbx20	Circulation	TBX20 Regulates Angiogenesis Through the Prokineticin 2-Prokineticin Receptor 1 Pathway.	2018	138(9):913-928	[Meng S et al.]	Houston Methodist Research Institute, TX, USA.	29545372	10.1161/CIRCULATIONAHA.118.033939	BACKGROUND: Angiogenesis is integral for embryogenesis, and targeting angiogenesis improves the outcome of many pathological conditions in patients. TBX20 is a crucial transcription factor for embryonic development, and its deficiency is associated with congenital heart disease. However, the role of TBX20 in angiogenesis has not been described. METHODS: Loss- and gain-of-function approaches were used to explore the role of TBX20 in angiogenesis both in vitro and in vivo. Angiogenesis gene array was used to identify key downstream targets of TBX20. RESULTS: Unbiased gene array survey showed that TBX20 knockdown profoundly reduced angiogenesis-associated PROK2 (prokineticin 2) gene expression. Indeed, loss of TBX20 hindered endothelial cell migration and in vitro angiogenesis. In a murine angiogenesis model using subcutaneously implanted Matrigel plugs, we observed that TBX20 deficiency markedly reduced PROK2 expression and restricted intraplug angiogenesis. Furthermore, recombinant PROK2 administration enhanced angiogenesis and blood flow recovery in murine hind-limb ischemia. In zebrafish, transient knockdown of tbx20 by morpholino antisense oligos or genetic disruption of tbx20 by CRISPR/Cas9 impaired angiogenesis. Furthermore, loss of prok2 or its cognate receptor prokr1a also limited angiogenesis. In contrast, overexpression of prok2 or prokr1a rescued the impaired angiogenesis in tbx20-deficient animals. CONCLUSIONS: Our study identifies TBX20 as a novel transcription factor regulating angiogenesis through the PROK2-PROKR1 (prokineticin receptor 1) pathway in both development and disease and reveals a novel mode of angiogenic regulation whereby the TBX20-PROK2-PROKR1 signaling cascade may act as a "biological capacitor" to relay and sustain the proangiogenic effect of vascular endothelial growth factor. This pathway may be a	fish
187	animal (fish)	zebrafish	CRISPR;Cas9;	nodal modulator (nomo)	Current molecular medicine	Loss of the Nodal modulator Nomo results in chondrodysplasia in zebrafish.	2018	18(7):448-458	[Cao L et al.]	Hunan Normal University, Changsha, Hunan, China.	30539698	10.2174/1566524019666181212095307	BACKGROUND: Transforming growth factor-beta (TGF-beta)/nodal signaling is involved in early embryonic patterning in vertebrates. Nodal modulator (Nomo, also called pM5) is a negative regulator of nodal signaling. Currently, the role of nomo gene in cartilage development in vertebrates remains unknown. METHODS: Nomo mutants were generated in a knockout model of zebrafish by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) targeting of the fibronectin type III domain. The expression of related genes, which are critical for chondrogenesis, was analyzed by whole-mount in situ hybridization and qRT-PCR. Whole-mount alcian staining was performed to analyze the cartilage structure. RESULTS: nomo is highly expressed in various tissues including the cartilage. We successfully constructed a zebrafish nomo knockout model. nomo homozygous mutants exhibited varying degrees of hypoplasia and dysmorphism on 4 and 5 dpf, which is similar to chondrodysplasia in humans. The key genes of cartilage and skeletal development, including sox9a, sox9b, dlx1a, dlx2a, osx, col10a1, and col11a2 were all downregulated in nomo mutants compared with the wildtype. CONCLUSION: The nomo gene positively regulates the expression of the master regulator and other key development genes involved in bone formation and cartilage	fish

188	animal (fish)	zebrafish	CRISPR;Cas9;	spastin	Development	BMP- and neuropilin 1-mediated motor axon navigation relies on spastin alternative translation.	2018	145(17)	[Jardin N et al.]	Sorbonne Universites, UPMC Universite Paris 06, INSERM, CNRS, Paris, France.	30082270	10.1242/dev.162701	Functional analyses of genes responsible for neurodegenerative disorders have unveiled crucial links between neurodegenerative processes and key developmental signalling pathways. Mutations in SPG4-encoding spastin cause hereditary spastic paraplegia (HSP). Spastin is involved in diverse cellular processes that couple microtubule severing to membrane remodelling. Two main spastin isoforms are synthesised from alternative translational start sites (M1 and M87). However, their specific roles in neuronal development and homeostasis remain largely unknown. To selectively unravel their neuronal function, we blocked spastin synthesis from each initiation codon during zebrafish development and performed rescue analyses. The knockdown of each isoform led to different motor neuron and locomotion defects, which were not rescued by the selective expression of the other isoform. Notably, both morphant neuronal phenotypes were observed in a CRISPR/Cas9 spastin mutant. We next showed that M1 spastin, together with HSP proteins atlastin 1 and NIPA1, drives motor axon targeting by repressing BMP signalling, whereas M87 spastin acts downstream of neuropilin 1 to control motor neuron migration. Our data therefore suggest that defective BMP and neuropilin 1 signalling may contribute to the motor	fish
189	animal (fish)	zebrafish	CRISPR;TALENs;	pcdh10a; pcdh10b	Developmental biology	Requirement of zebrafish pcdh10a and pcdh10b in melanocyte precursor migration.	2018	444 Suppl 1:S274-S286	[Williams JS et al.]	University of Colorado Anschutz Medical Campus, Aurora, CO, USA.	29604249	10.1016/j.ydbio.2018.03.022	Melanocytes derive from neural crest cells, which are a highly migratory population of cells that play an important role in pigmentation of the skin and epidermal appendages. In most vertebrates, melanocyte precursor cells migrate solely along the dorsolateral pathway to populate the skin. However, zebrafish melanocyte precursors also migrate along the ventromedial pathway, in route to the yolk, where they interact with other neural crest derivative populations. Here, we demonstrate the requirement for zebrafish paralogs pcdh10a and pcdh10b in zebrafish melanocyte precursor migration. pcdh10a and pcdh10b are expressed in a subset of melanocyte precursor and somatic cells respectively, and knockdown and TALEN mediated gene disruption of pcdh10a results in aberrant migration of melanocyte precursors resulting in fully melanized melanocytes that differentiate precociously in the ventromedial pathway. Live cell imaging analysis demonstrates that loss of pchd10a results in a reduction of directed cell migration of melanocyte precursors, caused by both increased adhesion and a loss of cell-cell contact with other migratory neural crest cells. Also, we determined that the paralog pcdh10b is upregulated and can compensate for the genetic loss of pcdh10a. Disruption of pcdh10b alone by CRISPR mutagenesis results in somite defects, while the loss of both paralogs results in enhanced migratory melanocyte precursor phenotype and embryonic lethality. These results reveal a novel role for pcdh10a and pcdh10b in zebrafish melanocyte precursor migration and suggest that pcdh10 paralogs potentially interact for proper transient migration along the ventromedial pathway.	fish
190	animal (fish)	zebrafish	TALENs;	wnt8a	Developmental biology	Roles of maternal wnt8a transcripts in axis formation in zebrafish.	2018	434(1):96-107	[Hino H et al.]	Nagoya University, Nagoya, Aichi, Japan.	29208373	10.1016/j.ydbio.2017.11.016	In early zebrafish development, the program for dorsal axis formation begins soon after fertilization. Previous studies suggested that dorsal determinants (DDs) localize to the vegetal pole, and are transported to the dorsal blastomeres in a microtubule-dependent manner. The DDs activate the canonical Wnt pathway and induce dorsal-specific genes that are required for dorsal axis formation. Among wnt-family genes, only the wnt8a mRNA is reported to localize to the vegetal pole in oocytes and to induce the dorsal axis, suggesting that Wnt8a is a candidate DD. Here, to reveal the roles of maternal wnt8a, we generated wnt8a mutants by transcription activator-like effector nucleases (TALENs), and established zygotic, maternal, and maternal zygotic wnt8a mutants by germ-line replacement. Zebrafish wnt8a has two open reading frames (ORF1 and ORF2) that are tandemly located in the genome. Although the zygotic ORF1 or ORF2 wnt8a mutants showed little or no axis-formation defects, the ORF1/2 compound mutants showed antero-dorsalized phenotypes, indicating that ORF1 and ORF2 have redundant roles in ventrolateral and posterior tissue formation. Unexpectedly, the maternal wnt8a ORF1/2 mutants showed no axis-formation defects. The maternal-zygotic wnt8a ORF1/2 mutants showed more severe antero-dorsalized phenotypes than the zygotic mutants. These results indicated that maternal wnt8a is dispensable for the initial dorsal determination, but cooperates with zygotic wnt8a for ventrolateral and posterior tissue formation. Finally, we re-examined the maternal wnt	fish
191	animal (fish)	zebrafish	CRISPR;Cas9;	zbtb16a etc.	Developmental cell	A Rapid Method for Directed Gene Knockout for Screening in G0 Zebrafish.	2018	46(1):112-125.e4	[Wu RS et al.]	University of California, San Francisco, San Francisco, CA, USA.	29974860	10.1016/j.devcel.2018.06.003	Zebrafish is a powerful model for forward genetics. Reverse genetic approaches are limited by the time required to generate stable mutant lines. We describe a system for gene knockout that consistently produces null phenotypes in G0 zebrafish. Yolk injection of sets of four CRISPR/Cas9 ribonucleoprotein complexes redundantly targeting a single gene recapitulated germline-transmitted knockout phenotypes in >90% of G0 embryos for each of 8 test genes. Early embryonic (6 hpf) and stable adult phenotypes were produced. Simultaneous multi-gene knockout was feasible but associated with toxicity in some cases. To facilitate use, we generated a lookup table of four-guide sets for 21,386 zebrafish genes and validated several. Using this resource, we targeted 50 cardiomyocyte transcriptional regulators and uncovered a role of zbtb16a in cardiac development. This system provides a platform for rapid screening of genes of interest in development, physiology, and disease models in	fish
192	animal (fish)	zebrafish	CRISPR;Cas9;	rfx4	Developmental dynamics	Zebrafish Rfx4 controls dorsal and ventral midline formation in the neural tube.	2018	247(4):650-659	[Sedykh I et al.]	University of Wisconsin, Madison, WI, USA.	29243319	10.1002/dvdy.24613	BACKGROUND: Rfx winged-helix transcription factors, best known as key regulators of core ciliogenesis, also play ciliogenesis-independent roles during neural development. Mammalian Rfx4 controls neural tube morphogenesis via both mechanisms. RESULTS: We set out to identify conserved aspects of rfx4 gene function during vertebrate development and to establish a new genetic model in which to analyze these mechanisms further. To this end, we have generated frame-shift alleles in the zebrafish rfx4 locus using CRISPR/Cas9 mutagenesis. Using RNAseq-based transcriptome analysis, in situ hybridization and immunostaining we identified a requirement for zebrafish rfx4 in the forming midlines of the caudal neural tube. These functions are mediated, least in part, through transcriptional regulation of several zic genes in the dorsal hindbrain and of foxa2 in the ventral hindbrain and spinal cord (floor plate). CONCLUSIONS: The midline patterning functions of rfx4 are conserved, because rfx4 regulates transcription of foxa2 and zic2 in zebrafish and in mouse. In contrast, zebrafish rfx4 function is dispensable for forebrain morphogenesis, while mouse rfx4 is required for normal formation of forebrain ventricles in a ciliogenesis-dependent manner. Collectively, this report identifies conserved aspects of rfx4 function and establishes a robust new genetic model for in-depth dissection of these mechanisms.	fish

193	animal (fish)	zebrafish	CRISPR;Cas9;	pore-forming subunits of an ATP-sensitive potassium channel (Kir6.1, KCNJ8); regulatory subunits of an ATP-sensitive potassium channel (SUR2, ABCC9)	Disease models & mechanisms	Effective CRISPR/Cas9-based nucleotide editing in zebrafish to model human genetic cardiovascular disorders.	2018	11(10)	[Tessadori F et al.]	Hubrecht Institute-KNAW and UMC Utrecht, Utrecht, the Netherlands.	30355756	10.1242/dmm.035469	The zebrafish (Danio rerio) has become a popular vertebrate model organism to study organ formation and function due to its optical clarity and rapid embryonic development. The use of genetically modified zebrafish has also allowed identification of new putative therapeutic drugs. So far, most studies have relied on broad overexpression of transgenes harboring patient-derived mutations or loss-of-function mutants, which incompletely model the human disease allele in terms of expression levels or cell-type specificity of the endogenous gene of interest. Most human genetically inherited conditions are caused by alleles carrying single nucleotide changes resulting in altered gene function. Introduction of such point mutations in the zebrafish genome would be a prerequisite to recapitulate human disease but remains challenging to this day. We present an effective approach to introduce small nucleotide changes in the zebrafish genome. We generated four different knock-in lines carrying distinct human cardiovascular-disorder-causing missense mutations in their zebrafish orthologous genes by combining CRISPR/Cas9 with a short template oligonucleotide. Three of these lines carry gain-of-function mutations in genes encoding the pore-forming (Kir6.1, KCNJ8) and regulatory (SUR2, ABCC9) subunits of an ATP-sensitive potassium channel (KATP) linked to Cantu syndrome (CS). Our heterozygous zebrafish knock-in lines display significantly enlarged ventricles with enhanced cardiac output and contractile function, and distinct cerebral vasodilation, demonstrating the causality of the introduced mutations for CS. These results demonstrate that introducing patient alleles in their zebrafish orthologs promises a broad application for modeling human	fish
194	animal (fish)	zebrafish	CRISPR;Cas9;	pbx3	Disease models & mechanisms	Functional testing of a human PBX3 variant in zebrafish reveals a potential modifier role in congenital heart defects.	2018	11(10)	[Farr GH 3rd et al.]	Seattle Children's Research Institute, Seattle, WA, USA.	30355621	10.1242/dmm.035972	Whole-genome and exome sequencing efforts are increasingly identifying candidate genetic variants associated with human disease. However, predicting and testing the pathogenicity of a genetic variant remains challenging. Genome editing allows for the rigorous functional testing of human genetic variants in animal models. Congenital heart defects (CHDs) are a prominent example of a human disorder with complex genetics. An inherited sequence variant in the human PBX3 gene (PBX3 p.A136V) has previously been shown to be enriched in a CHD patient cohort, indicating that the PBX3 p.A136V variant could be a modifier allele for CHDs. Pbx genes encode three-amino-acid loop extension (TALE)-class homeodomain-containing DNA-binding proteins with diverse roles in development and disease, and are required for heart development in mouse and zebrafish. Here, we used CRISPR-Cas9 genome editing to directly test whether this Pbx gene variant acts as a genetic modifier in zebrafish heart development. We used a single-stranded oligodeoxynucleotide to precisely introduce the human PBX3 p.A136V variant in the homologous zebrafish pbx4 gene (pbx4 p.A131V). We observed that zebrafish that are homozygous for pbx4 p.A131V are viable as adults. However, the pbx4 p.A131V variant enhances the embryonic cardiac morphogenesis phenotype caused by loss of the known cardiac specification factor, Hand2. Our study is the first example of using precision genome editing in zebrafish to demonstrate a function for a human disease-associated single nucleotide variant of unknown significance. Our work underscores the importance of testing the roles of inherited variants, not just de novo variants, as genetic modifiers of CHDs. Our study provides a novel approach toward advancing our understanding of the complex genetics of CHDs.	fish
195	animal (fish)	zebrafish	CRISPR;Cas9;		Disease models & mechanisms	CRISPR/Cas9-mediated homology-directed repair by ssODNs in zebrafish induces complex mutational patterns resulting from genomic integration of repair-template fragments.	2018	11(10)	[Boel A et al.]	Ghent University, Ghent, Belgium.	30355591	10.1242/dmm.035352	Targeted genome editing by CRISPR/Cas9 is extremely well fitted to generate gene disruptions, although precise sequence replacement by CRISPR/Cas9-mediated homology-directed repair (HDR) suffers from low efficiency, impeding its use for high-throughput knock-in disease modeling. In this study, we used next-generation sequencing (NGS) analysis to determine the efficiency and reliability of CRISPR/Cas9-mediated HDR using several types of single-stranded oligodeoxynucleotide (ssODN) repair templates for the introduction of disease-relevant point mutations in the zebrafish genome. Our results suggest that HDR rates are strongly determined by repair-template composition, with the most influential factor being homology-arm length. However, we found that repair using ssODNs does not only lead to precise sequence replacement but also induces integration of repair-template fragments at the Cas9 cut site. We observed that error-free repair occurs at a relatively constant rate of 1-4% when using different repair templates, which was sufficient for transmission of point mutations to the F1 generation. On the other hand, erroneous repair mainly accounts for the variability in repair rate between the different repair templates. To further improve error-free HDR rates, elucidating the mechanism behind this erroneous repair is essential. We show that the error-prone nature of ssODN-mediated repair, believed to act via synthesis-dependent strand annealing (SDSA), is most likely due to DNA synthesis errors. In conclusion, caution is warranted when using ssODNs for the generation of knock-in models or for therapeutic applications. We recommend the application of in-depth NGS analysis to examine both the efficiency and error-free nature of HDR events.This article has an associated First Person	fish
196	animal (fish)	zebrafish	CRISPR;Cas9;	Rb1	Disease models & mechanisms	Cancer modeling by Transgene Electroporation in Adult Zebrafish (TEAZ).	2018	11(9)	[Callahan SJ et al.]	Memorial Sloan Kettering Cancer Center, New York, NY, USA.	30061297	10.1242/dmm.034561	Transgenic animals are invaluable for modeling cancer genomics, but often require complex crosses of multiple germline alleles to obtain the desired combinations. Zebrafish models have advantages in that transgenes can be rapidly tested by mosaic expression, but typically lack spatial and temporal control of tumor onset, which limits their utility for the study of tumor progression and metastasis. To overcome these limitations, we have developed a method referred to as Transgene Electroporation in Adult Zebrafish (TEAZ). TEAZ can deliver DNA constructs with promoter elements of interest to drive fluorophores, oncogenes or CRISPR-Cas9-based mutagenic cassettes in specific cell types. Using TEAZ, we created a highly aggressive melanoma model via Cas9-mediated inactivation of Rb1 in the context of BRAF(V600E) in spatially constrained melanocytes. Unlike prior models that take approximately 4 months to develop, we found that TEAZ leads to tumor onset in approximately 7 weeks, and these tumors develop in fully immunocompetent animals. As the resulting tumors initiated at highly defined locations, we could track their progression via fluorescence, and documented deep invasion into tissues and metastatic deposits. TEAZ can be deployed to other tissues and cell types, such as the heart, with the use of suitable transgenic promoters. The versatility of TEAZ makes it widely accessible for rapid modeling of somatic gene alterations and cancer progression at a scale not achievable in other in	fish

197	animal (fish)	zebrafish	CRISPR;Cas9;	Niemann-Pick disease type C1 (NPC1)	Disease models & mechanisms	Modeling Niemann-Pick disease type C1 in zebrafish: a robust platform for in vivo screening of candidate therapeutic compounds.	2018	11(9)	[Tseng WC et al.]	National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA.	30135069	10.1242/dmm.034165	Niemann-Pick disease type C1 (NPC1) is a rare autosomal recessive lysosomal storage disease primarily caused by mutations in NPC1 NPC1 is characterized by abnormal accumulation of unesterified cholesterol and glycolipids in late endosomes and lysosomes. Common signs include neonatal jaundice, hepatosplenomegaly, cerebellar ataxia, seizures and cognitive decline. Both mouse and feline models of NPC1 mimic the disease progression in humans and have been used in preclinical studies of 2-hydroxypropyl-beta-cyclodextrin (2HPbetaCD; VTS-270), a drug that appeared to slow neurological progression in a Phase 1/2 clinical trial. However, there remains a need to identify additional therapeutic agents. High-throughput drug screens have been useful in identifying potential therapeutic compounds; however, current preclinical testing is time and labor intensive. Thus, development of a high-capacity in vivo platform suitable for screening candidate drugs/compounds would be valuable for compound optimization and prioritizing subsequent in vivo testing. Here, we generated and characterize two zebrafish npc1-null mutants using CRISPR/Cas9-mediated gene targeting. The npc1 mutants model both the early liver and later neurological disease phenotypes of NPC1. LysoTracker staining of npc1 mutant larvae was notable for intense staining of lateral line neuromasts, thus providing a robust in vivo screen for lysosomal storage. As a proof of principle, we were able to show that treatment of the npc1 mutant larvae with 2HPbetaCD significantly reduced neuromast LysoTracker staining. These data demonstrate the potential value of using this zebrafish NPC1 model for efficient and rapid in vivo optimization and screening of potential therapeutic compounds.This article has an associated First Person interview with the first author of the paper.	fish
198	animal (fish)	zebrafish	CRISPR;Cas9;	kyphoscoliosis peptidase	Disease models & mechanisms	Transcriptional upregulation of Bag3, a chaperone-assisted selective autophagy factor, in animal models of KY-deficient hereditary myopathy.	2018	11(7)	[Jokl EJ et al.]	University of York, York, UK.	29914939	10.1242/dmm.033225	The importance of kyphoscoliosis peptidase (KY) in skeletal muscle physiology has recently been emphasised by the identification of novel human myopathies associated with KY deficiency. Neither the pathogenic mechanism of KY deficiency nor a specific role for KY in muscle function have been established. However, aberrant localisation of filamin C (FLNC) in muscle fibres has been shown in humans and mice with loss-of-function mutations in the KY gene. FLNC turnover has been proposed to be controlled by chaperone-assisted selective autophagy (CASA), a client-specific and tension-induced pathway that is required for muscle maintenance. Here, we have generated new C2C12 myoblast and zebrafish models of KY deficiency by CRISPR/Cas9 mutagenesis. To obtain insights into the pathogenic mechanism caused by KY deficiency, expression of the co-chaperone BAG3 and other CASA factors was analyzed in the cellular, zebrafish and ky/ky mouse models. Ky-deficient C2C12-derived clones show trends of higher transcription of CASA factors in differentiated myotubes. The ky-deficient zebrafish model (ky(yo1)/ky(yo1)) lacks overt signs of pathology, but shows significantly increased bag3 and flnca/b expression in embryos and adult muscle. Additionally, ky(yo1)/ky(yo1) embryos challenged by swimming in viscous media show an inability to further increase expression of these factors in contrast with wild-type controls. The ky/ky mouse shows elevated expression of Bag3 in the non-pathological exterior digitorum longus (EDL) and evidence of impaired BAG3 turnover in the pathological soleus. Thus, upregulation of CASA factors appears to be	fish
199	animal (fish)	zebrafish	CRISPR;	histone-H2A.F/Z-GFP fusion protein	Disease models & mechanisms	Epigenetic regulators Rbbp4 and Hdac1 are overexpressed in a zebrafish model of RB1 embryonal brain tumor, and are required for neural progenitor survival and proliferation.	2018	11(6)	[Schultz LE et al.]	Iowa State University, Ames, IA, USA.	29914980	10.1242/dmm.034124	In this study, we used comparative genomics and developmental genetics to identify epigenetic regulators driving oncogenesis in a zebrafish retinoblastoma 1 (rb1) somatic-targeting model of RB1 mutant embryonal brain tumors. Zebrafish rb1 brain tumors caused by TALEN or CRISPR targeting are histologically similar to human central nervous system primitive neuroectodermal tumors (CNS-PNETs). Like the human oligoneural OLIG2+/SOX10+ CNS-PNET subtype, zebrafish rb1 tumors show elevated expression of neural progenitor transcription factors olig2, sox10, sox8b and the receptor tyrosine kinase erbb3a oncogene. Comparison of rb1 tumor and rb1/rb1 germline mutant larval transcriptomes shows that the altered oligoneural precursor signature is specific to tumor tissue. More than 170 chromatin regulators were differentially expressed in rb1 tumors, including overexpression of chromatin remodeler components histone deacetylase 1 (hdac1) and retinoblastoma binding protein 4 (rbbp4). Germline mutant analysis confirms that zebrafish rb1, rbbp4 and hdac1 are required during brain development. rb1 is necessary for neural precursor cell cycle exit and terminal differentiation, rbbp4 is required for survival of postmitotic precursors, and hdac1 maintains proliferation of the neural stem cell/progenitor pool. We present an in vivo assay using somatic CRISPR targeting plus live imaging of histone-H2A.F/Z-GFP fusion protein in developing larval brain to rapidly test the role of chromatin remodelers in neural stem and progenitor cells. Our somatic assay recapitulates germline mutant phenotypes and reveals a dynamic view of their roles in neural cell populations. Our study provides new insight into the epigenetic processes that might drive pathogenesis in RB1 brain tumors, and identifies Rbbp4 and its associated chromatin remodeling complexes as potential target pathways to induce apoptosis in RB1 mutant brain cancer cells.This article has an associated First Person interview with the first author	fish
200	animal (fish)	zebrafish	CRISPR;Cas9;	mitochondrial DNA polymerase (polg); sod1	Disease models & mechanisms	Neutrophil-specific knockout demonstrates a role for mitochondria in regulating neutrophil motility in zebrafish.	2018	11(3)	[Zhou W et al.]	Purdue University, West Lafayette, IN, USA.	29590639	10.1242/dmm.033027	Neutrophils are fast-moving cells essential for host immune functions. Although they primarily rely on glycolysis for ATP, isolated primary human neutrophils depend on mitochondrial membrane potential for chemotaxis. However, it is not known whether mitochondria regulate neutrophil motility in vivo, and the underlying molecular mechanisms remain obscure. Here, we visualized mitochondria in an interconnected network that localizes to the front and rear of migrating neutrophils using a novel transgenic zebrafish line. To disrupt mitochondrial function genetically, we established a gateway system harboring the CRISPR/Cas9 elements for tissue-specific knockout. In a transgenic line, neutrophil-specific disruption of mitochondrial DNA polymerase, polg, significantly reduced the velocity of neutrophil interstitial migration. In addition, inhibiting the mitochondrial electron transport chain or the enzymes that reduce mitochondrial reactive oxygen species also inhibited neutrophil motility. The reduced cell motility that resulted from neutrophil-specific knockout of sod1 was rescued with sod1 mRNA overexpression, or by treating with scavengers of reactive oxygen species. Together, our work has provided the first in vivo evidence that mitochondria regulate neutrophil motility, as well as tools for the functional characterization of mitochondria-related genes in neutrophils and insights into immune deficiency seen in patients with primary mitochondrial disorders.This article has an associated First Person interview	fish

201	animal (fish)	zebrafish	CRISPR;Cas9;	oocyte-specific transcription factor (figla)	Endocrinology	Roles of Figla/figla in Juvenile Ovary Development and Follicle Formation During Zebrafish Gonadogenesis.	2018	159(11):3699-3722	[Qin M et al.]	University of Macau, Macau, China.	30184072	10.1210/en.2018-00648	Sex determination and differentiation are complex processes. As a juvenile hermaphrodite or undifferentiated gonochorist, zebrafish undergo a special juvenile ovarian phase during sex differentiation, making it an excellent model for studying early oogenesis and folliculogenesis. We provide lines of evidence at morphological, molecular, and genetic levels for roles of factor in the germline alpha (Figla), an oocyte-specific transcription factor, in early zebrafish gonadogenesis. As in mammals, Figla/figla was also expressed in the gonads and its expression in the ovary was also restricted to early oocytes. Disruption of figla gene by CRISPR/Cas9 led to an all-male phenotype in the mutant. Detailed analysis of early gonadal development showed that the germ cells in the mutant were clustered in cysts and underwent meiosis, forming oocytes at prefollicular chromatin nucleolar (CN) stage (stage IA). However, the subsequent transition from cystic CN oocytes to individual follicular perinucleolar oocytes (stage IB) was blocked, resulting in an all-male phenotype in the mutant. The phenotype of figla mutant could not be rescued by estrogen treatment, in contrast to cyp19a1a mutant, and introduction of tp53 mutation also had no effect, unlike in fancd1 and fancl mutants. Transcriptome analysis revealed that many biological processes and pathways related to germ cell development, especially oogenesis, were upregulated in the presence of Figla and that the regulation of figla expression may involve heat shock proteins. Our results strongly suggest important roles for Figla in	fish
202	animal (fish)	zebrafish	CRISPR;	NMDA receptor (NMDAR) NR1.1 subunit (grin1a)	eNeuro	The Midline Axon Crossing Decision Is Regulated through an Activity-Dependent Mechanism by the NMDA Receptor.	2018	5(2)	[Gao J et al.]	University of Utah School of Medicine Salt Lake City, UT, USA.	29766040	10.1523/ENEURO.0389-17.2018	Axon guidance in vertebrates is controlled by genetic cascades as well as by intrinsic activity-dependent refinement of connections. Midline axon crossing is one of the best studied pathfinding models and is fundamental to the establishment of bilaterally symmetric nervous systems. However, it is not known whether crossing requires intrinsic activity in axons, and what controls that activity. Further, a mechanism linking neuronal activity and gene expression has not been identified for axon pathfinding. Using embryonic zebrafish, we found that the NMDA receptor (NMDAR) NR1.1 subunit (grin1a) is expressed in commissural axons. Pharmacological inhibition of grin1a, hypoxia exposure reduction of grin1a expression, or CRISPR knock-down of grin1a leads to defects in midline crossing. Inhibition of neuronal activity phenocopies the effects of grin1a loss on midline crossing. By combining pharmacological inhibition of the NMDAR with optogenetic stimulation to precisely restore neuronal activity, we observed rescue of midline crossing. This suggests that the NMDAR controls pathfinding by an activity-dependent mechanism. We further show that the NMDAR may act, via modulating activity, on the transcription factor arxa (mammalian Arx), a known regulator of midline pathfinding. These findings uncover a novel role for the NMDAR in controlling activity to regulate commissural pathfinding and identify arxa as	fish
203	animal (fish)	zebrafish	CRISPR;Cas9;	ush2a	Experimental eye research	Usherin defects lead to early-onset retinal dysfunction in zebrafish.	2018	173:148-159	[Dona M et al.]	Radboud University Medical Center, Nijmegen, The Netherlands.	29777677	10.1016/j.exer.2018.05.015	Mutations in USH2A are the most frequent cause of Usher syndrome and autosomal recessive nonsyndromic retinitis pigmentosa. To unravel the pathogenic mechanisms underlying USH2A-associated retinal degeneration and to evaluate future therapeutic strategies that could potentially halt the progression of this devastating disorder, an animal model is needed. The available Ush2a knock-out mouse model does not mimic the human phenotype, because it presents with only a mild and late-onset retinal degeneration. Using CRISPR/Cas9-technology, we introduced protein-truncating germline lesions into the zebrafish ush2a gene (ush2a(rmc1): c.2337_2342delinsAC; p.Cys780GlnfsTer32 and ush2a(b1245): c.15520_15523delinsTG; p.Ala5174fsTer). Homozygous mutants were viable and displayed no obvious morphological or developmental defects. Immunohistochemical analyses with antibodies recognizing the N- or C-terminal region of the ush2a-encoded protein, usherin, demonstrated complete absence of usherin in photoreceptors of ush2a(rmc1), but presence of the ectodomain of usherin at the periciliary membrane of ush2a(b1245)-derived photoreceptors. Furthermore, defects of usherin led to a reduction in localization of USH2 complex members, whirlin and Adgrv1, at the photoreceptor periciliary membrane of both mutants. Significantly elevated levels of apoptotic photoreceptors could be observed in both mutants when kept under constant bright illumination for three days. Electroretinogram (ERG) recordings revealed a significant and similar decrease in both a- and b-wave amplitudes in ush2a(rmc1) as well as ush2a(b1245) larvae as compared to strain- and age-matched wild-type larvae. In conclusion, this study shows that mutant ush2a zebrafish models present with early-onset retinal dysfunction that is exacerbated by light exposure. These models provide a better understanding of the pathophysiology underlying USH2A-associated RP and a unique opportunity to	fish
204	animal (fish)	zebrafish	CRISPR;Cas9;	glia maturation factor beta	Experimental neurology	Glia maturation factor beta is required for reactive gliosis after traumatic brain injury in zebrafish.	2018	305:129-138	[Yin G et al.]	Southern Medical University, Guangzhou, China.	29655639	10.1016/j.expneurol.2018.04.008	Gliosis is a hallmark of neural pathology that occurs after most forms of central nervous system (CNS) injuries including traumatic brain injury (TBI). Identification of genes that control gliosis may provide novel treatment targets for patients with diverse CNS injuries. Glia maturation factor beta (GMFB) is crucial in brain development and stress response. In the present study, GMFB was found to be widely expressed in adult zebrafish telencephalon. A gmfb mutant zebrafish was created using CRISPR/cas9. In the uninjured zebrafish telencephalon, glial fibrillary acidic protein (GFAP) fibers in gmfb mutants were disorganized and shorter than wild type zebrafish. After TBI, transformation of quiescent type I radial glial cells (RGC) to proliferative type II RGCs was significantly suppressed in the gmfb mutant. RGC proliferation and hypertrophy post-TBI was reduced in gmfb mutants, indicating that reactive gliosis was attenuated. TBI-induced acute inflammation was also found to be alleviated in the gmfb mutant. Morphological changes also suggest attenuation of microglial reactive gliosis. In a mouse model of TBI, GMFB expression was increased around the injury site. These GMFB+ cells were identified as astrocytes and microglia. Taken together, the data suggests that GMFB is not only required for normal development of GFAP fibers in the zebrafish telencephalon, but also promotes reactive gliosis after TBI. Our findings provide novel information to help better understand the reactive gliosis process	fish

205	animal (fish)	zebrafish	CRISPR;Cas9;	mCherry; etc.	FASEB journal	CRISPR/Cas9-based genome engineering of zebrafish using a seamless integration strategy.	2018	32(9):5132-5142	[Luo JJ et al.]	Shantou University Medical College, Shantou, China.	29812974	10.1096/fj.201800077RR	Numerous feasible methods for inserting large fragments of exogenous DNA sequences into the zebrafish genome have been developed, as has genome editing technology using programmable nucleases. However, the coding sequences of targeted endogenous genes are disrupted, and the expression patterns of inserted exogenous genes cannot completely recapitulate those of endogenous genes. Here we describe the establishment of a novel strategy for endogenous promoter-driven and microhomology-mediated end-joining-dependent integration of a donor vector using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9. We successfully integrated mCherry into the final coding sequence of targeted genes to generate seamless transgenic zebrafish lines with high efficiency. This novel seamless transgenesis technique not only maintained the integrity of the endogenous gene but also did not disrupt the function of targeted gene. Therefore, our microhomology-mediated end-joining-mediated transgenesis strategy may have broader applications in gene therapy. Moreover, this novel seamless gene-editing strategy in zebrafish provides a valuable new transgenesis technique, which was driven by endogenous promoters and in vivo animal reporter modes for translational medicine. It is expected to be a standard gene-editing technique in the field of zebrafish, leading to some important breakthroughs for studies in early embryogenesis.-Luo, J.-J., Bian, W.-P., Liu, Y., Huang, H.-Y., Yin, Q., Yang, X.-J., Pei, D.-S. CRISPR/Cas9-based genome	fish
206	animal (fish)	zebrafish	CRISPR;Cas9;	esf1	FEBS journal	The ribosome biogenesis protein Esf1 is essential for pharyngeal cartilage formation in zebrafish.	2018	285(18):3464-3484	[Chen JY et al.]	Key Laboratory of Marine Drugs (Ocean University of China), Chinese Ministry of Education, Qingdao, China.	30073783	10.1111/febs.14622	Craniofacial malformations are common congenital birth defects and usually caused by abnormal development of the cranial neural crest cells. Some nucleolar ribosome biogenesis factors are implicated in neural crest disorders also known as neurocristopathies. However, the underlying mechanisms linking ribosome biogenesis and neural crest cell (NCC) development remain to be elucidated. Here we report a novel zebrafish model with a CRISPR/Cas9-generated esf1 mutation, which exhibits severe NCC-derived pharyngeal cartilage loss and defects in the eyes, brain, and heart. The expression of several typical NCC markers, including sox10, dlx2a, nrp2b, crestin, vgll2a, and sox9a, was reduced in the head of the esf1 mutants, which indicates that esf1 plays a role in the development of zebrafish NCCs. We demonstrate that, similar to the yeast, loss of esf1 in zebrafish leads to defects in 18S rRNA biogenesis and ribosome biogenesis. We also show strong upregulation of p53 signaling as well as apoptosis, and poor proliferation in mutants. Inactivation of p53 rescues the early tissue defects and pharyngeal cartilage loss observed in esf1 mutants, indicating that increased cell death and pharyngeal cartilage defects observed in esf1 mutants are mediated via upregulated p53 signaling pathways. Based on transplantation analysis, we found esf1 functions in NCC in a cell autonomous fashion. Together, our results suggest that esf1 is required for NCC development and pharyngeal cartilage formation. These studies provide a potential model for investigating the relationship between	fish
207	animal (fish)	zebrafish	CRISPR;Cas9;	myostatin (mstna; mstnb)	Fish & shellfish immunology	Deletion of mstna and mstnb impairs the immune system and affects growth performance in zebrafish.	2018	72:572-580	[Wang C et al.]	South China Agricultural University, Guangzhou, China.	29175471	10.1016/j.fsi.2017.11.040	Myostatin (Mstn) is a negative regulator of muscle development in vertebrates. Although its function in muscle growth has been well studied in mammals and fish, it remains unclear whether or how mstn functions in the immune system. In this study, mstna(-/-) and mstnb(-/-) homozygous zebrafish were firstly generated using CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9). Deletion of mstnb but not mstna enhanced growth performance. Although survival rates under normal conditions were slightly decreased in both strains, mortality after dexamethasone-induced stress was increased by approximately 30%. Furthermore, transcriptional levels of several critical immune-related genes were decreased, and the ability to withstand exposure to pathogenic E. tarda was decreased, compared with that of controls. In mstnb(-/-) but not mstna(-/-) zebrafish, expression of NF-kappaB subunits and several pro-inflammatory cytokines failed to respond to E. tarda exposure except nfkb1, c-rel and tnfalpha. Taken together, these results indicate that mstnb but not mstna plays a key role in zebrafish muscle growth. While each paralogue contributes to the response to bacterial insult, mstnb affects the immune system through activation of the NF-kappaB pathway, and mstna is likely to act	fish
208	animal (fish)	zebrafish	CRISPR;Cas9;	over 300 genes implicated in retinal regeneration or degeneration	Frontiers in cell and developmental biology	Multiplexed CRISPR/Cas9 Targeting of Genes Implicated in Retinal Regeneration and Degeneration.	2018	6:88	[Unal Eroglu A et al.]	Johns Hopkins University School of Medicine, Baltimore, MD, USA.	30186835	10.3389/fcell.2018.00088	Thousands of genes have been implicated in retinal regeneration, but only a few have been shown to impact the regenerative capacity of Muller glia-an adult retinal stem cell with untapped therapeutic potential. Similarly, among nearly 300 genetic loci associated with human retinal disease, the majority remain untested in animal models. To address the large-scale nature of these problems, we are applying CRISPR/Cas9-based genome modification strategies in zebrafish to target over 300 genes implicated in retinal regeneration or degeneration. Our intent is to enable large-scale reverse genetic screens by applying a multiplexed gene disruption strategy that markedly increases the efficiency of the screening process. To facilitate large-scale phenotyping, we incorporate an automated reporter quantification-based assay to identify cellular degeneration and regeneration-deficient phenotypes in transgenic fish. Multiplexed gene targeting strategies can address mismatches in scale between "big data" bioinformatics and wet lab experimental capacities, a critical shortfall limiting comprehensive functional analyses of factors implicated in ever-expanding multiomics datasets. This report details the progress we have made to date with a multiplexed CRISPR/Cas9-based gene targeting strategy and discusses how the methodologies applied can further our understanding of the genes that predispose to retinal	fish

209	animal (fish)	zebrafish	CRISPR;Cas9;	cystathionine ss-synthase (cbsb)	Frontiers in cell and developmental biology	Cystathionine beta-Synthase Is Necessary for Axis Development in Vivo.	2018	6:14	[Prabhudesai S et al.]	Medical College of Wisconsin, Milwaukee, WI, USA.	29503817	10.3389/fcell.2018.00014	The cystathionine ss-synthase (CBS) is a critical enzyme in the transsulfuration pathway and is responsible for the synthesis of cystathionine from serine and homocysteine. Cystathionine is a precursor to amino acid cysteine. CBS is also responsible for generation of hydrogen sulfide (H2S) from cysteine. Mutation in CBS enzyme causes homocysteine levels to rise, and gives rise to a condition called hyperhomocysteinuria. To date, numerous mouse knockout models for CBS enzyme has been generated, which show panoply of defects, reflecting the importance of this enzyme in development. In zebrafish, we and others have identified two orthologs of cbs, which we call cbsa and cbsb. Previous gene knockdown studies in zebrafish have reported a function for cbsb ortholog in maintaining ion homeostasis in developing embryos. However, its role in maintaining H2S homeostasis in embryos is unknown. Here, we have performed RNA analysis in whole zebrafish embryos that showed a wide expression pattern for cbsa and cbsb primarily along the embryonic axis of the developing embryo. Loss-of-function analysis using a combination of approaches which include splice morpholinos and CRISPR/Cas9 genomic engineering show evidence that cbsb ortholog is responsible for anterior-posterior axis development, and cbsa function is redundant. Cbsb loss of function fish embryos show shortened and bent axis, along with less H2S and more homocysteine, effects resulting from loss of Cbsb. Using a chemical biology approach, we rescued the axis defects with betaine, a compound known to reduce homocysteine levels in plasma, and GYY4137, a long term H2S donor. These results collectively argue that cells along the axis of a developing embryo are sensitive to changes in homocysteine and H2S levels, pathways that are controlled by	fish
210	animal (fish)	zebrafish	CRISPR;Cas9;	tyr; gol	G3	Comparison of Various Nuclear Localization Signal-Fused Cas9 Proteins and Cas9 mRNA for Genome Editing in Zebrafish.	2018	8(3):823-831	[Hu P et al.]	Shanghai Ocean University, China.	29295818	10.1534/g3.117.300359	The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has been proven to be an efficient and precise genome editing technology in various organisms. However, the gene editing efficiencies of Cas9 proteins with a nuclear localization signal (NLS) fused to different termini and Cas9 mRNA have not been systematically compared. Here, we compared the ability of Cas9 proteins with NLS fused to the N-, C-, or both the N- and C-termini and N-NLS-Cas9-NLS-C mRNA to target two sites in the tyr gene and two sites in the gol gene related to pigmentation in zebrafish. Phenotypic analysis revealed that all types of Cas9 led to hypopigmentation in similar proportions of injected embryos. Genome analysis by T7 Endonuclease I (T7E1) assays demonstrated that all types of Cas9 similarly induced mutagenesis in four target sites. Sequencing results further confirmed that a high frequency of indels occurred in the target sites (tyr1 > 66%, tyr2 > 73%, gol1 > 50%, and gol2 > 35%), as well as various types (more than six) of indel mutations observed in all four types of Cas9-injected embryos. Furthermore, all types of Cas9 showed efficient targeted mutagenesis on multiplex genome editing, resulting in multiple phenotypes simultaneously. Collectively, we conclude that various NLS-fused Cas9 proteins and Cas9 mRNAs have similar genome editing efficiencies on targeting single or multiple genes, suggesting that the efficiency of CRISPR/Cas9 genome editing is highly dependent on guide RNAs (gRNAs) and gene loci. These findings may help to simplify	fish
211	animal (fish)	zebrafish	CRISPR;	ASCL1a; BCL6a; HSP70	Gene	Spatiotemporal control of zebrafish (Danio rerio) gene expression using a light-activated CRISPR activation system.	2018	677:273-279	[Putri RR et al.]	Shanghai Ocean University, Shanghai, China.	30077009	10.1016/j.gene.2018.07.077	CRISPR activation (CRISPRa) system is the convenient tool for targeted-gene activation, it has been developed and combined with a lighting-based system that can control transcription initiation spatially and temporally by utilizing photoreceptor derived from plant Arabidopsis thaliana. A blue light photoreceptor the Cryptochrome 2 (CRY2), and its binding partner CIB1 will dimerize by exposure to the blue light and it has been applied to human cells. However, the application of a combination of these two systems to zebrafish cell is still not explored. We performed zebrafish gene activation using p65 and VP64 activators in the zebrafish cells (ZF4). Our study demonstrated that we have successfully controlled the transcription level of ASCL1a, BCL6a, and HSP70 genes using blue light-activated CRISPR activation system. The result showed that using this system, mRNA level expression of ASCL1a, BCL6a, and HSP70 genes increased after irradiated under blue light for several hours and	fish
212	animal (fish)	zebrafish	CRISPR;Cas9;	heterotaxy	Genome medicine	Rare copy number variants analysis identifies novel candidate genes in heterotaxy syndrome patients with congenital heart defects.	2018	10(1):40	[Liu C et al.]	Shanghai Jiao Tong University, Shanghai, China.	29843777	10.1186/s13073-018-0549-y	BACKGROUND: Heterotaxy (Htx) syndrome comprises a class of congenital disorders resulting from malformations in left-right body patterning. Approximately 90% of patients with heterotaxy have serious congenital heart diseases; as a result, the survival rate and outcomes of Htx patients are not satisfactory. However, the underlying etiology and mechanisms in the majority of Htx cases remain unknown. The aim of this study was to investigate the function of rare copy number variants (CNVs) in the pathogenesis of Htx. METHODS: We collected 63 sporadic Htx patients with congenital heart defects and identified rare CNVs using an Affymetrix CytoScan HD microarray and real-time polymerase chain reaction. Potential candidate genes associated with the rare CNVs were selected by referring to previous literature related to left-right development. The expression patterns and function of candidate genes were further analyzed by whole mount in situ hybridization, morpholino knockdown, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated mutation, and over-expressing methods with zebrafish models. RESULTS: Nineteen rare CNVs were identified for the first time in patients with Htx. These CNVs include 5 heterozygous genic deletions, 4 internal genic duplications, and 10 complete duplications of at least one gene. Further analyses of the 19 rare CNVs identified six novel potential candidate genes (NUMB, PACRG, TCTN2, DANH10, RNF115, and TTC40) linked to left-right patterning. These candidate genes exhibited early expression patterns in zebrafish embryos. Functional testing revealed that downregulation and over-expression of five candidate genes (numb, pacrg, tctn2, dnah10, and rnf115) in zebrafish resulted in disruption of cardiac looping and abnormal expression of lefty2 or pitx2, molecular markers of left-right patterning. CONCLUSIONS: Our findings show that Htx with congenital heart defects in some sporadic patients may be attributed to rare CNVs. Furthermore, DNAH10 and RNF115 are Htx candidate genes involved in left-right patterning which have not previously	fish

213	animal (fish)	zebrafish	CRISPR;Cas9;	adenosine triphosphate-binding cassette B11 (abcb11b)	Hepatology	Zebrafish abcb11b mutant reveals strategies to restore bile excretion impaired by bile salt export pump deficiency.	2018	67(4):1531-1545	[Ellis JL et al.]	Cincinnati Children's Hospital Medical Center, Cincinnati, OH., USA	29091294	10.1002/hep.29632	Bile salt export pump (BSEP) adenosine triphosphate-binding cassette B11 (ABCB11) is a liver-specific ABC transporter that mediates canalicular bile salt excretion from hepatocytes. Human mutations in ABCB11 cause progressive familial intrahepatic cholestasis type 2. Although over 150 ABCB11 variants have been reported, our understanding of their biological consequences is limited by the lack of an experimental model that recapitulates the patient phenotypes. We applied CRISPR/Cas9-based genome editing technology to knock out abcb11b, the ortholog of human ABCB11, in zebrafish and found that these mutants died prematurely. Histological and ultrastructural analyses showed that abcb11b mutant zebrafish exhibited hepatocyte injury similar to that seen in patients with progressive familial intrahepatic cholestasis type 2. Hepatocytes of mutant zebrafish failed to excrete the fluorescently tagged bile acid that is a substrate of human BSEP. Multidrug resistance protein 1, which is thought to play a compensatory role in Abcb11 knockout mice, was mislocalized to the hepatocyte cytoplasm in abcb11b mutant zebrafish and in a patient lacking BSEP protein due to nonsense mutations in ABCB11. We discovered that BSEP deficiency induced autophagy in both human and zebrafish hepatocytes. Treatment with rapamycin restored bile acid excretion, attenuated hepatocyte damage, and extended the life span of abcb11b mutant zebrafish, correlating with the recovery of canalicular multidrug resistance protein 1 localization. CONCLUSIONS: Collectively, these data suggest a model that rapamycin rescues BSEP-deficient phenotypes by prompting alternative transporters to excrete bile salts; multidrug resistance protein 1 is a candidate for such an alternative transporter. (Hepatology 2018;67:1531-1545).	fish
214	animal (fish)	zebrafish	TALENs;	ush2a	Human genetics	Knockout of ush2a gene in zebrafish causes hearing impairment and late onset rod-cone dystrophy.	2018	137(10):779-794	[Han S et al.]	Huazhong University of Science and Technology, Wuhan, Hubei, China.	30242501	10.1007/s00439-018-1936-6	Most cases of Usher syndrome type II (USH2) are due to mutations in the USH2A gene. There are no effective treatments or ideal animal models for this disease, and the pathological mechanisms of USH2 caused by USH2A mutations are still unknown. Here, we constructed a ush2a knockout (ush2a(-/-)) zebrafish model using TALEN technology to investigate the molecular pathology of USH2. An early onset auditory disorder and abnormal morphology of inner ear stereocilia were identified in the ush2a(-/-) zebrafish. Consequently, the disruption of Ush2a in zebrafish led to a hearing impairment, like that in mammals. Electroretinography (ERG) test indicated that deletion of Ush2a affected visual function at an early stage, and histological analysis revealed that the photoreceptors progressively degenerated. Rod degeneration occurred prior to cone degeneration in ush2a(-/-) zebrafish, which is consistent with the classical description of the progression of retinitis pigmentosa (RP). Destruction of the outer segments (OSs) of rods led to the down-regulation of phototransduction cascade proteins at late stage. The expression of Ush1b and Ush1c was up-regulated when Ush2a was null. We also found that disruption of fibronectin assembly at the retinal basement membrane weakened cell adhesion in ush2a(-/-) mutants. In summary, for the first time, we generated a ush2a knockout zebrafish line with auditory disorder and retinal degeneration which mimicked the symptoms of patients, and revealed that disruption of fibronectin assembly may be one of the factors underlying RP. This model may help us to better understand the pathogenic mechanism and find	fish
215	animal (fish)	zebrafish	CRISPR;Cas9;	Forkhead box E3 (foxe3)	Human genetics	A zebrafish model of foxe3 deficiency demonstrates lens and eye defects with dysregulation of key genes involved in cataract formation in humans.	2018	137(4):315-328	[Krall M et al.]	University of California San Francisco, San Francisco, CA, USA.	29713869	10.1007/s00439-018-1884-1	The Forkhead box E3 (FOXE3) gene encodes a transcription factor with a forkhead/winged helix domain that is critical for development of the lens and anterior segment of the eye. Monoallelic and biallelic deleterious sequence variants in FOXE3 cause aphakia, cataracts, sclerocornea and microphthalmia in humans. We used clustered regularly interspaced short palindromic repeats/Cas9 injections to target the foxe3 transcript in zebrafish in order to create an experimental model of loss of function for this gene. Larvae that were homozygous for an indel variant, c.296_300delTGCAG, predicting p.(Val99Alafs*2), demonstrated severe eye defects, including small or absent lenses and microphthalmia. The lenses of the homozygous foxe3 indel mutants showed more intense staining with zl-1 antibody compared to control lenses, consistent with increased lens fiber cell differentiation. Whole genome transcriptome analysis (RNA-Seq) on RNA isolated from wildtype larvae and larvae with eye defects that were putative homozygotes for the foxe3 indel variant found significant dysregulation of genes expressed in the lens and eye whose orthologues are associated with cataracts in human patients, including cryba2a, cryba1l1, mipa and hsf4. Comparative analysis of this RNA-seq data with iSyTE data identified several lens-enriched genes to be down-regulated in foxe3 indel mutants. We also noted upregulation of lgsn and crygmxl2 and downregulation of fmodb and cx43.4, genes that are expressed in the zebrafish lens, but that are not yet associated with an eye phenotype in humans. These findings demonstrate that this new zebrafish foxe3 mutant model is highly relevant to the study of the gene regulatory networks conserved in	fish
216	animal (fish)	zebrafish	CRISPR;	myomaker	Human molecular genetics	Knockout of myomaker results in defective myoblast fusion, reduced muscle growth and increased adipocyte infiltration in zebrafish skeletal muscle.	2018	27(20):3542-3554	[Shi J et al.]	University of Maryland School of Medicine, Baltimore, MD, USA.	30016436	10.1093/hmg/ddy268	The fusion of myoblasts into multinucleated muscle fibers is vital to skeletal muscle development, maintenance and regeneration. Genetic mutations in the Myomaker (mymk) gene cause Carey-Fineman-Ziter syndrome (CFZS) in human populations. To study the regulation of mymk gene expression and function, we generated three mymk mutant alleles in zebrafish using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology and analyzed the effects of mymk knockout on muscle development and growth. Our studies demonstrated that knockout of mymk resulted in defective myoblast fusion in zebrafish embryos and increased mortality at larval stage around 35-45 days post-fertilization. The viable homozygous mutants were smaller in size and weighed approximately one-third the weight of the wild type (WT) sibling at 3 months old. The homozygous mutants showed craniofacial deformities, resembling the facial defect observed in human populations with CFZS. Histological analysis revealed that skeletal muscles of mymk mutants contained mainly small-size fibers and substantial intramuscular adipocyte infiltration. Single fiber analysis revealed that myofibers in mymk mutant were predominantly single-nucleated fibers. However, myofibers with multiple myonuclei were observed, although the number of nuclei per fiber was much less compared with that in WT fibers. Overexpression of sonic Hedgehog inhibited mymk expression in zebrafish embryos and blocked myoblast fusion. Collectively, these studies demonstrated that mymk is essential for myoblast fusion	fish

217	animal (fish)	zebrafish	TALENs;	paired-like homeodomain 2 (pitx2)	Human molecular genetics	PITX2 deficiency and associated human disease: insights from the zebrafish model.	2018	27(10):1675-1695	[Hendee KE et al.]	Medical College of Wisconsin and Children's Hospital of Wisconsin, Milwaukee, WI, USA.	29506241	10.1093/hmg/ddy074	The PITX2 (paired-like homeodomain 2) gene encodes a bicoid-like homeodomain transcription factor linked with several human disorders. The main associated congenital phenotype is Axenfeld-Rieger syndrome, type 1, an autosomal dominant condition characterized by variable defects in the anterior segment of the eye, an increased risk of glaucoma, craniofacial dysmorphism and dental and umbilical anomalies; in addition to this, one report implicated PITX2 in ring dermoid of the cornea and a few others described cardiac phenotypes. We report three novel PITX2 mutations-c.271C > T, p.(Arg91Trp); c.259T > C, p.(Phe87Leu); and c.356delA, p.(Gln119Argfs*36)-identified in independent families with typical Axenfeld-Rieger syndrome characteristics and some unusual features such as corneal guttata, Wolf-Parkinson-White syndrome, and hyperextensibility. To gain further insight into the diverse roles of PITX2/pitx2 in vertebrate development, we generated various genetic lesions in the pitx2 gene via TALEN-mediated genome editing. Affected homozygous zebrafish demonstrated congenital defects consistent with the range of PITX2-associated human phenotypes: abnormal development of the cornea, iris and iridocorneal angle; corneal dermoids; and craniofacial dysmorphism. In addition, via comparison of pitx2M64* and wild-type embryonic ocular transcriptomes we defined molecular changes associated with pitx2 deficiency, thereby implicating processes potentially underlying disease pathology. This analysis identified numerous affected factors including several members of the Wnt pathway and collagen types I and V gene families. These data further support the link between PITX2 and the WNT pathway and	fish
218	animal (fish)	zebrafish	CRISPR;Cas9;	cdc14a	Human molecular genetics	CDC14A phosphatase is essential for hearing and male fertility in mouse and human.	2018	27(5):780-798	[Imtiaz A et al.]	National Institute on Deafness and Other Communication Disorders, NIH, Bethesda, MD, USA.	29293958	10.1093/hmg/ddx440	The Cell Division-Cycle-14 gene encodes a dual-specificity phosphatase necessary in yeast for exit from mitosis. Numerous disparate roles of vertebrate Cell Division-Cycle-14 (CDC14A) have been proposed largely based on studies of cultured cancer cells in vitro. The in vivo functions of vertebrate CDC14A are largely unknown. We generated and analyzed mutations of zebrafish and mouse CDC14A, developed a computational structural model of human CDC14A protein and report four novel truncating and three missense alleles of CDC14A in human families segregating progressive, moderate-to-profound deafness. In five of these families segregating pathogenic variants of CDC14A, deaf males are infertile, while deaf females are fertile. Several recessive mutations of mouse Cdc14a, including a CRISPR/Cas9-edited phosphatase-dead p.C278S substitution, result in substantial perinatal lethality, but survivors recapitulate the human phenotype of deafness and male infertility. CDC14A protein localizes to inner ear hair cell kinocilia, basal bodies and sound-transducing stereocilia. Auditory hair cells of postnatal Cdc14a mutants develop normally, but subsequently degenerate causing deafness. Kinocilia of germ-line mutants of mouse and zebrafish have normal lengths, which does not recapitulate the published cdc14aa knockdown morphant phenotype of short kinocilia. In mutant male mice, degeneration of seminiferous tubules and spermiation defects result in low sperm count, and abnormal sperm motility and morphology. These findings for the first time define a new monogenic syndrome of deafness and male infertility revealing an absolute requirement	fish
219	animal (fish)	zebrafish	CRISPR;Cas9;	WDR63	Human mutation	Targeted copy number screening highlights an intragenic deletion of WDR63 as the likely cause of human occipital encephalocele and abnormal CNS development in zebrafish.	2018	39(4):495-505	[Hofmeister W et al.]	Karolinska Institutet, Stockholm, Sweden.	29285825	10.1002/humu.23388	Congenital malformations affecting the neural tube can present as isolated malformations or occur in association with other developmental abnormalities and syndromes. Using high-resolution copy number screening in 66 fetuses with neural tube defects, we identified six fetuses with likely pathogenic mutations, three aneuploidies (one trisomy 13 and two trisomy 18) and three deletions previously reported in NTDs (one 22q11.2 deletion and two 1p36 deletions) corresponding to 9% of the cohort. In addition, we identified five rare deletions and two duplications of uncertain significance including a rare intragenic heterozygous in-frame WDR63 deletion in a fetus with occipital encephalocele. Whole genome sequencing verified the deletion and excluded known pathogenic variants. The deletion spans exons 14-17 resulting in the expression of a protein missing the third and fourth WD-repeat domains. These findings were supported by CRISPR/Cas9-mediated somatic deletions in zebrafish. Injection of two different sgRNA-pairs targeting relevant intronic regions resulted in a deletion mimicking the human deletion and a concomitant increase of abnormal embryos with body and brain malformations (41%, n = 161 and 62%, n = 224, respectively), including a sac-like brain protrusion (7% and 9%, $P < 0.01$). Similar results were seen with overexpression of RNA encoding the deleted variant in zebrafish (total abnormal; 46%, n = 255, $P < 0.001$) compared with the overexpression of an equivalent amount of wild-type RNA (total abnormal; 3%, n = 177). We predict the in-frame WDR63 deletion to result in a dominant negative or gain-of-function form of WDR63. These are	fish
220	animal (fish)	zebrafish	TALENs;	family with sequence similarity 60, member A, like (fam60al)	International journal of biological sciences	Fam60al as a novel factor involved in reprogramming of somatic cell nuclear transfer in zebrafish (Danio rerio).	2018	14(1):78-86	[Hu H et al.]	Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.	29483827	10.7150/ijbs.22426	The main reason for abnormal development of cloned animals or embryos, and inefficient animal cloning, is a poor understanding of the reprogramming mechanism. To better comprehend reprogramming and subsequent generation of pluripotent stem cells, we must investigate factors related to reprogramming of somatic cells as nuclear donors. As we know, fam60al (family with sequence similarity 60, member A, like) is a coding gene only found in zebrafish and frog (Xenopus laevis) among vertebrates. However, until now, its functions have remained unknown. Here, we generated a zebrafish fam60al(-/-) mutant line using transcription activator-like effector nucleases (TALENs), and found that both nanog and klf4b expression significantly decreased while myca expression significantly increased in fam60al(-/-) mutant embryos. Concurrently, we also uncovered that in developmentally arrested embryos of somatic cell nuclear transfer, nanog, klf4b and myca expression was down-regulated, accompanying a decrease of fam60al expression. Interestingly, we identified a long noncoding RNA (lncRNA) of fam60al, named fam60al-AS, which negatively regulated fam60al by forming double-stranded RNA (dsRNA). RNase protection assay and real-time PCR confirmed these findings. Taken together, these results suggest that fam60al is a novel factor related to the reprogramming of somatic cell nuclear transfer in zebrafish, which is	fish

221	animal (fish)	zebrafish	CRISPR;Cas9;	lbx2	International journal of cardiology	Identification of LBX2 as a novel causal gene of atrial septal defect.	2018	265:188–194	[Wang J et al.]	Capital Medical University, Beijing, China.	29669692	10.1016/j.ijcard.2018.04.038	BACKGROUND: Atrial septal defect (ASD) is one of the most common cardiac malformations worldwide. Several genes have been identified so far, which can merely explain small proportion of all the cases, therefore, it is anticipated that there are additional genes causing ASD. The aims of this study were to identify the causal gene of ostium secundum atrial septal defect (ASDII) in a Chinese family. METHODS: Whole exome sequencing was performed in three affected members and one control in the ASDII family. We screened mutations of LBX2 in 300 unrelated ASD patients and validated in 400 normal controls by Sanger sequencing. LBX2 knockout zebrafish was generated by CRISPR/Cas9 to detect whether lbx2 deficiency influenced cardiac development. RESULTS: A rare missense mutation in LBX2 (c.A403G: p.K135E) was identified as the pathogenic cause of ASD. Subsequent mutation screening revealed two missense variants in 3 of 300 sporadic patients. We observed expanded size of atrium and ventricle in LBX2 knockout zebrafish through hematoxylin–eosin staining. more incompact distribution of cardiac myocytes was also discovered in homozygote compared with in wildtype. Furthermore, we performed in situ hybridization of crip2 gene to trace the cardiac neural crest cells in the embryo stage and found that the migration of neural crest cells was obviously delayed in the homozygotes. CONCLUSIONS: We identified LBX2 for the first time as a pathogenic gene of ASDII. LBX2 deficiency may cause abnormal development of heart through influencing the	fish
222	animal (fish)	zebrafish	TALENs;	leptin a	International journal of molecular sciences	Zebrafish Mutants Carrying Leptin a (lepa) Gene Deficiency Display Obesity, Anxiety, Less Aggression and Fear, and Circadian Rhythm and Color Preference Dysregulation.	2018	19(12)	[Audira G et al.]	Chung Yuan Christian University, Chung-Li, Taiwan.	30551684	10.3390/ijms19124038	Leptin, a hormone secreted by peripheral adipose tissues, regulates the appetite in animals. Recently, evidence has shown that leptin also plays roles in behavioral response in addition to controlling appetite. In this study, we examined the potential function of leptin on non-appetite behaviors in zebrafish model. By using genome editing tool of Transcription activator-like effector nuclease (TALEN), we successfully knocked out leptin a (lepa) gene by deleting 4 bp within coding region to create a premature-translation stop. Morphological and appetite analysis showed the lepa KO fish display a phenotype with obese, good appetite and elevation of Agouti-related peptide (AgRP) and Ghrelin hormones, consistent with the canonical function of leptin in controlling food intake. By multiple behavior endpoint analyses, including novel tank, mirror biting, predator avoidance, social interaction, shoaling, circadian rhythm, and color preference assay, we found the lepa KO fish display an anxiogenic phenotype showing hyperactivity with rapid swimming, less freezing time, less fear to predator, loose shoaling area forming, and circadian rhythm and color preference dysregulations. Using biochemical assays, melatonin, norepinephrine, acetylcholine and serotonin levels in the brain were found to be significantly reduced in lepa KO fish, while the levels of dopamine, glycine and cortisol in the brain were significantly elevated. In addition, the brain ROS level was elevated, and the anti-oxidative enzyme catalase level was reduced. Taken together, by performing loss-of-function multiple behavior endpoint testing and biochemical analysis, we provide strong evidence for a critical role of lepa gene in modulating anxiety, aggression, fear, and circadian rhythm behaviors in	fish
223	animal (fish)	zebrafish	CRISPR;Cas9;	aquaporin 0a; aquaporin 0b	Investigative ophthalmology & visual science	Aqp0a Regulates Suture Stability in the Zebrafish Lens.	2018	59(7):2869–2879	[Vorontsova I et al.]	University of California, Irvine, CA, USA.	30025131	10.1167/iovs.18-24044	Purpose: To investigate the roles of Aquaporin 0a (Aqp0a) and Aqp0b in zebrafish lens development and transparency. Methods: CRISPR/Cas9 gene editing was used to generate loss-of-function deletions in zebrafish aqp0a and/or aqp0b. Wild type (WT), single mutant, and double mutant lenses were analyzed from embryonic to adult stages. Lens transparency, morphology, and growth were assessed. Immunohistochemistry was used to map protein localization as well as to assess tissue organization and distribution of cell nuclei. Results: aqp0a-/- and/or aqp0b-/- cause embryonic cataracts with variable penetrance. While lenses of single mutants of either gene recover transparency in juveniles, double mutants consistently form dense cataracts that persist in adults, indicating partially redundant functions. Double mutants also reveal redundant Aqp0 functions in lens growth. The nucleus of WT lenses moves from the anterior pole to the lens center with age. In aqp0a-/- mutants, the nucleus fails to centralize as it does in WT or aqp0b-/- lenses, and in double mutant lenses there is no consistent lens nuclear position. In addition, the anterior sutures of aqp0a-/-, but not aqp0b-/- mutants, are unstable resulting in failure of suture maintenance at older stages and anterior polar opacity. Conclusions. Zebrafish Aqp0s have partially redundant functions, but only Aqp0a promotes suture stability, which directs the lens nucleus to centralize, failure of which results in anterior polar opacity. These studies support the hypothesis that the two Aqp0s subfunctionalized during fish evolution and	fish
224	animal (fish)	zebrafish	CRISPR;Cas9;	beta subunit of chaperonin containing TCP-1 (cct2)	Investigative ophthalmology & visual science	Mutation in the Zebrafish cct2 Gene Leads to Abnormalities of Cell Cycle and Cell Death in the Retina: A Model of CCT2-Related Leber Congenital Amaurosis.	2018	59(2):995–1004	[Minegishi Y et al.]	National Eye Institute, National Institutes of Health, Bethesda, MD, USA.	29450543	10.1167/iovs.17-22919	Purpose: The compound heterozygous mutations in the beta subunit of chaperonin containing TCP-1 (CCT), encoded by CCT2, lead to the Leber congenital amaurosis (LCA). In this study, a cct2 mutant line of zebrafish was established to investigate the role of CCT2 mutations in LCA in vertebrates. Methods: A cct2 mutant zebrafish line was produced using the CRISPR-Cas9 system. Changes in the eyes of developing wild-type and mutant larvae were monitored using microscopy, immunostaining, TUNEL, and EdU assays. Phenotypic rescue of mutant phenotype was investigated by injection of CCT2 RNA into zebrafish embryos. Results: The cct2 mutation (L394H-7del) led to the synthesis of a mutated cctbeta protein with the L394H replacement and deletion of 7 amino acid residues (positions 395-401). The homozygous cct2-L394H-7del mutant exhibited a small eye phenotype at 2 days post fertilization (dpf) and was embryonically lethal after 5 dpf. In homozygous cct2-L394H-7del mutants, the retinal ganglion cell differentiation was attenuated, retinal cell cycle was affected, and the neural retinal cell death was significantly increased at 2 dpf compared with wild-type. Injection of RNA encoding wild-type human CCTbeta rescued the small eye phenotype, reduced retinal cell death, and restored the levels of CCTbeta protein and the major client protein Gbeta1 that were significantly reduced in the homozygous cct2-L394H-7del mutant compared with wild-type. These results indicate that cct2 plays an essential role in retinal development by regulating the cell cycle. Conclusions: The retinal pathology observed in the homozygous cct2-L394H-7del mutants resembles the	fish

225	animal (fish)	zebrafish	CRISPR;Cas9;	glycine decarboxyla (gldc)	JCI insight	Glycine decarboxylase deficiency-induced motor dysfunction in zebrafish is rescued by counterbalancing glycine synaptic level.	2018	3(21)	[Riche R et al.]	Universite de Montreal, Montreal, Quebec, Canada.	30385710	10.1172/jci.insight.124642	Glycine encephalopathy (GE), or nonketotic hyperglycinemia (NKH), is a rare recessive genetic disease caused by defective glycine cleavage and characterized by increased accumulation of glycine in all tissues. Here, based on new case reports of GLDC loss-of-function mutations in GE patients, we aimed to generate a zebrafish model of severe GE in order to unravel the molecular mechanism of the disease. Using CRISPR/Cas9, we knocked out the gldc gene and showed that gldc-/- fish recapitulate GE on a molecular level and present a motor phenotype reminiscent of severe GE symptoms. The molecular characterization of gldc-/- mutants showed a broad metabolic disturbance affecting amino acids and neurotransmitters other than glycine, with lactic acidosis at stages preceding death. Although a transient imbalance was found in cell proliferation in the brain of gldc-/- zebrafish, the main brain networks were not affected, thus suggesting that GE pathogenicity is mainly due to metabolic defects. We confirmed that the gldc-/- hypotonic phenotype is due to NMDA and glycine receptor overactivation, and demonstrated that gldc-/- larvae depict exacerbated hyperglycinemia at these synapses. Remarkably, we were able to rescue the motor dysfunction of gldc-/- larvae by counterbalancing pharmacologically or genetically the	fish
226	animal (fish)	zebrafish	CRISPR;Cas9;	transcobalamin homolog (tcn2)	Journal of biological chemistry	Functional and phylogenetic characterization of noncanonical vitamin B12-binding proteins in zebrafish suggests involvement in cobalamin transport.	2018	293(45):17606-17621	[Benoit CR et al.]	National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA.	30237171	10.1074/jbc.RA118.005323	In humans, transport of food-derived cobalamin (vitamin B12) from the digestive system into the bloodstream involves three paralogous proteins: transcobalamin (TC), haptocorrin (HC), and intrinsic factor (IF). Each of these proteins contains two domains, an alpha-domain and a beta-domain, which together form a cleft in which cobalamin binds. Zebrafish (Danio rerio) are thought to possess only a single cobalamin transport protein, referred to as Tcn2, which is a transcobalamin homolog. Here, we used CRISPR/Cas9 mutagenesis to create null alleles of tcn2 in zebrafish. Fish homozygous for tcn2-null alleles were viable and exhibited no obvious developmentally or behaviorally abnormal phenotypes. For this reason, we hypothesized that previously unidentified cobalamin-carrier proteins encoded in the zebrafish genome may provide an additional pathway for cobalamin transport. We identified genes predicted to code for two such proteins, Tcn-beta-a (Tcnba) and Tcn-beta-b (Tcnbb), which differ from all previously characterized cobalamin transport proteins as they lack the alpha-domain. These beta-domain-only proteins are representative of an undescribed class of cobalamin-carrier proteins that are highly conserved throughout the ray-finned fishes. We observed that the genes encoding the three cobalamin transport homologs, tcn2, tcnba, and tcnbb, are expressed in unique spatial and temporal patterns in the developing zebrafish. Moreover, exogenously expressed recombinant Tcnba and Tcnbb bound cobalamin with high affinity, comparable with binding by full-length Tcn2. Taken together, our results suggest that this noncanonical protein structure has evolved to fully function as a cobalamin-carrier protein, thereby allowing for a compensatory	fish
227	animal (fish)	zebrafish	CRISPR;Cas9;	factor inhibiting HIF (FIH)	Journal of biological chemistry	Deletion of the fih gene encoding an inhibitor of hypoxia-inducible factors increases hypoxia tolerance in zebrafish.	2018	293(40):15370-15380	[Cai X et al.]	Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China	30126845	10.1074/jbc.RA118.003004	Many aerobic organisms have developed molecular mechanism to tolerate hypoxia, but the specifics of these mechanisms remain poorly understood. It is important to develop genetic methods that confer increased hypoxia tolerance to intensively farmed aquatic species, as these are maintained in environments with limited available oxygen. As an asparaginyl hydroxylase of hypoxia-inducible factors (HIFs), factor inhibiting HIF (FIH) inhibits transcriptional activation of hypoxia-inducible genes by blocking the association of HIFs with the transcriptional coactivators CREB-binding protein (CBP) and p300. Therefore, here we sought to test whether fih is involved in regulating hypoxia tolerance in the commonly used zebrafish model. Overexpressing the zebrafish fih gene in epithelioma papulosum cyprini (EPC) cells and embryos, we found that fih inhibits the transcriptional activation of zebrafish HIF-alpha proteins. Using CRISPR/Cas9 to obtain fih-null zebrafish mutants, we noted that the fih deletion makes zebrafish more tolerant of hypoxic conditions than their WT siblings, but does not result in oxygen consumption rates that significantly differ from those of WT fish. Of note, we identified fewer apoptotic cells in adult fih-null zebrafish brains and in fih-null embryos, possibly explaining why the fih-null mutant had greater hypoxia tolerance than the WT. Moreover, the fih deletion up-regulated several hypoxia-inducible genes in fih-null zebrafish exposed to hypoxia. The findings of our study suggest that fih plays a role in hypoxia tolerance by affecting the rate of cellular apoptosis in zebrafish.	fish
228	animal (fish)	zebrafish	CRISPR;Cas9;	Y box-binding protein 1	Journal of biological chemistry	An efficient platform for generating somatic point mutations with germline transmission in the zebrafish by CRISPR/Cas9-mediated gene editing.	2018	293(17):6611-6622	[Zhang Y et al.]	University of Macau, Macau, China.	29500194	10.1074/jbc.RA117.001080	Homology-directed recombination (HDR)-mediated genome editing is a powerful approach for both basic functional study and disease modeling. Although some studies have reported HDR-mediated precise editing in nonrodent models, the efficiency of establishing pure mutant animal lines that carry specific amino acid substitutions remains low. Furthermore, because the efficiency of nonhomologous end joining (NHEJ)-induced insertion and deletion (indel) mutations is normally much higher than that of HDR-induced point mutations, it is often difficult to identify the latter in the background of indel mutations. Using zebrafish as the model organism and Y box-binding protein 1 (Ybx1/ybx1) as the model molecule, we have established an efficient platform for precise CRISPR/Cas9-mediated gene editing in somatic cells, yielding an efficiency of up to 74% embryos. Moreover, we established a procedure for screening germline transmission of point mutations out of indel mutations even when germline transmission efficiency was low (<2%). To further improve germline transmission of HDR-induced point mutations, we optimized several key factors that may affect HDR efficiency, including the type of DNA donor, suppression of NHEJ, stimulation of HDR pathways, and use of Cas9 protein instead of mRNA. The optimized combination of these factors significantly increased the efficiency of germline transmission of point mutation up to 25%. In summary, we have developed an efficient procedure for creating point mutations and differentiating mutant individuals from those carrying knockouts of	fish

229	animal (fish)	zebrafish	CRISPR;Cas9;	alphaB-crystallin (alphaBa; alphaBb)	Journal of biological chemistry	Loss of alphaB-crystallin function in zebrafish reveals critical roles in the development of the lens and stress resistance of the heart.	2018	293(2):740-753	[Mishra S et al.]	Vanderbilt University School of Medicine, Nashville, TN, USA.	29162721	10.1074/jbc.M117.808634	Genetic mutations in the human small heat shock protein alphaB-crystallin have been implicated in autosomal cataracts and skeletal myopathies, including heart muscle diseases (cardiomyopathy). Although these mutations lead to modulation of their chaperone activity in vitro, the in vivo functions of alphaB-crystallin in the maintenance of both lens transparency and muscle integrity remain unclear. This lack of information has hindered a mechanistic understanding of these diseases. To better define the functional roles of alphaB-crystallin, we generated loss-of-function zebrafish mutant lines by utilizing the CRISPR/Cas9 system to specifically disrupt the two alphaB-crystallin genes, alphaBa and alphaBb We observed lens abnormalities in the mutant lines of both genes, and the penetrance of the lens phenotype was higher in alphaBa than alphaBb mutants. This finding is in contrast with the lack of a phenotype previously reported in alphaB-crystallin knock-out mice and suggests that the elevated chaperone activity of the two zebrafish orthologs is critical for lens development. Besides its key role in the lens, we uncovered another critical role for alphaB-crystallin in providing stress tolerance to the heart. The alphaB-crystallin mutants exhibited hypersusceptibility to develop pericardial edema when challenged by crowding stress or exposed to elevated cortisol stress, both of which activate glucocorticoid receptor signaling. Our work illuminates the involvement of alphaB-crystallin in stress tolerance of the heart presumably through the proteostasis network and reinforces the critical role of the chaperone activity of alphaB-crystallin in the maintenance of lens	fish
230	animal (fish)	zebrafish	CRISPR;Cas9;	nup107; nup85	Journal of clinical investigation	Mutations in multiple components of the nuclear pore complex cause nephrotic syndrome.	2018	128(10):4313-4328	[Braun DA et al.]	Boston Children's Hospital, Harvard Medical School, Boston, MA, USA.	30179222	10.1172/JCI98688	Steroid-resistant nephrotic syndrome (SRNS) almost invariably progresses to end-stage renal disease. Although more than 50 monogenic causes of SRNS have been described, a large proportion of SRNS remains unexplained. Recently, it was discovered that mutations of NUP93 and NUP205, encoding 2 proteins of the inner ring subunit of the nuclear pore complex (NPC), cause SRNS. Here, we describe mutations in genes encoding 4 components of the outer rings of the NPC, namely NUP107, NUP85, NUP133, and NUP160, in 13 families with SRNS. Using coimmunoprecipitation experiments, we showed that certain pathogenic alleles weakened the interaction between neighboring NPC subunits. We demonstrated that morpholino knockdown of nup107, nup85, or nup133 in Xenopus disrupted glomerulogenesis. Re-expression of WT mRNA, but not of mRNA reflecting mutations from SRNS patients, mitigated this phenotype. We furthermore found that CRISPR/Cas9 knockout of NUP107, NUP85, or NUP133 in podocytes activated Cdc42, an important effector of SRNS pathogenesis. CRISPR/Cas9 knockout of nup107 or nup85 in zebrafish caused developmental anomalies and early lethality. In contrast, an in-frame mutation of nup107 did not affect survival, thus mimicking the allelic effects seen in humans. In conclusion, we discovered here that mutations in 4 genes encoding components of the outer ring subunits of the NPC cause SRNS and thereby provide further evidence that specific hypomorphic mutations in these essential genes cause a distinct, organ-specific	fish
231	animal (fish)	zebrafish	CRISPR;Cas9;	norepinephrine transporter gene (slc6a2)	Journal of comparative neurology	Features of the structure, development, and activity of the zebrafish noradrenergic system explored in new CRISPR transgenic lines.	2018	526(15):2493-2508	[Farrar MJ et al.]	Cornell University, Ithaca, NY, USA.	30070695	10.1002/cne.24508	The noradrenergic (NA) system of vertebrates is implicated in learning, memory, arousal, and neuroinflammatory responses, but is difficult to access experimentally. Small and optically transparent, larval zebrafish offer the prospect of exploration of NA structure and function in an intact animal. We made multiple transgenic zebrafish lines using the CRISPR/Cas9 system to insert fluorescent reporters upstream of slc6a2, the norepinephrine transporter gene. These lines faithfully express reporters in NA cell populations, including the locus coeruleus (LC), which contains only about 14 total neurons. We used the lines in combination with two-photon microscopy to explore the structure and projections of the NA system in the context of the columnar organization of cell types in the zebrafish hindbrain. We found robust alignment of NA projections with glutamatergic neurotransmitter stripes in some hindbrain segments, suggesting orderly relations to neuronal cell types early in life. We also quantified neurite density in the rostral spinal cord in individual larvae with as much as 100% difference in the number of LC neurons, and found no correlation between neuronal number in the LC and projection density in the rostral spinal cord. Finally, using light sheet microscopy, we performed bilateral calcium imaging of the entire LC. We found that large-amplitude calcium responses were evident in all LC neurons and showed bilateral synchrony, whereas small-amplitude events were more likely to show interhemispheric asynchrony, supporting the potential for targeted LC neuromodulation. Our	fish
232	animal (fish)	zebrafish	CRISPR;Cas9;	Nodal-related protein (ndr2)	Journal of genetics	Analysis of novel domain-specific mutations in the zebrafish ndr2/cyclops gene generated using CRISPR-Cas9 RNPs.	2018	97(5):1315-1325.	[Turner AN et al.]	University of Alabama at Birmingham, Birmingham, AL, USA.	30555080	10.1007/s12041-018-1033-6	Nodal-related protein (ndr2) is amember of the transforming growth factor type beta superfamily of factors and is required for ventral midline patterning of the embryonic central nervous system in zebrafish. In humans, mutations in the gene encoding nodal cause holoprosencephaly and heterotaxy. Mutations in the ndr2 gene in the zebrafish (Danio rerio) lead to similar phenotypes, including loss of the medial floor plate, severe deficits in ventral forebrain development and cyclopia. Alleles of the ndr2 gene have been useful in studying patterning of ventral structures of the central nervous system. Fifteen different ndr2 alleles have been reported in zebrafish, of which eight were generated using chemical mutagenesis, four were radiation-induced and the remaining alleles were obtained via random insertion, gene targeting (TALEN) or unknown methods. Therefore, most mutation sites were random and could not be predicted a priori. Using the CRISPR-Cas9 system from Streptococcus pyogenes, we targeted distinct regions in all three exons of zebrafish ndr2 and observed cyclopia in the injected (G0) embryos.We show that the use of sgRNA-Cas9 ribonucleoprotein (RNP) complexes can cause penetrant cyclopic phenotypes in injected (G0) embryos. Targeted polymerase chain reaction amplicon analysis using Sanger sequencing showed that most of the alleles had small indels resulting in frameshifts. The sequence information correlates with the loss of ndr2 activity. In this study, we validate multiple CRISPR targets using an in vitro nuclease assay and in vivo analysis using embryos. We describe one specific mutant allele resulting in the loss of conserved terminal cysteine-coding sequences. This study is another demonstration of the utility of the CRISPR-Cas9 system in generating domain-specific mutations and provides further	fish

233	animal (fish)	zebrafish	TALENs;	galactose-1-phosphate uridylyltransferase	Journal of inherited metabolic disease	Impaired fertility and motor function in a zebrafish model for classic galactosemia.	2018	41(1):117-127	[Vanoevelen JM et al.]	Maastricht University Medical Centre, Maastricht, The Netherlands.	28913702	10.1007/s10545-017-0071-1	Classic galactosemia is a genetic disorder of galactose metabolism, caused by severe deficiency of galactose-1-phosphate uridylyltransferase (GALT) enzyme activity due to mutations of the GALT gene. Its pathogenesis is still not fully elucidated, and a therapy that prevents chronic impairments is lacking. In order to move research forward, there is a high need for a novel animal model, which allows organ studies throughout development and high-throughput screening of pharmacologic compounds. Here, we describe the generation of a galt knockout zebrafish model and present its phenotypical characterization. Using a TALEN approach, a galt knockout line was successfully created. Accordingly, biochemical assays confirm essentially undetectable galt enzyme activity in homozygotes. Analogous to humans, galt knockout fish accumulate galactose-1-phosphate upon exposure to exogenous galactose. Furthermore, without prior exposure to exogenous galactose, they exhibit reduced motor activity and impaired fertility (lower egg quantity per mating, higher number of unsuccessful crossings), resembling the human phenotype(s) of neurological sequelae and subfertility. In conclusion, our galt knockout zebrafish model for classic galactosemia mimics the human phenotype(s) at biochemical and clinical levels. Future studies in our model will contribute to improved understanding and management of this disorder.	fish
234	animal (fish)	zebrafish	Cas9;	abcc6a	Journal of investigative dermatology	Generation and Validation of a Complete Knockout Model of abcc6a in Zebrafish.	2018	138(11):2333-2342	[Van Gils M et al.]	Ghent University Hospital, Ghent, Belgium.	30030150	10.1016/j.jid.2018.06.183	Pseudoxanthoma elasticum is an ectopic mineralization disease due to biallelic ABCC6 mutations. As no treatment options are currently available, a reliable zebrafish model is invaluable for high throughput compound screening. However, data from previously reported knockdown and mutant zebrafish models for abcc6a, the functional orthologue of ABCC6, showed phenotypic discrepancies. To address this, we developed a complete abcc6a knockout model using Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 and compared its phenotype to that of a mutant model (Sa963) and a splice junction morpholino model. Our data showed that abcc6a is not required for embryonic survival, but rather that it has an essential role in controlling mineralization. The three models developed very similar hypermineralization of spine and ribs starting embryonically and progressing in adulthood with development of scoliosis. Our results indicate a direct relation between loss of abcc6a expression and dysregulated osteogenesis. As such, our models recapitulate part of the human phenotype in which ectopic mineralization and pro-osteogenic signaling have been reported. Because of its reproducibility in three models and its ease of quantification, we consider this phenotype to be unequivocally the result of abcc6 deficiency and, as	fish
235	animal (fish)	zebrafish	CRISPR;	histone acetyltransferase (kat2a; kat2b)	Journal of molecular and cellular cardiology	Acetylation of TBX5 by KAT2B and KAT2A regulates heart and limb development.	2018	114:185-198	[Ghosh TK et al.]	University of Nottingham, Nottingham, UK.	29174768	10.1016/j.yjmcc.2017.11.013	TBX5 plays a critical role in heart and forelimb development. Mutations in TBX5 cause Holt-Oram syndrome, an autosomal dominant condition that affects the formation of the heart and upper-limb. Several studies have provided significant insight into the role of TBX5 in cardiogenesis; however, how TBX5 activity is regulated by other factors is still unknown. Here we report that histone acetyltransferases KAT2A and KAT2B associate with TBX5 and acetylate it at Lys339. Acetylation potentiates its transcriptional activity and is required for nuclear retention. Morpholino-mediated knockdown of kat2a and kat2b transcripts in zebrafish severely perturb heart and limb development, mirroring the tbx5a knockdown phenotype. The phenotypes found in MO-injected embryos were also observed when we introduced mutations in the kat2a or kat2b genes using the CRISPR-Cas system. These studies highlight the importance of KAT2A and KAT2B modulation of TBX5 and their impact on heart and limb	fish
236	animal (fish)	zebrafish	Cas9;	ntla; gata5	Journal of molecular cell biology	The genetic program of oocytes can be modified in vivo in the zebrafish ovary.	2018	10(6):479-493	[Wu X et al.]	Tsinghua University, Beijing, China.	30060229	10.1093/jmcb/mjy044	Oocytes, the irreplaceable gametes for generating a new organism, are matured in the ovary of living female animals. It is unknown whether any genetic manipulations can be applied to immature oocytes inside the living ovaries. As a proof-of-concept, we here demonstrate genetic amendments of zebrafish immature oocytes within the ovary. Oocyte microinjection in situ (OMIS) stimulates tissue repair responses, but some of the microinjected immature oocytes are matured, ovulated and fertilizable. By OMIS-mediated Cas9 approach, ntla and gata5 loci of oocytes arrested at prophase I of meiosis are successfully edited before fertilization. Through OMIS, high efficiency of biallelic mutations in single or multiple loci using Cas9/gRNAs allows immediate manifestation of mutant phenotypes in F0 embryos and multiple transgenes can co-express the reporters in F0 embryos with patterns similar to germline transgenic embryos. Furthermore, maternal knockdown of dnmt1 by antisense morpholino via OMIS results in a dramatic decrease of global DNA methylation level at the dome stage and causes embryonic lethality prior to segmentation period. Therefore, OMIS opens a door to efficiently modify the genome and provides a possibility to repair genetically	fish
237	animal (fish)	zebrafish	CRISPR;Cas9;	NADPH oxidase (nox1, nox2/cybb, nox5, duox)	Journal of neuroscience	nox2/cybb Deficiency Affects Zebrafish Retinotectal Connectivity.	2018	38(26):5854-5871	[Weaver CJ et al.]	Purdue University, West Lafayette, IN, USA.	29793976	10.1523/JNEUROSCI.1483-16.2018	NADPH oxidase (Nox)-derived reactive oxygen species (ROS) have been linked to neuronal polarity, axonal outgrowth, cerebellar development, regeneration of sensory axons, and neuroplasticity. However, the specific roles that individual Nox isoforms play during nervous system development in vivo remain unclear. To address this problem, we investigated the role of Nox activity in the development of retinotectal connections in zebrafish embryos. Zebrafish broadly express four nox genes (nox1, nox2/cybb, nox5, and duox) throughout the CNS during early development. Application of a pan-Nox inhibitor, celastrol, during the time of optic nerve (ON) outgrowth resulted in significant expansion of the ganglion cell layer (GCL), thinning of the ON, and a decrease in retinal axons reaching the optic tectum (OT). With the exception of GCL expansion, these effects were partially ameliorated by the addition of H2O2, a key ROS involved in Nox signaling. To address isoform-specific Nox functions, we used CRISPR/Cas9 to generate mutations in each zebrafish nox gene. We found that nox2/cybb chimeric mutants displayed ON thinning and decreased OT innervation. Furthermore, nox2/cybb homozygous mutants (nox2/cybb(-/-)) showed significant GCL expansion and mistargeted retinal axons in the OT. Neurite outgrowth from cultured zebrafish retinal ganglion cells was reduced by Nox inhibitors, suggesting a cell-autonomous role for Nox in these neurons. Collectively, our results show that Nox2/Cybb is important for retinotectal development in zebrafish.SIGNIFICANCE STATEMENT Most isoforms of NADPH oxidase (Nox) only produce reactive oxygen species (ROS) when activated by an upstream signal, making them ideal candidates for ROS signaling. Nox enzymes are present in neurons and their activity has been shown to be important for neuronal development and function largely by in vitro studies. However, whether Nox is involved in the development of axons and formation of neuronal connections in vivo has remained unclear. Using mutant zebrafish embryos, this study shows that a specific Nox isoform, Nox2/Cybb, is important for the	fish

238	animal (fish)	zebrafish	CRISPR;		Journal of visualized experiments : JoVE	Efficient Production and Identification of CRISPR/Cas9-generated Gene Knockouts in the Model System Danio rerio.	2018	(138)	[Sorlien EL et al.]	Purdue University, USA.	30222157	10.3791/56969	Characterization of the clustered, regularly interspaced, short, palindromic repeat (CRISPR) system of Streptococcus pyogenes has enabled the development of a customizable platform to rapidly generate gene modifications in a wide variety of organisms, including zebrafish. CRISPR-based genome editing uses a single guide RNA (sgRNA) to target a CRISPR-associated (Cas) endonuclease to a genomic DNA (gDNA) target of interest, where the Cas endonuclease generates a double-strand break (DSB). Repair of DSBs by error-prone mechanisms lead to insertions and/or deletions (indels). This can cause frameshift mutations that often introduce a premature stop codon within the coding sequence, thus creating a protein-null allele. CRISPR-based genome engineering requires only a few molecular components and is easily introduced into zebrafish embryos by microinjection. This protocol describes the methods used to generate CRISPR reagents for zebrafish microinjection and to identify fish exhibiting germline transmission of CRISPR-modified genes. These methods include in vitro transcription of sgRNAs, microinjection of CRISPR reagents, identification of indels induced at the target site using a PCR-based method called a heteroduplex mobility assay (HMA), and characterization of the indels using both a low throughput and a powerful next-generation sequencing (NGS)-based approach that can analyze multiple PCR products collected from heterozygous fish. This protocol is streamlined to minimize both the number of fish required and the types of equipment needed to perform the analyses. Furthermore, this protocol is designed to be amenable for use by laboratory personal of all levels of experience including undergraduates, enabling this powerful tool to be economically employed by any research group interested in	fish
239	animal (fish)	zebrafish	CRISPR;Cas9;	ptch1	Leukemia	Hedgehog pathway mutations drive oncogenic transformation in high-risk T-cell acute lymphoblastic leukemia.	2018	32(10):2126-2137	[Burns MA et al.]	Boston Children's Hospital, Boston, MA, USA.	29654263	10.1038/s41375-018-0097-x	The role of Hedgehog signaling in normal and malignant T-cell development is controversial. Recently, Hedgehog pathway mutations have been described in T-ALL, but whether mutational activation of Hedgehog signaling drives T-cell transformation is unknown, hindering the rationale for therapeutic intervention. Here, we show that Hedgehog pathway mutations predict chemotherapy resistance in human T-ALL, and drive oncogenic transformation in a zebrafish model of the disease. We found Hedgehog pathway mutations in 16% of 109 childhood T-ALL cases, most commonly affecting its negative regulator PTCH1. Hedgehog mutations were associated with resistance to induction chemotherapy (P = 0.009). Transduction of wild-type PTCH1 into PTCH1-mutant T-ALL cells induced apoptosis (P = 0.005), a phenotype that was reversed by downstream Hedgehog pathway activation (P = 0.007). Transduction of most mutant PTCH1, SUFU, and GLI alleles into mammalian cells induced aberrant regulation of Hedgehog signaling, indicating that these mutations are pathogenic. Using a CRISPR/Cas9 system for lineage-restricted gene disruption in transgenic zebrafish, we found that ptch1 mutations accelerated the onset of notch1-induced T-ALL (P = 0.0001), and pharmacologic Hedgehog pathway inhibition had therapeutic activity. Thus, Hedgehog-activating mutations are driver oncogenic alterations in high-risk T-ALL.	fish
240	animal (fish)	zebrafish	CRISPR;Cas9;	red fluorescence protein	Marine biotechnology	Zebrafish Embryonic Slow Muscle Is a Rapid System for Genetic Analysis of Sarcomere Organization by CRISPR/Cas9, but Not NgAgo.	2018	20(2):168-181	[Cai M et al.]	University of Maryland School of Medicine, Baltimore, MD, USA.	29374849	10.1007/s10126-018-9794-8	Zebrafish embryonic slow muscle cells, with their superficial localization and clear sarcomere organization, provide a useful model system for genetic analysis of muscle cell differentiation and sarcomere assembly. To develop a quick assay for testing CRISPR-mediated gene editing in slow muscles of zebrafish embryos, we targeted a red fluorescence protein (RFP) reporter gene specifically expressed in slow muscles of myomesin-3-RFP (Myom3-RFP) zebrafish embryos. We demonstrated that microinjection of RFP-sgRNA with Cas9 protein or Cas9 mRNA resulted in a mosaic pattern in loss of RFP expression in slow muscle fibers of the injected zebrafish embryos. To uncover gene functions in sarcomere organization, we targeted two endogenous genes, slow myosin heavy chain-1 (smyhc1) and heat shock protein 90 alpha1 (hsp90alpha1), which are specifically expressed in zebrafish muscle cells. We demonstrated that injection of Cas9 protein or mRNA with respective sgRNAs targeted to smyhc1 or hsp90a1 resulted in a mosaic pattern of myosin thick filament disruption in slow myofibers of the injected zebrafish embryos. Moreover, Myom3-RFP expression and M-line localization were also abolished in these defective myofibers. Given that zebrafish embryonic slow muscles are a rapid in vivo system for testing genome editing and uncovering gene functions in muscle cell differentiation, we investigated whether microinjection of Natronobacterium gregoryi Argonaute (NgAgo) system could induce genetic mutations and muscle defects in zebrafish embryos. Single-strand guide DNAs targeted to RFP, Smyhc1, or Hsp90alpha1 were injected with NgAgo mRNA into Myom3-RFP zebrafish embryos. Myom3-RFP expression was analyzed in the injected embryos. The results showed that, in contrast to the CRISPR/Cas9 system, injection of the NgAgo-gDNA system did not affect Myom3-RFP expression and sarcomere organization in myofibers of the injected embryos. Sequence analysis failed to detect genetic mutations at the target genes. Together, our studies demonstrate that zebrafish embryonic slow muscle is a rapid model for testing gene editing technologies	fish
241	animal (fish)	zebrafish	CRISPR;Cas9;	CCCTC-binding factor	Mechanisms of development	CTCF knockout reveals an essential role for this protein during the zebrafish development.	2018	154:51-59	[Carmona-Aldana F et al.]	Universidad Nacional Autonoma de Mexico, Ciudad de Mexico, Mexico.	29723654	10.1016/j.mod.2018.04.006	Chromatin regulation and organization are essential processes that regulate gene activity. The CCCTC-binding factor (CTCF) is a protein with different and important molecular functions related with chromatin dynamics. It is conserved since invertebrates to vertebrates, posing it as a factor with an important role in the physiology. In this work, we aimed to understand the distribution and functional relevance of CTCF during the embryonic development of the zebrafish (Danio rerio). We generated a zebrafish specific anti-Ctcf antibody, and found this protein to be ubiquitous, through different stages and tissues. We used the CRISPR-Cas9 system to induce molecular alterations in the locus. This resulted in early lethality. We delayed the lethality performing knockdown morpholino experiments, and found an aberrant embryo morphology involving malformations in structures through all the length of the embryo. These phenotypes were rescued with human CTCF mRNA injections, showing the specificity of the morpholinos and a partial functional conservation between the fish and the human proteins. Lastly, we found that the pro-apoptotic genes p53 and bbc3/PUMA are deregulated in the ctcf morpholino-injected embryos. In conclusion, CTCF is a ubiquitous factor during the zebrafish development, which regulates the correct formation of different structures of the embryo, and its deregulation impacts on essential cell survival genes. Overall, this work provides a basis to look for the	fish

242	animal (fish)	zebrafish	CRISPR;Cas9;	contactin2	Mechanisms of development	Distinct roles for the cell adhesion molecule Contactin2 in the development and function of neural circuits in zebrafish.	2018	152:1–12	[Gurung S et al.]	University of Missouri, Columbia, MO, USA.	29777776	10.1016/j.mod.2018.05.005	Contactin2 (Cntn2)/Transient Axonal Glycoprotein 1 (Tag1), a neural cell adhesion molecule, has established roles in neuronal migration and axon fasciculation in chick and mouse. In zebrafish, antisense morpholino-based studies have indicated roles for cntn2 in the migration of facial branchiomotor (FBM) neurons, the guidance of the axons of the nucleus of the medial longitudinal fascicle (nucMLF), and the outgrowth of Rohon-Beard (RB) central axons. To study functions of Cntn2 in later stages of neuronal development, we generated cntn2 mutant zebrafish using CRISPR-Cas9. Using a null mutant allele, we detected genetic interactions between cntn2 and the planar cell polarity gene vangl2, as shown previously with cntn2 morphants, demonstrating a function for cntn2 during FBM neuron migration in a sensitized background of reduced planar cell polarity signaling. In addition, maternal-zygotic (MZ) cntn2 mutant larvae exhibited aberrant touch responses and swimming, suggestive of defects in sensorimotor circuits, consistent with studies in mice. However, the nucMLF axon convergence, FBM neuron migration, and RB outgrowth defects seen in morphants were not seen in the mutants, and we show here that they are likely off-target effects of morpholinos. However, MLF axons exhibited local defasciculation in MZcntn2 mutants, consistent with a role for Cntn2 in axon fasciculation. These data demonstrate distinct roles for zebrafish cntn2 in neuronal migration and axon	fish
243	animal (fish)	zebrafish	CRISPR;Cas9;		Methods	Programmable base editing in zebrafish using a modified CRISPR-Cas9 system.	2018	150:19–23	[Qin W et al.]	Peking University Shenzhen Graduate School, Shenzhen, China.	30076894	10.1016/j.ymeth.2018.07.010	The use of CRISPR/Cas9 to knockout genes in zebrafish has been well established. However, to better model many human diseases that are caused by point mutations, a robust methodology for generating desirable DNA base changes is still needed. Recently, Cas9-linked cytidine deaminases (base editors) evolved as a strategy to introduce single base mutations in model organisms. They have been used to convert cytidine to thymine at specific genomic loci. Here we describe a protocol for using the base editing system in zebrafish and its application to reproduce a single base mutation observed in human Ablepharon-Macrostomia Syndrome.	fish
244	animal (fish)	zebrafish	CRISPR;Cpf1;		Methods	Optimized CRISPR-Cpf1 system for genome editing in zebrafish.	2018	150:11–18	[Fernandez JP et al.]	Yale University School of Medicine, New Haven, CT, USA.	29964176	10.1016/j.ymeth.2018.06.014	The impact of the CRISPR-Cas biotechnological systems has recently broadened the genome editing toolbox available to different model organisms further with the addition of new efficient RNA-guided endonucleases. We have recently optimized CRISPR-Cpf1 (renamed Cas12a) system in zebrafish. We showed that (i) in the absence of Cpf1 protein, crRNAs are unstable and degraded in vivo, and CRISPR-Cpf1 RNP complexes efficiently mutagenize the zebrafish genome; and (ii) temperature modulates Cpf1 activity especially affecting AsCpf1, which experiences a reduced performance below 37 degrees C. Here, we describe a step-by-step protocol on how to easily design and generate crRNAs in vitro, purify recombinant Cpf1 proteins, and assemble ribonucleoprotein complexes to carry out efficient mutagenesis in zebrafish in a constitutive and temperature-controlled manner. Finally, we explain how to induce Cpf1-mediated homology-directed repair using single-stranded DNA oligonucleotides. In summary, this protocol includes the steps to efficiently modify the zebrafish genome and other ectothermic organisms using the CRISPR-Cpf1 system.	fish
245	animal (fish)	zebrafish	CRISPR;Cas9;	shank3b	Molecular autism	CRISPR/Cas9-induced shank3b mutant zebrafish display autism-like behaviors.	2018	9:23	[Liu CX et al.]	Fudan University, Shanghai, China.	29619162	10.1186/s13229-018-0204-x	Background: Human genetic and genomic studies have supported a strong causal role of SHANK3 deficiency in autism spectrum disorder (ASD). However, the molecular mechanism underlying SHANK3 deficiency resulting in ASD is not fully understood. Recently, the zebrafish has become an attractive organism to model ASD because of its high efficiency of genetic manipulation and robust behavioral phenotypes. The orthologous gene to human SHANK3 is duplicated in the zebrafish genome and has two homologs, shank3a and shank3b. Previous studies have reported shank3 morphants in zebrafish using the morpholino method. Here, we report the generation and characterization of shank3b mutant zebrafish in larval and adult stages using the CRISPR/Cas9 genome editing technique. Methods: CRISPR/Cas9 was applied to generate a shank3b loss-of-function mutation (shank3b(-/-)) in zebrafish. A series of morphological measurements, behavioral tests, and molecular analyses were performed to systematically characterize the behavioral and molecular changes in shank3b mutant zebrafish. Results: shank3b(-/-) zebrafish exhibited abnormal morphology in early development. They showed reduced locomotor activity both as larvae and adults, reduced social interaction and time spent near conspecifics, and significant repetitive swimming behaviors. Additionally, the levels of both postsynaptic homer1 and presynaptic synaptophysin were significantly reduced in the adult brain of shank3b-deficient zebrafish. Conclusions: We generated the first inheritable shank3b mutant zebrafish model using CRISPR/Cas9 gene editing approach. shank3b(-/-) zebrafish displayed robust autism-like behaviors and altered levels of the synaptic proteins homer1 and synaptophysin. The versatility of zebrafish as a model for studying neurodevelopment and conducting drug screening will likely have a significant	fish
246	animal (fish)	zebrafish	CRISPR;Cas9;	erythropoietin; erythropoietin receptor	Molecular metabolism	Targeting erythropoietin protects against proteinuria in type 2 diabetic patients and in zebrafish.	2018	8:189–202	[She J et al.]	First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China.	29203238	10.1016/j.molmet.2017.11.006	OBJECTIVE: Adult human kidneys produce erythropoietin (EPO), which regulates red blood cell formation; however, whether EPO also functions directly on kidney development and controls diabetic kidney disease remains unknown. Here we analyzed the role of EPO in kidney development and under hyperglycemic conditions in zebrafish and in humans. METHODS: Diabetic patients and respective controls were enrolled in two cohorts. Serum EPO level and urine protein change upon human EPO administration were then analyzed. Transient knockdown and permanent knockout of EPO and EPOR in renal TG(WT1B:EGFP) zebrafish were established using the morpholino technology and CRISPR/Cas9 technology. Zebrafish embryos were phenotypically analyzed using fluorescence microscopy, and functional assays were carried out with the help of TexasRed labeled 70 kDa Dextran. Apoptosis was determined using the TUNEL assay and Annexin V staining, and caspase inhibitor zVADfmk was used for rescue experiments. RESULTS: In type 2 diabetic patients, serum EPO level decreased with the duration of diabetes, which was linked to reduced kidney function. Human recombinant EPO supplementation ameliorated proteinuria in diabetic nephropathy patients. In zebrafish, loss-of-function studies for EPO and EPOR, showed morphological and functional alterations within the pronephros, adversely affecting pronephric structure, leading to slit diaphragm dysfunction by increasing apoptosis within the pronephros. Induction of hyperglycemia in zebrafish embryos induced pronephros alterations which were further worsened upon silencing of EPO expression. CONCLUSIONS: EPO was identified as a direct renal protective	fish

247	animal (fish)	zebrafish	CRISPR;Cas9;	rhodopsin	Molecular vision	Targeted disruption of the endogenous zebrafish rhodopsin locus as models of rapid rod photoreceptor degeneration.	2018	24:587-602. eCollection 2018.	[Zelinka CP et al.]	Florida State University, Tallahassee, FL, USA.	30210230		Purpose: Retinitis pigmentosa (RP) is a collection of genetic disorders that results in the degeneration of light-sensitive photoreceptor cells, leading to blindness. RP is associated with more than 70 loci that may display dominant or recessive modes of inheritance, but mutations in the gene encoding the visual pigment rhodopsin (RHO) are the most frequent cause. In an effort to develop precise mutations in zebrafish as novel models of photoreceptor degeneration, we describe the generation and germline transmission of a series of novel clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-induced insertion and deletion (indel) mutations in the major zebrafish rho locus, rh1-1. Methods: One- or two-cell staged zebrafish embryos were microinjected with in vitro transcribed mRNA encoding Cas9 and a single guide RNA (gRNA). Mutations were detected by restriction fragment length polymorphism (RFLP) and DNA sequence analyses in injected embryos and offspring. Immunolabeling with rod- and cone-specific antibodies was used to test for histological and cellular changes. Results: Using gRNAs that targeted highly conserved regions of rh1-1, a series of dominant and recessive alleles were recovered that resulted in the rapid degeneration of rod photoreceptors. No effect on cones was observed. Targeting the 5'-coding sequence of rh1-1 led to the recovery of several indels similar to disease-associated alleles. A frame shift mutation leading to a premature stop codon (T17*) resulted in rod degeneration when brought to homozygosity. Immunoblot and fluorescence labeling with a Rho-specific antibody suggest that this is indeed a null allele, illustrating that the Rho expression is essential for rod survival. Two in-frame mutations were recovered that disrupted the highly conserved N-linked glycosylation consensus sequence at N15. Larvae heterozygous for either of the alleles demonstrated rapid rod degeneration. Targeting of the 3'-coding region of rh1-1 resulted in the recovery of an allele encoding a premature stop codon (S347*) upstream of the conserved VSPA sorting sequence and a second in-frame allele that disrupted the putative phosphorylation site at S339. Both alleles resulted in rod death in a dominant inheritance pattern. Following the loss of the targeting sequence, immunolabeling for Rho was no longer restricted to the rod outer segment, but it was also localized to the plasma membrane. Conclusions: The efficiency of CRISPR/Cas9 for gene targeting, coupled with the large number of mutations associated with RP, provided a backdrop for the rapid isolation of novel alleles in zebrafish that phenocopy	fish
248	animal (fish)	zebrafish	CRISPR;Cas9;	titin	Nature communications	Rare truncating variants in the sarcomeric protein titin associate with familial and early-onset atrial fibrillation.	2018	9(1):4316	[Ahlberg G et al.]	Rigshospitalet, University Hospital of Copenhagen, Copenhagen, Denmark.	30333491	10.1038/s41467-018-06618-y	A family history of atrial fibrillation constitutes a substantial risk of developing the disease, however, the pathogenesis of this complex disease is poorly understood. We perform whole-exome sequencing on 24 families with at least three family members diagnosed with atrial fibrillation (AF) and find that titin-truncating variants (TTNtv) are significantly enriched in these patients (P = 1.76 x 10(-6)). This finding is replicated in an independent cohort of early-onset lone AF patients (n = 399; odds ratio = 36.8; P = 4.13 x 10(-6)). A CRISPR/Cas9 modified zebrafish carrying a truncating variant of titin is used to investigate TTNtv effect in atrial development. We observe compromised assembly of the sarcomere in both atria and ventricle, longer PR interval, and heterozygous adult zebrafish have a higher degree of fibrosis in the atria, indicating that TTNtv are important risk factors for AF. This aligns with the early onset of the disease and adds an important dimension to the understanding of the molecular	fish
249	animal (fish)	zebrafish	CRISPR;Cas9;	mtu1	Nucleic acids research	Deletion of Mtu1 (Trmu) in zebrafish revealed the essential role of tRNA modification in mitochondrial biogenesis and hearing function.	2018	46(20):10930-10945	[Zhang Q et al.]	Zhejiang University School of Medicine, Hangzhou, Zhejiang, China.	30137487	10.1093/nar/gky758	Mtu1(Trmu) is a highly conserved tRNA modifying enzyme responsible for the biosynthesis of taum5s2U at the wobble position of tRNAGln, tRNAGlu and tRNALys. Our previous investigations showed that MTU1 mutation modulated the phenotypic manifestation of deafness-associated mitochondrial 12S rRNA mutation. However, the pathophysiology of MTU1 deficiency remains poorly understood. Using the mtu1 knock-out zebrafish generated by CRISPR/Cas9 system, we demonstrated the abolished 2-thiouridine modification of U34 of mitochondrial tRNALys, tRNAGlu and tRNAGln in the mtu1 knock-out zebrafish. The elimination of this post-transcriptional modification mediated mitochondrial tRNA metabolisms, causing the global decreases in the levels of mitochondrial tRNAs. The aberrant mitochondrial tRNA metabolisms led to the impairment of mitochondrial translation, respiratory deficiencies and reductions of mitochondrial ATP production. These mitochondria dysfunctions caused the defects in hearing organs. Strikingly, mtu1-/- mutant zebrafish displayed the abnormal startle response and swimming behaviors, significant decreases in the sizes of saccular otolith and numbers of hair cells in the auditory and vestibular organs. Furthermore, mtu1-/- mutant zebrafish exhibited the significant reductions in the hair bundle densities in utricle, saccule and lagena. Therefore, our findings may provide new insights into the pathophysiology of deafness, which was manifested by the deficient	fish
250	animal (fish)	zebrafish	CRISPR;Cas9;		Nucleic acids research	Optimized knock-in of point mutations in zebrafish using CRISPR/Cas9.	2018	46(17):e102	[Prykhozhij SV et al.]	Dalhousie University, Halifax, NS, Canada.	29905858	10.1093/nar/gky512	We have optimized point mutation knock-ins into zebrafish genomic sites using clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 reagents and single-stranded oligodeoxynucleotides. The efficiency of knock-ins was assessed by a novel application of allele-specific polymerase chain reaction and confirmed by high-throughput sequencing. Anti-sense asymmetric oligo design was found to be the most successful optimization strategy. However, cut site proximity to the mutation and phosphorothioate oligo modifications also greatly improved knock-in efficiency. A previously unrecognized risk of off-target trans knock-ins was identified that we obviated through the development of a workflow for correct knock-in detection. Together these strategies greatly facilitate the study of human genetic diseases in zebrafish, with additional applicability to enhance CRISPR-based approaches in other	fish

251	animal (fish)	zebrafish	CRISPR;Cas9;	forkhead box 1	PeerJ	foxr1 is a novel maternal-effect gene in fish that is required for early embryonic success.	2018	6:e5534	[Cheung CT et al.]	INRA, Rennes, France.	30155373	10.7717/peerj.5534	The family of forkhead box (Fox) transcription factors regulates gonadogenesis and embryogenesis, but the role of foxr1 in reproduction is unknown. Evolutionary history of foxr1 in vertebrates was examined and the gene was found to exist in most vertebrates, including mammals, ray-finned fish, amphibians, and sauropsids. By quantitative PCR and RNA-seq, we found that foxr1 had an ovarian-specific expression in zebrafish, a common feature of maternal-effect genes. In addition, it was demonstrated using in situ hybridization that foxr1 was a maternally-inherited transcript that was highly expressed even in early-stage oocytes and accumulated in the developing eggs during oogenesis. We also analyzed the function of foxr1 in female reproduction using a zebrafish CRISPR/cas9 knockout model. It was observed that embryos from the foxr1-deficient females had a significantly lower survival rate whereby they either failed to undergo cell division or underwent abnormal division that culminated in growth arrest at around the mid-blastula transition and early death. These mutant-derived eggs contained dramatically increased levels of p21, a cell cycle inhibitor, and reduced rictor, a component of mTOR and regulator of cell survival, which were in line with the observed growth arrest phenotype. Our study shows for the first time that foxr1 is an essential maternal-effect gene and may be required for proper cell division and survival via the p21 and mTOR pathways. These novel findings will broaden our knowledge on the functions of specific maternal factors stored in the developing	fish
252	animal (fish)	zebrafish	CRISPR;Cas9;	GIP receptor	Peptides	Evaluation of the insulinotropic and glucose-lowering actions of zebrafish GIP in mammalian systems: Evidence for involvement of the GLP-1 receptor.	2018	100:182-189	[Graham GV et al.]	Ulster University, Coleraine, Northern Ireland, UK.	29157578	10.1016/j.peptides.2017.11.007	The insulinotropic properties of zebrafish GIP (zfGIP) were assessed in vitro using clonal pancreatic beta-cell lines and isolated mouse islets and acute effects on glucose tolerance and insulin release in vivo were evaluated in mice. The peptide produced a dose-dependent increase in the rate of insulin release from BRIN-BD11 rat clonal beta-cells at concentrations >/=30nM. Insulin release from 1.1 B4 human clonal beta-cells and mouse islets was significantly increased by zfGIP (10nM and 1muM). The in vitro insulinotropic activity of zfGIP was decreased after incubating BRIN-BD11 cells with the GLP-1 receptor antagonist, exendin-4(9-39) ($p<0.001$) and the GIP receptor antagonist, GIP (6-30) Cex-K(40)[Pal] ($p<0.05$) but the glucagon receptor antagonist [des-His(1),Pro(4),Glu(9)]glucagon amide was without effect. zfGIP (10nM and 1muM) produced significant increases in cAMP concentration in CHL cells transfected with the human GLP-1 receptor but was without effect on HEK293 cells transfected with the human glucagon receptor. Conversely, zfGIP, but not human GIP, significantly stimulated insulin release from CRISPR/Cas9-engineered INS-1 clonal beta-cells from which the GIP receptor had been deleted. Intraperitoneal administration of zfGIP (25 and 75nmol/kg body weight) to mice together with an intraperitoneal glucose load (18mmol/kg body weight) produced a significant decrease in plasma glucose concentrations concomitant with an increase in insulin concentrations. The study provides evidence that the insulinotropic action of zfGIP in mammalian systems	fish
253	animal (fish)	zebrafish	CRISPR;Cas9;	fanca; fancb; fancc; fancd1/brca2; fancd2; fance; fancf; fancg; fanci; fancj/brip1; fancl; fancm; fancn/palb2; fanco/rad51c; fancp/slx4; fancq/ercc4; fanct/ube2t; faap100; faap24	PLoS genetics	Multiplexed CRISPR/Cas9-mediated knockout of 19 Fanconi anemia pathway genes in zebrafish revealed their roles in growth, sexual development and fertility.	2018	14(12):e1007821	[Ramanagoudr-Bhojappa R et al.]	National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA.	30540754	10.1371/journal.pgen.1007821	Fanconi Anemia (FA) is a genomic instability syndrome resulting in aplastic anemia, developmental abnormalities, and predisposition to hematological and other solid organ malignancies. Mutations in genes that encode proteins of the FA pathway fail to orchestrate the repair of DNA damage caused by DNA interstrand crosslinks. Zebrafish harbor homologs for nearly all known FA genes. We used multiplexed CRISPR/Cas9-mediated mutagenesis to generate loss-of-function mutants for 17 FA genes: fanca, fancb, fancc, fancd1/brca2, fancd2, fance, fancf, fancg, fanci, fancj/brip1, fancl, fancm, fancn/palb2, fanco/rad51c, fancp/slx4, fancq/ercc4, fanct/ube2t, and two genes encoding FA-associated proteins: faap100 and faap24. We selected two indel mutations predicted to cause premature truncations for all but two of the genes, and a total of 36 mutant lines were generated for 19 genes. Generating two independent mutant lines for each gene was important to validate their phenotypic consequences. RT-PCR from homozygous mutant fish confirmed the presence of transcripts with indels in all genes. Interestingly, 4 of the indel mutations led to aberrant splicing, which may produce a different protein than predicted from the genomic sequence. Analysis of RNA is thus critical in proper evaluation of the consequences of the mutations introduced in zebrafish genome. We used fluorescent reporter assay, and western blots to confirm loss-of-function for several mutants. Additionally, we developed a DEB treatment assay by evaluating morphological changes in embryos and confirmed that homozygous mutants from all the FA genes that could be tested (11/17), displayed hypersensitivity and thus were indeed null alleles. Our multiplexing strategy helped us to evaluate 11 multiple gene knockout combinations without additional breeding. Homozygous zebrafish for all 19 single and 11 multi-gene knockouts were adult viable, indicating FA genes in zebrafish are generally not essential for early development. None of the mutant fish displayed gross developmental abnormalities except for fancp-/- fish, which were significantly smaller in length than their wildtype clutch mates. Complete female-to-male sex reversal was observed in knockouts for 12/17 FA genes, while partial sex reversal was seen for the other five gene knockouts. All adult females were fertile, and among the adult males, all were fertile except for the fancd1 mutants and one of the fancj mutants. We report here generation and characterization of zebrafish knockout mutants for 17 FA disease-causing genes, providing an integral	fish
254	animal (fish)	zebrafish	CRISPR;Cas9;	tbx20; fleer; aldh1a2; tcf21	PLoS genetics	Conditional mutagenesis by oligonucleotide-mediated integration of loxP sites in zebrafish.	2018	14(11):e1007754	[Burg L et al.]	Temple University, Philadelphia, PA, USA.	30427827	10.1371/journal.pgen.1007754	Many eukaryotic genes play essential roles in multiple biological processes in several different tissues. Conditional mutants are needed to analyze genes with such pleiotropic functions. In vertebrates, conditional gene inactivation has only been feasible in the mouse, leaving other model systems to rely on surrogate experimental approaches such as overexpression of dominant negative proteins and antisense-based tools. Here, we have developed a simple and straightforward method to integrate loxP sequences at specific sites in the zebrafish genome using the CRISPR/Cas9 technology and oligonucleotide templates for homology directed repair. We engineered conditional (floxed) mutants of tbx20 and fleer, and demonstrate excision of exons flanked by loxP sites using tamoxifen-inducible CreERT2 recombinase. To demonstrate broad applicability of our method, we also integrated loxP sites into two additional genes, aldh1a2 and tcf21. The ease of this approach will further expand the use of zebrafish to study various aspects of vertebrate biology, especially post-embryonic	fish

255	animal (fish)	zebrafish	CRISPR;Cas9;	eTw5-7	PLoS genetics	Unraveling the transcriptional regulation of TWIST1 in limb development.	2018	14(10):e1007738	[Hirsch N et al.]	Ben-Gurion University of the Negev, Beer-Sheva, Israel.	30372441	10.1371/journal.pgen.1007738	The transcription factor TWIST1 plays a vital role in mesoderm development, particularly in limb and craniofacial formation. Accordingly, haploinsufficiency of TWIST1 can cause limb and craniofacial malformations as part of Saethre-Chotzen syndrome. However, the molecular basis of TWIST1 transcriptional regulation during development has yet to be elucidated. Here, we characterized active enhancers in the TWIST1-HDAC9 locus that drive transcription in the developing limb and branchial arches. Using available p300 and H3K27ac ChIP-seq data, we identified 12 enhancer candidates, located both within and outside the coding sequences of the neighboring gene, Histone deacetyase 9 (HDAC9). Using zebrafish and mouse enhancer assays, we showed that eight of these candidates have limb/fin and branchial arch enhancer activity that resemble Twist1 expression. Using 4C-seq, we showed that the Twist1 promoter region interacts with three enhancers (eTw-5, 6, 7) in the limb bud and branchial arch of mouse embryos at day 11.5. Furthermore, we found that two transcription factors, LMX1B and TFAP2, bind these enhancers and modulate their enhancer activity. Finally, using CRISPR/Cas9 genome editing, we showed that homozygous deletion of eTw5-7 enhancers reduced Twist1 expression in the limb bud and caused pre-axial polydactyly, a phenotype observed in Twist1+/- mice. Taken together, our findings reveal that each enhancer has a discrete activity pattern, and together comprise a spatiotemporal regulatory network of Twist1 transcription in the developing limbs/fins and branchial arches. Our study suggests that mutations in TWIST1 enhancers could lead to reduced TWIST1 expression, resulting in phenotypic	fish
256	animal (fish)	zebrafish	TALENs;	clcc1	PLoS genetics	Mutation in the intracellular chloride channel CLCC1 associated with autosomal recessive retinitis pigmentosa.	2018	14(8):e1007504	[Li L et al.]	Shanghai JiaoTong University School of Medicine, Shanghai, China.	30157172	10.1371/journal.pgen.1007504	We identified a homozygous missense alteration (c.75C>A, p.D25E) in CLCC1, encoding a presumptive intracellular chloride channel highly expressed in the retina, associated with autosomal recessive retinitis pigmentosa (arRP) in eight consanguineous families of Pakistani descent. The p.D25E alteration decreased CLCC1 channel function accompanied by accumulation of mutant protein in granules within the ER lumen, while siRNA knockdown of CLCC1 mRNA induced apoptosis in cultured ARPE-19 cells. TALEN KO in zebrafish was lethal 11 days post fertilization. The depressed electroretinogram (ERG) cone response and cone spectral sensitivity of 5 dpf KO zebrafish and reduced eye size, retinal thickness, and expression of rod and cone opsins could be rescued by injection of wild type CLCC1 mRNA. Clcc1+/- KO mice showed decreased ERGs and photoreceptor number. Together these results strongly suggest that intracellular chloride transport by CLCC1 is a critical process in	fish
257	animal (fish)	zebrafish	CRISPR;Cas9;	smyd4	PLoS genetics	The roles of SMYD4 in epigenetic regulation of cardiac development in zebrafish.	2018	14(8):e1007578	[Xiao D et al.]	Fudan University, Shanghai, China.	30110327	10.1371/journal.pgen.1007578	SMYD4 belongs to a family of lysine methyltransferases. We analyzed the role of smyd4 in zebrafish development by generating a smyd4 mutant zebrafish line (smyd4L544Efs*1) using the CRISPR/Cas9 technology. The maternal and zygotic smyd4L544Efs*1 mutants demonstrated severe cardiac malformations, including defects in left-right patterning and looping and hypoplastic ventricles, suggesting that smyd4 was critical for heart development. Importantly, we identified two rare SMYD4 genetic variants in a 208-patient cohort with congenital heart defects. Both biochemical and functional analyses indicated that SMYD4(G345D) was pathogenic. Our data suggested that smyd4 functions as a histone methyltransferase and, by interacting with HDAC1, also serves as a potential modulator for histone acetylation. Transcriptome and bioinformatics analyses of smyd4L544Efs*1 and wild-type developing hearts suggested that smyd4 is a key epigenetic regulator involved in regulating endoplasmic reticulum-mediated protein processing and several important	fish
258	animal (fish)	zebrafish	CRISPR;Cas9;	krox20	PLoS genetics	Cooperation, cis-interactions, versatility and evolutionary plasticity of multiple cis-acting elements underlie krox20 hindbrain regulation.	2018	14(8):e1007581	[Torbey P et al.]	Ecole normale superieure, CNRS, Inserm, PSL Universite, Paris, France.	30080860	10.1371/journal.pgen.1007581	Cis-regulation plays an essential role in the control of gene expression, and is particularly complex and poorly understood for developmental genes, which are subject to multiple levels of modulation. In this study, we performed a global analysis of the cis-acting elements involved in the control of the zebrafish developmental gene krox20. krox20 encodes a transcription factor required for hindbrain segmentation and patterning, a morphogenetic process highly conserved during vertebrate evolution. Chromatin accessibility analysis reveals a cis-regulatory landscape that includes 6 elements participating in the control of initiation and autoregulatory aspects of krox20 hindbrain expression. Combining transgenic reporter analyses and CRISPR/Cas9-mediated mutagenesis, we assign precise functions to each of these 6 elements and provide a comprehensive view of krox20 cis-regulation. Three important features emerged. First, cooperation between multiple cis-elements plays a major role in the regulation. Cooperation can surprisingly combine synergy and redundancy, and is not restricted to transcriptional enhancer activity (for example, 4 distinct elements cooperate through different modes to maintain autoregulation). Second, several elements are unexpectedly versatile, which allows them to be involved in different aspects of control of gene expression. Third, comparative analysis of the elements and their activities in several vertebrate species reveals that this versatility is underlain by major plasticity across evolution, despite the high conservation of the gene expression pattern. These characteristics are likely to be of broad significance for developmental	fish
259	animal (fish)	zebrafish	TALENs;	regulatory subunit of protein phosphatase 2A (pr72)	PloS one	Deletion of Pr72 causes cardiac developmental defects in Zebrafish.	2018	13(11):e0206883	[Song G et al.]	Zhongnan Hospital of Wuhan University, Wuhan, China.	30481179	10.1371/journal.pone.0206883	The alpha regulator subunit B" of protein phosphatase 2 (PPP2R3A), a regulatory subunit of protein phosphatase 2A (PP2A), was reported to present a special subcellular localization in cardiomyocytes and elevate in non-ischemia failing hearts. PPP2R3A has two transcriptions PR72 and PR130. PR72 acts as a negative regulator of the Wnt signaling cascade, while the Wnt signaling cascade plays a pivotal role in cardiac development. And PR130 was found to be involved in cardiac development of zebrafish in our previous study. Thus, to investigate the function of PR72 in heart, two stable pr72 knockout (KO) zebrafish lines were generated using Transcription Activator-Like Effector Nuclease (TALEN) technology. Homozygous pr72 KO fish struggled to survive to adulthood and exhibited cardiac developmental defects, including enlarged ventricular chambers, reduced cardiomyocytes and decreased cardiac function. And the defective sarcomere ultrastructure that affected mitochondria, I bands, Z lines, and intercalated disks was also observed. Furthermore, the abnormal heart looping was detected in mutants which could be rescued by injection with wild type pr72 mRNA. Additionally, it was found that Wnt effectors were elevated in mutants. Those indicated that deletion of pr72 in zebrafish interrupted	fish

260	animal (fish)	zebrafish	CRISPR;Cas9;	eysrmc101/rmc101	PloS one	Eyes shut homolog is important for the maintenance of photoreceptor morphology and visual function in zebrafish.	2018	13(7):e0200789	[Messchaert M et al.]	Radboud University Medical Center, Nijmegen, The Netherlands.	30052645	10.1371/journal.pone.0200789	Mutations in eyes shut homolog (EYS), a gene predominantly expressed in the photoreceptor cells of the retina, are among the most frequent causes of autosomal recessive (ar) retinitis pigmentosa (RP), a progressive retinal disorder. Due to the absence of EYS in several rodent species and its retina-specific expression, still little is known about the exact function of EYS and the pathogenic mechanism underlying EYS-associated RP. We characterized eys in zebrafish, by RT-PCR analysis on zebrafish eye-derived RNA, which led to the identification of a 8,715 nucleotide coding sequence that is divided over 46 exons. The transcript is predicted to encode a 2,905-aa protein that contains 39 EGF-like domains and five laminin A G-like domains, which overall shows 33% identity with human EYS. To study the function of EYS, we generated a stable eysrmc101/rmc101 mutant zebrafish model using CRISPR/Cas9 technology. The introduced lesion is predicted to result in premature termination of protein synthesis and lead to loss of Eys function. Immunohistochemistry on retinal sections revealed that Eys localizes at the region of the connecting cilium and that both rhodopsin and cone transducin are mislocalized in the absence of Eys. Electroretinogram recordings showed diminished b-wave amplitudes in eysrmc101/rmc101 zebrafish (5 dpf) compared to age- and strain-matched wild-type larvae. In addition, decreased locomotor activity in response to light stimuli was observed in eys mutant larvae. Altogether, our study shows that absence of Eys leads	fish
261	animal (fish)	zebrafish	TALENs;	Islet2a	PloS one	Investigation of Islet2a function in zebrafish embryos: Mutants and morphants differ in morphologic phenotypes and gene expression.	2018	13(6):e0199233	[Moreno RL et al.]	University of Colorado School of Medicine, Aurora, CO, USA.	29927984	10.1371/journal.pone.0199233	Zebrafish primary motor neurons differ from each other with respect to morphology, muscle targets and electrophysiological properties. For example, CaP has 2-3-fold larger densities of both inward and outward currents than do other motor neurons. We tested whether the transcription factor Islet2a, uniquely expressed in CaP, but not other primary motor neurons, plays a role in specifying its stereotypic electrophysiological properties. We used both TALEN-based gene editing and antisense morpholino approaches to disrupt Islet2a function. Our electrophysiology results do not support a specific role for Islet2a in determining CaP's unique electrical properties. However, we also found that the morphological phenotypes of CaP and a later-born motor neuron differed between islet2a mutants and morphants. Using microarrays, we tested whether the gene expression profiles of whole embryo morphants, mutants and controls also differed. Morphants had 174 and 201 genes that were differentially expressed compared to mutants and controls, respectively. Further, islet2a was identified as a differentially expressed gene. To examine how mutation of islet2a affected islet gene expression specifically in CaPs, we performed RNA in situ hybridization. We detected no obvious differences in expression of islet1, islet2a, or islet2b in CaPs of mutant versus sibling control embryos. However, immunolabeling studies revealed that an Islet protein persisted in CaPs of mutants, albeit at a reduced level compared to controls. While we cannot exclude requirement for some Islet protein, we conclude that differentiation of the CaP's stereotypic large inward and	fish
262	animal (fish)	zebrafish	CRISPR;	starmaker	PloS one	Candidate gene identification of ovulation-inducing genes by RNA sequencing with an in vivo assay in zebrafish.	2018	13(5):e0196544	[Klangnurak W et al.]	National University Corporation Shizuoka University, Shizuoka, Japan.	29715317	10.1371/journal.pone.0196544	We previously reported the microarray-based selection of three ovulation-related genes in zebrafish. We used a different selection method in this study, RNA sequencing analysis. An additional eight up-regulated candidates were found as specifically up-regulated genes in ovulation-induced samples. Changes in gene expression were confirmed by qPCR analysis. Furthermore, up-regulation prior to ovulation during natural spawning was verified in samples from natural pairing. Gene knock-out zebrafish strains of one of the candidates, the starmaker gene (stm), were established by CRISPR genome editing techniques. Unexpectedly, homozygous mutants were fertile and could spawn eggs. However, a high percentage of unfertilized eggs and abnormal embryos were produced from these homozygous females. The results suggest that the stm gene is necessary for fertilization. In this study, we selected additional ovulation-inducing candidate genes, and a novel function of the stm gene was investigated.	fish
263	animal (fish)	zebrafish	CRISPR;Cas9;		PloS one	Chromatin accessibility is associated with CRISPR-Cas9 efficiency in the zebrafish (Danio rerio).	2018	13(4):e0196238	[Uusi-Makela MIE et al.]	University of Tampere, Tampere, Finland.	29684067	10.1371/journal.pone.0196238	CRISPR-Cas9 technology is routinely applied for targeted mutagenesis in model organisms and cell lines. Recent studies indicate that the prokaryotic CRISPR-Cas9 system is affected by eukaryotic chromatin structures. Here, we show that the likelihood of successful mutagenesis correlates with transcript levels during early development in zebrafish (Danio rerio) embryos. In an experimental setting, we found that guide RNAs differ in their onset of mutagenesis activity in vivo. Furthermore, some guide RNAs with high in vitro activity possessed poor mutagenesis activity in vivo, suggesting the presence of factors that limit the mutagenesis in vivo. Using open access datasets generated from early developmental stages of the zebrafish, and guide RNAs selected from the CRISPRz database, we provide further evidence for an association between gene expression during early development and the success of CRISPR-Cas9 mutagenesis in zebrafish embryos. In order to further inspect the effect of chromatin on CRISPR-Cas9 mutagenesis, we analysed the relationship of selected chromatin features on CRISPR-Cas9 mutagenesis efficiency using publicly available data from zebrafish embryos. We found a correlation between chromatin openness and the efficiency of CRISPR-Cas9 mutagenesis. These results indicate that CRISPR-	fish
264	animal (fish)	zebrafish	CRISPR;Cas9;	foxq1a; foxq1b	PloS one	Genetic analysis of zebrafish homologs of human FOXQ1, foxq1a and foxq1b, in innate immune cell development and bacterial host response.	2018	13(3):e0194207	[Earley AM et al.]	University of North Carolina at Chapel Hill, Chapel Hill, NC, USA.	29534099	10.1371/journal.pone.0194207	FOXQ1 is a member of the forkhead-box transcription factor family that has important functions in development, cancer, aging, and many cellular processes. The role of FOXQ1 in cancer biology has raised intense interest, yet much remains poorly understood. We investigated the possible function of the two zebrafish orthologs (foxq1a and foxq1b) of human FOXQ1 in innate immune cell development and function. We employed CRISPR-Cas9 targeted mutagenesis to create null mutations of foxq1a and foxq1b in zebrafish. Using a combination of molecular, cellular, and embryological approaches, we characterized single and double foxq1a bcz11 and foxq1b bcz18 mutants. This study provides the first genetic mutant analyses of zebrafish foxq1a and foxq1b. Interestingly, we found that foxq1a, but not foxq1b, was transcriptionally regulated during a bacterial response, while the expression of foxq1a was detected in sorted macrophages and upregulated in foxq1a-deficient mutants. However, the transcriptional response to E. coli challenge of foxq1a and foxq1b mutants was not significantly different from that of their wildtype control siblings. Our data shows that foxq1a may have a role in modulating bacterial response, while both foxq1a and foxq1b are not required for the development of macrophages, neutrophils, and microglia. Considering the implicated role of FOXQ1 in a vast number of cancers and biological processes, the foxq1a and foxq1b null mutants from this study provide useful genetic	fish

265	animal (fish)	zebrafish	CRISPR;Cas9;	aryl hydrocarbon receptor (AHR) 2	PloS one	AHR2 required for normal behavioral responses and proper development of the skeletal and reproductive systems in zebrafish.	2018	13(3):e0193484	[Garcia GR et al.]	Oregon State University, Corvallis, OR, USA.	29494622	10.1371/journal.pone.0193484	The aryl hydrocarbon receptor (AHR) is a conserved ligand-activated transcription factor required for proper vertebrate development and homeostasis. The inappropriate activation of AHR by ubiquitous pollutants can lead to adverse effects on wildlife and human health. The zebrafish is a powerful model system that provides a vertebrate data stream that anchors hypothesis at the genetic and cellular levels to observations at the morphological and behavioral level, in a high-throughput format. In order to investigate the endogenous functions of AHR, we generated an AHR2 (homolog of human AHR)-null zebrafish line (ahr2osu1) using the clustered, regulatory interspaced, short palindromic repeats (CRISPR)-Cas9 precision genome editing method. In zebrafish, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) mediated toxicity requires AHR2. The AHR2-null line was resistant to TCDD-induced toxicity, indicating the line can be used to investigate the biological and toxicological functions of AHR2. The AHR2-null zebrafish exhibited decreased survival and fecundity compared to the wild type line. At 36 weeks, histological evaluations of the AHR2-null ovaries revealed a reduction of mature follicles when compared to wild type ovaries, suggesting AHR2 regulates follicle growth in zebrafish. AHR2-null adults had malformed cranial skeletal bones and severely damaged fins. Our data suggests AHR2 regulates some aspect(s) of neuromuscular and/or sensory system development, with impaired behavioral responses observed in larval and adult AHR2-null zebrafish. This study increases our understanding of the endogenous functions of AHR, which may help foster a better understanding of the target organs and molecular mechanisms involved in AHR-	fish
266	animal (fish)	zebrafish	CRISPR;	osgep; tprkb	PloS one	Acute multi-sgRNA knockdown of KEOPS complex genes reproduces the microcephaly phenotype of the stable knockout zebrafish model.	2018	13(1):e0191503	[Jobst-Schwan T et al.]	Boston Children's Hospital, Harvard Medical School, Boston, MA, USA.	29346415	10.1371/journal.pone.0191503	Until recently, morpholino oligonucleotides have been widely employed in zebrafish as an acute and efficient loss-of-function assay. However, off-target effects and reproducibility issues when compared to stable knockout lines have compromised their further use. Here we employed an acute CRISPR/Cas approach using multiple single guide RNAs targeting simultaneously different positions in two exemplar genes (osgep or tprkb) to increase the likelihood of generating mutations on both alleles in the injected F0 generation and to achieve a similar effect as morpholinos but with the reproducibility of stable lines. This multi single guide RNA approach resulted in median likelihoods for at least one mutation on each allele of >99% and sgRNA specific insertion/deletion profiles as revealed by deep-sequencing. Immunoblot showed a significant reduction for Osgep and Tprkb proteins. For both genes, the acute multi-sgRNA knockout recapitulated the microcephaly phenotype and reduction in survival that we observed previously in stable knockout lines, though milder in the acute multi-sgRNA knockout. Finally, we quantify the degree of mutagenesis by deep sequencing, and provide a mathematical model to quantitate the chance for a biallelic loss-of-function mutation. Our findings can be generalized to acute and stable CRISPR/Cas	fish
267	animal (fish)	zebrafish	CRISPR;Cas9;	lymphocyte cytosolic protein 1 (actin-bundling protein L-plastin)	PloS one	Targeted deletion of the zebrafish actin-bundling protein L-plastin (lcp1).	2018	13(1):e0190353	[Kell MJ et al.]	Northwestern University Feinberg School of Medicine / Stanley Manne Children's Research Center, Chicago, IL, USA.	29293625	10.1371/journal.pone.0190353	Regulation of the cytoskeleton is essential for cell migration in health and disease. Lymphocyte cytosolic protein 1 (lcp1, also called L-plastin) is a hematopoietic-specific actin-bundling protein that is highly conserved in zebrafish, mice and humans. In addition, L-plastin expression is documented as both a genetic marker and a cellular mechanism contributing to the invasiveness of tumors and transformed cell lines. Despite L-plastin's role in both immunity and cancer, in zebrafish there are no direct studies of its function, and no mutant, knockout or reporter lines available. Using CRISPR-Cas9 genome editing, we generated null alleles of zebrafish lcp1 and examined the phenotypes of these fish throughout the life cycle. Our editing strategy used gRNA to target the second exon of lcp1, producing F0 mosaic fish that were outcrossed to wild types to confirm germline transmission. F1 heterozygotes were then sequenced to identify three unique null alleles, here called 'Charlie', 'Foxtrot' and 'Lima'. In silico, each allele truncates the endogenous protein to less than 5% normal size and removes both essential actin-binding domains (ABD1 and ABD2). Although none of the null lines express detectable LCP1 protein, homozygous mutant zebrafish (-/-) can develop and reproduce normally, a finding consistent with that of the L-plastin null mouse (LPL -/-). However, such mice do have a profound immune defect when challenged by lung bacteria. Interestingly, we observed reduced long-term survival of zebrafish lcp1 -/- homozygotes (~30% below the expected numbers) in all three of our knockout lines, with greatest mortality corresponding to the period (4-6 weeks post-fertilization) when the innate immune system is functional, but the adaptive immune system is not yet mature. This suggests that null zebrafish may have reduced capacity to combat opportunistic infections, which are more easily transmissible in the aquatic environment. Overall, our novel mutant lines establish a sound genetic model and an enhanced platform for further studies of L-plastin gene function in hematopoiesis and cancer.	fish
268	animal (fish)	zebrafish	CRISPR;	rac3b/rfng/sgca regulatory cluster	Proceedings of the National Academy of Sciences of the United States of America	Evolutionary emergence of the rac3b/rfng/sgca regulatory cluster refined mechanisms for hindbrain boundaries formation.	2018	115(16):E3731-E3740	[Letelier J et al.]	Centro Andaluz de Biologia del Desarrollo, Consejo Superior de Investigaciones Cientificas/Universidad Pablo Olavide, Sevilla, Spain.	29610331	10.1073/pnas.1719885115	Developmental programs often rely on parallel morphogenetic mechanisms that guarantee precise tissue architecture. While redundancy constitutes an obvious selective advantage, little is known on how novel morphogenetic mechanisms emerge during evolution. In zebrafish, rhombomeric boundaries behave as an elastic barrier, preventing cell intermingling between adjacent compartments. Here, we identify the fundamental role of the small-GTPase Rac3b in actomyosin cable assembly at hindbrain boundaries. We show that the novel rac3b/rfng/sgca regulatory cluster, which is specifically expressed at the boundaries, emerged in the Ostariophysi superorder by chromosomal rearrangement that generated new cis-regulatory interactions. By combining 4C-seq, ATAC-seq, transgenesis, and CRISPR-induced deletions, we characterized this regulatory domain, identifying hindbrain boundary-specific cis-regulatory elements. Our results suggest that the capacity of boundaries to act as an elastic mesh for segregating rhombomeric cells evolved by cooption of	fish

269	animal (fish)	zebrafish	CRISPR;Cas9;	cystic fibrosis transmembrane conductance regulator	Reproduction	CFTR is required for the migration of primordial germ cells during zebrafish early embryogenesis.	2018	156(3):261-268	[Liao H et al.]	Sichuan University, Chengdu, China.	29930176	10.1530/REP-17-0681	Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene affect fertility in both sexes. However, the involvement of CFTR in regulating germ cell development remains largely unknown. Here, we used zebrafish model to investigate the role of CFTR in primordial germ cells (PGCs) development. We generated a cftr frameshift mutant zebrafish line using CRISPR/Cas9 technique and investigated the migration of PGCs during early embryo development. Our results showed that loss of Cftr impairs the migration of PGCs from dome stages onward. The migration of PGCs was also perturbed by treatment of CFTRinh-172, a gating-speci fi c CFTR channel inhibitor. Moreover, defected PGCs migration in cftr mutant embryos can be partially rescued by injection of WT but not other channel-defective mutant cftr mRNAs. Finally, we observed the elevation of cxcr4b, cxcl12a, rgs14a and ca15b, key factors involved in zebrafish PGCs migration, in cftr-mutant zebrafish embryos. Taken together, the present study revealed an important role of CFTR acting as an ion channel in regulating PGCs migration during early embryogenesis. Defect of which may impair germ cell development through elevation of key factors involved in cell motility and response to	fish
270	animal (fish)	zebrafish	CRISPR;Cas9;	stem-loop binding protein (slbp2)	RNA	Oocyte-specific maternal Slbp2 is required for replication-dependent histone storage and early nuclear cleavage in zebrafish oogenesis and embryogenesis.	2018	24(12):1738-1748	[He WX et al.]	Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.	30185624	10.1261/rna.067090.118	Stem-loop binding protein (SLBP) is required for replication-dependent histone mRNA metabolism in mammals. Zebrafish possesses two slbps, and slbp1 is necessary for retinal neurogenesis. However, the detailed expression and function of slbp2 in zebrafish are still unknown. In this study, we first identified zebrafish slbp2 as an oocyte-specific maternal factor and then generated a maternal-zygotic slbp2 F3 homozygous mutant (MZslbp2Delta4(-/-)) using CRISPR/Cas9. The depletion of maternal Slbp2 disrupted early nuclear cleavage, which resulted in developmental arrest at the MBT stage. The developmental defects could be rescued in slbp2 transgenic MZslbp2Delta4(-/-) embryos. However, homozygous mutant MZslbp1Delta1(-/-) developed normally, indicating slbp1 is dispensable for zebrafish early embryogenesis. Through comparative proteome and transcriptome profiling between WT and MZslbp2Delta4(-/-) embryos, we identified many differentially expressed proteins and genes. In comparison with those in WT embryos, four replication-dependent histones, including H2a, H2b, H3, and H4, all reduced their expression, while histone variant h2afx significantly increased in MZslbp2Delta4(-/-) embryos at the 256-cell stage and high stage. Zebrafish Slbp2 can bind histone mRNA stem-loop in vitro, and the defects of MZslbp2Delta4(-/-) embryos can be partially rescued by overexpression of H2b. The current data indicate that maternal Slbp2 plays	fish
271	animal (fish)	zebrafish	CRISPR;	SPRED1	Science	Human tumor genomics and zebrafish modeling identify SPRED1 loss as a driver of mucosal melanoma.	2018	362(6418):1055-1060	[Ablain J et al.]	Boston Children's Hospital and Dana-Farber Cancer Institute, Boston, MA, USA.	30385465	10.1126/science.aau6509	Melanomas originating from mucosal surfaces have low mutation burden, genomic instability, and poor prognosis. To identify potential driver genes, we sequenced hundreds of cancer-related genes in 43 human mucosal melanomas, cataloging point mutations, amplifications, and deletions. The SPRED1 gene, which encodes a negative regulator of mitogen-activated protein kinase (MAPK) signaling, was inactivated in 37% of the tumors. Four distinct genotypes were associated with SPRED1 loss. Using a rapid, tissue-specific CRISPR technique to model these genotypes in zebrafish, we found that SPRED1 functions as a tumor suppressor, particularly in the context of KIT mutations. SPRED1 knockdown caused MAPK activation, increased cell proliferation, and conferred resistance to drugs inhibiting KIT tyrosine kinase activity. These findings provide a rationale for MAPK inhibition in SPRED1-deficient melanomas and introduce a zebrafish modeling approach that can be used more generally to dissect genetic	fish
272	animal (fish)	zebrafish	CRISPR;Cas9;	smarce1	Scientific reports	smarce1 mutants have a defective endocardium and an increased expression of cardiac transcription factors in zebrafish.	2018	8(1):15369	[Castillo-Robles J et al.]	Universidad Nacional Autonoma de Mexico, Cuernavaca, Morelos, Mexico.	30337622	10.1038/s41598-018-33746-8	SWI/SNF or BAF chromatin-remodeling complexes are polymorphic assemblies of homologous subunit families that remodel nucleosomes and facilitate tissue-specific gene regulation during development. BAF57/SMARCE1 is a BAF complex subunit encoded in animals by a single gene and is a component of all mammalian BAF complexes. In vivo, the loss of SMARCE1 would lead to the formation of deficient combinations of the complex which might present limited remodeling activities. To address the specific contribution of SMARCE1 to the function of the BAF complex, we generated CRISPR/Cas9 mutations of smarce1 in zebrafish. Smarce1 mutants showed visible defects at 72 hpf, including smaller eyes, abnormal body curvature and heart abnormalities. Gene expression analysis revealed that the mutant embryos displayed defects in endocardial development since early stages, which led to the formation of a misshapen heart tube. The severe morphological and functional cardiac problems observed at 4 dpf were correlated with the substantially increased expression of different cardiac transcription factors. Additionally, we showed that Smarce1 binds to cis-regulatory regions of the gata5 gene and is necessary for the recruitment of the	fish
273	animal (fish)	zebrafish	CRISPR;Cas9;	slc45a2	Scientific reports	A tRNA-based multiplex sgRNA expression system in zebrafish and its application to generation of transgenic albino fish.	2018	8(1):13366	[Shiraki T et al.]	National Institute of Genetics, Mishima, Shizuoka, Japan.	30190522	10.1038/s41598-018-31476-5	The CRISPR/Cas9 system can be introduced into zebrafish as transgenes. Namely, expression of single-guide RNA (sgRNA) and controlled expression of Cas9 in transgenic zebrafish enables the study of gene functions in specific cell types. This transgenic CRISPR/Cas9 approach would be more useful if multiple sgRNAs could be expressed simultaneously since we could knock-out a gene more efficiently or disrupt multiple genes simultaneously. Here we describe a novel system to express multiple sgRNAs efficiently in zebrafish, that relies on the endogenous tRNA processing machinery. We cloned nine endogenous zebrafish tRNA genes, fused them to sgRNAs, and demonstrated that an active sgRNA can be produced from a precursor transcript containing either of these tRNAs. To show a proof of principle, we constructed transgenic fish expressing Cas9 under the control of the ubiquitin promoter and a single transcript containing three distinct sgRNAs, that targeted the slc45a2 (albino) gene, fused to tRNAs under the control of the U6 promoter. We found that the Tg(ubb:SpCas9,u6c:3xslc45a2-sgRNA) harbored mutations in all of the target sites in the albino gene and showed nearly complete albino phenotypes, which were amenable to imaging experiments. Thus, the tRNA-based multiplex sgRNA expression system	fish

274	animal (fish)	zebrafish	CRISPR;Cas9;	photoreceptor cilium actin regulator 1	Scientific reports	C2orf71a/pcare1 is important for photoreceptor outer segment morphogenesis and visual function in zebrafish.	2018	8(1):9675	[Corral-Serrano JC et al.]	Radboud University Medical Center, Nijmegen, The Netherlands.	29946172	10.1038/s41598-018-27928-7	Mutations in C2orf71 are causative for autosomal recessive retinitis pigmentosa and occasionally cone-rod dystrophy. We have recently discovered that the protein encoded by this gene is important for modulation of the ciliary membrane through the recruitment of an actin assembly module, and have therefore renamed the gene to PCARE (photoreceptor cilium actin regulator). Here, we report on the identification of two copies of the c2orf71/pcare gene in zebrafish, pcare1 and pcare2. To study the role of the gene most similar to human PCARE, pcare1, we have generated a stable pcare1 mutant zebrafish model (designated pcare1 (rmc100/rmc100)) in which the coding sequence was disrupted using CRISPR/Cas9 technology. Retinas of both embryonic (5 dpf) and adult (6 mpf) pcare1 (rmc100/rmc100) zebrafish display a clear disorganization of photoreceptor outer segments, resembling the phenotype observed in Pcare(-/-) mice. Optokinetic response and visual motor response measurements indicated visual impairment in pcare1 (rmc100/rmc100) zebrafish larvae at 5 dpf. In addition, electroretinogram measurements showed decreased b-wave amplitudes in pcare1 (rmc100/rmc100) zebrafish as compared to age- and strain-matched wild-type larvae, indicating a defect in the transretinal current. Altogether, our data show that lack of pcare1 causes a retinal phenotype in zebrafish and indicate that the function of	fish
275	animal (fish)	zebrafish	CRISPR;	pax2a; nkx2.4b; duox; duoxa; tshr	Scientific reports	A Rapid CRISPR/Cas-based Mutagenesis Assay in Zebrafish for Identification of Genes Involved in Thyroid Morphogenesis and Function.	2018	8(1):5647	[Trubiroha A et al.]	Universite Libre de Bruxelles, Brussels, Belgium.	29618800	10.1038/s41598-018-24036-4	The foregut endoderm gives rise to several organs including liver, pancreas, lung and thyroid with important roles in human physiology. Understanding which genes and signalling pathways regulate their development is crucial for understanding developmental disorders as well as diseases in adulthood. We exploited unique advantages of the zebrafish model to develop a rapid and scalable CRISPR/Cas-based mutagenesis strategy aiming at the identification of genes involved in morphogenesis and function of the thyroid. Core elements of the mutagenesis assay comprise bi-allelic gene invalidation in somatic mutants, a non-invasive monitoring of thyroid development in live transgenic fish, complementary analyses of thyroid function in fixed specimens and quantitative analyses of mutagenesis efficiency by Illumina sequencing of individual fish. We successfully validated our mutagenesis-phenotyping strategy in experiments targeting genes with known functions in early thyroid morphogenesis (pax2a, nkx2.4b) and thyroid functional differentiation (duox, duoxa, tshr). We also demonstrate that duox and duoxa crispants phenocopy thyroid phenotypes previously observed in human patients with bi-allelic DUOX2 and DUOXA2 mutations. The proposed combination of efficient mutagenesis protocols, rapid non-invasive phenotyping and sensitive genotyping holds great potential to systematically characterize the function of larger candidate gene panels during thyroid development and is applicable to other organs and	fish
276	animal (fish)	zebrafish	CRISPR;Cas9;	ush2a	Zebrafish	Poor Splice-Site Recognition in a Humanized Zebrafish Knockin Model for the Recurrent Deep-Intronic c.7595-2144A>G Mutation in USH2A.	2018	15(6):597-609	[Slijkerman R et al.]	Radboud University Medical Center , Nijmegen, the Netherlands .	30281416	10.1089/zeb.2018.1613	The frequent deep-intronic c.7595-2144A>G mutation in intron 40 of USH2A generates a high-quality splice donor site, resulting in the incorporation of a pseudoexon (PE40) into the mature transcript that is predicted to prematurely terminate usherin translation. Aberrant USH2A pre-mRNA splicing could be corrected in patient-derived fibroblasts using antisense oligonucleotides. With the aim to study the effect of the c.7595-2144A>G mutation and USH2A splice redirection on retinal function, a humanized zebrafish knockin model was generated, in which 670 basepairs of ush2a intron 40 were exchanged for 557 basepairs of the corresponding human sequence using an optimized CRISPR/Cas9-based protocol. However, in the retina of adult homozygous humanized zebrafish, only 7.4% +/- 3.9% of ush2a transcripts contained the human PE40 sequence and immunohistochemical analyses revealed no differences in the usherin expression and localization between the retina of humanized and wild-type zebrafish larvae. Nevertheless, we were able to partially correct aberrant ush2a splicing using a PE40-targeting antisense morpholino. Our results indicate a clear difference in splice-site recognition by the human and zebrafish splicing machinery. Therefore, we propose a protocol in which the effect of human splice-modulating mutations is studied in a zebrafish-specific cell-based splice assay before the	fish
277	animal (fish)	zebrafish; sharks; ray-finned fish	CRISPR;Cas9;	Nucleoplasmin 2 (npm2a; npm2b)	BMC evolutionary biology	Double maternal-effect: duplicated nucleoplasmin 2 genes, npm2a and npm2b, with essential but distinct functions are shared by fish and tetrapods.	2018	18(1):167	[Cheung CT et al.]	INRA LPGP UR1037, Campus de Beaulieu, Rennes, France.	30419815	10.1186/s12862-018-1281-3	BACKGROUND: Nucleoplasmin 2 (npm2) is an essential maternal-effect gene that mediates early embryonic events through its function as a histone chaperone that remodels chromatin. Recently, two npm2 (npm2a and npm2b) genes have been annotated in zebrafish. Thus, we examined the evolution of npm2a and npm2b in a variety of vertebrates, their potential phylogenetic relationships, and their biological functions using knockout models via the CRISPR/cas9 system. RESULTS: We demonstrated that the two npm2 duplicates exist in a wide range of vertebrates, including sharks, ray-finned fish, amphibians, and sauropsids, while npm2a was lost in coelacanth and mammals, as well as some specific teleost lineages. Using phylogeny and synteny analyses, we traced their origins to the early stages of vertebrate evolution. Our findings suggested that npm2a and npm2b resulted from an ancient local gene duplication, and their functions diverged although key protein domains were conserved. We then investigated their functions by examining their tissue distribution in a wide variety of species and found that they shared ovarian-specific expression, a key feature of maternal-effect genes. We also demonstrated that both npm2a and npm2b are maternally-inherited transcripts in vertebrates, and that they play essential, but distinct, roles in early embryogenesis using zebrafish knockout models. Both npm2a and npm2b function early during oogenesis and may play a role in cortical granule function that impact egg activation and fertilization, while npm2b is also involved in early embryogenesis. CONCLUSION: These novel findings will broaden our knowledge on the evolutionary history of maternal-effect genes and underlying mechanisms that contribute to vertebrate reproductive success. In addition, our results demonstrate the existence of a newly described maternal-effect gene, npm2a, that contributes to egg	fish

278	animal (fish)	Amia calva (bowfin), Oncorhynchus mykiss (trout), Petromyzon marinus (sea lamprey) and Scaphirhynchus albus (sturgeon)	CRISPR;Cas9;	npyr1	Diabetes, obesity & metabolism	Peptide YY (1-36) peptides from phylogenetically ancient fish targeting mammalian neuropeptide Y1 receptors demonstrate potent effects on pancreatic beta-cell function, growth and survival.	2019		[Lafferty RA et al.]	University of Ulster, Coleraine, UK.	31692207	10.1111/dom.13908	AIM: To investigate the antidiabetic efficacy of enzymatically stable Peptide YY (PYY) peptides from phylogenetically ancient fish. MATERIALS AND METHODS: N-terminally stabilized, PYY (1-36) sequences from Amia calva (bowfin), Oncorhynchus mykiss (trout), Petromyzon marinus (sea lamprey) and Scaphirhynchus albus (sturgeon), were synthesized, and both biological actions and antidiabetic therapeutic efficacy were assessed. RESULTS: All fish PYY (1-36) peptides were resistant to dipeptidyl peptidase-4 (DPP-4) degradation and inhibited glucose- and alanine-induced (P < 0.05 to P < 0.001) insulin secretion. In addition, PYY (1-36) peptides imparted significant (P < 0.05 to P < 0.001) beta-cell proliferative and anti-apoptotic benefits. Proliferative effects were almost entirely absent in beta cells with CRISPR-Cas9-induced knockout of Npyr1. In contrast to human PYY (1-36), the piscine-derived peptides lacked appetite-suppressive actions. Twice-daily administration of sea lamprey PYY (1-36), the superior bioactive peptide, for 21 days significantly (P < 0.05 to P < 0.001) decreased fluid intake, non-fasting glucose and glucagon in streptozotocin (STZ)-induced diabetic mice. In addition, glucose tolerance, insulin sensitivity, pancreatic insulin and glucagon content were significantly improved. Metabolic benefits were linked to positive changes in pancreatic islet morphology as a result of augmented (P < 0.001) proliferation and decreased apoptosis of beta cells. Sturgeon PYY (1-36) exerted similar but less impressive effects in STZ mice. CONCLUSION: These observations reveal, for the first time, that PYY (1-36) peptide sequences from phylogenetically ancient fish replicate the pancreatic beta-cell benefits of human PYY (1-36) and have	fish
279	animal (fish)	atlantic salmon	CRISPR;	very long chain fatty acyl elongase (elovl2)	Scientific reports	CRISPR/Cas9-mediated ablation of elovl2 in Atlantic salmon (Salmo salar L.) inhibits elongation of polyunsaturated fatty acids and induces Srebp-1 and target genes.	2019	9(1):7533	[Datsomor AK et al.]	Norwegian University of Science and Technology, Trondheim, Norway.	31101849	10.1038/s41598-019-43862-8	Atlantic salmon can synthesize polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (20:5n-3), arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) via activities of very long chain fatty acyl elongases (Elovls) and fatty acyl desaturases (Fads), albeit to a limited degree. Understanding molecular mechanisms of PUFA biosynthesis and regulation is a pre-requisite for sustainable use of vegetable oils in aquafeeds as current sources of fish oils are unable to meet increasing demands for omega-3 PUFAs. By generating CRISPR-mediated elovl2 partial knockout (KO), we have shown that elovl2 is crucial for multi-tissue synthesis of 22:6n-3 in vivo and that endogenously synthesized PUFAs are important for transcriptional regulation of lipogenic genes in Atlantic salmon. The elovl2-KOs showed reduced levels of 22:6n-3 and accumulation of 20:5n-3 and docosapentaenoic acid (22:5n-3) in the liver, brain and white muscle, suggesting inhibition of elongation. Additionally, elovl2-KO salmon showed accumulation of 20:4n-6 in brain and white muscle. The impaired synthesis of 22:6n-3 induced hepatic expression of sterol regulatory element binding protein-1 (srebp-1), fatty acid synthase-b, Delta6fad-a, Delta5fad and elovl5. Our study demonstrates key roles of elovl2 at two penultimate steps of PUFA synthesis in vivo and suggests Srebp-1 as a main regulator of endogenous PUFA synthesis in Atlantic	fish
280	animal (fish)	Atlantic salmon (Salmo salar L.)	CRISPR;	fatty acyl desaturase (delta5; delta6)	Scientific reports	CRISPR/Cas9-mediated editing of Delta5 and Delta6 desaturases impairs Delta8-desaturation and docosahexaenoic acid synthesis in Atlantic salmon (Salmo salar L.).	2019	9(1):16888	[Datsomor AK et al.]	Norwegian University of Science and Technology, ITrondheim, Norway.	31729437	10.1038/s41598-019-53316-w	The in vivo functions of Atlantic salmon fatty acyl desaturases (fads2), Delta6fads2-a, Delta6fads2-b, Delta6fads2-c and Delta5fads2 in long chain polyunsaturated fatty acid (LC-PUFA) synthesis in salmon and fish in general remains to be elucidated. Here, we investigate in vivo functions and in vivo functional redundancy of salmon fads2 using two CRISPR-mediated partial knockout salmon, Delta6abc/5(Mt) with mutations in Delta6fads2-a, Delta6fads2-b, Delta6fads2-c and Delta5fads2, and Delta6bc(Mt) with mutations in Delta6fads2-b and Delta6fads2-c. F0 fish displaying high degree of gene editing (50-100%) were fed low LC-PUFA and high LC-PUFA diets, the former containing reduced levels of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids but higher content of linoleic (18:2n-6) and alpha-linolenic (18:3n-3) acids, and the latter containing high levels of 20:5n-3 and 22:6n-3 but reduced compositions of 18:2n-6 and 18:3n-3. The Delta6abc/5(Mt) showed reduced 22:6n-3 levels and accumulated Delta6-desaturation substrates (18:2n-6, 18:3n-3) and Delta5-desaturation substrate (20:4n-3), demonstrating impaired 22:6n-3 synthesis compared to wildtypes (WT). Delta6bc(Mt) showed no effect on Delta6-desaturation compared to WT, suggesting Delta6 Fads2-a as having the predominant Delta6-desaturation activity in salmon, at least in the tissues analyzed. Both Delta6abc/5(Mt) and Delta6bc(Mt) demonstrated significant accumulation of Delta8-desaturation substrates (20:2n-6, 20:3n-3) when fed low LC-PUFA diet. Additionally, Delta6abc/5(Mt) demonstrated significant upregulation of the lipogenic transcription regulator, sterol regulatory element binding protein-1 (srebp-1) in liver and pyloric caeca under reduced dietary LC-PUFA. Our data suggest a combined effect of endogenous LC-PUFA synthesis and dietary LC-PUFA levels on srebp-1 expression which ultimately affects LC-PUFA synthesis in salmon. Our data also suggest Delta8-desaturation activities for salmon	fish
281	animal (fish)	Atlantic Salmon (Salmo salar)	CRISPR;Cpf1;		Molecular ecology resources	The application of CRISPR-Cas for single species identification from environmental DNA.	2019	19(5):1106-1114	[Williams MA et al.]	Dublin City University, Dublin, Ireland.	31177615	10.1111/1755-0998.13045	We report the first application of CRISPR-Cas technology to single species detection from environmental DNA (eDNA). Organisms shed and excrete DNA into their environment such as in skin cells and faeces, referred to as environmental DNA (eDNA). Utilising eDNA allows noninvasive monitoring with increased specificity and sensitivity. Current methods primarily employ PCR-based techniques to detect a given species from eDNA samples, posing a logistical challenge for on-site monitoring and potential adaptation to biosensor devices. We have developed an alternative method; coupling isothermal amplification to a CRISPR-Cas12a detection system. This utilises the collateral cleavage activity of Cas12a, a ribonuclease guided by a highly specific single CRISPR RNA. We used the target species Salmo salar as a proof-of-concept test of the specificity of the assay among closely related species and to show the assay is successful at a single temperature of 37 degrees C with signal detection at 535 nM. The specific assay, detects at attomolar sensitivity with rapid detection rates (<2.5 hr). This approach simplifies the challenge of building a biosensor device for rapid target species detection in the field and can be easily adapted to detect any species from eDNA samples from a variety of sources enhancing the capabilities of eDNA as a	fish

282	animal (fish)	chinook salmon (Oncorhynchus tshawytscha)	CRISPR;Cas9;	stat2	Journal of immunology	Viral Resistance and IFN Signaling in STAT2 Knockout Fish Cells.	2019	203(2):465-475	[Dehler CE et al.]	University of Aberdeen, Aberdeen, UK.	31142600	10.4049/jimmunol.1801376	IFN belong to a group of cytokines specialized in the immunity to viruses. Upon viral infection, type I IFN is produced and alters the transcriptome of responding cells through induction of a set of IFN stimulated genes (ISGs) with regulatory or antiviral function, resulting in a cellular antiviral state. Fish genomes have both type I IFN and type II IFN (IFN-gamma), but no type III (lambda) IFN has been identified. Their receptors are not simple counterparts of the mammalian type I/II IFN receptors, because alternative chains are used in type I IFN receptors. The mechanisms of the downstream signaling remain partly undefined. In mammals, members of the signal transducer and activator of family of transcription factors are responsible for the transmission of the signal from cytokine receptors, and STAT2 is required for type I but not type II IFN signaling. In fish, its role in IFN signaling in fish remains unclear. We isolated a Chinook salmon (Oncorhynchus tshawytscha) cell line, GS2, with a stat2 gene knocked out by CRISPR/Cas9 genome editing. In this cell line, the induction of ISGs by stimulation with a recombinant type I IFN is completely obliterated as evidenced by comparative RNA-seq analysis of the transcriptome of GS2 and its parental counterpart, EC. Despite a complete absence of ISGs induction, the GS2 cell line has a remarkable ability to resist to viral infections. Therefore, other STAT2-independent pathways may be induced by the viral infection, illustrating the robustness	fish
283	animal (fish)	clownfish (Amphiprion ocellaris)	CRISPR;Cas9;	fhl2a; fhl2b; saiyan; gpnmb; apoD1a	Pigment cell & melanoma research	Developmental and comparative transcriptomic identification of iridophore contribution to white barring in clownfish.	2019	32(3):391-402	[Salis P et al.]	UMR CNRS 7232 BIOM, Sorbonne Universite, Banyuls-sur-Mer, France.	30633441	10.1111/pcmr.12766	Actinopterygian fishes harbor at least eight distinct pigment cell types, leading to a fascinating diversity of colors. Among this diversity, the cellular origin of the white color appears to be linked to several pigment cell types such as iridophores or leucophores. We used the clownfish Amphiprion ocellaris, which has a color pattern consisting of white bars over a darker body, to characterize the pigment cells that underlie the white hue. We observe by electron microscopy that cells in white bars are similar to iridophores. In addition, the transcriptomic signature of clownfish white bars exhibits similarities with that of zebrafish iridophores. We further show by pharmacological treatments that these cells are necessary for the white color. Among the top differentially expressed genes in white skin, we identified several genes (fhl2a, fhl2b, saiyan, gpnmb, and apoD1a) and show that three of them are expressed in iridophores. Finally, we show by CRISPR/Cas9 mutagenesis that these genes are critical for iridophore development in zebrafish. Our analyses provide clues to the genomic underpinning of color diversity and allow identification of new iridophore genes	fish
284	animal (fish)	japanese anchovy	CRISPR;Cas9;TALENs;		Scientific reports	Comprehensive Experimental System for a Promising Model Organism Candidate for Marine Teleosts.	2019	9(1):4948	[Sakaguchi K et al.]	Kyushu University, Saga, Japan.	30894668	10.1038/s41598-019-41468-8	A comprehensive experimental system for Japanese anchovy, a promising candidate model organism for marine teleosts, was established. Through the design of a rearing/spawning facility that controls the photoperiod and water temperature, one-cell eggs were continuously obtained shortly after spawning throughout the rearing period. The stages of eggs are indispensable for microinjection experiments, and we developed an efficient and robust microinjection system for the Japanese anchovy. Embryos injected with GFP mRNA showed strong whole-body GFP fluorescence and the survival rates of injected- and non-injected embryos were not significantly different, 87.5% (28 in 32 embryos) and 90.0% (45 in 50 embryos), respectively. We verified that the Tol2 transposon system, which mediates gene transfer in vertebrates, worked efficiently in the Japanese anchovy using the transient transgenesis protocol, with GFP or DsRed as the reporter gene. Finally, we confirmed that genome-editing technologies, namely Transcription Activator-Like Effector Nucleases (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9, were applicable to the Japanese anchovy. In practice, specific gene-disrupted fishes were generated in the F1 generation. These results demonstrated the establishment of a basic, yet comprehensive, experimental system, which could be employed to undertake	fish
285	animal (fish)	large-scale loach (Paramisgurnus dabryanus)	CRISPR;Cas9;	tyrosinase	Transgenic research	Production of a mutant of large-scale loach Paramisgurnus dabryanus with skin pigmentation loss by genome editing with CRISPR/Cas9 system.	2019	28(3-4):341-356	[Xu X et al.]	Huazhong Agricultural University, Wuhan, Hubei, China.	31183663	10.1007/s11248-019-00125-6	CRISPR/Cas9 system has been developed as a highly efficient genome editing technology to specifically induce mutations in a few aquaculture species. In this study, we described induction of targeted gene (namely tyrosinase, tyr) mutations in large-scale loach Paramisgurnus dabryanus, an important aquaculture fish species and a potential model organism for studies of intestinal air-breathing function, using the CRISPR/Cas9 system. Tyr gene in large-scale loach was firstly cloned and then its expressions were investigated. Two guide RNAs (gRNAs) were designed and separately transformed with Cas9 in the loach. 89.4% and 96.1% of injected loach juveniles respectively displayed a graded loss of pigmentation for the two gRNAs, in other words, for target 1 and target 2. We classified the injected loach juveniles into five groups according to their skin color phenotypes, including four albino groups and one wild-type-like group. And one of them was clear albino group, which was of high ornamental and commercial value. More than 50 clones for each albino transformant with a visible phenotype in each target were randomly selected and sequenced. Results obtained here showed that along with the increase of pigmentation, wild-type alleles appeared in the injected loach juveniles more often and insertion/deletion alleles less frequently. This study demonstrated that CRISPR/Cas9 system could be practically performed to modify large-scale loach tyr to produce an albino mutant of high ornamental and commercial value, and for the first time showed successful use of the CRISPR/Cas9	fish
286	animal (fish)	medaka	CRISPR;Cas9;	corticotropin-releasing hormone b receptor (crhr1; crhr2)	Development	The central nervous system acts as a transducer of stress-induced masculinization through corticotropin-releasing hormone B.	2019	146(8)	[Castaneda Cortes DC et al.]	Instituto Tecnologico de Chascomus, INTECH (CONICET-UNSAM), Chascomus, Argentina.	30936180	10.1242/dev.172866	Exposure to environmental stressors, such as high temperature (HT), during early development of fish induces sex reversal of genotypic females. Nevertheless, the involvement of the brain in this process is not well clarified. In the present work, we investigated the mRNA levels of corticotropin-releasing hormone b (crhb) and its receptors (crhr1 and crhr2), and found that they were upregulated at HT during the crucial period of gonadal sex determination in medaka. In order to clarify their roles in sex reversal, biallelic mutants for crhr1 and crhr2 were produced by CRISPR/Cas9 technology. Remarkably, biallelic mutants of both loci (crhr1 and crhr2) did not undergo female-to-male sex reversal upon exposure to HT. Inhibition of this process in double corticotropin-releasing hormone receptor mutants could be successfully rescued through the administration of the downstream effector of the hypothalamic-pituitary-interrenal axis, cortisol. Taken together, these results reveal for the first time that the CNS acts as a transducer of masculinization induced by thermal stress.	fish

287	animal (fish)	medaka	CRISPR;Cas9;	cdkn2ab	Pigment cell & melanoma research	Analysis of the putative tumor suppressor gene cdkn2ab in pigment cells and melanoma of Xiphophorus and medaka.	2019	32(2):248-258	[Regneri J et al.]	University of Wurzburg, Biozentrum, Wurzburg, Germany.	30117276	10.1111/pcmr.12729	In humans, the CDKN2A locus encodes two transcripts, INK4A and ARF. Inactivation of either one by mutations or epigenetic changes is a frequent signature of malignant melanoma and one of the most relevant entry points for melanomagenesis. To analyze whether cdkn2ab, the fish ortholog of CDKN2A, has a similar function as its human counterpart, we studied its action in fish models for human melanoma. Overexpression of cdkn2ab in a Xiphophorus melanoma cell line led to decreased proliferation and induction of a senescence-like phenotype, indicating a melanoma-suppressive function analogous to mammals. Coexpression of Xiphophorus cdkn2ab in medaka transgenic for the mitfa:xmrk melanoma-inducing gene resulted in full suppression of melanoma development, whereas CRISPR/Cas9 knockout of cdkn2ab resulted in strongly enhanced tumor growth. In summary, this provides the first functional evidence that cdkn2ab acts as a potent tumor suppressor gene in fish melanoma models.	fish
288	animal (fish)	medaka	CRISPR;Cas9;	oca2; pnp4a	PloS one	Enhanced in vivo-imaging in medaka by optimized anaesthesia, fluorescent protein selection and removal of pigmentation.	2019	14(3):e0212956	[Lischik CQ et al.]	Heidelberg University, Heidelberg, Germany.	30845151	10.1371/journal.pone.0212956	Fish are ideally suited for in vivo-imaging due to their transparency at early stages combined with a large genetic toolbox. Key challenges to further advance imaging are fluorophore selection, immobilization of the specimen and approaches to eliminate pigmentation. We addressed all three and identified the fluorophores and anaesthesia of choice by high throughput time-lapse imaging. Our results indicate that eGFP and mCherry are the best conservative choices for in vivo-fluorescence experiments, when availability of well-established antibodies and nanobodies matters. Still, mVenusNB and mGFPmut2 delivered highest absolute fluorescence intensities in vivo. Immobilization is of key importance during extended in vivo imaging. Here, traditional approaches are outperformed by mRNA injection of alpha-Bungarotoxin which allows a complete and reversible, transient immobilization. In combination with fully transparent juvenile and adult fish established by the targeted inactivation of both, oca2 and pnp4a via CRISPR/Cas9-mediated gene editing in medaka we could dramatically improve the state-of-the art imaging conditions in post-embryonic fish, now enabling light-sheet microscopy of the growing retina, brain, gills and inner organs in the absence of side	fish
289	animal (fish)	medaka (Japanese killifish, Oryzias latipes)	CRISPR;Cas9;	H3K27 methyltranferase Ezh2; dCas9; H2K27me3	Epigenetics & chromatin	Targeted in vivo epigenome editing of H3K27me3.	2019	12(1):17	[Fukushima HS et al.]	University of Tokyo, Tokyo, Japan.	30871638	10.1186/s13072-019-0263-z	BACKGROUND: Epigenetic modifications have a central role in transcriptional regulation. While several studies using next-generation sequencing have revealed genome-wide associations between epigenetic modifications and transcriptional states, a direct causal relationship at specific genomic loci has not been fully demonstrated, due to a lack of technology for targeted manipulation of epigenetic modifications. Recently, epigenome editing techniques based on the CRISPR-Cas9 system have been reported to directly manipulate specific modifications at precise genomic regions. However, the number of editable modifications as well as studies applying these techniques in vivo is still limited. RESULTS: Here, we report direct modification of the epigenome in medaka (Japanese killifish, Oryzias latipes) embryos. Specifically, we developed a method to ectopically induce the repressive histone modification, H3K27me3 in a locus-specific manner, using a fusion construct of Oryzias latipes H3K27 methyltransferase Ezh2 (olEzh2) and dCas9 (dCas9-olEzh2). Co-injection of dCas9-olEzh2 mRNA with single guide RNAs (sgRNAs) into one-cell-stage embryos induced specific H3K27me3 accumulation at the targeted loci and induced downregulation of gene expression. CONCLUSION: In this study, we established the in vivo epigenome editing of H3K27me3 using medaka embryos. The locus-specific manipulation of the epigenome in living organisms will lead to a previously inaccessible	fish
290	animal (fish)	medaka (Oryzias latipes); zebrafish (Danio rerio)	CRISPR;Cas9;		PloS one	Swift Large-scale Examination of Directed Genome Editing.	2019	14(3):e0213317	[Hammouda OT et al.]	Heidelberg University, Heidelberg, Germany.	30835740	10.1371/journal.pone.0213317	In the era of CRISPR gene editing and genetic screening, there is an increasing demand for quick and reliable nucleic acid extraction pipelines for rapid genotyping of large and diverse sample sets. Despite continuous improvements of current workflows, the handling-time and material costs per sample remain major limiting factors. Here we present a robust method for low-cost DIY-pipet tips addressing these needs; i.e. using a cellulose filter disc inserted into a regular pipet tip. These filter-in-tips allow for a rapid, stand-alone four-step genotyping workflow by simply binding the DNA contained in the primary lysate to the cellulose filter, washing it in water and eluting it directly into the buffer for the downstream application (e.g. PCR). This drastically cuts down processing time to maximum 30 seconds per sample, with the potential for parallelizing and automation. We show the ease and sensitivity of our procedure by genotyping genetically modified medaka (Oryzias latipes) and zebrafish (Danio rerio) embryos (targeted by CRISPR/Cas9 knock-out and knock-in) in a 96-well plate format. The robust isolation and detection of multiple alleles of various abundancies in a mosaic genetic background allows phenotype-genotype correlation already in the injected generation, demonstrating the reliability and sensitivity of the protocol. Our method is applicable across kingdoms to samples ranging from cells to tissues i. e. plant	fish
291	animal (fish)	nile tilapia	CRISPR;Cas9;	foxh1	Biology of reproduction	Homozygous mutation of foxh1 arrests oogenesis causing infertility in female Nile tilapia.	2019		[Tao W et al.]	Guangdong Ocean University, Zhanjiang, China.	31837141	10.1093/biolre/ioz225	Foxh1, a member of fox gene family, was first characterized as a transcriptional partner in the formation of the Smad protein complex. Recent studies have shown foxh1 is highly expressed in the cytoplasm of oocytes in both tilapia and mouse. However, its function in oogenesis remains unexplored. In the present study, foxh1-/- tilapia was created by CRISPR/Cas9. At 180 dah (days after hatching), the foxh1-/- XX fish showed oogenesis arrest and a significantly lower GSI. The transition of oocytes from phase II to phase III and follicle cells from one to two layers was blocked, resulting in infertility of the mutant. Transcriptomic analysis revealed that expression of genes involved in estrogen synthesis and oocyte growth were altered in the foxh1-/- ovaries. Loss of foxh1 resulted in significantly decreased Cyp19a1a and increased Cyp11b2 expression, consistent with significantly lower concentrations of serum estradiol-17beta (E2) and higher concentrations of 11-Ketotestosterone (11-KT). Moreover, administration of E2 rescued the phenotypes of foxh1-/- XX fish, as indicated by the appearance of phase III and IV oocytes and absence of Cyp11b2 expression. Taken together, these results suggest that foxh1 functions in the oocytes to regulate oogenesis by promoting cyp19a1a expression, and therefore estrogen production. Disruption of foxh1 may block the estrogen synthesis and oocyte growth,	fish

292	animal (fish)	nile tilapia	CRISPR;Cas9;	esr1; esr2a; esr2b	Journal of steroid biochemistry and molecular biology	Establishment of three estrogen receptors (esr1, esr2a, esr2b) knockout lines for functional study in Nile tilapia.	2019	191:105379	[Yan L et al.]	Southwest University, Chongqing, China.	31078694	10.1016/j.jsbmb.2019.105379	Estrogens play fundamental roles in regulating reproductive activities and they act through estrogen receptors (ESRs) in all vertebrates. To date, distinct roles of estrogen receptors have been characterized only in human and model organisms, including mouse, rat, zebrafish and medaka. Physiological role of estrogen/receptor signaling in reproduction remains poorly defined in non-model organisms. In the present study, we successfully generated esr1, esr2a and esr2b mutant lines in tilapia by CRISPR/Cas9 and examined their phenotypes. Surprisingly, the esr1 mutants showed no phenotypes of reproductive development and function in both females and males. The esr2a mutant females showed significantly delayed ovarian development and follicle growth at 90 and 180 dah, and the development caught up later at 360 dah. The esr2a mutant males showed no phenotypes at 90 dah, and displayed smaller gonads and efferent ducts, less spermatogonia and more abnormal sperms at 180 dah. In contrast, the esr2b mutants displayed abnormal development of ovarian ducts and efferent ducts which failed to connect to the genital orifice, and which in turn, resulted in infertility in female and male, respectively, although they produced gametes in their gonads. Taken together, our study provides evidence for differential functions of esr1, esr2a and esr2b	fish
293	animal (fish)	Nile tilapia (Oreochromis niloticus)	CRISPR;Cas9;	relaxin3	Journal of steroid biochemistry and molecular biology	Rln3a is a prerequisite for spermatogenesis and fertility in male fish.	2019	197:105517	[Yang L et al.]	Southwest University, Chongqing, China.	31678357	10.1016/j.jsbmb.2019.105517	The essential roles of Relaxin3 (RLN3) in energy homeostasis had been well investigated, while the mechanisms of RLN3 regulating reproduction remain to be elusive in mammals. Although two rln3 paralogues have been characterized in several teleosts, their functions still remain largely unknown. In this study, two paralogous rln3 genes, represented as rln3a and rln3b, were identified from the testis of Nile tilapia (Oreochromis niloticus). Rln3a was dominantly expressed in testis, while the most abundant rln3b expression was in brain. In situ hybridization demonstrated that rln3a is abundantly expressed in the Leydig cells of the testis. To understand the role of Rln3 in the testicular development, homologous null-rln3a gene mutant line was constructed by CRISPR/Cas9 technology. Morphological observation demonstrated that null mutation of rln3a gene caused testicular hypertrophy and a significant increase of GSI. However, a significant decrease of spermatogenic cells at different phases, i.e. spermatogonia, spermatocytes, spermatids and sperms was found. Silencing of rln3a gene repressed the expression of key genes in germ cell and Leydig cell. Deficiency of Rln3a led to the significant decrease of 11-KT production, which stimulated the up-regulation of both FSH and LH production in the pituitary via a negative feedback manner possibly. Mutation of rln3a in XY fish led to the hypogonadism with sperm deformation, significant decrease of fertility, and sperm motility, revealing as the high mortality of the offspring obtained by crossing the wild type female and rln3a(-/-) XY fish. Interestingly, recombinant human RLN3 injection significantly enhanced the sperm motility in rln3a(-/-) XY fish. Moreover, hCG treatment stimulated the expression of steroidogenic enzyme genes and 11-KT production, which were repressed by rln3a mutation in XY fish. Taken together, this study, for the first time by using a gene knockout model, proved that Rln3a is an indispensable mediator for androgen	fish
294	animal (fish)	pufferfish	CRISPR;Cas9;	ectodysplastin	iScience	Evolution and Developmental Diversity of Skin Spines in Pufferfishes.	2019	19:1248-1259	[Shono T et al.]	University of Sheffield, Sheffield, UK.	31353167	10.1016/j.isci.2019.06.003	Teleost fishes develop remarkable varieties of skin ornaments. The developmental basis of these structures is poorly understood. The order Tetraodontiformes includes diverse fishes such as the ocean sunfishes, triggerfishes, and pufferfishes, which exhibit a vast assortment of scale derivatives. Pufferfishes possess some of the most extreme scale derivatives, dermal spines, erected during their characteristic puffing behavior. We demonstrate that pufferfish scale-less spines develop through conserved gene interactions that underlie general vertebrate skin appendage formation, including feathers and hair. Spine development retains conservation of the EDA (ectodysplasin) signaling pathway, important for the development of diverse vertebrate skin appendages, including these modified scale-less spines of pufferfish. Further modification of genetic signaling from both CRISPR-Cas9 and small molecule inhibition leads to loss or reduction of spine coverage, providing a mechanism for skin appendage diversification observed throughout the pufferfishes. Pufferfish spines have evolved broad variations in body coverage, enabling adaptation to diverse ecological niches.	fish
295	animal (fish)	tilapia	CRISPR;Cas9;	non-coding sequences including microRNA and 3' untranslated region	G3	High Efficiency Targeting of Non-coding Sequences Using CRISPR/Cas9 System in Tilapia.	2019	9(1):287-295	[Li M et al.]	Southwest University, Chongqing, China.	30482801	10.1534/g3.118.200883	The CRISPR/Cas9 has been successfully applied for disruption of protein coding sequences in a variety of organisms. The majority of the animal genome is actually non-coding sequences, which are key regulators associated with various biological processes. In this study, to understand the biological significance of these sequences, we used one or dual gRNA guided Cas9 nuclease to achieve specific deletion of non-coding sequences including microRNA and 3' untranslated region (UTR) in tilapia, which is an important fish for studying sex determination and evolution. Co-injection of fertilized eggs with single gRNA targeting seed region of miRNA and Cas9 mRNA resulted in indel mutations. Further, co-injection of fertilized eggs with dual gRNAs and Cas9 mRNA led to the removal of the fragment between the two target loci, yielding maximum efficiency of 11%. This highest genomic deletion efficiency was further improved up to 19% using short ssDNA as a donor. The deletions can be transmitted through the germline to the next generation at average efficiency of 8.7%. Cas9-vasa 3'-UTR was used to increase the efficiency of germline transmission of non-coding sequence deletion up to 14.9%. In addition, the 3'-UTR of the vasa gene was successfully deleted by dual gRNAs. Deletion of vasa 3'-UTR resulted in low expression level of vasa mRNA in the gonad when compared with the control. To summarize, the improved CRISPR/Cas9 system provided a powerful platform that can assist to easily generate desirable non-coding sequences mutants in non-model fish	fish

296	animal (fish)	weakly electric fish (mormyrid species Brienomyrus brachyistius and the gymnotiform Brachyhypopomus gauderio)	CRISPR;Cas9;	sodium channel gene (scn4aa)	Journal of visualized experiments	Silencing the Spark: CRISPR/Cas9 Genome Editing in Weakly Electric Fish.	2019	(152)	[Constantinou SJ et al.]	Michigan State University, MI, USA.	31710047	10.3791/60253	Electroreception and electrogenesis have changed in the evolutionary history of vertebrates. There is a striking degree of convergence in these independently derived phenotypes, which share a common genetic architecture. This is perhaps best exemplified by the numerous convergent features of gymnotiforms and mormyrids, two species-rich teleost clades that produce and detect weak electric fields and are called weakly electric fish. In the 50 years since the discovery that weakly electric fish use electricity to sense their surroundings and communicate, a growing community of scientists has gained tremendous insights into evolution of development, systems and circuits neuroscience, cellular physiology, ecology, evolutionary biology, and behavior. More recently, there has been a proliferation of genomic resources for electric fish. Use of these resources has already facilitated important insights with regards to the connection between genotype and phenotype in these species. A major obstacle to integrating genomics data with phenotypic data of weakly electric fish is a present lack of functional genomics tools. We report here a full protocol for performing CRISPR/Cas9 mutagenesis that utilizes endogenous DNA repair mechanisms in weakly electric fish. We demonstrate that this protocol is equally effective in both the mormyrid species Brienomyrus brachyistius and the gymnotiform Brachyhypopomus gauderio by using CRISPR/Cas9 to target indels and point mutations in the first exon of the sodium channel gene scn4aa. Using this protocol, embryos from both species were obtained and genotyped to confirm that the predicted mutations in the first exon of the sodium channel scn4aa were present. The knock-out success phenotype was confirmed with recordings showing reduced electric organ discharge amplitudes when	fish
297	animal (fish)	white crucian carp (Carassius auratus cuvieri, WCC); WWC-red crucian carp hybrid	CRISPR;Cas9;	tyrosinase	Science China. Life sciences	Targeted disruption of tyrosinase causes melanin reduction in Carassius auratus cuvieri and its hybrid progeny.	2019	62(9):1194-1202	[Liu Q et al.]	Hunan Normal University, Changsha, China.	30593611	10.1007/s11427-018-9404-7	The white crucian carp (Carassius auratus cuvieri, WCC) not only is one of the most economically important fish in Asia, characterized by strong reproductive ability and rapid growth rates, but also represents a good germplasm to produce hybrid progenies with heterosis. Gene knockout technique provides a safe and acceptant way for fish breeding. Achieving gene knockout in WCC and its hybrid progeny will be of great importance for both genetic studies and hybridization breeding. Tyrosinase (TYR) is a key enzyme in melanin synthesis. Depletion of tyr in zebrafish and mice results in mosaic pigmentation or total albinism. Here, we successfully used CRISPR-Cas9 to target tyr in WCC and its hybrid progeny (WR) derived from the cross of WCC (female symbol) and red crucian carp (Carassius auratus red var., RCC, male symbol). The level of TYR protein was significantly reduced in mutant WCC. Both the mutant WCC and the mutant WR showed different degrees of melanin reduction compared with the wild-type sibling control fish, resulting from different mutation efficiency ranging from 60% to 90%. In addition, the transcriptional expression profiles of a series of pivotal pigment synthesis genes, i.e. tyrp1, mitfa, mitfb, dct and sox10, were down-regulated in tyr-CRISPR WCC, which ultimately caused a reduction in melanin synthesis. These results demonstrated that tyr plays a key role in melanin synthesis in WCC and WR, and CRISPR-Cas9 is an effective tool for modifying the genome of economical fish. Furthermore, the tyr-CRISPR models could be valuable in understanding fundamental	fish
298	animal (fish)	zebrafish	CRISPR;Cas9;	ncapg2; nphp1	American journal of human genetics	Mutations in NCAPG2 Cause a Severe Neurodevelopmental Syndrome that Expands the Phenotypic Spectrum of Condensinopathies.	2019	104(1):94-111	[Khan TN et al.]	Duke University, Durham, NC, USA.	30609410	10.1016/j.ajhg.2018.11.017	The use of whole-exome and whole-genome sequencing has been a catalyst for a genotype-first approach to diagnostics. Under this paradigm, we have implemented systematic sequencing of neonates and young children with a suspected genetic disorder. Here, we report on two families with recessive mutations in NCAPG2 and overlapping clinical phenotypes that include severe neurodevelopmental defects, failure to thrive, ocular abnormalities, and defects in urogenital and limb morphogenesis. NCAPG2 encodes a member of the condensin II complex, necessary for the condensation of chromosomes prior to cell division. Consistent with a causal role for NCAPG2, we found abnormal chromosome condensation, augmented anaphase chromatin-bridge formation, and micronuclei in daughter cells of proband skin fibroblasts. To test the functional relevance of the discovered variants, we generated an ncapg2 zebrafish model. Morphants displayed clinically relevant phenotypes, such as renal anomalies, microcephaly, and concomitant increases in apoptosis and altered mitotic progression. These could be rescued by wild-type but not mutant human NCAPG2 mRNA and were recapitulated in CRISPR-Cas9 F0 mutants. Finally, we noted that the individual with a complex urogenital defect also harbored a heterozygous NPHP1 deletion, a common contributor to nephronophthisis. To test whether sensitization at the NPHP1 locus might contribute to a more severe renal phenotype, we co-suppressed nphp1 and ncapg2, which resulted in significantly more dysplastic renal tubules in zebrafish larvae. Together, our data suggest that impaired function of NCAPG2 results in a severe condensinopathy, and they highlight the potential utility of	fish
299	animal (fish)	zebrafish	TALENs;	zip6	Biochemical and biophysical research communications	SLC39A6/ZIP6 is essential for zinc homeostasis and T-cell development in zebrafish.	2019	511(4):896-902	[Zhao L et al.]	Huazhong University of Science and Technology, Wuhan, China.	30851936	10.1016/j.bbrc.2019.02.148	Zinc (Zn) is an essential trace element that modulate innate and acquired immune responses, and its deficiency triggers lymphopenia. However, the precise mechanisms underlying zinc-mediated lymphocyte maintenance have not been well clarified. Here, we have successfully generated a zip6-null mutant zebrafish line using TALENs. The Zip6-null mutant zebrafish developed normally during gastrulation. Loss of zip6 in zebrafish resulted in significant T lymphocyte reduction and a decrease in intracellular Zn levels. And the zip6 deficiency increases caspase-related cell apoptosis in both zebrafish cells and human T cells. Our results suggest that ZIP6 plays a critical part in T cell development, and enhance our understanding of Zn homeostasis and immune	fish

300	animal (fish)	zebrafish	CRISPR;	photoreceptor cell-specific nuclear receptor gene (Nr2e3)	Biochimica et biophysica acta. Molecular basis of disease	Knockout of Nr2e3 prevents rod photoreceptor differentiation and leads to selective L-/M-cone photoreceptor degeneration in zebrafish.	2019	1865(6):1273-1283	[Xie S et al.]	Huazhong University of Science and Technology, Wuhan, Hubei, China.	30684641	10.1016/j.bbadis.2019.01.022	Mutations in the photoreceptor cell-specific nuclear receptor gene Nr2e3 increased the number of S-cone photoreceptors in human and murine retinas and led to retinal degeneration that involved photoreceptor and non-photoreceptor cells. The mechanisms underlying these complex phenotypes remain unclear. In the hope of understanding the precise role of Nr2e3 in photoreceptor cell fate determination and differentiation, we generated a line of Nr2e3 knockout zebrafish using CRISPR technology. In these Nr2e3-null animals, rod precursors undergo terminal mitoses but fail to differentiate as rods. Rod-specific genes are not expressed and the outer segment (OS) fails to form. Formation and differentiation of cone photoreceptors is normal. Specifically, there is no increase in the number of UV-cone or S-cone photoreceptors. Laminated retinal structure is maintained. After normal development, L-/M-cones selectively degenerate, with progressive shortening of OS that starts at age 1month. The amount of cone phototransduction proteins is concomitantly reduced, whereas UV- and S-cones have normal OS lengths even at age 10months. In vitro studies show Nr2e3 synergizes with Crx and Nrl to enhance rhodopsin gene expression. Nr2e3 does not affect cone opsin expression. Our results extend the knowledge of Nr2e3's roles and have specific implications for the interpretation of the phenotypes observed in human and murine retinas. Furthermore, our model may offer new opportunities in finding treatments for enhanced S-cone syndrome (ESCS) and	fish
301	animal (fish)	zebrafish	CRISPR;Cas9;	ptprja	Blood	Loss-of-function mutations in PTPRJ cause a new form of inherited thrombocytopenia.	2019	133(12):1346-1357	[Marconi C et al.]	University of Bologna, Bologna, Italy.	30591527	10.1182/blood-2018-07-859496	Inherited thrombocytopenias (ITs) are a heterogeneous group of disorders characterized by low platelet count that may result in bleeding tendency. Despite progress being made in defining the genetic causes of ITs, nearly 50% of patients with familial thrombocytopenia are affected with forms of unknown origin. Here, through exome sequencing of 2 siblings with autosomal-recessive thrombocytopenia, we identified biallelic loss-of-function variants in PTPRJ . This gene encodes for a receptor-like PTP, PTPRJ (or CD148), which is expressed abundantly in platelets and megakaryocytes. Consistent with the predicted effects of the variants, both probands have an almost complete loss of PTPRJ at the messenger RNA and protein levels. To investigate the pathogenic role of PTPRJ deficiency in hematopoiesis in vivo, we carried out CRISPR/Cas9-mediated ablation of ptprja (the ortholog of human PTPRJ) in zebrafish, which induced a significantly decreased number of CD41(+) thrombocytes in vivo. Moreover, megakaryocytes of our patients showed impaired maturation and profound defects in SDF1-driven migration and formation of proplatelets in vitro. Silencing of PTPRJ in a human megakaryocytic cell line reproduced the functional defects observed in patients' megakaryocytes. The disorder caused by PTPRJ mutations presented as a nonsyndromic thrombocytopenia characterized by spontaneous bleeding, small-sized platelets, and impaired platelet responses to the GPVI agonists collagen and convulxin. These platelet functional defects could be attributed to reduced activation of Src family kinases. Taken together, our data identify	fish
302	animal (fish)	zebrafish	TALENs;	C-lectin family 14 Member A (clec14a)	BMC developmental biology	Clec14a genetically interacts with Etv2 and Vegf signaling during vasculogenesis and angiogenesis in zebrafish.	2019	19(1):6	[Pociute K et al.]	Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA.	30953479	10.1186/s12861-019-0188-6	BACKGROUND: C-lectin family 14 Member A (Clec14a) is a transmembrane protein specifically expressed in vascular endothelial cells during embryogenesis. Previous in vitro and in vivo studies have provided conflicting data regarding Clec14a role in promoting or inhibiting angiogenesis, therefore its functional role in vascular development remains poorly understood. RESULTS: Here we have generated a novel clec14a mutant allele in zebrafish embryos using TALEN genome editing. clec14a mutant embryos exhibit partial defects and delay in the sprouting of intersegmental vessels. These defects in angiogenesis are greatly increased upon the knockdown of a structurally related C1qr protein. Furthermore, a partial knockdown of an ETS transcription factor Etv2 results in a synergistic effect with the clec14a mutation and inhibits expression of early vascular markers in endothelial progenitor cells, arguing that clec14a is involved in promoting vasculogenesis. In addition, Clec14a genetically interacts with Vegfa signaling. A partial knockdown of Vegfaa function in the clec14a mutant background resulted in a synergistic inhibition of intersegmental vessel sprouting. CONCLUSIONS: These results argue that clec14a is involved in both vasculogenesis and angiogenesis, and suggest that Clec14a genetically interacts with	fish
303	animal (fish)	zebrafish	CRISPR;Cas9;	otulina; slc29a1a	BMC genomics	What makes a bad egg? Egg transcriptome reveals dysregulation of translational machinery and novel fertility genes important for fertilization.	2019	20(1):584	[Cheung CT et al.]	INRA, Rennes cedex, France.	31307377	10.1186/s12864-019-5930-8	BACKGROUND: Egg quality can be defined as the egg ability to be fertilized and subsequently develop into a normal embryo. Previous research has shed light on factors that can influence egg quality. Large gaps however remain including a comprehensive view of what makes a bad egg. Initial development of the embryo relies on maternally-inherited molecules, such as transcripts, deposited in the egg during its formation. Bad egg quality is therefore susceptible to be associated with alteration or dysregulation of maternally-inherited transcripts. We performed transcriptome analysis on a large number (N = 136) of zebrafish egg clutches, each clutch being split to monitor developmental success and perform transcriptome analysis in parallel. We aimed at drawing a molecular portrait of the egg in order to characterize the relation between egg transcriptome and developmental success and to subsequently identify new candidate genes involved in fertility. RESULTS: We identified 66 transcript that were differentially abundant in eggs of contrasted phenotype (low or high developmental success). Statistical modeling using partial least squares regression and genetics algorithm demonstrated that gene signatures from transcriptomic data can be used to predict developmental success. The identity and function of differentially expressed genes indicate a major dysregulation of genes of the translational machinery in poor quality eggs. Two genes, otulina and slc29a1a, predominantly expressed in the ovary and dysregulated in poor quality eggs were further investigated using CRISPR/Cas9 mediated genome editing. Mutants of each gene revealed remarkable subfertility whereby the majority of their eggs were unfertilizable. The Wnt pathway appeared to be dysregulated in the otulina mutant-derived eggs. CONCLUSIONS: Here we show that egg transcriptome contains molecular signatures, which can be used to predict developmental success. Our results also indicate that poor egg quality in zebrafish is associated with a dysregulation of (i) the translational machinery genes and (ii) novel fertility genes, otulina and slc29a1a, playing an important role for fertilization. Together, our observations highlight the diversity of the possible causes of egg quality defects and reveal mechanisms of maternal origin behind the lack of fertilization and	fish

304	animal (fish)	zebrafish	CRISPR;Cas9;	G protein-coupled receptor 137b (gpr137ba)	Bone	A role for G protein-coupled receptor 137b in bone remodeling in mouse and zebrafish.	2019	127:104-113	[Urso K et al.]	Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA.	31173907	10.1016/j.bone.2019.06.002	G protein-coupled receptor 137b (GPR137b) is an orphan seven-pass transmembrane receptor of unknown function. In mouse, Gpr137b is highly expressed in osteoclasts in vivo and is upregulated during in vitro differentiation. To elucidate the role that GPR137b plays in osteoclasts, we tested the effect of GPR137b deficiency on osteoclast maturation and resorbing activity. We used CRISPR/Cas9 gene editing in mouse-derived ER-Hoxb8 immortalized myeloid progenitors to generate GPR137b-deficient osteoclast precursors. Decreasing Gpr137b in these precursors led to increased osteoclast differentiation and bone resorption activity. To explore the role of GPR137b during skeletal development, we generated zebrafish deficient for the ortholog gpr137ba. Gpr137ba-deficient zebrafish are viable and fertile and do not display overt morphological defects as adults. However, analysis of osteoclast function in gpr137ba(-/-) mutants demonstrated increased bone resorption. Micro-computed tomography evaluation of vertebral bone mass and morphology demonstrated that gpr137ba-deficiency altered the angle of the neural arch, a skeletal site with high osteoclast activity. Vital staining of gpr137ba(-/-) fish with calcein and alizarin red indicated that bone formation in the mutants is also increased, suggesting high bone turnover. These results identify GPR137b as a conserved negative regulator of osteoclast activity essential for normal resorption and patterning of the skeleton. Further, these data suggest that coordination of osteoclast and osteoblast activity is a	fish
305	animal (fish)	zebrafish	CRISPR;Cas9;	microtubule associated protein 11	Brain	Mutations in the microtubule-associated protein MAP11 (C7orf43) cause microcephaly in humans and zebrafish.	2019	142(3):574-585	[Perez Y et al.]	Ben-Gurion University of the Negev, Beer Sheva, Israel.	30715179	10.1093/brain/awz004	Microtubule associated protein 11 (MAP11, previously termed C7orf43) encodes a highly conserved protein whose function is unknown. Through genome-wide linkage analysis combined with whole exome sequencing, we demonstrate that human autosomal recessive primary microcephaly is caused by a truncating mutation in MAP11. Moreover, homozygous MAP11-orthologue CRISPR/Cas9 knock-out zebrafish presented with microcephaly and decreased neuronal proliferation, recapitulating the human phenotype. We demonstrate that MAP11 is ubiquitously transcribed with high levels in brain and cerebellum. Immunofluorescence and co-immunoprecipitation studies in SH-SY5Y cells showed that MAP11 associates with mitotic spindles, co-localizing and physically associating with alpha-tubulin during mitosis. MAP11 expression precedes alpha-tubulin in gap formation of cell abscission at the midbody and is co-localized with PLK1, a key regulator of cytokinesis, at the edges of microtubule extensions of daughter cells post cytokinesis abscission, implicating a role in mitotic spindle dynamics and in regulation of cell abscission during cytokinesis. Finally, lentiviral-mediated silencing of MAP11 diminished SH-SY5Y cell viability, reducing proliferation rather than affecting apoptosis. Thus, MAP11 encodes a microtubule-associated protein that plays a role in spindle dynamics and cell division,	fish
306	animal (fish)	zebrafish	CRISPR;Cas9;	plphp	Brain	PLPHP deficiency: clinical, genetic, biochemical, and mechanistic insights.	2019	142(3):542-559	[Johnstone DL et al.]	Children's Hospital of Eastern Ontario Research Institute, Ottawa, ON, Canada.	30668673	10.1093/brain/awy346	Biallelic pathogenic variants in PLPBP (formerly called PROSC) have recently been shown to cause a novel form of vitamin B6-dependent epilepsy, the pathophysiological basis of which is poorly understood. When left untreated, the disease can progress to status epilepticus and death in infancy. Here we present 12 previously undescribed patients and six novel pathogenic variants in PLPBP. Suspected clinical diagnoses prior to identification of PLPBP variants included mitochondrial encephalopathy (two patients), folinic acid-responsive epilepsy (one patient) and a movement disorder compatible with AADC deficiency (one patient). The encoded protein, PLPHP is believed to be crucial for B6 homeostasis. We modelled the pathogenicity of the variants and developed a clinical severity scoring system. The most severe phenotypes were associated with variants leading to loss of function of PLPBP or significantly affecting protein stability/PLP-binding. To explore the pathophysiology of this disease further, we developed the first zebrafish model of PLPHP deficiency using CRISPR/Cas9. Our model recapitulates the disease, with plpbp-/- larvae showing behavioural, biochemical, and electrophysiological signs of seizure activity by 10 days post-fertilization and early death by 16 days post-fertilization. Treatment with pyridoxine significantly improved the epileptic phenotype and extended lifespan in plpbp-/- animals. Larvae had disruptions in amino acid metabolism as well as GABA and catecholamine biosynthesis, indicating impairment of PLP-dependent enzymatic activities. Using mass spectrometry, we observed significant B6 vitamer level changes in plpbp-/- zebrafish, patient fibroblasts and PLPHP-deficient HEK293 cells. Additional studies in human cells and yeast provide the first empirical evidence that PLPHP is localized in mitochondria and may play a role in mitochondrial metabolism. These	fish
307	animal (fish)	zebrafish	TALENs;	fzd4	Developmental dynamics	Frizzled 4 regulates ventral blood vessel remodeling in the zebrafish retina.	2019	248(12):1243-1256	[Caceres L et al.]	IWK Health Centre/Dalhousie University, Halifax, Nova Scotia, Canada.	31566834	10.1002/dvdy.117	BACKGROUND: Familial exudative vitreoretinopathy (FEVR) is a rare congenital disorder characterized by a lack of blood vessel growth to the periphery of the retina with secondary fibrovascular proliferation at the vascular-avascular junction. These structurally abnormal vessels cause leakage and hemorrhage, while the fibroproliferative scarring results in retinal dragging, detachment and blindness. Mutations in the FZD4 gene represent one of the most common causes of FEVR. METHODS: A loss of function mutation resulting from a 10-nucleotide insertion into exon 1 of the zebrafish fzd4 gene was generated using transcription activator-like effector nucleases (TALENs). Structural and functional integrity of the retinal vasculature was examined by fluorescent microscopy and optokinetic responses. RESULTS: Zebrafish retinal vasculature is asymmetrically distributed along the dorsoventral axis, with active vascular remodeling on the ventral surface of the retina throughout development. fzd4 mutants exhibit disorganized ventral retinal vasculature with discernable tubular fusion by week 8 of development. Furthermore, fzd4 mutants have impaired optokinetic responses requiring increased illumination. CONCLUSION: We have generated a visually impaired zebrafish FEVR model exhibiting abnormal retinal vasculature. These fish provide a tractable system for studying vascular biology in retinovascular disorders, and demonstrate the feasibility of using zebrafish for	fish

308	animal (fish)	zebrafish	CRISPR;Cas9;	miR-18a	Developmental neurobiology	The MicroRNA, miR-18a, Regulates NeuroD and Photoreceptor Differentiation in the Retina of Zebrafish.	2019	79(2):202-219	[Taylor SM et al.]	University of West Florida, Pensacola, FL, USA.	30615274	10.1002/dneu.22666	During embryonic retinal development, six types of retinal neurons are generated from multipotent progenitors in a strict spatiotemporal pattern. This pattern requires cell cycle exit (i.e. neurogenesis) and differentiation to be precisely regulated in a lineage-specific manner. In zebrafish, the bHLH transcription factor NeuroD governs photoreceptor genesis through Notch signaling but also governs photoreceptor differentiation though distinct mechanisms that are currently unknown. Also unknown are the mechanisms that regulate NeuroD and the spatiotemporal pattern of photoreceptor development. Members of the miR-17-92 microRNA cluster regulate CNS neurogenesis, and a member of this cluster, miR-18a, is predicted to target neuroD mRNA. The purpose of this study was to determine if, in the developing zebrafish retina, miR-18a regulates NeuroD and if it plays a role in photoreceptor development. Quantitative RT-PCR showed that, of the three miR-18 family members (miR-18a, b, and c), miR-18a expression most closely parallels neuroD expression. Morpholino oligonucleotides and CRISPR/Cas9 gene editing were used for miR-18a loss-of-function (LOF) and both resulted in larvae with more mature photoreceptors at 70 hpf without affecting cell proliferation. Western blot showed that miR-18a LOF increases NeuroD protein levels and in vitro dual luciferase assay showed that miR-18a directly interacts with the 3' UTR of neuroD. Finally, tgif1 mutants have increased miR-18a expression, less NeuroD protein and fewer mature photoreceptors, and the photoreceptor deficiency is rescued by miR-18a knockdown. Together, these results show that, independent of neurogenesis, miR-18a regulates the timing of photoreceptor	fish
309	animal (fish)	zebrafish	CRISPR;Cas9;	il34; csf1	Disease models & mechanisms	Reverse genetic screen reveals that Il34 facilitates yolk sac macrophage distribution and seeding of the brain.	2019	12(3)	[Kuil LE et al.]	Erasmus University Medical Center, Rotterdam, The Netherlands.	30765415	10.1242/dmm.037762	Microglia are brain-resident macrophages, which have specialized functions important in brain development and in disease. They colonize the brain in early embryonic stages, but few factors that drive the migration of yolk sac macrophages (YSMs) into the embryonic brain, or regulate their acquisition of specialized properties, are currently known. Here, we present a CRISPR/Cas9-based in vivo reverse genetic screening pipeline to identify new microglia regulators using zebrafish. Zebrafish larvae are particularly suitable due to their external development, transparency and conserved microglia features. We targeted putative microglia regulators, by Cas9/gRNA complex injections, followed by Neutral-Red-based visualization of microglia. Microglia were quantified automatically in 3-day-old larvae using a software tool we called SpotNGlia. We identified that loss of zebrafish colony-stimulating factor 1 receptor (Csf1r) ligand, Il34, caused reduced microglia numbers. Previous studies on the role of IL34 in microglia development in vivo were ambiguous. Our data, and a concurrent paper, show that, in zebrafish, il34 is required during the earliest seeding of the brain by microglia. Our data also indicate that Il34 is required for YSM distribution to other organs. Disruption of the other Csf1r ligand, Csf1, did not reduce microglia numbers in mutants, whereas overexpression increased the number of microglia. This shows that Csf1 can influence microglia numbers, but might not be essential for the early seeding of the brain. In all, we identified il34 as a modifier of microglia colonization, by affecting distribution of YSMs to target organs, validating our reverse genetic screening pipeline	fish
310	animal (fish)	zebrafish	CRISPR;Cas9;	MiR-125a	EBioMedicine	Dysregulated miR-125a promotes angiogenesis through enhanced glycolysis.	2019	47:402-413	[Wade SM et al.]	Trinity College Dublin, Dublin, Ireland.	31466915	10.1016/j.ebiom.2019.08.043	BACKGROUND: Although neoangiogenesis is a hallmark of chronic inflammatory diseases such as inflammatory arthritis and many cancers, therapeutic agents targeting the vasculature remain elusive. Here we identified miR-125a as an important regulator of angiogenesis. METHODS: MiRNA levels were quantified in Psoriatic Arthritis (PsA) synovial-tissue by RT-PCR and compared to macroscopic synovial vascularity. HMVEC were transfected with anti-miR-125a and angiogenic mechanisms quantified using tube formation assays, transwell invasion chambers, wound repair, RT-PCR and western blot. Real-time analysis of EC metabolism was assessed using the XF-24 Extracellular-Flux Analyzer. Synovial expression of metabolic markers was assessed by immunohistochemistry and immunofluorescent staining. MiR-125a CRISPR/Cas9-based knock-out zebrafish were generated and vascular development assessed. Finally, glycolytic blockade using 3PO, which inhibits Phosphofructokinase-fructose-2,6-bisphophatase 3 (PFKFB3), was assessed in miR-125a-/- ECs and zebrafish embryos. FINDINGS: MiR-125a is significantly decreased in PsA synovium and inversely associated with macroscopic vascularity. In-vivo, CRISPR/cas9 miR-125a(-/-) zebrafish displayed a hyper-branching phenotype. In-vitro, miR-125a inhibition promoted EC tube formation, branching, migration and invasion, effects paralleled by a shift in their metabolic profile towards glycolysis. This metabolic shift was also observed in the PsA synovial vasculature where increased expression of glucose transporter 1 (GLUT1), PFKFB3 and Pyruvate kinase muscle isozyme M2 (PKM2) were demonstrated. Finally, blockade of PFKFB3 significantly inhibited anti-miR-125a-induced angiogenic mechanisms in-vitro, paralleled by normalisation of vascular development of CRISPR/cas9 miR-125a(-/-) zebrafish embryos. INTEPRETATION: Our results provide evidence that miR-125a deficiency enhances angiogenic processes through metabolic reprogramming of endothelial cells. FUND: Irish Research Council,	fish

311	animal (fish)	zebrafish	CRISPR;Cas9;	Tumour necrosis factor receptor associated factor 6 (TRAF6)	EBioMedicine	TRAF6 function as a novel co-regulator of Wnt3a target genes in prostate cancer.	2019	45:192-207	[Aripaka K et al.]	Umea University, Umea, Sweden.	31262711	10.1016/j.ebiom.2019.06.046	BACKGROUND: Tumour necrosis factor receptor associated factor 6 (TRAF6) promotes inflammation in response to various cytokines. Aberrant Wnt3a signals promotes cancer progression through accumulation of beta-Catenin. Here we investigated a potential role for TRAF6 in Wnt signaling. METHODS: TRAF6 expression was silenced by siRNA in human prostate cancer (PC3U) and human colorectal SW480 cells and by CRISPR/Cas9 in zebrafish. Several biochemical methods and analyses of mutant phenotype in zebrafish were used to analyse the function of TRAF6 in Wnt signaling. FINDINGS: Wnt3a-treatment promoted binding of TRAF6 to the Wnt co-receptors LRP5/LRP6 in PC3U and LNCaP cells in vitro. TRAF6 positively regulated mRNA expression of beta-Catenin and subsequent activation of Wnt target genes in PC3U cells. Wnt3a-induced invasion of PC3U and SW480 cells were significantly reduced when TRAF6 was silenced by siRNA. Database analysis revealed a correlation between TRAF6 mRNA and Wnt target genes in patients with prostate cancer, and high expression of LRP5, TRAF6 and c-Myc correlated with poor prognosis. By using CRISPR/Cas9 to silence TRAF6 in zebrafish, we confirm TRAF6 as a key molecule in Wnt3a signaling for expression of Wnt target genes. INTERPRETATION: We identify TRAF6 as an important component in Wnt3a signaling to promote activation of Wnt target genes, a finding important for understanding mechanisms driving prostate cancer progression. FUND: KAW 2012.0090, CAN 2017/544, Swedish Medical Research Council (2016-02513), Prostatacancerforbundet, Konung Gustaf V:s Frimurarestiftelse and Cancerforskningsfonden Norrland. The funders did not play a role in manuscript	fish
312	animal (fish)	zebrafish	CRISPR;Cas9;	gh1	Endocrinology	Loss of Growth Hormone Gene (gh1) in Zebrafish Arrests Folliculogenesis in Females and Delays Spermatogenesis in Males.	2019	160(3):568-586	[Hu Z et al.]	University of Macau, Macau, China.	30668682	10.1210/en.2018-00878	As a master hormone controlling growth and metabolism, GH is also known to regulate reproduction. Studies in mammals have shown that mutations in GH or its receptor (GHR) not only result in retardation in body growth but also reproductive dysfunctions in both sexes. However, the roles of GH in reproduction of other vertebrates are poorly defined. In this study, we created two zebrafish GH (gh1) mutant lines using CRISPR/Cas9. The mutant developed normally up to 14 days postfertilization (dpf); however, a high rate of mortality was observed afterward in both lines, and only a small number of mutant fish could survive to adult stage. The body growth of the mutants was significantly retarded in both sexes in a gene dose-dependent manner compared with their wild-type siblings. A severe dysfunction of gonadal development was observed in survived mutant females, with ovarian folliculogenesis being arrested completely at primary growth stage until 100 dpf. Interestingly, the folliculogenesis in the mutant resumed after months of delay with a certain number of follicles entering vitellogenic growth. As for male reproduction, although the spermatogenesis in mutant males seemed normal in adults, the GH-insufficient heterozygote showed an obvious delay of spermatogenesis (puberty onset) at early developmental stages. The adult mutant males could not breed with wild-type females through natural spawning; however, the sperm isolated from the mutant testes could fertilize eggs through artificial fertilization. This study provides further genetic evidence for the dependence	fish
313	animal (fish)	zebrafish	CRISPR;Cas9;	pxr; nrf2	Environmental pollution	Pxr- and Nrf2- mediated induction of ABC transporters by heavy metal ions in zebrafish embryos.	2019	255(Pt 2):113329	[Hu J et al.]	Soochow University, Suzhou, Jiangsu, China.	31600704	10.1016/j.envpol.2019.113329	Transcription factors including pregnane X receptor (Pxr) and nuclear factor-erythroid 2-related factor-2 (Nrf2) are important modulators of Adenosine triphosphate-binding cassette (ABC) transporters in mammalian cells. However, whether such modulation is conserved in zebrafish embryos remains largely unknown. In this manuscript, pxr- and nrf2-deficient models were constructed with CRISPR/Cas9 system, to evaluate the individual function of Pxr and Nrf2 in the regulation of ABC transporters and detoxification of heavy metal ions like Cd(2+) and Ag(+). As a result, both Cd(2+) and Ag(+) conferred extensive interactions with ABC transporters in wild type (WT) embryos: their accumulation and toxicity were affected by the activity of ABC transporters, and they significantly induced the mRNA expressions of ABC transporters. These induction effects were reduced by the mutation of pxr and nrf2, but elevations in the basal expression of ABC transporters compensated for the loss of their inducibility. This could be an explanation for remaining transporter function in both mutant models as well as the unaltered toxicity of metal ions in pxr-deficient embryos. However, mutation of nrf2 disrupted the production of glutathione (GSH), resulting in the enhanced toxicity of Cd(2+)/Ag(+) in zebrafish embryos. In addition, elevated expressions of other transcription factors like aryl hydrocarbon receptor (ahr) 1b, peroxisome proliferator-activated receptor (ppar)-beta, and nrf2 were found in pxr-deficient models without any treatment, while enhanced induction of ahr1b, ppar-beta and pxr could only be seen in nrf2-deficient embryos after the treatment of metal ions, indicating different compensation phenomena for the absence of transcription factors. After all, pxr-deficient and nrf2-deficient zebrafish embryos are useful tools in the functional investigation of Pxr and Nrf2 in the early life stages of aquatic organisms. However, the compensatory mechanisms should be taken into consideration when	fish
314	animal (fish)	zebrafish	CRISPR;Cas9;	scleraxis homolog a; scleraxis homolog b	FASEB journal	Scleraxis genes are required for normal musculoskeletal development and for rib growth and mineralization in zebrafish.	2019	33(8):9116-9130	[Kague E et al.]	University of Bristol, Bristol, UK.	31100023	10.1096/fj.201802654RR	Tendons are an essential part of the musculoskeletal system, connecting muscle and skeletal elements to enable force generation. The transcription factor scleraxis marks vertebrate tendons from early specification. Scleraxis-null mice are viable and have a range of tendon and bone defects in the trunk and limbs but no described cranial phenotype. We report the expression of zebrafish scleraxis orthologs: scleraxis homolog (scx)-a and scxb in cranial and intramuscular tendons and in other skeletal elements. Single mutants for either scxa or scxb, generated by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), are viable and fertile as adult fish. Although scxb mutants show no obvious phenotype, scxa mutant embryos have defects in cranial tendon maturation and muscle misalignment. Mutation of both scleraxis genes results in more severe defects in cranial tendon differentiation, muscle and cartilage dysmorphogenesis and paralysis, and lethality by 2-5 wk, which indicates an essential function of scleraxis for craniofacial development. At juvenile and adult stages, ribs in scxa mutants fail to mineralize and/or are small and heavily fractured. Scxa mutants also have smaller muscle volume, abnormal swim movement, and defects in bone growth and composition. Scleraxis function is therefore essential for normal craniofacial form and function and vital for fish development.-Kague, E., Hughes, S. M., Lawrence, E. A., Cross, S., Martin-Silverstone, E., Hammond, C. L., Hinits, Y. Scleraxis genes are required for normal musculoskeletal development and	fish

315	animal (fish)	zebrafish	CRISPR;Cas9;	(54 puative ciliary genes)	FASEB journal	Mutagenesis of putative ciliary genes with the CRISPR/Cas9 system in zebrafish identifies genes required for retinal development.	2019	33(4):5248-5256	[Hu R et al.]	Tongji University, Shanghai, China.	30624971	10.1096/fj.201802140R	Cilia are conserved microtubule-based organelles that function as mechanical and chemical sensors in various cell types. By bioinformatic, genomic, and proteomic studies, more than 2000 proteins have been identified as cilium-associated proteins or putative ciliary proteins; these proteins are referred to as the ciliary proteome or the ciliome. However, little is known about the function of these numerous putative ciliary proteins in cilia. To identify the possible new functional proteins or pathways in cilia, we carried out a small-scale genetic screen targeting 54 putative ciliary genes by using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system. We successfully constructed 54 zebrafish mutants, and 8 of them displayed microphthalmias. Three of these 8 genes encode proteins for protein transport, suggesting the important roles of protein transport in retinal development. In situ hybridization revealed that all these genes are expressed in zebrafish eyes. Furthermore, polo-like kinase 1 was required for ciliogenesis in neural tube. We uncovered the potential function of the ciliary genes for the retinal development of zebrafish.-Hu, R., Huang, W., Liu, J., Jin, M., Wu, Y., Li, J., Wang, J., Yu, Z., Wang, H., Cao, Y. Mutagenesis of putative ciliary genes with the CRISPR/Cas9	fish
316	animal (fish)	zebrafish	CRISPR;Cas9;	fibroblast growth factor receptor 1 oncogene partner-related protein of 20 kDa (for20)	FASEB journal	Centrosomal protein FOR20 is essential for cilia-dependent development in zebrafish embryos.	2019	33(3):3613-3622	[Xie S et al.]	Zhejiang University School of Medicine, Hangzhou, China.	30475641	10.1096/fj.201801235RR	Centrosomal proteins play critical roles in ciliogenesis. Mutations in many centrosomal proteins have been documented to contribute to developmental defects and cilium-related diseases. Centrosomal protein fibroblast growth factor receptor 1 oncogene partner-related protein of 20 kDa (FOR20) is crucial for ciliogenesis in mammalian cells and the unicellular eukaryote Paramecium; however, the biologic significance of FOR20 in vertebrate development remains unclear. We cloned the zebrafish homolog of the for20 gene and found that for20 mRNA is enriched in ciliated tissues during early zebrafish development. Knockdown of for20 by morpholino oligonucleotides in zebrafish results in multiple ciliary phenotypes, including curved body, hydrocephaly, pericardial edema, kidney cysts, and left-right asymmetry defects. for20 morphants show reduced number and length of cilia in Kupffer's vesicle and pronephric ducts. High-speed video microscopy reveals that cilia in most for20 morphants are consistently paralyzed or beat arrhythmically. To confirm the ciliary phenotypes of for20 morphants, we used the CRISPR/Cas9 system to disrupt for20 gene in zebrafish. for20 mutants exhibit multiple ciliary phenotypes resembling the defects in for20 morphants. All of these phenotypes in for20 morphants and mutants are significantly reversed by exogenous expression of for20 mRNA. Taken together, these data suggest that FOR20 is required for cilium-mediated processes during zebrafish embryogenesis.-Xie, S., Jin, J., Xu, Z., Huang, Y., Zhang, W., Zhao, L., Lo, L. J., Peng, J., Liu, W., Wang, F., Shu, Q., Zhou, T. Centrosomal protein FOR20 is essential for cilia-dependent development in zebrafish embryos.	fish
317	animal (fish)	zebrafish	CRISPR;Cas9;	tlx1	Fish & shellfish immunology	Congenital asplenia due to a tlx1 mutation reduces resistance to Aeromonas hydrophila infection in zebrafish.	2019	95:538-545	[Xie L et al.]	Southwest University, Chongqing, China.	31678534	10.1016/j.fsi.2019.10.065	It is documented that tlx1, an orphan homeobox gene, plays critical roles in the regulation of early spleen developmental in mammalian species. However, there is no direct evidence supporting the functions of tlx1 in non-mammalian species, especially in fish. In this study, we demonstrated that tlx1 is expressed in the splenic primordia as early as 52hours post-fertilization (hpf) in zebrafish. A tlx1(-/-) homozygous mutant line was generated via CRISPR/Cas9 to elucidate the roles of tlx1 in spleen development in zebrafish. In the tlx1(-/-) background, tlx1(-/-) cells persisted in the splenic primordia until 52 hpf but were no longer detectable after 53 hpf, suggesting perturbation of early spleen development. The zebrafish also exhibited congenital asplenia caused by the tlx1 mutation. Asplenic zebrafish can survive and breed normally under standard laboratory conditions, but the survival rate of animals infected with Aeromonas hydrophila was significantly lower than that of wild-type (WT) zebrafish. In asplenic zebrafish, the mononuclear phagocyte system was partially impaired, as demonstrated by retarded b7r expression and reduced ccr2 expression after injection with an inactivated A. hydrophila vaccine. Furthermore, the expression of MHCII/IgM was significantly reduced in the congenitally asplenic fish compared with that of the WT zebrafish. Taken together, our data suggest that tlx1 is a crucial regulator of spleen development in fish, as it is in mammals. We have also provided a	fish
318	animal (fish)	zebrafish	CRISPR;Cas9;	Notch1a	Fish & shellfish immunology	Notch1a can widely mediate innate immune responses in zebrafish larvae infected with Vibrio parahaemolyticus.	2019	92:680-689	[Ji C et al.]	Shanghai Ocean University, Shanghai, China.	31271837	10.1016/j.fsi.2019.06.058	The Notch signaling pathway is known to regulate innate immunity by influencing macrophage function and interacting with the Toll-like receptor (TLR) signaling pathway. However, the comprehensive role of the Notch signaling pathway in the innate immune response remains unknown. To assess the function of Notch1a in immunity, we examined the innate immune responses to Vibrio parahaemolyticus strain Vp13 of wild-type (WT) and notch1a(-/-) zebrafish larvae generated using the clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) system. The median lethal dose (LD50) of V. parahaemolyticus was significantly lower in notch1a(-/-) larvae than in WT larvae 3 days post fertilization (dpf). Transcriptome data analysis revealed 359 significantly differentially expressed genes (DEGs), including 246 significantly down-regulated genes and 113 significantly up-regulated genes, in WT infected groups compared with WT control groups. In contrast, 986 significantly DEGs were found in notch1a(-/-) infected groups compared with notch1a(-/-) control groups, of which 82 genes were significantly down-regulated and 904 genes were significantly up-regulated. These DEGs belonged to the tumor necrosis factor (TNF), complement, nuclear factor kappa B (NF-kappaB), cathepsin, interleukin (IL), chemokine, serpin peptidase inhibitor, matrix metallopeptidase, innate immune cells, pattern recognition receptor (PRR), and other cytokine families. Our results indicate that Notch1a plays roles in inhibiting many immunity-related genes and could comprehensively mediate the innate immune response by regulating TLRs, nucleotide-binding-oligomerization-domain-like receptors (NLRs), lectins, complement, ILs, chemokines, TNF, cathepsin, and serpin. Further studies are required to understand the	fish

319	animal (fish)	zebrafish	CRISPR;Cas9;	gcsfr	Fish & shellfish immunology	Neutrophil plays critical role during Edwardsiella piscicida immersion infection in zebrafish larvae.	2019	87:565-572	[Wang Z et al.]	East China University of Science and Technology, Shanghai, China.	30742890	10.1016/j.fsi.2019.02.008	Edwardsiella piscicida is a facultative intracellular pathogen that causes hemorrhagic septicemia and haemolytic ascites disease in aquaculture fish. During bacterial infection, macrophages and neutrophils are the first line of host innate immune system. However, the role of neutrophils in response to E. piscicida infection in vivo remains poorly understood. Here, through developing an immersion infection model in the 5 day-post fertilization (dpf) zebrafish larvae, we found that E. piscicida was mainly colonized in intestine, and resulted into significant pathological changes in paraffin sections. Moreover, a dynamic up-regulation of inflammatory cytokines (TNF-alpha, IL-1beta, GCSFb, CXCL8 and MMP9) was detected in zebrafish larvae during E. piscicida infection. Furthermore, a significant recruitment of neutrophils was observed during the E. piscicida infection in Tg(mpx:eGFP) zebrafish larvae. Thus, we utilized the CRISPR/Cas9 system to generate the neutrophil-knockdown (gcsfr(-/-) crispants) larvae, and found a comparative higher mortality and bacterial colonization in gcsfr(-/-) crispants, which reveals the critical role of fish neutrophils in bacterial clearance. Taken together, our results developed an effective E. piscicida immersion challenge model in zebrafish larvae to clarify the dynamic of bacterial infection in vivo, which would provide a better understanding of the action about innate immune cells during	fish
320	animal (fish)	zebrafish	CRISPR;Cas9;	ghrelin	Fish physiology and biochemistry	Brain transcriptome profile after CRISPR-induced ghrelin mutations in zebrafish.	2019		[Blanco AM et al.]	Universidad Complutense de Madrid, Madrid, Spain.	31673996	10.1007/s10695-019-00687-6	Ghrelin (GRL) is a gut-brain hormone with a role in a wide variety of physiological functions in mammals and fish, which points out the ghrelinergic system as a key element for the appropriate biological functioning of the organism. However, many aspects of the multifunctional nature of GRL remain to be better explored, especially in fish. In this study, we used the CRISPR/Cas9 genome editing technique to generate F0 zebrafish in which the expression of grl is compromised. Then, we employed high-throughput mRNA sequencing (RNA-seq) to explore changes in the brain transcriptome landscape associated with the silencing of grl. The CRISPR/Cas9 technique successfully edited the genome of F0 zebrafish resulting in individuals with considerably lower levels of GRL mRNAs and protein and ghrelin O-acyl transferase (goat) mRNAs in the brain, intestine, and liver compared to wild-type (WT) zebrafish. Analysis of brain transcriptome revealed a total of 1360 differentially expressed genes (DEGs) between the grl knockdown (KD) and WT zebrafish, with 664 up- and 696 downregulated DEGs in the KD group. Functional enrichment analysis revealed that DEGs are highly enriched for terms related to morphogenesis, metabolism (especially of lipids), entrainment of circadian clocks, oxygen transport, apoptosis, and response to stimulus. The present study offers valuable information on the central genes and pathways implicated in functions of GRL, and points out the possible involvement of	fish
321	animal (fish)	zebrafish	CRISPR;Cas9;	lymphocyte cytosolic protein 1 (lcp1, also called L-plastin)	Gene expression patterns	Identification of regulatory elements recapitulating early expression of L-plastin in the zebrafish enveloping layer and embryonic periderm.	2019	32:53-66	[Baumgartner EA et al.]	DePaul University, USA.	30940554	10.1016/j.gep.2019.03.001	We have cloned and characterized an intronic fragment of zebrafish lymphocyte cytosolic protein 1 (lcp1, also called L-plastin) that drives expression to the zebrafish enveloping layer (EVL). L-plastin is a calcium-dependent actin-bundling protein belonging to the plastin/fimbrin family of proteins, and is necessary for the proper migration and attachment of several adult cell types, including leukocytes and osteoclasts. However, in zebrafish lcp1 is abundantly expressed much earlier, during differentiation of the EVL. The cells of this epithelial layer migrate collectively, spreading vegetally over the yolk. L-plastin expression persists into the larval periderm, a transient epithelial tissue that forms the first larval skin. This finding establishes that L-plastin is activated in two different embryonic waves, with a distinct regulatory switch between the early EVL and the later leukocyte. To better study L-plastin expressing cells we attempted CRISPR/Cas9 homology-driven recombination (HDR) to insert a self-cleaving peptide (Cre-P2A-EGFP-CAAX) downstream of the native lcp1 promoter. This produced a stable zebrafish line expressing Cre recombinase in EVL nuclei and green fluorescence in EVL cell membranes. In vivo tracking of these labeled cells provided enhanced views of EVL migration behavior, membrane extensions, and mitotic events. Finally, we experimentally dissected key elements of the targeted lcp1 locus, discovering a approximately 300 bp intronic sequence sufficient to drive EVL expression. The lcp1: Cre-P2A-EGFP-CAAX zebrafish should be useful for studying enveloping layer specification, gastrulation movements and periderm development in this widely used vertebrate model. In addition, the conserved regulatory sequences we have isolated predict that L-plastin orthologs may have a similar early expression	fish
322	animal (fish)	zebrafish	CRISPR;Cas9;	A disintegrin and metalloprotease with thrombospondin type-1 motif, member 9 (adamts9)	General and comparative endocrinology	Adamts9 is necessary for ovarian development in zebrafish.	2019	277:130-140	[Carter NJ et al.]	East Carolina University, Greenville, NC, USA.	30951722	10.1016/j.ygcen.2019.04.003	Expression of adamts9 (A disintegrin and metalloprotease with thrombospondin type-1 motif, member 9) increases dramatically in the somatic cells surrounding oocytes during ovulation in vertebrates from zebrafish to human. However, the function of Adamts9 during ovulation has not been determined due to the embryonic lethality of knockouts in mice and Drosophila. To identify the role of Adamts9 during ovulation we generated knockout (adamts9(-/-)) zebrafish using CRISPR/Cas9 and characterized the effects of the mutation. From 1047 fish generated by crossing adamts9(+/-) pairs, we found significantly fewer adult adamts9(-/-) fish (4%) than predicted by Mendelian ratios (25%). Of the mutants found, there was a significant male bias (82%). Only 3 female mutants were identified (7%), and they had small ovaries with few stage III and IV oocytes compared to wildtype (wt) counterparts of comparable size and age. Astoundingly, the remaining mutants (11%) did not appear to have normal testis or ovaries. Instead there was a pair of transparent, ovarian-like membranous shells that filled the abdominal cavity. Histological examination confirmed that shells were largely empty with no internal structure. Surprisingly, seminiferous tubules and various spermatocytes including mature spermatozoa were observed on the periphery of these transparent shells. No female or female like knockouts were observed to release eggs, and no ovulated oocytes were observed in histological sections. To our knowledge, this is the first report of an adamts9 global knockout model in any adult vertebrates and the first description of how gonadal sex and structure are affected- highlighting the importance of Adamts9 during gonadal development and the value of zebrafish as a	fish

323	animal (fish)	zebrafish	CRISPR;Cas9;	integrin alpha4	Genes & genetic systems	Disruption of integrin alpha4 in zebrafish leads to cephalic hemorrhage during development.	2019	94(4):177-179	[Iida A et al.]	Kyoto University, Kyoto, Japan.	31582646	10.1266/ggs.19-00033	Integrins, transmembrane molecules that facilitate cell-to-cell and cell-to-extracellular matrix interactions, are heterodimers that consist of an alpha- and beta-subunit. The integrin alpha4 gene (itgalpha4) is expressed in various type of cells and tissues. Its biochemical functions and physiological roles have been revealed using cultured cell assays. In contrast, the primary effect caused by itgalpha4 deletion on vertebrate development is poorly understood, because knockout mice exhibit multiple defects that can lead to embryonic lethality in the uterus. Zebrafish are a convenient vertebrate model to investigate morphogenesis during embryogenesis, because of their external fertilization and subsequent development outside the female's body. Here, we generated a zebrafish mutant line named itgalpha4 (ko108) using the CRISPR/Cas9 genome editing system; the mutant genome harbored an approximately 2.0-kb deletion in the itgalpha4 locus. A truncated transcript was detected in itgalpha4 (+/-) or (-/-) fish but not in (+/+) fish. The mutant transcript was hypothesized to encode a truncated Itgalpha4 protein due to a premature stop codon. itgalpha4 (-/-) embryos obtained from the mating of heterozygous parents exhibited no apparent phenotype during development at 24 hours post-fertilization (hpf). However, approximately half of them exhibited cephalic hemorrhage at 48 hpf. The incidence ratio was significantly higher than that in (+/+) or (+/-) embryos. Embryonic hemorrhage has also been reported previously in Itgalpha4 knockout mice. In contrast, embryonic lethality with the other defects reported in the knockout mice was not observed in our zebrafish model. Therefore, the mutant line itgalpha4 (ko108) should be a useful model to investigate a	fish
324	animal (fish)	zebrafish	CRISPR;	41 genes involved in various aspects of MG cell morphogenesis; etc.	Glia	Genetic control of cellular morphogenesis in Muller glia.	2019	67(7):1401-1411	[Charlton-Perkins M et al.]	University of Cambridge, Cambridge, UK.	30924555	10.1002/glia.23615	Cell shape is critical for the proper function of every cell in every tissue in the body. This is especially true for the highly morphologically diverse neural and glia cells of the central nervous system. The molecular processes by which these, or indeed any, cells gain their particular cell-specific morphology remain largely unexplored. To identify the genes involved in the morphogenesis of the principal glial cell type in the vertebrate retina, the Muller glia (MG), we used genomic and CRISPR based strategies in zebrafish (Danio rerio). We identified 41 genes involved in various aspects of MG cell morphogenesis and revealed a striking concordance between the sequential steps of anatomical feature addition and the expression of cohorts of functionally related genes that regulate these steps. We noted that the many of the genes preferentially expressed in zebrafish MG showed conservation in glia across species suggesting	fish
325	animal (fish)	zebrafish	CRISPR;Cas9;	premelanosome protein	Human molecular genetics	Non-Synonymous variants in premelanosome protein (PMEL) cause ocular pigment dispersion and pigmentary glaucoma.	2019	28(8):1298-1311	[Lahola-Chomiak AA et al.]	University of Alberta, Edmonton AB, Canada.	30561643	10.1093/hmg/ddy429	Pigmentary glaucoma (PG) is a common glaucoma subtype that results from release of pigment from the iris, called pigment dispersion syndrome (PDS), and its deposition throughout the anterior chamber of the eye. Although PG has a substantial heritable component, no causative genes have yet been identified. We used whole exome sequencing of two independent pedigrees to identify two premelanosome protein (PMEL) variants associated with heritable PDS/PG. PMEL encodes a key component of the melanosome, the organelle essential for melanin synthesis, storage and transport. Targeted screening of PMEL in three independent cohorts (n = 394) identified seven additional PDS/PG-associated non-synonymous variants. Five of the nine variants exhibited defective processing of the PMEL protein. In addition, analysis of PDS/PG-associated PMEL variants expressed in HeLa cells revealed structural changes to pseudomelanosomes indicating altered amyloid fibril formation in five of the nine variants. Introduction of 11-base pair deletions to the homologous pmela in zebrafish by the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 method caused profound pigmentation defects and enlarged anterior segments in the eye, further supporting PMEL's role in ocular pigmentation and function. Taken together, these data support a model in which missense PMEL variants represent dominant negative mutations that impair the ability of PMEL to form functional amyloid fibrils. While PMEL mutations have previously been shown to cause pigmentation and ocular defects in animals, this research is the first report of mutations in PMEL	fish
326	animal (fish)	zebrafish	CRISPR;Cas9;	deoxyguanosine kinase (dguok)	Human molecular genetics	Nucleoside supplementation modulates mitochondrial DNA copy number in the dguok -/- zebrafish.	2019	28(5):796-803	[Munro B et al.]	Newcastle University, Newcastle upon Tyne, UK.	30428046	10.1093/hmg/ddy389	Deoxyguanosine kinase (dGK) is an essential rate-limiting component of the mitochondrial purine nucleotide salvage pathway, encoded by the nuclear gene encoding deoxyguanosine kinase (DGUOK). Mutations in DGUOK lead to mitochondrial DNA (mtDNA) depletion typically in the liver and brain, causing a hepatocerebral phenotype. Previous work has shown that in cultured DGUOK patient cells it is possible to rescue mtDNA depletion by increasing substrate amounts for dGK. In this study we developed a mutant dguok zebrafish (Danio rerio) line using CRISPR/Cas9 mediated mutagenesis; dguok-/- fish have significantly reduced mtDNA levels compared with wild-type (wt) fish. When supplemented with only one purine nucleoside (dGuo), mtDNA copy number in both mutant and wt juvenile animals was significantly reduced, contrasting with previous cell culture studies, possibly because of nucleotide pool imbalance. However, in adult dguok-/- fish we detected a significant increase in liver mtDNA copy number when supplemented with both purine nucleosides. This study further supports the idea that nucleoside supplementation has a potential therapeutic benefit in mtDNA depletion syndromes by substrate enhancement of the purine	fish
327	animal (fish)	zebrafish	CRISPR;Cas9;	RhoB	International journal of radiation oncology, biology, physics	The Critical Role of Dysregulated RhoB Signaling Pathway in Radioresistance of Colorectal Cancer.	2019	104(5):1153-1164	[Liu N et al.]	Linkoping University, Linkoping, Sweden.	31039421	10.1016/j.ijrobp.2019.04.021	PURPOSE: To explore whether the Rho protein is involved in the radioresistance of colorectal cancer and investigate the underlying mechanisms. METHODS AND MATERIALS: Rho GTPase expression was measured after radiation treatment in colon cancer cells. RhoB knockout cell lines were established using the CRISPR/Cas9 system. In vitro assays and zebrafish embryos were used for analyzing radiosensitivity and invasive ability. Mass cytometry was used to detect RhoB downstream signaling factors. RhoB and Forkhead box M1 (FOXM1) expression were detected by immunohistochemistry in rectal cancer patients who participated in a radiation therapy trial. RESULTS: RhoB expression was related to radiation resistance. Complete depletion of the RhoB protein increased radiosensitivity and impaired radiation-enhanced metastatic potential in vitro and in zebrafish models. Probing signaling using mass cytometry-based single-cell analysis showed that the Akt phosphorylation level was inhibited by RhoB depletion after radiation. FOXM1 was downregulated in RhoB knockout cells, and the inhibition of FOXM1 led to lower survival rates and attenuated migration and invasion abilities of the cells after radiation. In the patients who underwent radiation therapy, RhoB overexpression was related to high FOXM1, late Tumor, Node, Metastasis stage, high distant recurrence, and poor survival independent of other clinical factors. CONCLUSIONS: RhoB plays a critical role in radioresistance of	fish

328	animal (fish)	zebrafish	TALENs;	autotaxin (atxa)	Journal of biochemistry	Identification and biochemical characterization of a second zebrafish autotaxin gene.	2019	165(3):269–275	[Kise R et al.]	Tohoku University, Sendai, Japan.	30629186	10.1093/jb/mvy114	Autotaxin (ATX) is a secreted enzyme that produces a bioactive lysophospholipid, lysophosphatidic acid (LPA). ATX plays a role in vascular and neural development in embryos but its mechanisms remain unclear. At the beginning of this study, only one zebrafish atx gene (atxa) was known and had been investigated. In this study, we generated ATX knockout (KO) fish by TALEN targeting atxa. Unexpectedly, atxa KO fish showed neither vascular defects nor reduction of ATX activity, implying the existence of one or more other ATXs in the genome. By a BLAST search using ATXa protein fragments as a query, we found a genomic sequence that closely resembled atxa exons 13, 14 and 15. Consequently, we cloned a cDNA encoding a second zebrafish autotaxin (ATXb), and found that it was transcribed in various tissues. The atxb gene encoded a protein of 832 amino acids (compared to 850 amino acids in ATXa) with 60% amino acid identity to ATXa and clustered with ATXs from other species. A recombinant ATXb protein showed lysophospholipase D (lysoPLD) activities with substrate specificities similar to those of ATXa and mammalian ATXs. These results indicate that ATXb is a second zebrafish ATX, which possibly shares redundant	fish
329	animal (fish)	zebrafish	CRISPR;	zinc finger transcription factor 1 (znfl1s)	Journal of cellular physiology	Zebrafish znfl1s regulate left-right asymmetry patterning through controlling the expression of fgfr1a.	2019	234(3):1987–1995	[Li J et al.]	Women's Hospital of Nanjing Medical University, China.	30317609	10.1002/jcp.27564	Proper left–right (LR) axis establishment is critical for organogenesis in vertebrates. Previously, we reported that zinc finger transcription factors zinc finger transcription factor 1 (znfl1s) are expressed in the tailbud and axial mesoderm in zebrafish. However, a role of znfl1s in LR axis development has not been demonstrated. Here, we discovered that the knockdown of znfl1s using morpholino (MO) in whole embryos or dorsal forerunner cells (DFCs) interrupted LR asymmetry and normal development of the heart, liver, and pancreas. Whole-embryo knockdown of znfl1s by MO or clustered regularly interspaced short palindromic repeat (CRISPR) interference (CRISPRi) resulted in the absent expression of nodal gene spaw and Nodal signaling–related genes lft1, lft2, and pitx2c in the left lateral plate mesoderm (LPM), and Spaw, Lft1, Lft2, and Pitx2c play important roles in LR axis development in zebrafish. However, specific knockdown of znfl1s in DFCs resulted in random expression of spaw, lft1, lft2, and pitx2c. Knockdown of znfl1s led to abnormal cilia formation by the downregulation of fgfr1a and foxj1a expression. The expression of spaw, lft1, lft2, and pitx2c was partially rescued by the overexpression of fgfr1a mRNA in znfl1s morphants. Taken together, our results suggest that znfl1s regulate laterality development in zebrafish embryos	fish
330	animal (fish)	zebrafish	CRISPR;Cas9;	cyp11a2	Journal of endocrinology	The P450 side chain cleavage enzyme Cyp11a2 facilitates steroidogenesis in zebrafish.	2019		[Li N et al.]	University of Sheffield, Sheffield, UK.	31693487	10.1530/JOE-19-0384	The cytochrome P450 side-chain cleavage enzyme, encoded by the CYP11A1 gene, catalyzes the first and rate-limiting step of steroid hormone biosynthesis. Previous morpholino knockdown studies in zebrafish suggested cyp11a2 is a functional equivalent of human CYP11A1 and is essential for interrenal steroidogenesis in zebrafish larvae. The role of Cyp11a2 in adult zebrafish, particularly in gonadal steroidogenesis, remains elusive. To explore the role of Cyp11a2 in adults, we developed zebrafish mutant lines by creating deletions in cyp11a2 using the CRISPR/Cas9 genomic engineering approach. Homozygous mutant zebrafish larvae showed an upregulation of the hypothalamic–pituitary–interrenal axis. Furthermore, Cyp11a2-deficient zebrafish demonstrated profound glucocorticoid and androgen deficiencies. Cyp11a2 homozygotes only developed into males with feminized secondary sex characteristics. Adult cyp11a2−/− mutant fish showed a lack of natural breeding behaviors. Histological characterization revealed disorganized testicular structure and significantly decreased numbers of mature spermatozoa. These findings are further supported by the downregulation of the expression of several pro-male genes in the testes of cyp11a2 homozygous zebrafish, including sox9a, dmrt1 and amh. Moreover, the spermatogonia markers nanos2 and piwil1 were upregulated, while the spermatocytes marker sycp3 and spermatids marker odf3b were downregulated in the testes of cyp11a2 homozygous mutants. Our expression analysis is consistent with our histological studies, suggesting that spermatogonia are the predominant cell types in the testes of cyp11a2 homozygous mutants. Our work thus demonstrates the crucial	fish
331	animal (fish)	zebrafish	CRISPR;Cas9;	gba1; gba2	Journal of lipid research	Role of beta-glucosidase 2 in aberrant glycosphingolipid metabolism: model of glucocerebrosidase deficiency in zebrafish.	2019	60(11):1851–1867	[Lelieveld LT et al.]	Leiden Institute of Chemistry, Leiden, The Netherlands.	31562193	10.1194/jlr.RA119000154	beta-glucosidases [GBA1 (glucocerebrosidase) and GBA2] are ubiquitous essential enzymes. Lysosomal GBA1 and cytosol-facing GBA2 degrade glucosylceramide (GlcCer); GBA1 deficiency causes Gaucher disease, a lysosomal storage disorder characterized by lysosomal accumulation of GlcCer, which is partly converted to glucosylsphingosine (GlcSph). GBA1 and GBA2 also may transfer glucose from GlcCer to cholesterol, yielding glucosylated cholesterol (GlcChol). Here, we aimed to clarify the role of zebrafish Gba2 in glycosphingolipid metabolism during Gba1 deficiency in zebrafish (Danio rerio), which are able to survive total Gba1 deficiency. We developed Gba1 (gba1 (−/−)), Gba2 (gba2 (−/−)), and double (gba1 (−/−) ;gba2 (−/−)) zebrafish knockouts using CRISPR/Cas9 and explored the effects of both genetic and pharmacological interventions on GlcCer metabolism in individual larvae. Activity-based probes and quantification of relevant glycolipid metabolites confirmed enzyme deficiency. GlcSph increased in gba1 (−/−) larvae (0.09 pmol/fish) but did not increase more in gba1 (−/−) ;gba2 (−/−) larvae. GlcCer was comparable in gba1 (−/−) and WT larvae but increased in gba2 (−/−) and gba1 (−/−) ;gba2 (−/−) larvae. Independent of Gba1 status, GlcChol was low in all gba2 (−/−) larvae (0.05 vs. 0.18 pmol/fish in WT). Pharmacologic inactivation of zebrafish Gba1 comparably increased GlcSph. Inhibition of GlcCer synthase (GCS) in Gba1-deficient larvae reduced GlcCer and GlcSph, and concomitant inhibition of GCS and Gba2 with iminosugars also reduced excessive GlcChol. Finally, overexpression of human GBA1 and injection of recombinant GBA1 both decreased GlcSph. We determined that zebrafish larvae offer an attractive model to study glucosidase actions in glycosphingolipid metabolism in vivo, and we identified	fish

332	animal (fish)	zebrafish	CRISPR;Cas9;	tardbpl	Journal of neurophysiology	Neuromuscular junction abnormalities in a zebrafish loss-of-function model of TDP-43.	2019	121(1):285-297	[Bose P et al.]	Centre de Recherche du Centre Hospitalier de l'Universite de Montreal , Montreal, Quebec , Canada.	30461368	10.1152/jn.00265.2018	Almost 90% of amyotrophic lateral sclerosis (ALS) cases are characterized by the presence of aggregates of insoluble, misfolded cytoplasmic TAR DNA binding protein of 43 kDa (TDP-43). Distal axonopathy with impaired neuromuscular junctions (NMJs) before motor neuron degeneration or clinical onset of symptoms has been hypothesized as an early pathology in ALS. However, synaptic defects at the NMJ caused by TDP-43 mutations have not been characterized. In this study, we examined a previously reported zebrafish line expressing the tardbp(Y220X/Y220X) variant, which results in an unstable and degraded protein. These tardbp(-/-) larvae, however, mature normally due to the upregulated expression of an alternative splice variant of the tardbp paralog tardbp-like, or tardbpl. We generated a mutant line with a CRISPR/Cas9-mediated 5-base pair deletion encompassing the ATG start codon of tardbpl and in-crossed these with tardbp(-/-) mutants to obtain tardbp(-/-) and tardbpl(-/-) double mutants, herein referred to as hom/hom. We subsequently characterized morphological, coiling, locomotor, synaptic, and NMJ structural abnormalities in the hom/hom mutants and in their genotypic controls. We observed that hom/hom mutants displayed gross morphological defects, early lethality, reduced locomotor function, aberrant quantal transmission, and perturbed synapse architecture at the NMJ. We further employed pharmacological manipulations in an effort to rescue phenotypic defects and observed that tardbp(+/-); tardbpl(-/-) (herein referred to as het/hom) mutants, but not hom/hom mutants, were sensitive to chronic treatments of BAY K 8644, an L-type calcium channel agonist. This result highlights the importance of partial vs. complete loss of allelic functions of TDP-43. NEW & NOTEWORTHY This study highlights the importance of partial vs. complete loss of allelic functions of TDP-	fish
333	animal (fish)	zebrafish	CRISPR;	nuclear factor erythroid 2-related factor 2a	Journal of pharmacological and toxicological methods	Increased susceptibility to oxidative stress-induced toxicological evaluation by genetically modified nrf2a-deficient zebrafish.	2019	96:34-45	[Yamashita A et al.]	Mie University Graduate School of Medicine, Mie, Japan.	30594530	10.1016/j.vascn.2018.12.006	INTRODUCTION: Oxidative stress plays an important role in drug-induced toxicity. Oxidative stress-mediated toxicities can be detected using conventional animal models but their sensitivity is insufficient, and novel models to improve susceptibility to oxidative stress have been researched. In recent years, gene targeting methods in zebrafish have been developed, making it possible to generate homozygous null mutants. In this study, we established zebrafish deficient in the nuclear factor erythroid 2-related factor 2a (nrf2a), a key antioxidant-responsive gene, and its potential to detect oxidative stress-mediated toxicity was examined. METHODS: Nrf2a-deficient zebrafish were generated using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 technique. The loss of nrf2a function was confirmed by the tolerability to hydrogen peroxide and hydrogen peroxide-induced gene expression profiles being related to antioxidant response element (ARE)-dependent signaling. Subsequently, vulnerability of nrf2a-deficient zebrafish to acetaminophen (APAP)- or doxorubicin (DOX)-induced toxicity was investigated. RESULTS: Nrf2a-deficient zebrafish showed higher mortality than wild type accompanied by less induction of ARE-dependent genes with hydrogen peroxide treatment. Subsequently, this model showed increased severity and incidence of APAP-induced hepatotoxicity or DOX-induced cardiotoxicity than wild type. DISCUSSION: Our results demonstrated that anti-oxidative response might not fully function in this model, and resulted in higher sensitivity to drug-induced oxidative stress. Our data support the usefulness of nrf2a-deficient model as a tool for	fish
334	animal (fish)	zebrafish	CRISPR;Cas9;	col14a1a	Matrix biology	Gene profile of zebrafish fin regeneration offers clues to kinetics, organization and biomechanics of basement membrane.	2019	75-76:82-101	[Nauroy P et al.]	Universite de Lyon, ENSL, CNRS, Lyon, France.	30031067	10.1016/j.matbio.2018.07.005	How some animals regenerate missing body parts is not well understood. Taking advantage of the zebrafish caudal fin model, we performed a global unbiased time-course transcriptomic analysis of fin regeneration. Biostatistics analyses identified extracellular matrix (ECM) as the most enriched gene sets. Basement membranes (BMs) are specialized ECM structures that provide tissues with structural cohesion and serve as a major extracellular signaling platform. While the embryonic formation of BM has been extensively investigated, its regeneration in adults remains poorly studied. We therefore focused on BM gene expression kinetics and showed that it recapitulates many aspects of development. As such, the re-expression of the embryonic col14a1a gene indicated that col14a1a is part of the regeneration-specific program. We showed that laminins and col14a1a genes display similar kinetics and that the corresponding proteins are spatially and temporally controlled during regeneration. Analysis of our CRISPR/Cas9-mediated col14a1a knockout fish showed that collagen XIV-A contributes to timely deposition of laminins. As changes in ECM organization can affect tissue mechanical properties, we analyzed the biomechanics of col14a1a(-/-) regenerative BM using atomic force microscopy (AFM). Our data revealed a thinner BM accompanied by a substantial increase of the stiffness when compared to controls. Further AFM 3D-reconstructions showed that BM is organized as a checkerboard made of alternation of soft and rigid regions that is compromised in mutants leading to a more compact structure. We conclude that collagen XIV-A transiently acts as a molecular spacer responsible for BM structure and biomechanics possibly by helping	fish

335	animal (fish)	zebrafish	CRISPR;Cas9;	shank3a; shank3b	Molecular autism	Intestinal dysmotility in a zebrafish (Danio rerio) shank3a;shank3b mutant model of autism.	2019	10:3	[James DM et al.]	University of Miami, Coral Gables, FL, USA.	30733854	10.1186/s13229-018-0250-4	Background and aims: Autism spectrum disorder (ASD) is currently estimated to affect more than 1% of the world population. For people with ASD, gastrointestinal (GI) distress is a commonly reported but a poorly understood co-occurring symptom. Here, we investigate the physiological basis for GI distress in ASD by studying gut function in a zebrafish model of Phelan-McDermid syndrome (PMS), a condition caused by mutations in the SHANK3 gene. Methods: To generate a zebrafish model of PMS, we used CRISPR/Cas9 to introduce clinically related C-terminal frameshift mutations in shank3a and shank3b zebrafish paralogues (shank3abDeltaC). Because PMS is caused by SHANK3 haploinsufficiency, we assessed the digestive tract (DT) structure and function in zebrafish shank3abDeltaC (+/-) heterozygotes. Human SHANK3 mRNA was then used to rescue DT phenotypes in larval zebrafish. Results: Significantly slower rates of DT peristaltic contractions ($p < 0.001$) with correspondingly prolonged passage time ($p < 0.004$) occurred in shank3abDeltaC (+/-) mutants. Rescue injections of mRNA encoding the longest human SHANK3 isoform into shank3abDeltaC (+/-) mutants produced larvae with intestinal bulb emptying similar to wild type (WT), but still deficits in posterior intestinal motility. Serotonin-positive enteroendocrine cells (EECs) were significantly reduced in both shank3abDeltaC (+/-) and shank3abDeltaC (-/-) mutants ($p < 0.05$) while enteric neuron counts and overall structure of the DT epithelium, including goblet cell number, were unaffected in shank3abDeltaC (+/-) larvae. Conclusions: Our data and rescue experiments support mutations in SHANK3 as causal for GI transit and motility abnormalities. Reductions in serotonin-positive EECs and serotonin-filled ENS boutons suggest an endocrine/neural component to this dysmotility. This is the first study to date demonstrating DT dysmotility in a zebrafish	fish
336	animal (fish)	zebrafish	CRISPR;Cas9;	erbb4a	Molecular biology of the cell	ErbB4 tyrosine kinase inhibition impairs neuromuscular development in zebrafish embryos.	2019	30(2):209-218	[Paatero I et al.]	University of Turku, Turku, Finland.	30462579	10.1091/mbc.E18-07-0460	Tyrosine kinase inhibitors are widely used in the clinic, but limited information is available about their toxicity in developing organisms. Here, we tested the effect of tyrosine kinase inhibitors targeting the ErbB receptors for their effects on developing zebrafish (Danio rerio) embryos. Embryos treated with wide-spectrum pan-ErbB inhibitors or erbb4a-targeting antisense oligonucleotides demonstrated reduced locomotion, reduced diameter of skeletal muscle fibers, and reduced expression of muscle-specific genes, as well as reduced motoneuron length. The phenotypes in the skeletal muscle, as well as the defect in motility, were rescued both by microinjection of human ERBB4 mRNA and by transposon-mediated muscle-specific ERBB4 overexpression. The role of ErbB4 in regulating motility was further controlled by targeted mutation of the endogenous erbb4a locus in the zebrafish genome by CRISPR/Cas9. These observations demonstrate a potential for the ErbB tyrosine kinase inhibitors to induce neuromuscular toxicity in a developing organism via a	fish
337	animal (fish)	zebrafish	CRISPR;Cas9;	dj-1	Molecular neurobiology	Dysregulation in the Brain Protein Profile of Zebrafish Lacking the Parkinson's Disease-Related Protein DJ-1.	2019	56(12):8306-8322	[Edson AJ et al.]	University of Bergen, Bergen, Norway.	31218647	10.1007/s12035-019-01667-w	DJ-1 is a protein with a wide range of functions importantly related to redox regulation in the cell. In humans, dysfunction of the PARK7 gene is associated with neurodegeneration and Parkinson's disease. Our objective was to establish a novel DJ-1 knockout zebrafish line and to identify early brain proteome changes, which could be linked to later pathology. The CRISPR-Cas9 method was used to target exon 1 of the park7-/- gene to produce a transgenic DJ-1-deficient zebrafish model of Parkinson's disease. Label-free mass spectrometry was employed to identify altered protein expression in the DJ-1 null brain of early adult animals. The park7(-/-) line appears to develop normally at young adult and larval stages. With aging however, DJ-1 null fish exhibit lower tyrosine hydroxylase levels, respiratory failure in skeletal muscle, and lower body mass which is especially prevalent among male fish. By proteomic analysis of early adult brains, we determined that less than 5% of the 4091 identified proteins were influenced by the lack of DJ-1. The dysregulated proteins were mainly proteins known to be involved in mitochondrial metabolism, mitophagy, stress response, redox regulation, and inflammation. This dysregulation in protein networks of our novel DJ-1-deficient zebrafish model occurs in the early adult stage preceding a Parkinson's disease-related phenotype and the reduction of tyrosine hydroxylase level. The identified protein changes provide new mechanistic background for DJ-1 function. The experimental power of zebrafish makes this model a highly valuable tool to understand	fish
338	animal (fish)	zebrafish	CRISPR;Cas9;	type-I vitellogenins (vtf1, 4, 5, 6, 7), type-III vitellogenin (vtg3)	Molecular reproduction and development	Genome editing reveals reproductive and developmental dependencies on specific types of vitellogenin in zebrafish (Danio rerio).	2019	86(9):1168-1188	[Yilmaz O et al.]	INRA, UR1037, Rennes Cedex, France.	31380595	10.1002/mrd.23231	Oviparous vertebrates produce multiple forms of vitellogenin (Vtg), the major source of yolk nutrients, but little is known about their individual contributions to reproduction and development. This study utilized clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) genome editing to assess essentiality and functionality of zebrafish (Danio rerio) type-I and type-III Vtgs. A multiple CRISPR approach was employed to knockout (KO) all genes encoding type-I vtgs (vtg1, 4, 5, 6, and 7) simultaneously (vtg1-KO), and the type-III vtg (vtg3) individually (vtg3-KO). Results of polymerase chain reaction (PCR) genotyping and sequencing, quantitative PCR, liquid chromatography-tandem mass spectrometry, and Western blot analysis showed that only vtg6 and vtg7 escaped Cas9 editing. In fish whose remaining type-I vtgs were incapacitated (vtg1-KO), and in vtg3-KO fish, significant increases in Vtg7 transcript and protein levels occurred in liver and eggs, revealing a heretofore-unknown mechanism of genetic compensation regulating Vtg homeostasis. Egg numbers per spawn were elevated more than 2-fold in vtg1-KO females, and egg fertility was approximately halved in vtg3-KO females. Substantial mortality was evident in vtg3-KO eggs/embryos after only 8 hr of incubation and in vtg1-KO embryos after 5 days. Hatching rate and timing were markedly impaired in embryos from vtg mutant mothers and pericardial and yolk sac/abdominal edema and spinal lordosis were evident in the larvae, with feeding and motor activities also being absent in vtg1-KO larvae. By late larval stages, vtg mutations were either completely lethal (vtg1-KO) or nearly so (vtg3-KO). These novel findings offer the first experimental evidence that different types of vertebrate Vtg are essential and have	fish

339	animal (fish)	zebrafish	CRISPR;Cas9;	miR-155	Neurobiology of disease	Ablation of the pro-inflammatory master regulator miR-155 does not mitigate neuroinflammation or neurodegeneration in a vertebrate model of Gaucher's disease.	2019	127:563-569	[Watson L et al.]	University of Sheffield, Sheffield, UK.	30981829	10.1016/j.nbd.2019.04.008	Bi-allelic mutations in the glucocerebrosidase gene (GBA1) cause Gaucher's disease, the most common human lysosomal storage disease. We previously reported a marked increase in miR-155 transcript levels and early microglial activation in a zebrafish model of Gaucher's disease (gba1(-/-)). miR-155 is a master regulator of inflammation and has been implicated in a wide range of different neurodegenerative disorders. The observed miR-155 upregulation preceded the subsequent development of widespread pathology with marked neuroinflammation, closely resembling human Gaucher's disease pathology. We now report similar increases of miR-155 expression in mammalian models of GD, confirming that miR-155 upregulation is a shared feature in glucocerebrosidase (GCase) deficiency across different species. Using CRISPR/Cas9 mutagenesis we then generated a miR-155 mutant zebrafish line (miR-155(-/-)) with completely abolished miR-155 expression. Unexpectedly, loss of miR-155 did not mitigate either the reduced lifespan or the robust inflammatory phenotypes of gba1(-/-) mutant zebrafish. Our data demonstrate that neither neuroinflammation nor disease progression in GCase deficiency are dependent on miR-155 and suggest that miR-155 inhibition would not be a promising therapeutic target in Gaucher's disease.	fish
340	animal (fish)	zebrafish	CRISPR;Cas9;	gtpbp3	Nucleic acids research	Deletion of Gtpbp3 in zebrafish revealed the hypertrophic cardiomyopathy manifested by aberrant mitochondrial tRNA metabolism.	2019	47(10):5341-5355	[Chen D et al.]	Zhejiang University School of Medicine, Hangzhou, Zhejiang, China.	30916346	10.1093/nar/gkz218	GTPBP3 is a highly conserved tRNA modifying enzyme for the biosynthesis of taum5U at the wobble position of mitochondrial tRNAGlu, tRNAGln, tRNALys, tRNATrp and tRNALeu(UUR). The previous investigations showed that GTPBP3 mutations were associated with hypertrophic cardiomyopathy (HCM). However, the pathophysiology of GTPBP3 deficiency remains elusively. Using the gtpbp3 knockout zebrafish generated by CRISPR/Cas9 system, we demonstrated the aberrant mitochondrial tRNA metabolism in gtpbp3 knock-out zebrafish. The deletion of gtpbp3 may alter functional folding of tRNA, indicated by conformation changes and sensitivity to S1-mediated digestion of tRNAGlu, tRNALys, tRNATrp and tRNALeu(UUR). Strikingly, gtpbp3 knock-out zebrafish displayed the global increases in the aminoacylated efficiencies of mitochondrial tRNAs. The aberrant mitochondrial tRNA metabolisms impaired mitochondrial translation, produced proteostasis stress and altered activities of respiratory chain complexes. These mitochondria dysfunctions caused the alterations in the embryonic heart development and reduced fractional shortening of ventricles in mutant zebrafish. Notably, the gtpbp3 knock-out zebrafish exhibited hypertrophy of cardiomyocytes and myocardial fiber disarray in ventricles. These cardiac defects in the gtpbp3 knock-out zebrafish recapitulated the clinical phenotypes in HCM patients carrying the GTPBP3 mutation(s). Our findings highlight the fundamental role of defective nucleotide modifications of tRNAs in mitochondrial biogenesis and their	fish
341	animal (fish)	zebrafish	CRISPR;Cas12a(Cpf1)		Nucleic acids research	Enhanced Cas12a editing in mammalian cells and zebrafish.	2019	47(8):4169-4180	[Liu P et al.]	University of Massachusetts Medical School, Worcester, MA, USA.	30892626	10.1093/nar/gkz184	Type V CRISPR-Cas12a systems provide an alternate nuclease platform to Cas9, with potential advantages for specific genome editing applications. Here we describe improvements to the Cas12a system that facilitate efficient targeted mutagenesis in mammalian cells and zebrafish embryos. We show that engineered variants of Cas12a with two different nuclear localization sequences (NLS) on the C terminus provide increased editing efficiency in mammalian cells. Additionally, we find that pre-crRNAs comprising a full-length direct repeat (full-DR-crRNA) sequence with specific stem-loop G-C base substitutions exhibit increased editing efficiencies compared with the standard mature crRNA framework. Finally, we demonstrate in zebrafish embryos that the improved LbCas12a and FnoCas12a nucleases in combination with these modified crRNAs display high mutagenesis efficiencies and low toxicity when delivered as ribonucleoprotein complexes at high concentration. Together, these results define a set of enhanced Cas12a components with broad utility in vertebrate systems.	fish
342	animal (fish)	zebrafish	CRISPR;Cas9;	Histone deacetylase 4 (hdac4)	PeerJ	hdac4 mediates perichondral ossification and pharyngeal skeleton development in the zebrafish.	2019	7:e6167	[DeLaurier A et al.]	University of South Carolina-Aiken, Aiken, SC, USA.	30643696	10.7717/peerj.6167	Background: Histone deacetylases (HDACs) are epigenetic factors that function to repress gene transcription by removing acetyl groups from the N-terminal of histone lysines. Histone deacetylase 4 (HDAC4), a class IIa HDAC, has previously been shown to regulate the process of endochondral ossification in mice via repression of Myocyte enhancer factor 2c (MEF2C), a transcriptional activator of Runx2, which in turn promotes chondrocyte maturation and production of bone by osteoblasts. Methods & Materials: In this study, we generated two zebrafish lines with mutations in hdac4 using CRISPR/Cas9 and analyzed mutants for skeletal phenotypes and expression of genes known to be affected by Hdac4 expression. Results: Lines have insertions causing a frameshift in a proximal exon of hdac4 and a premature stop codon. Mutations are predicted to result in aberrant protein sequence and a truncated protein, eliminating the Mef2c binding domain and Hdac domain. Zygotic mutants from two separate lines show a significant increase in ossification of pharyngeal ceratohyal cartilages at 7 days post fertilization (dpf) ($p < 0.01$, $p < 0.001$). At 4 dpf, mutant larvae have a significant increase of expression of runx2a and runx2b in the ceratohyal cartilage ($p < 0.05$ and $p < 0.01$, respectively). A subset of maternal-zygotic (mz) mutant and heterozygote larvae (40%) have dramatically increased ossification at 7 dpf compared to zygotic mutants, including formation of a premature anguloarticular bone and mineralization of the first and second ceratobranchial cartilages and symplectic cartilages, which normally does not occur until fish are approximately 10 or 12 dpf. Some maternal-zygotic mutants and heterozygotes show loss of pharyngeal first arch elements (25.9% and 10.2%, respectively) and neurocranium defects (30.8% and 15.2%, respectively). Analysis of RNA-seq mRNA transcript levels and in situ hybridizations from zygotic stages to 75-90% epiboly indicates that hdac4 is highly expressed in early embryos, but diminishes by late epiboly, becoming expressed again in larval stages. Discussion: Loss of function of hdac4 in zebrafish is associated with increased expression of runx2a and runx2b targets indicating that a role for hdac4 in zebrafish is to repress activation of ossification of cartilage. These findings are consistent with observations of precocious cartilage ossification in Hdac4 mutant mice, demonstrating that the function of Hdac4 in skeletal development is conserved among vertebrates. Expression of hdac4 mRNA in embryos younger than 256-512 cells indicates that there is a maternal contribution of hdac4 to the early embryo. The increase in ossification and profound loss of first pharyngeal arch elements and anterior neurocranium in a subset of maternal-zygotic mutant and heterozygote larvae suggests that maternal hdac4 functions in cartilage	fish

343	animal (fish)	zebrafish	CRISPR;Cas9;	melanocortin 1 receptor (mc1r)	Pigment cell & melanoma research	Loss-of-function mutations in the melanocortin 1 receptor cause disruption of dorso-ventral countershading in teleost fish.	2019	32(6):817-828	[Cal L et al.]	Institute of Marine Research (IIM-CSIC), Vigo, Spain.	31251842	10.1111/pcmr.12806	The melanocortin 1 receptor (MC1R) is the central melanocortin receptor involved in vertebrate pigmentation. Mutations in this gene cause variations in coat coloration in amniotes. Additionally, in mammals MC1R is the main receptor for agouti-signaling protein (ASIP), making it the critical receptor for the establishment of dorsal-ventral countershading. In fish, Mc1r is also involved in pigmentation, but it has been almost exclusively studied in relation to melanosome dispersion activity and as a putative genetic factor involved in dark/light adaptation. However, its role as the crucial component for the Asip1-dependent control of dorsal-ventral pigmentation remains unexplored. Using CRISPR/Cas9, we created mc1r homozygous knockout zebrafish and found that loss-of-function of mc1r causes a reduction of countershading and a general paling of the animals. We find ectopic development of melanophores and xanthophores, accompanied by a decrease in iridophore numbers in the ventral region of mc1r mutants. We also reveal subtle differences in the role of mc1r in repressing pigment cell development between the skin and scale niches in ventral regions.	fish
344	animal (fish)	zebrafish	CRISPR;Cas9;	SWI/SNF-family chromatin remodeling protein atrx	PLoS genetics	Loss of atrx cooperates with p53-deficiency to promote the development of sarcomas and other malignancies.	2019	15(4):e1008039	[Oppel F et al.]	Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA.	30970016	10.1371/journal.pgen.1008039	The SWI/SNF-family chromatin remodeling protein ATRX is a tumor suppressor in sarcomas, gliomas and other malignancies. Its loss of function facilitates the alternative lengthening of telomeres (ALT) pathway in tumor cells, while it also affects Polycomb repressive complex 2 (PRC2) silencing of its target genes. To further define the role of inactivating ATRX mutations in carcinogenesis, we knocked out atrx in our previously reported p53/nf1-deficient zebrafish line that develops malignant peripheral nerve sheath tumors and gliomas. Complete inactivation of atrx using CRISPR/Cas9 was lethal in developing fish and resulted in an alpha-thalassemia-like phenotype including reduced alpha-globin expression. In p53/nf1-deficient zebrafish neither peripheral nerve sheath tumors nor gliomas showed accelerated onset in atrx+/- fish, but these fish developed various tumors that were not observed in their atrx+/+ siblings, including epithelioid sarcoma, angiosarcoma, undifferentiated pleomorphic sarcoma and rare types of carcinoma. These cancer types are included in the AACR Genie database of human tumors associated with mutant ATRX, indicating that our zebrafish model reliably mimics a role for ATRX-loss in the early pathogenesis of these human cancer types. RNA-seq of p53/nf1- and p53/nf1/atrx-deficient tumors revealed that down-regulation of telomerase accompanied ALT-mediated lengthening of the telomeres in atrx-mutant samples. Moreover, inactivating mutations in atrx disturbed PRC2-target gene silencing, indicating a connection between ATRX loss and PRC2 dysfunction in	fish
345	animal (fish)	zebrafish	CRISPR;Cas9;	glycine receptor alpha subunits (glra1, glra2, glra3, glra4a, glra4b)	PloS one	Individual knock out of glycine receptor alpha subunits identifies a specific requirement of glra1 for motor function in zebrafish.	2019	14(5):e0216159	[Samarut E et al.]	Universite de Montreal, Montreal, QC, Canada.	31048868	10.1371/journal.pone.0216159	Glycine receptors (GlyRs) are ligand-gated chloride channels mediating inhibitory neurotransmission in the brain stem and spinal cord. They function as pentamers composed of alpha and beta subunits for which 5 genes have been identified in human (GLRA1, GLRA2, GLRA3, GLRA4, GLRB). Several in vitro studies showed that the pentameric subtype composition as well as its stoichiometry influence the distribution and the molecular function of the receptor. Moreover, mutations in some of these genes are involved in different human conditions ranging from tinnitus to epilepsy and hyperekplexia, suggesting distinct functions of the different subunits. Although the beta subunit is essential for synaptic clustering of the receptor, the specific role of each alpha subtype is still puzzling in vivo. The zebrafish genome encodes for five glycine receptor alpha subunits (glra1, glra2, glra3, glra4a, glra4b) thus offering a model of choice to investigate the respective role of each subtype on general motor behaviour. After establishing a phylogeny of GlyR subunit evolution between human and zebrafish, we checked the temporal expression pattern of these transcripts during embryo development. Interestingly, we found that glra1 is the only maternally transmitted alpha subunit. We also showed that the expression of the different GlyR subunits starts at different time points during development. Lastly, in order to decipher the role of each alpha subunit on the general motor behaviour of the fish, we knocked out individually each alpha subunit by CRISPR/Cas9-targeted mutagenesis. Surprisingly, we found that knocking out any of the alpha2, 3, a4a or a4b subunit did not lead to any obvious developmental or motor phenotype. However, glra1-/- (hitch) embryos depicted a strong motor dysfunction from 3 days, making them incapable to swim and thus leading to their premature death. Our results infer a strong functional redundancy between alpha subunits and confirm the central role played by glra1 for proper inhibitory neurotransmission controlling locomotion. The genetic tools we developed here will be of general interest for further studies aiming at dissecting the role of GlyRs in	fish
346	animal (fish)	zebrafish	CRISPR;Cas9;		PloS one	Deep learning image recognition enables efficient genome editing in zebrafish by automated injections.	2019	14(1):e0202377	[Cordero-Maldonado ML et al.]	University of Luxembourg, Belvaux, Luxembourg.	30615627	10.1371/journal.pone.0202377	One of the most popular techniques in zebrafish research is microinjection. This is a rapid and efficient way to genetically manipulate early developing embryos, and to introduce microbes, chemical compounds, nanoparticles or tracers at larval stages. Here we demonstrate the development of a machine learning software that allows for microinjection at a trained target site in zebrafish eggs at unprecedented speed. The software is based on the open-source deep-learning library Inception v3. In a first step, the software distinguishes wells containing embryos at one-cell stage from wells to be skipped with an accuracy of 93%. A second step was developed to pinpoint the injection site. Deep learning allows to predict this location on average within 42 mum to manually annotated sites. Using a Graphics Processing Unit (GPU), both steps together take less than 100 milliseconds. We first tested our system by injecting a morpholino into the middle of the yolk and found that the automated injection efficiency is as efficient as manual injection (~ 80%). Next, we tested both CRISPR/Cas9 and DNA construct injections into the zygote and obtained a comparable efficiency to that of an experienced experimentalist. Combined with a higher throughput, this results in a higher yield. Hence, the automated injection of CRISPR/Cas9 will allow high-throughput applications to knock out and knock in relevant genes to study their mechanisms or	fish

347	animal (fish)	zebrafish	CRISPR;Cas9;	il7	Proceedings of the National Academy of Sciences of the United States of America	Evolutionary transition from degenerate to nonredundant cytokine signaling networks supporting intrathymic T cell development.	2019		[Lawir DF et al.]	Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany.	31822609	10.1073/pnas.1915223116	In mammals, T cell development critically depends on the IL-7 cytokine signaling pathway. Here we describe the identification of the zebrafish ortholog of mammalian IL-7 based on chromosomal localization, deduced protein sequence, and expression patterns. To examine the biological role of il7 in teleosts, we generated an il7 allele lacking most of its coding exons using CRISPR/Cas9-based mutagenesis. il7-deficient animals are viable and exhibit no obvious signs of immune disorder. With respect to intrathymic T cell development, il7 deficiency is associated with only a mild reduction of thymocyte numbers, contrasting with a more pronounced impairment of T cell development in il7r-deficient fish. Genetic interaction studies between il7 and il7r mutants, and il7 and crlf2(tslpr) mutants suggest the contribution of additional, as-yet unidentified cytokines to intrathymic T cell development. Such activities were also ascertained for other cytokines, such as il2 and il15, collectively indicating that in contrast to the situation in mammals, T cell development in the thymus of teleosts is driven by a degenerate multicomponent network of gammac cytokines; this explains why deficiencies of single components have little detrimental effect. In contrast, the dependence on a single cytokine in the mammalian thymus has catastrophic consequences in cases of congenital deficiencies in genes affecting the IL-7 signaling pathway. We speculate that the transition from a degenerate to a nonredundant cytokine network supporting intrathymic T cell development emerged as a consequence of repurposing evolutionarily ancient constitutive cytokine pathways for	fish
348	animal (fish)	zebrafish	TALENs;	sine oculis homeobox 6b; sine oculis homeobox 7	Proceedings of the National Academy of Sciences of the United States of America	Six6 and Six7 coordinately regulate expression of middle-wavelength opsins in zebrafish.	2019	116(10):4651-4660	[Ogawa Y et al.]	University of Tokyo, Tokyo, Japan.	30765521	10.1073/pnas.1812884116	Color discrimination in the vertebrate retina is mediated by a combination of spectrally distinct cone photoreceptors, each expressing one of multiple cone opsins. The opsin genes diverged early in vertebrate evolution into four classes maximally sensitive to varying wavelengths of light: UV (SWS1), blue (SWS2), green (RH2), and red (LWS) opsins. Although the tetrachromatic cone system is retained in most nonmammalian vertebrate lineages, the transcriptional mechanism underlying gene expression of the cone opsins remains elusive, particularly for SWS2 and RH2 opsins, both of which have been lost in the mammalian lineage. In zebrafish, which have all four cone subtypes, rh2 opsin gene expression depends on a homeobox transcription factor, sine oculis homeobox 7 (Six7). However, the six7 gene is found only in the ray-finned fish lineage, suggesting the existence of another evolutionarily conserved transcriptional factor(s) controlling rh2 opsin expression in vertebrates. Here, we found that the reduced rh2 expression caused by six7 deficiency was rescued by forced expression of six6b, which is a six7-related transcription factor conserved widely among vertebrates. The compensatory role of six6b was reinforced by ChIP-sequencing analysis, which revealed a similar pattern of Six6b- and Six7-binding sites within and near the cone opsin genes. TAL effector nuclease-induced genetic ablation of six6b and six7 revealed that they coordinately regulate SWS2 opsin gene expression. Mutant larvae deficient for these transcription factors showed severely impaired visually driven foraging behavior. These results demonstrate that in zebrafish, six6b and six7 govern expression of the SWS2 and RH2 opsins responsible for middle-wavelength sensitivity, which	fish
349	animal (fish)	zebrafish	CRISPR;Cas9;	lin28b	Scientific reports	LIN28B affects gene expression at the hypothalamic-pituitary axis and serum testosterone levels.	2019	9(1):18060	[Leinonen JT et al.]	University of Helsinki, Helsinki, Finland.	31792362	10.1038/s41598-019-54475-6	Genome-wide association studies (GWAS) have recurrently associated sequence variation nearby LIN28B with pubertal timing, growth and disease. However, the biology linking LIN28B with these traits is still poorly understood. With our study, we sought to elucidate the mechanisms behind the LIN28B associations, with a special focus on studying LIN28B function at the hypothalamic-pituitary (HP) axis that is ultimately responsible for pubertal onset. Using CRISPR-Cas9 technology, we first generated lin28b knockout (KO) zebrafish. Compared to controls, the lin28b KO fish showed both accelerated growth tempo, reduced adult size and increased expression of mitochondrial genes during larval development. Importantly, data from the knockout zebrafish models and adult humans imply that LIN28B expression has potential to affect gene expression in the HP axis. Specifically, our results suggest that LIN28B expression correlates positively with the expression of ESR1 in the hypothalamus and POMC in the pituitary. Moreover, we show how the pubertal timing advancing allele (T) for rs7759938 at the LIN28B locus associates with higher testosterone levels in the UK Biobank data. Overall, we provide novel evidence that LIN28B contributes to the regulation of sex hormone pathways, which might help explain why the gene associates	fish
350	animal (fish)	zebrafish	CRISPR;Cas9;	fibronectin domain containing protein 3a (fndc3a)	Scientific reports	ECM alterations in Fndc3a (Fibronectin Domain Containing Protein 3A) deficient zebrafish cause temporal fin development and regeneration defects.	2019	9(1):13383	[Liedtke D et al.]	Julius-Maximilians-University, Wurzburg, Germany.	31527654	10.1038/s41598-019-50055-w	Fin development and regeneration are complex biological processes that are highly relevant in teleost fish. They share genetic factors, signaling pathways and cellular properties to coordinate formation of regularly shaped extremities. Especially correct tissue structure defined by extracellular matrix (ECM) formation is essential. Gene expression and protein localization studies demonstrated expression of fndc3a (fibronectin domain containing protein 3a) in both developing and regenerating caudal fins of zebrafish (Danio rerio). We established a hypomorphic fndc3a mutant line (fndc3a(wue1/wue1)) via CRISPR/Cas9, exhibiting phenotypic malformations and changed gene expression patterns during early stages of median fin fold development. These developmental effects are mostly temporary, but result in a fraction of adults with permanent tail fin deformations. In addition, caudal fin regeneration in adult fndc3a(wue1/wue1) mutants is hampered by interference with actinotrichia formation and epidermal cell organization. Investigation of the ECM implies that loss of epidermal tissue structure is a common cause for both of the observed defects. Our results thereby provide a molecular link between these developmental processes and foreshadow Fndc3a as a novel temporal regulator of epidermal cell properties during	fish

351	animal (fish)	zebrafish	CRISPR;Cas9;	androgen receptor	Scientific reports	Liver-specific androgen receptor knockout attenuates early liver tumor development in zebrafish.	2019	9(1):10645	[Li H et al.]	National University of Singapore, Singapore, Singapore.	31337771	10.1038/s41598-019-46378-3	Hepatocellular carcinoma (HCC) is one of the most severe cancer types and many genetic and environmental factors contribute to the development of HCC. Androgen receptor (AR) signaling is increasingly recognized as one of the important factors associated with HCC. Previously, we have developed an inducible HCC model in kras transgenic zebrafish. In the present study, to investigate the role of AR in liver tumor development, we specifically knocked out ar gene in the liver of zebrafish via the CRISPR/Cas9 system and the knockout zebrafish was named L-ARKO for liver-specific ar knockout. We observed that liver-specific knockout of ar attenuated liver tumor development in kras transgenic zebrafish at the early stage (one week of tumor induction). However, at the late stage (two weeks of tumor induction), essentially all kras transgenic fish continue to develop HCC irrespective of the absence or presence of ar gene, indicating an overwhelming role of the driver oncogene kras over ar knockout. Consistently, cell proliferation was reduced at the early stage, but not the late stage, of liver tumor induction in the kras/L-ARKO fish, indicating that the attenuant effect of ar knockout was at least in part via cell proliferation. Furthermore, androgen treatment showed acceleration of HCC progression in kras fish but not in kras/L-ARKO fish, further indicating the abolishment of ar signalling. Therefore, we have established a tissue-specific ar knockout zebrafish and it should be a valuable	fish
352	animal (fish)	zebrafish	TALENs;	Polycomb Repressive Complex 2 catalytic subunit (ezh1)	Scientific reports	Ezh1 arises from Ezh2 gene duplication but its function is not required for zebrafish development.	2019	9(1):4319	[Volkel P et al.]	Inserm U908, Lille, France.	30867490	10.1038/s41598-019-40738-9	Trimethylation on H3K27 mediated by Polycomb Repressive Complex 2 (PRC2) is required to control gene repression programs involved in development, regulation of tissue homeostasis or maintenance and lineage specification of stem cells. In Drosophila, the PRC2 catalytic subunit is the single protein E(z), while in mammals this function is fulfilled by two proteins, Ezh1 and Ezh2. Based on database searches, we propose that Ezh1 arose from an Ezh2 gene duplication that has occurred in the common ancestor to elasmobranchs and bony vertebrates. Expression studies in zebrafish using in situ hybridization and RT-PCR followed by the sequencing of the amplicon revealed that ezh1 mRNAs are maternally deposited. Then, ezh1 transcripts are ubiquitously distributed in the entire embryo at 24 hpf and become more restricted to anterior part of the embryo at later developmental stages. To unveil the function of ezh1 in zebrafish, a mutant line was generated using the TALEN technology. Ezh1-deficient mutant fish are viable and fertile, but the loss of ezh1 function is responsible for the earlier death of ezh2 mutant larvae indicating that ezh1 contributes to zebrafish development in absence of zygotic ezh2 gene function. Furthermore, we show that presence of ezh1 transcripts from the maternal origin accounts for the delayed	fish
353	animal (fish)	zebrafish	CRISPR;Cas9;	interlectin 3	Scientific reports	Intelectin 3 is dispensable for resistance against a mycobacterial infection in zebrafish (Danio rerio).	2019	9(1):995	[Ojanen MJT et al.]	University of Tampere, Tampere, Finland.	30700796	10.1038/s41598-018-37678-1	Tuberculosis is a multifactorial bacterial disease, which can be modeled in the zebrafish (Danio rerio). Abdominal cavity infection with Mycobacterium marinum, a close relative of Mycobacterium tuberculosis, leads to a granulomatous disease in adult zebrafish, which replicates the different phases of human tuberculosis, including primary infection, latency and spontaneous reactivation. Here, we have carried out a transcriptional analysis of zebrafish challenged with low-dose of M. marinum, and identified intelectin 3 (itln3) among the highly up-regulated genes. In order to clarify the in vivo significance of Itln3 in immunity, we created nonsense itln3 mutant zebrafish by CRISPR/Cas9 mutagenesis and analyzed the outcome of M. marinum infection in both zebrafish embryos and adult fish. The lack of functional itln3 did not affect survival or the mycobacterial burden in the zebrafish. Furthermore, embryonic survival was not affected when another mycobacterial challenge responsive intelectin, itln1, was silenced using morpholinos either in the WT or itln3 mutant fish. In addition, M. marinum infection in dexamethasone-treated adult zebrafish, which have lowered lymphocyte counts, resulted in similar bacterial burden in both WT fish and homozygous itln3 mutants. Collectively, although itln3 expression is induced upon M. marinum infection in zebrafish, it is dispensable for protective mycobacterial immune response.	fish
354	animal (fish)	zebrafish	CRISPR;Cas9;	kif15	Traffic	Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9-mediated kif15 mutations accelerate axonal outgrowth during neuronal development and regeneration in zebrafish.	2019	20(1):71-81	[Dong Z et al.]	Nantong University, Nantong, China.	30411440	10.1111/tra.12621	KIF15, the vertebrate kinesin-12, is best known as a mitotic motor protein, but continues to be expressed in neurons. Like KIF11 (the vertebrate kinesin-5), KIF15 interacts with microtubules in the axon to limit their sliding relative to one another. Unlike KIF11, KIF15 also regulates interactions between microtubules and actin filaments at sites of axonal branch formation and in growth cones. Our original work on these motors was done on cultured rat neurons, but we are now using zebrafish to extend these studies to an in vivo model. We previously studied kif15 in zebrafish by injecting splice-blocking morpholinos injected into embryos. Consistent with the cell culture work, these studies demonstrated that axons grow faster and longer when KIF15 levels are reduced. In the present study, we applied CRISPR/Cas9-based knockout technology to create kif15 mutants and labeled neurons with Tg(mnx1:GFP) transgene or transient expression of elavl3:EGFP-alpha tubulin. We then compared by live imaging the homozygotic, heterozygotic mutants to their wildtype siblings to ascertain the effects of depletion of kif15 during Caudal primary motor neuron and Rohon-Beard (R-B) sensory neuron development. The results showed, compared to the kif15 wildtype, the number of branches was reduced while axon outgrowth was accelerated in kif15 homozygotic and heterozygotic mutants. In R-B sensory neurons, after laser irradiation, injured axons with loss of kif15 displayed significantly greater regenerative velocity. Given these results and the fact that kif15 drugs are currently under development, we posit kif15 as a novel target for therapeutically augmenting	fish

355	animal (fish)	zebrafish	CRISPR;	foxc1b	Vision research	Loss of foxc1 in zebrafish reduces optic nerve size and cell number in the retinal ganglion cell layer.	2019	156:66-72	[Umali J et al.]	Memorial University of Newfoundland, Canada.	30684501	10.1016/j.visres.2019.01.008	Mutation of FOXC1 causes Axenfeld-Rieger Syndrome (ARS) with early onset or congenital glaucoma. We assessed retinal ganglion cell (RGC) number in zebrafish due to CRISPR-mediated mutation and antisense inhibition of two-forkhead box transcription factors, foxc1a and foxc1b. These genes represent duplicated homologues of human FOXC1. Using a CRISPR induced null mutation in foxc1b, in combination with antisense inhibition of foxc1a, we demonstrate reduced cell number in the retinal ganglion cell layer of developing zebrafish eyes. As early as 5days post fertilization (dpf), fewer RGCs are found in foxc1b homozygous mutants injected with foxc1a morpholinos, and a thinner optic nerve results. Our data illustrates that foxc1 is required for the expression of atonal homolog 7 (atoh7), a gene that is necessary for RGC differentiation. As markers of differentiated RGCs (pou4f2) are downregulated in foxc1b-/- mutants injected with foxc1a morpholinos and no cell death is observed, our results are consistent with defects in the differentiation of RGCs leading to reduced cell number, as opposed to increased cell death of RGCs or off targets effects of morpholino injection. Our zebrafish model demonstrates that aberrant regulation of RGC number could act in concert with other known glaucoma risk factors to influence	fish
356	animal (fish)	zebrafish	CRISPR/Cas9		Zebrafish	Fluorescently Labeled TracrRNA Improves Work Flow and Facilitates Successful Genome Editing in Zebrafish.	2019	16(1):135-137	[Hamimi M et al.]	Monash University, Clayton, Australia.	30585775	10.1089/zeb.2018.1669	Gene editing using clustered regularly interspaced short palindromic repeats (CRISPR) is widely used throughout the zebrafish community for the generation of knockouts and knockins. One of the bottlenecks that exists during the process is the laborious screening of injected embryos for F0 founder fish or CRISPants, weeks after the injection date. In this study we show that the use of fluorescently tagged tracrRNA and sorting for fluorescent embryos as early as the 512-cell stage using stereomicroscope significantly improve yield of fish with successfully CRISPR/Cas9-edited genomes. This is a cost-effective strategy that significantly improves workflow and efficacy in genome editing in particular for less experienced researchers.	fish
357	animal (fish)	zebrafish	CRISPR;Cas9;	KIAA0196	Zhong nan da xue xue bao. Yi xue ban = Journal of Central South University. Medical sciences	[Establishment and preliminary mechanism study of the zebrafish strain of KIAA0196: A candidate pathogenic gene for heart development].	2019	44(9):968-975	[Bu H et al.]	Central South University, Changsha, China.	31645484	10.11817/j.issn.1672-7347.2019.180360	OBJECTIVE: To explore the effects of KIAA0196 gene on cardiac development and the establishment of zebrafish strain. Methods: Peripheral blood and gDNA from patients were extracted. Copy number variation analysis and target sequencing were conducted to screen candidate genes. The KIAA0196 knockout zebra fi sh was generated by CRISPR/Cas9 to detect whether KIAA0196 de fi ciency could affect cardiac development. Finally, the wild-type and mutant zebrafish were anatomized and histologically stained to observe the phenotype of heart defects. Results: The KIAA0196 knockout zebrafish strain was successfully constructed using CRISPR/Cas9 technology. After 60 hours fertilization, microscopic examination of KIAA0196 knockout zebrafish (heterozygote + homozygote) showed pericardial effusion, cardiac compression and severely curly tail. Compared with wild-type zebrafish, the hearts of mutant KIAA0196 zebra fi sh had cardiac defects including smaller atrium and larger ventricle, and the myocardial cells were looser. Conclusion: KIAA0196 gene plays an important regulatory role in the development of heart. It might be a candidate gene for	fish
358	animal (fish)		CRISPR;Cas9;		Cells	Development of a Bicistronic Vector for the Expression of a CRISPR/Cas9-mCherry System in Fish Cell Lines.	2019	8(1)	[Escobar-Aguirre S et al.]	Universidad de Chile, Santiago, Chile.	30669572	10.3390/cells8010075	The clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system has been widely used in animals as an efficient genome editing tool. In fish cells, the technique has been difficult to implement due to the lack of proper vectors that use active promoters to drive the expression of both small guide RNA (sgRNA) and the S. pyogenes Cas9 (spCas9) protein within a single expression platform. Until now, fish cells have been modified using co-transfection of the mRNA of both the sgRNA and the spCas9. In the present study, we describe the optimization of a new vector for the expression of a CRISPR/Cas9 system, designed to edit the genome of fish cell lines, that combines a gene reporter (mCherry), sgRNA, and spCas9 in a single vector, facilitating the study of the efficiency of piscine and non-piscine promoters. A cassette containing the zebrafish U6 RNA III polymerase (U6ZF) promoter was used for the expression of the sgRNA. The new plasmid displayed the expression of spCas9, mCherry, and sgRNA in CHSE/F fish cells. The results demonstrate the functionality of the mammalian promoter and the U6ZF promoter in fish cell lines. This is the first approach aimed at developing a unified genome editing system in fish cells using bicistronic vectors, thus creating a powerful biotechnological	fish
359	plant	apple; grapevine	CRISPR;Cas9;		Nature protocols	CRISPR-Cas9-mediated genome editing in apple and grapevine.	2018	13(12):2844-2863	[Osakabe Y et al.]	Tokushima University, Tokushima, Japan.	30390050	10.1038/s41596-018-0067-9	The CRISPR-Cas9 genome-editing tool and the availability of whole-genome sequences from plant species have revolutionized our ability to introduce targeted mutations into important crop plants, both to explore genetic changes and to introduce new functionalities. Here, we describe protocols adapting the CRISPR-Cas9 system to apple and grapevine plants, using both plasmid-mediated genome editing and the direct delivery of CRISPR-Cas9 ribonucleoproteins (RNPs) to achieve efficient DNA-free targeted mutations in apple and grapevine protoplasts. We provide a stepwise protocol for the design and transfer of CRISPR-Cas9 components to apple and grapevine protoplasts, followed by verification of highly efficient targeted mutagenesis, and regeneration of plants following the plasmid-mediated delivery of components. Our plasmid-mediated procedure and the direct delivery of CRISPR-Cas9 RNPs can both be utilized to modulate traits of interest with high accuracy and efficiency in apple and grapevine, and could be extended to other crop species. The complete protocol employing the direct delivery of CRISPR-Cas9 RNPs takes as little as 2-3 weeks, whereas the plasmid-mediated procedure takes >3 months to regenerate plants and	apple; grapev
360	plant	Arabidopsis	CRISPR;Cas9;	target of monopteros 7 (tmo7)	Development	Regulation of intercellular TARGET OF MONOPTEROS 7 protein transport in the Arabidopsis root.	2018	145(2)	[Lu KJ et al.]	Wageningen University, Wageningen, The Netherlands.	29358212	10.1242/dev.152892	Intercellular communication coordinates hypophysis establishment in the Arabidopsis embryo. Previously, TARGET OF MONOPTEROS 7 (TMO7) was reported to be transported to the hypophysis, the founder cell of the root cap, and RNA suppression experiments implicated its function in embryonic root development. However, the protein properties and mechanisms mediating TMO7 protein transport, and the role the movement plays in development remained unclear. Here, we report that in the post-embryonic root, TMO7 and its close relatives are transported into the root cap through plasmodesmata in a sequence-dependent manner. We also show that nuclear residence is crucial for TMO7 transport, and postulate that modification, potentially phosphorylation, labels TMO7 for transport. Additionally, three novel CRISPR/Cas9-induced tmo7 alleles confirmed a role in hypophysis division, but suggest complex redundancies with close relatives in root formation. Finally, we demonstrate that TMO7 transport is biologically meaningful, as local expression partially restores hypophysis division in a plasmodesmal protein transport mutant. Our study identifies motifs and amino acids that are pivotal for TMO7 protein transport, and establishes the	Arabidopsis

361	plant	Arabidopsis	CRISPR;Cas9;	MYB-CC transcription factor (PHL4)	Frontiers in plant science	Functional Characterization of Arabidopsis PHL4 in Plant Response to Phosphate Starvation.	2018	9:1432	[Wang Z et al.]	Tsinghua University, Beijing, China.	30327661	10.3389/fpls.2018.01432	Plants have evolved an array of adaptive responses to cope with phosphate (Pi) starvation. These responses are mainly controlled at the transcriptional level. In Arabidopsis, PHR1, a member of the MYB-CC transcription factor family, is a key component of the central regulatory system controlling plant transcriptional responses to Pi starvation. Its homologs in the MYB-CC family, PHL1 (PHR1-LIKE 1), PHL2, and perhaps also PHL3, act redundantly with PHR1 to regulate plant Pi starvation responses. The functions of PHR1's closest homolog in this family, PHL4, however, have not been characterized due to the lack of its null mutant. In this work, we generated two phl4 null mutants using the CRISPR/Cas9 technique and investigated the functions of PHL4 in plant responses to Pi starvation. The results indicated that the major developmental, physiological, and molecular responses of the phl4 mutants to Pi starvation did not significantly differ from those of the wild type. By comparing the phenotypes of the phr1 single mutant and phr1phl1 and phr1phl4 double mutants, we found that PHL4 also acts redundantly with PHR1 to regulate plant Pi responses, but that its effects are weaker than those of PHL1. We also found that the overexpression of PHL4 suppresses plant development under both Pi-sufficient and -deficient conditions. Taken together, the results indicate that PHL4 has only a minor role in the regulation of plant responses to Pi starvation and is a negative regulator of plant	Arabidopsis
362	plant	Arabidopsis	CRISPR;Cas9;	AtDET2; AtDMC1	Frontiers in plant science	The Application of a Meiocyte-Specific CRISPR/Cas9 (MSC) System and a Suicide-MSC System in Generating Inheritable and Stable Mutations in Arabidopsis.	2018	9:1007	[Xu P et al.]	Fudan University, Shanghai, China.	30061908	10.3389/fpls.2018.01007	The CRISPR/Cas9 system has been widely used for generating targeted mutations in various species. In Arabidopsis, it largely relies on the edited cells where the Cas9 protein performs its activity to obtain heritable and stable mutated lines. Here, we designed an improved CRISPR/Cas9 system, named as the MSC (meiocyte-specific CRISPR/Cas9) system, in which the Cas9 expression is driven by an experimentally approved meiocyte-specific promoter (AtDMC1 promoter). Two endogenous genes, including vegetative gene AtDET2 and reproductive gene AtDMC1, were targeted. We obtained heterozygous T1 plants for targeted genes with high efficiency (64%). In the T2 generation, the homozygous plants were abundant with high efficiency (37%). Analysis of Sanger sequencing results of T2 generation revealed that heritable gene mutations were high (52%). Moreover, we showed that the MSC system could sufficiently delete a middle size DNA fragment (approximately 500 bp) between two cleavage sites with a high rate (64.15%) in the T1 plants, providing direct evidence for making complete knock-out or certain domain-depletion mutations. In addition, we further made a suicide-MSC system, which can edit the targeted endogenous gene and the exogenous Cas9 gene simultaneously, not only successfully avoiding the further destroy of alleles brought in by molecular complementary or genic allelic test, but also maintaining the stable mutated alleles for functional studies. In short, the two systems	Arabidopsis
363	plant	Arabidopsis	CRISPR;Cas9;	glabrous1	Frontiers in plant science	Homology-Directed Repair of a Defective Glabrous Gene in Arabidopsis With Cas9-Based Gene Targeting.	2018	9:424	[Hahn F et al.]	Heinrich Heine University Dusseldorf, Dusseldorf, Germany.	29675030	10.3389/fpls.2018.00424	The CRISPR/Cas9 system has emerged as a powerful tool for targeted genome editing in plants and beyond. Double-strand breaks induced by the Cas9 enzyme are repaired by the cell's own repair machinery either by the non-homologous end joining pathway or by homologous recombination (HR). While the first repair mechanism results in random mutations at the double-strand break site, HR uses the genetic information from a highly homologous repair template as blueprint for repair of the break. By offering an artificial repair template, this pathway can be exploited to introduce specific changes at a site of choice in the genome. However, frequencies of double-strand break repair by HR are very low. In this study, we compared two methods that have been reported to enhance frequencies of HR in plants. The first method boosts the repair template availability through the formation of viral replicons, the second method makes use of an in planta gene targeting (IPGT) approach. Additionally, we comparatively applied a nickase instead of a nuclease for target strand priming. To allow easy, visual detection of HR events, we aimed at restoring trichome formation in a glabrous Arabidopsis mutant by repairing a defective glabrous1 gene. Using this efficient visual marker, we were able to regenerate plants repaired by HR at frequencies of 0.12% using the IPGT approach, while both approaches using viral	Arabidopsis
364	plant	Arabidopsis	CRISPR;Cas9;	AGO1; AP1; TT4	Genome biology	Manipulating plant RNA-silencing pathways to improve the gene editing efficiency of CRISPR/Cas9 systems.	2018	19(1):149	[Mao Y et al.]	Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.	30266091	10.1186/s13059-018-1529-7	BACKGROUND: The CRISPR/Cas9 system, composed of a single-guide RNA for target recognition and a Cas9 protein for DNA cleavage, has the potential to revolutionize agriculture as well as medicine. Even though extensive work has been done to improve the gene editing activity of CRISPR/Cas9, little is known about the regulation of this bacterial system in eukaryotic host cells, especially at the post-transcriptional level. RESULTS: Here, we evaluate the expression levels of the two CRISPR/Cas9 components and the gene editing efficiency in a set of Arabidopsis mutants involved in RNA silencing. We find that mutants defective in the post-transcriptional gene-silencing pathway display significantly higher Cas9 and sgRNA transcript levels, resulting in higher mutagenesis frequencies than wild-type controls. Accordingly, silencing of AGO1 by introduction of an AGO1-RNAi cassette into the CRISPR/Cas9 vector provides an increase in gene editing efficiency. Co-expression of the viral suppressor p19 from the tomato bushy stunt virus to suppress the plant RNA-silencing pathway shows a strong correlation between the severity of the phenotypic effects caused by p19 and the gene editing efficiency of the CRISPR/Cas9 system for two different target genes, AP1 and TT4. CONCLUSIONS: This system has useful practical applications in facilitating the detection of CRISPR/Cas9-induced mutations in T1 plants as well as the identification of transgene-free T2 plants by simple visual observation of the symptom severity caused by p19. Our study shows that	Arabidopsis

365	plant	Arabidopsis	CRISPR;Cas9;		International journal of molecular sciences	A Highly Efficient Cell Division-Specific CRISPR/Cas9 System Generates Homozygous Mutants for Multiple Genes in Arabidopsis.	2018	19(12)	[Feng Z et al.]	Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.	30544514	10.3390/ijms19123925	The CRISPR/Cas9 system has been widely used for targeted genome editing in numerous plant species. In Arabidopsis, constitutive promoters usually result in a low efficiency of heritable mutation in the T1 generation. In this work, CRISPR/Cas9 gene editing efficiencies using different promoters to drive Cas9 expression were evaluated. Expression of Cas9 under the constitutive CaMV 35S promoter resulted in a 2.3% mutation rate in T1 plants and failed to produce homozygous mutations in the T1 and T2 generations. In contrast, expression of Cas9 under two cell division-specific promoters, YAO and CDC45, produced mutation rates of 80.9% to 100% in the T1 generation with nonchimeric mutations in the T1 (4.4(-)10%) and T2 (32.5(-)46.1%) generations. The pCDC45 promoter was used to modify a previously reported multiplex CRISPR/Cas9 system, replacing the original constitutive ubiquitin promoter. The multi-pCDC45-Cas9 system produced higher mutation efficiencies than the multi-pUBQ-Cas9 system in the T1 generation (60.17% vs. 43.71%) as well as higher efficiency of heritable mutations (11.30% vs. 4.31%). Sextuple T2 homozygous mutants were identified from a construct targeting seven individual loci. Our results demonstrate the advantage of using cell division promoters for CRISPR/Cas9 gene editing applications	Arabidopsis
366	plant	Arabidopsis	CRISPR;Cas9;	three catalase genes (CAT1; CAT2; CAT3)	Journal of integrative plant biology	The Arabidopsis catalase triple mutant reveals important roles of catalases and peroxisome-derived signaling in plant development.	2018	60(7):591-607	[Su T et al.]	Shandong Normal University, Jinan, China.	29575603	10.1111/jipb.12649	Hydrogen peroxide (H2 O2) is generated in many metabolic processes. As a signaling molecule, H2 O2 plays important roles in plant growth and development, as well as environmental stress response. In Arabidopsis, there are three catalase genes, CAT1, CAT2, and CAT3. The encoded catalases are predominately peroxisomal proteins and are critical for scavenging H2 O2 . Since CAT1 and CAT3 are linked on chromosome 1, it has been almost impossible to generate cat1/3 and cat1/2/3 mutants by traditional genetic tools. In this study, we constructed cat1/3 double mutants and cat1/2/3 triple mutants by CRISPR/Cas9 to investigate the role of catalases. The cat1/2/3 triple mutants displayed severe redox disturbance and growth defects under physiological conditions compared with wild-type and the cat2/3 double mutants. Transcriptome analysis showed a more profound transcriptional response in the cat1/2/3 triple mutants compared to the cat2/3 mutants. These differentially expressed genes are involved in plant growth regulation as well as abiotic and biotic stress responses. In addition, expression of OXI1 (OXIDATIVE SIGNAL INDUCIBLE 1) and several MAPK cascade genes were changed dramatically in the catalase triple mutant, suggesting that H2 O2 produced in peroxisomes could serve as a peroxisomal retrograde signal.	Arabidopsis
367	plant	Arabidopsis	CRISPR;Cas9;	AtRPL10A; AtRPL10B; AtRPL10C	Journal of integrative plant biology	Multigene editing via CRISPR/Cas9 guided by a single-sgRNA seed in Arabidopsis.	2018	60(5):376-381	[Yu Z et al.]	Hangzhou Normal University, Hangzhou, China.	29226588	10.1111/jipb.12622	We report that a solo single-guide RNA (sgRNA) seed is capable of guiding Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR -associated 9 (CRISRP/Cas9) to simultaneously edit multiple genes AtRPL10A, AtRPL10B and AtRPL10C in Arabidopsis. Our results also demonstrate that it is possible to use CRISPR/Cas9 technology to create AtRPL10 triple mutants which otherwise cannot be generated by conventional genetic crossing. Compared to other conventional multiplex CRISPR/Cas systems, a single sgRNA seed has the advantage of reducing off-target gene-editing. Such a gene editing system might be also applicable to modify other homologous genes, or even less-homologous sequences for multiple gene-editing	Arabidopsis
368	plant	Arabidopsis	Cas9;		Nature communications	CRISPR/Cas9-mediated gene targeting in Arabidopsis using sequential transformation.	2018	9(1):1967	[Miki D et al.]	Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.	29773790	10.1038/s41467-018-04416-0	Homologous recombination-based gene targeting is a powerful tool for precise genome modification and has been widely used in organisms ranging from yeast to higher organisms such as Drosophila and mouse. However, gene targeting in higher plants, including the most widely used model plant Arabidopsis thaliana, remains challenging. Here we report a sequential transformation method for gene targeting in Arabidopsis. We find that parental lines expressing the bacterial endonuclease Cas9 from the egg cell- and early embryo-specific DD45 gene promoter can improve the frequency of single-guide RNA-targeted gene knock-ins and sequence replacements via homologous recombination at several endogenous sites in the Arabidopsis genome. These heritable gene targeting can be identified by regular PCR. Our approach enables routine and fine manipulation of the Arabidopsis genome.	Arabidopsis
369	plant	Arabidopsis	CRISPR;Cas9;		Plant & cell physiology	BES1 and BZR1 Redundantly Promote Phloem and Xylem Differentiation.	2018	59(3):590-600	[Saito M et al.]	University of Tokyo, Tokyo, Japan.	29385529	10.1093/pcp/pcy012	Vascular development is a good model for studying cell differentiation in plants. Two conductive tissues, the xylem and phloem, are derived from common stem cells known as procambial/cambial cells. Glycogen synthase kinase 3 proteins (GSK3s) play crucial roles in maintaining procambial/cambial cells by suppressing their differentiation into xylem or phloem cells. We previously designed an in vitro culture system for analyzing vascular cell differentiation named VISUAL (Vascular cell Induction culture System Using Arabidopsis Leaves). Using this system, we found that the transcription factor BRI1-EMS-SUPPRESSOR 1 (BES1) functions as a downstream target of GSK3s during xylem differentiation. However, the function of BES1 in vascular development remains largely unknown. Here, we found that, in addition to xylem differentiation, BES1 positively regulates phloem differentiation downstream of GSK3s. Transcriptome analysis using VISUAL confirmed that BES1 promotes bi-directional differentiation of procambial cells into xylem and phloem cells. Genetic analysis of loss-of-function mutants newly generated using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system revealed that BRASSINAZOLE RESISTANT 1 (BZR1), the closest homolog of BES1, functions in vascular development redundantly with BES1. Notably, BZR1 has a weaker impact on vascular cell differentiation than BES1, suggesting that they contribute differentially to this process. In conclusion, our findings indicate that BES1 and BZR1 are key regulators of both xylem and phloem cell differentiation from vascular stem cells.	Arabidopsis
370	plant	Arabidopsis	CRISPR;Cas9;	DNA sequence-specific H3K27 demethylase (REF6)	Plant biotechnology journal	Verification of DNA motifs in Arabidopsis using CRISPR/Cas9-mediated mutagenesis.	2018	16(8):1446-1451	[Li C et al.]	Sun Yat-sen University, Guangzhou, China.	29331085	10.1111/pbi.12886	Transcription factors (TFs) and chromatin-modifying factors (CMFs) access chromatin by recognizing specific DNA motifs in their target genes. Chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) has been widely used to discover the potential DNA-binding motifs for both TFs and CMFs. Yet, an in vivo method for verifying DNA motifs captured by ChIP-seq is lacking in plants. Here, we describe the use of clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) to verify DNA motifs in their native genomic context in Arabidopsis. Using a single-guide RNA (sgRNA) targeting the DNA motif bound by REF6, a DNA sequence-specific H3K27 demethylase in plants, we generated stable transgenic plants where the motif was disrupted in a REF6 target gene. We also deleted a cluster of multiple motifs from another REF6 target gene using a pair of sgRNAs, targeting upstream and downstream regions of the cluster, respectively. We demonstrated that endogenous genes with motifs disrupted and/or deleted become inaccessible to REF6. This strategy should be widely applicable for in vivo verification	Arabidopsis

371	plant	Arabidopsis	CRISPR;Cas9;	RNA Polymerase II's largest subunit (RPB1)	Plant journal	Intact Arabidopsis RPB1 functions in stem cell niches maintenance and cell cycling control.	2018	95(1):150-167	[Zhang QQ et al.]	Shandong Agricultural University, Tai'an, China.	29752751	10.1111/tpj.13939	Plant meristem activity depends on accurate execution of transcriptional networks required for establishing optimum functioning of stem cell niches. An Arabidopsis mutant card1-1 (constitutive auxin response with DR5:GFP) that encodes a truncated RPB1 (RNA Polymerase II's largest subunit) with shortened C-terminal domain (CTD) was identified. Phosphorylation of the CTD repeats of RPB1 is coupled to transcription in eukaryotes. Here we uncover that the truncated CTD of RPB1 disturbed cell cycling and enlarged the size of shoot and root meristem. The defects in patterning of root stem cell niche in card1-1 indicates that intact CTD of RPB1 is necessary for fine-tuning the specific expression of genes responsible for cell-fate determination. The gene-edited plants with different CTD length of RPB1, created by CRISPR-CAS9 technology, confirmed that both the full length and the DK-rich tail of RPB1's CTD play roles in the accurate transcription of CYCB1;1 encoding a cell-cycle marker protein in root meristem and hence participate in maintaining root meristem size. Our experiment proves that the intact RPB1 CTD is necessary for stem cell niche maintenance, which is mediated by transcriptional regulation of cell cycling genes.	Arabidopsis
372	plant	Arabidopsis	CRISPR;Cas9;		Plant journal	A chromatin loop represses WUSCHEL expression in Arabidopsis.	2018	94(6):1083-1097	[Guo L et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Shijiazhuang, China.	29660180	10.1111/tpj.13921	WUSCHEL (WUS) is critical for plant meristem maintenance and determinacy in Arabidopsis, and the regulation of its spatiotemporal expression patterns is complex. We previously found that AGAMOUS (AG), a key MADS-domain transcription factor in floral organ identity and floral meristem determinacy, can directly suppress WUS expression through the recruitment of the Polycomb group (PcG) protein TERMINAL FLOWER 2 (TFL2, also known as LIKE HETEROCHROMATIN PROTEIN 1, LHP1) at the WUS locus; however, the mechanism by which WUS is repressed remains unclear. Here, using chromosome conformation capture (3C) and chromatin immunoprecipitation 3C, we found that two specific regions flanking the WUS gene body bound by AG and TFL2 form a chromatin loop that is directly promoted by AG during flower development in a manner independent of the physical distance and sequence content of the intervening region. Moreover, AG physically interacts with TFL2, and TFL2 binding to the chromatin loop is dependent on AG. Transgenic and CRISPR/Cas9-edited lines showed that the WUS chromatin loop represses gene expression by blocking the recruitment of RNA polymerase II at the locus. The findings uncover the WUS chromatin loop as another regulatory mechanism controlling WUS expression, and also	Arabidopsis
373	plant	Arabidopsis	Cas9;	acetolactate synthase	Plant journal	Efficient in planta gene targeting in Arabidopsis using egg cell-specific expression of the Cas9 nuclease of Staphylococcus aureus.	2018	94(4):735-746	[Wolter F et al.]	Karlsruhe Institute of Technology, Karlsruhe, Germany.	29573495	10.1111/tpj.13893	Gene targeting (GT), the programmed change of genomic sequences by homologous recombination (HR), is still a major challenge in plants. We previously developed an in planta GT strategy by simultaneously releasing from the genome a dsDNA donor molecule and creating a double-stranded break (DSB) at a specific site within the targeted gene. Using Cas9 form Streptococcus pyogenes (SpCas9) under the control of a ubiquitin gene promoter, we obtained seeds harbouring GT events, although at a low frequency. In the present research we tested different developmentally controlled promotors and different kinds of DNA lesions for their ability to enhance GT of the acetolactate synthase (ALS) gene of Arabidopsis. For this purpose, we used Staphylococcus aureus Cas9 (SaCas9) nuclease and the SpCas9 nickase in various combinations. Thus, we analysed the effect of single-stranded break (SSB) activation of a targeted gene and/or the HR donor region. Moreover, we tested whether DSBs with 5' or 3' overhangs can improve in planta GT. Interestingly, the use of the SaCas9 nuclease controlled by an egg cell-specific promoter was the most efficient: depending on the line, in the very best case 6% of all seeds carried GT events. In a third of all lines, the targeting occurred around the 1% range of the tested seeds. Molecular analysis revealed that in about half of the cases perfect HR of both DSB ends occurred. Thus, using the improved technology, it should now be feasible to introduce	Arabidopsis
374	plant	Arabidopsis	CRISPR;Cas9;		Plant molecular biology	Potential high-frequency off-target mutagenesis induced by CRISPR/Cas9 in Arabidopsis and its prevention.	2018	96(4-5):445-456	[Zhang Q et al.]	China Agricultural University, Beijing, China.	29476306	10.1007/s11103-018-0709-x	KEY MESSAGE: We present novel observations of high-specificity SpCas9 variants, sgRNA expression strategies based on mutant sgRNA scaffold and tRNA processing system, and CRISPR/Cas9-mediated T-DNA integrations. Specificity of CRISPR/Cas9 tools has been a major concern along with the reports of their successful applications. We report unexpected observations of high frequency off-target mutagenesis induced by CRISPR/Cas9 in T1 Arabidopsis mutants although the sgRNA was predicted to have a high specificity score. We also present evidence that the off-target effects were further exacerbated in the T2 progeny. To prevent the off-target effects, we tested and optimized two strategies in Arabidopsis, including introduction of a mCherry cassette for a simple and reliable isolation of Cas9-free mutants and the use of highly specific mutant SpCas9 variants. Optimization of the mCherry vectors and subsequent validation found that fusion of tRNA with the mutant rather than the original sgRNA scaffold significantly improves editing efficiency. We then examined the editing efficiency of eight high-specificity SpCas9 variants in combination with the improved tRNA-sgRNA fusion strategy. Our results suggest that highly specific SpCas9 variants require a higher level of expression than their wild-type counterpart to maintain high editing efficiency. Additionally, we demonstrate that T-DNA can be inserted into the cleavage sites of CRISPR/Cas9 targets with high frequency. Altogether, our results suggest that in plants, continuous attention should be paid to off-target effects induced by CRISPR/Cas9 in current and subsequent generations, and that the tools optimized in this report will be useful in improving genome editing efficiency and	Arabidopsis

375	plant	Arabidopsis	CRISPR;Cas9;	methionine (Met) adenosyltransferase 4 (MAT4)	Plant physiology	METHIONINE ADENOSYLTRANSFERASE4 Mediates DNA and Histone Methylation.	2018	177(2):652–670	[Meng J et al.]	China Agricultural University, Beijing, China.	29572390	10.1104/pp.18.00183	DNA and histone methylation coregulate heterochromatin formation and gene silencing in animals and plants. To identify factors involved in maintaining gene silencing, we conducted a forward genetic screen for mutants that release the silenced transgene Pro35S::NEOMYCIN PHOSPHOTRANSFERASE II in the transgenic Arabidopsis (Arabidopsis thaliana) line L119 We identified MAT4/SAMS3/MTO3/AT3G17390, which encodes methionine (Met) adenosyltransferase 4 (MAT4)/S-adenosyl-Met synthetase 3 that catalyzes the synthesis of S-adenosyl-Met (SAM) in the one-carbon metabolism cycle. mat4 mostly decreases CHG and CHH DNA methylation and histone H3K9me2 and reactivates certain silenced transposons. The exogenous addition of SAM partially rescues the epigenetic defects of mat4 SAM content and DNA methylation were reduced more in mat4 than in three other mat mutants. MAT4 knockout mutations generated by CRISPR/Cas9 were lethal, indicating that MAT4 is an essential gene in Arabidopsis. MAT1, 2, and 4 proteins exhibited nearly equal activity in an in vitro assay, whereas MAT3 exhibited higher activity. The native MAT4 promoter driving MAT1, 2, and 3 cDNA complemented the mat4 mutant. However, most mat4 transgenic lines carrying native MAT1, 2, and 3 promoters driving MAT4 cDNA did not complement the mat4 mutant because of their lower expression in seedlings. Genetic analyses indicated that the mat1mat4 double mutant is dwarfed and the mat2mat4 double mutant was nonviable, while mat1mat2 showed normal growth and fertility. These results indicate that MAT4 plays a predominant role in SAM production, plant growth, and development. Our findings provide direct evidence of the cooperative actions between metabolism	Arabidopsis
376	plant	Arabidopsis	CRISPR;	topoisomerase 3alpha (TOP3alpha)	PLoS genetics	The topoisomerase 3alpha zinc-finger domain T1 of Arabidopsis thaliana is required for targeting the enzyme activity to Holliday junction-like DNA repair intermediates.	2018	14(9):e1007674	[Dorn A et al.]	Karlsruhe Institute of Technology, Karlsruhe, Germany.	30222730	10.1371/journal.pgen.1007674	Topoisomerase 3alpha, a class I topoisomerase, consists of a TOPRIM domain, an active centre and a variable number of zinc-finger domains (ZFDs) at the C-terminus, in multicellular organisms. Whereas the functions of the TOPRIM domain and the active centre are known, the specific role of the ZFDs is still obscure. In contrast to mammals where a knockout of TOP3alpha leads to lethality, we found that CRISPR/Cas induced mutants in Arabidopsis are viable but show growth retardation and meiotic defects, which can be reversed by the expression of the complete protein. However, complementation with AtTOP3alpha missing either the TOPRIM-domain or carrying a mutation of the catalytic tyrosine of the active centre leads to embryo lethality. Surprisingly, this phenotype can be overcome by the simultaneous removal of the ZFDs from the protein. In combination with a mutation of the nuclease AtMUS81, the TOP3alpha knockout proved to be also embryo lethal. Here, expression of TOP3alpha without ZFDs, and in particular without the conserved ZFD T1, leads to only a partly complementation in root growth-in contrast to the complete protein, that restores root length to mus81-1 mutant level. Expressing the E. coli resolvase RusA in this background, which is able to process Holliday junction (HJ)-like recombination intermediates, we could rescue this root growth defect. Considering all these results, we conclude that the ZFD T1 is specifically required for targeting the topoisomerase activity to HJ like recombination intermediates to enable their processing. In the case of an inactivated enzyme, this leads to cell death due to the masking of these	Arabidopsis
377	plant	Arabidopsis	CRISPR;Cas9;	FLOWERING WAGENINGEN (FWA); CACTA1 transposon	Proceedings of the National Academy of Sciences of the United States of America	Targeted DNA demethylation of the Arabidopsis genome using the human TET1 catalytic domain.	2018	115(9):E2125–E2134	[Gallego-Bartolome J et al.]	University of California, Los Angeles, CA, USA.	29444862	10.1073/pnas.1716945115	DNA methylation is an important epigenetic modification involved in gene regulation and transposable element silencing. Changes in DNA methylation can be heritable and, thus, can lead to the formation of stable epialleles. A well-characterized example of a stable epiallele in plants is fwa, which consists of the loss of DNA cytosine methylation (5mC) in the promoter of the FLOWERING WAGENINGEN (FWA) gene, causing up-regulation of FWA and a heritable late-flowering phenotype. Here we demonstrate that a fusion between the catalytic domain of the human demethylase TEN-ELEVEN TRANSLOCATION1 (TET1cd) and an artificial zinc finger (ZF) designed to target the FWA promoter can cause highly efficient targeted demethylation, FWA up-regulation, and a heritable late-flowering phenotype. Additional ZF-TET1cd fusions designed to target methylated regions of the CACTA1 transposon also caused targeted demethylation and changes in expression. Finally, we have developed a CRISPR/dCas9-based targeted demethylation system using the TET1cd and a modified SunTag system. Similar to the ZF-TET1cd fusions, the SunTag-TET1cd system is able to target demethylation and activate gene expression when directed to the FWA or CACTA1 loci. Our study provides tools for targeted removal of 5mC at specific loci in the genome with high specificity and minimal off-target effects. These tools provide the opportunity to develop new epialleles for traits of interest, and to reactivate	Arabidopsis
378	plant	Arabidopsis	Cas9;	4 genes including HAB1.1 (type 2C phosphatase) and RS31A	Science China. Life sciences	Manipulating mRNA splicing by base editing in plants.	2018	61(11):1293–1300	[Xue C et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.	30267262	10.1007/s11427-018-9392-7	Precursor-mRNAs (pre-mRNA) can be processed into one or more mature mRNA isoforms through constitutive or alternative splicing pathways. Constitutive splicing of pre-mRNA plays critical roles in gene expressional regulation, such as intron-mediated enhancement (IME), whereas alternative splicing (AS) dramatically increases the protein diversity and gene functional regulation. However, the unavailability of mutants for individual spliced isoforms in plants has been a major limitation in studying the function of mRNA splicing. Here, we describe an efficient tool for manipulating the splicing of plant genes. Using a Cas9-directed base editor, we converted the 5' splice sites in four Arabidopsis genes from the activated GT form to the inactive AT form. Silencing the AS of HAB1.1 (encoding a type 2C phosphatase) validated its function in abscisic acid signaling, while perturbing the AS of RS31A revealed its functional involvement in plant response to genotoxic treatment for the first time. Lastly, altering the constitutive splicing of Act2 via base editing facilitated the analysis of IME. This strategy provides an efficient tool for investigating the function and regulation of gene	Arabidopsis

379	plant	Arabidopsis	CRISPR;Cas9;		Scientific reports	Highly efficient heritable targeted deletions of gene clusters and non-coding regulatory regions in Arabidopsis using CRISPR/Cas9.	2018	8(1):4443	[Durr J et al.]	University of Warwick, Coventry, UK.	29535386	10.1038/s41598-018-22667-1	Genome editing using CRISPR/Cas9 is considered the best instrument for genome engineering in plants. This methodology is based on the nuclease activity of Cas9 that is guided to specific genome sequences by single guide RNAs (sgRNAs) thus enabling researchers to engineer simple mutations or large chromosomal deletions. Current methodologies for targeted genome editing in plants using CRISPR/Cas9 are however largely inefficient, mostly due to low Cas9 activity, variable sgRNA efficiency and low heritability of genetic lesions. Here, we describe a newly developed strategy to enhance CRISPR/Cas9 efficiency in Arabidopsis thaliana focusing on the design of novel binary vectors (pUbiCAS9-Red and pEciCAS9-Red), the selection of highly efficient sgRNAs, and the use of direct plant regeneration from induced cell cultures. Our work demonstrates that by combining these three independent developments, heritable targeted chromosomal deletions of large gene clusters and intergenic regulatory sequences can be engineered at a high efficiency. Our results demonstrate that this improved CRISPR/Cas9 methodology can provide a fast, efficient and cost-effective tool to engineer targeted heritable chromosomal deletions, which will be instrumental for future high-throughput functional genomics studies in plants.	Arabidopsis
380	plant	Arabidopsis	CRISPR;Cas9;	glutamate:glyoxylate aminotransferase 1 (ggat1 (Ler background))	Transgenic research	Arabidopsis glutamate:glyoxylate aminotransferase 1 (Ler) mutants generated by CRISPR/Cas9 and their characteristics.	2018	27(1):61-74	[Liang Y et al.]	South-China Agricultural University, Guangzhou, China.	29392632	10.1007/s11248-017-0052-z	Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (CRISPR/Cas9) technology provides an efficient tool for editing the genomes of plants, animals and microorganisms. Glutamate:glyoxylate aminotransferase 1 (GGAT1) is a key enzyme in the photorespiration pathway; however, its regulation mechanism is largely unknown. Given that EMS-mutagenized ggat1 (Col-0 background) M2 pools have been generated, ggat1 (Ler background) should be very useful in the positional cloning of suppressor and/or enhancer genes of GGAT1. Unfortunately, such ggat1 (Ler) mutants are not currently available. In this study, CRISPR/Cas9 was used to generate ggat1 (Ler) mutants. Two GGAT1 target single-guide RNAs (sgRNAs) were constructed into pYLCRISPR/Cas9P35S-N, and flowering Arabidopsis (Ler) plants were transformed using an Agrobacterium tumefaciens-mediated floral dip protocol. Eleven chimeric and two heterozygous GGAT1-edited T1 lines of target 1 were separately screened from positive transgenic lines. Two ggat1 homozygous mutants, CTC-deletion and T-deletion at target 1, were generated from T2 generations of the 13 T1 lines. The edited mutation sites were found to be stable through generations regardless of whether the T-DNA was present. In addition, the genetic segregation of the mutation sites obeyed the Mendelian single gene segregation rule, and no mutations were detected at the possible off-target site. Also, the two independent ggat1 mutants had similar photorespiration phenotypes and down-regulated GGAT enzyme activity. Together, these results indicate that genetically stable ggat1 (Ler) mutants were generated by CRISPR/Cas9 genome editing, and these mutants will be used to promote the positional cloning of suppressor and/or enhancer genes of GGAT1 in our	Arabidopsis
381	plant	Arabidopsis thaliana	Cas9;	synthetic and modular hormone activated Cas9-based repressors that respond to auxin, gibberellins, jasmonates	eLife	Synthetic hormone-responsive transcription factors can monitor and re-program plant development.	2018	7	[Khakhar A et al.]	University of Washington, Seattle, WA, USA.	29714687	10.7554/eLife.34702	Developmental programs sculpt plant morphology to meet environmental challenges, and these same programs have been manipulated to increase agricultural productivity (Doebley et al., 1997; Khush, 2001). Hormones coordinate these programs, creating chemical circuitry (Vanstraelen and Benkova, 2012) that has been represented in mathematical models (Refahi et al., 2016; Prusinkiewicz et al., 2009); however, model-guided engineering of plant morphology has been limited by a lack of tools (Parry et al., 2009; Voytas and Gao, 2014). Here, we introduce a novel set of synthetic and modular hormone activated Cas9-based repressors (HACRs) in Arabidopsis thaliana that respond to three hormones: auxin, gibberellins and jasmonates. We demonstrate that HACRs are sensitive to both exogenous hormone treatments and local differences in endogenous hormone levels associated with development. We further show that this capability can be leveraged to reprogram development in an agriculturally relevant manner by changing how the hormonal circuitry regulates target genes. By deploying a HACR to re-parameterize the auxin-induced expression of the auxin transporter PIN-FORMED1 (PIN1), we decreased shoot branching and phyllotactic noise, as predicted by existing models (Refahi et al., 2016; Prusinkiewicz et al., 2009).	Arabidopsis
382	plant	Arabidopsis thaliana	CRISPR;Cas9;	nonsense mediated RNA decay factor SMG7	Frontiers in plant science	Functional Characterization of SMG7 Paralogs in Arabidopsis thaliana.	2018	9:1602	[Capitao C et al.]	Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Vienna, Austria.	30459790	10.3389/fpls.2018.01602	SMG7 proteins are evolutionary conserved across eukaryotes and primarily known for their function in nonsense mediated RNA decay (NMD). In contrast to other NMD factors, SMG7 proteins underwent independent expansions during evolution indicating their propensity to adopt novel functions. Here we characterized SMG7 and SMG7-like (SMG7L) paralogs in Arabidopsis thaliana. SMG7 retained its role in NMD and additionally appears to have acquired another function in meiosis. We inactivated SMG7 by CRISPR/Cas9 mutagenesis and showed that, in contrast to our previous report, SMG7 is not an essential gene in Arabidopsis. Furthermore, our data indicate that the N-terminal phosphoserine-binding domain is required for both NMD and meiosis. Phenotypic analysis of SMG7 and SMG7L double mutants did not indicate any functional redundancy between the two genes, suggesting neofunctionalization of SMG7L. Finally, protein sequence comparison together with a phenotyping of T-DNA insertion mutants identified several conserved regions specific for SMG7 that may underlie its role in NMD and meiosis. This information provides a framework for	Arabidopsis
383	plant	Arabidopsis thaliana	CRISPR;Cas9;	several different genes	G3	A Dual sgRNA Approach for Functional Genomics in Arabidopsis thaliana.	2018	8(8):2603-2615	[Pauwels L et al.]	Ghent University, Ghent, Belgium.	29884615	10.1534/g3.118.200046	Reverse genetics uses loss-of-function alleles to interrogate gene function. The advent of CRISPR/Cas9-based gene editing now allows the generation of knock-out alleles for any gene and entire gene families. Even in the model plant Arabidopsis thaliana, gene editing is welcomed as T-DNA insertion lines do not always generate null alleles. Here, we show efficient generation of heritable mutations in Arabidopsis using CRISPR/Cas9 with a workload similar to generating overexpression lines. We obtain for several different genes Cas9 null-segregants with bi-allelic mutations in the T2 generation. While somatic mutations were predominantly generated by the canonical non-homologous end joining (cNHEJ) pathway, we observed inherited mutations that were the result of synthesis-dependent microhomology-mediated end joining (SD-MMEJ), a repair pathway linked to polymerase theta (PolQ). We also demonstrate that our workflow is compatible with a dual sgRNA approach in which a gene is targeted by two sgRNAs simultaneously. This paired nuclease method results in more reliable loss-of-function alleles that lack a large essential part of the gene. The ease of the CRISPR/Cas9 workflow should help in the eventual generation of true null alleles of every gene in the Arabidopsis genome, which will advance both basic and applied plant	Arabidopsis

384	plant	Arabidopsis thaliana	CRISPR;Cas9;	Adaptor Protein Complex 2 (AP2) mu-adaptin gene (AP2M)	G3	Activation of Self-Incompatibility Signaling in Transgenic Arabidopsis thaliana Is Independent of AP2-Based Clathrin-Mediated Endocytosis.	2018	8(7):2231-2239	[Yamamoto M et al.]	Tohoku University, Sendai, Miyagi, Japan.	29720392	10.1534/g3.118.200231	Internalization of plasma membrane (PM)-localized ligand-activated receptor kinases and their trafficking to sorting endosomes have traditionally been viewed as functioning primarily in the down-regulation of receptor signaling, but are now considered to be also essential for signaling by some receptors. A major mechanism for internalization of PM proteins is clathrin-mediated endocytosis (CME). CME is mediated by the Adaptor Protein Complex 2 (AP2), which is involved in interaction of the AP2 mu-adaptin subunit with a tyrosine-based Yxxvarphi motif located in the cytoplasmic domain of the cargo protein. In this study, we investigated the role of AP2-mediated CME for signaling by the S-locus receptor kinase (SRK), a protein localized in the PM of stigma epidermal cells, which, together with its pollen coat-localized S-locus cysteine-rich (SCR) ligand, functions in the self-incompatibility (SI) response of the Brassicaceae. Using Arabidopsis thaliana plants that were made self-incompatible by transformation with an A. lyrata-derived SRK/SCR gene pair, we tested the effect on SI of site-directed mutations in each of the two Yxxvarphi motifs in SRK and of a CRISPR/Cas9-induced null mutation in the AP2 mu-adaptin gene AP2M Both in vitro SRK kinase activity and the in planta SI response were abolished by substitution of tyrosine in one of the two Yxxvarphi motifs, but were unaffected by elimination of either the second Yxxvarphi motif or AP2M function. Thus, AP2-mediated CME is considered to be unnecessary for	Arabidopsis
385	plant	Arabidopsis thaliana	CRISPR;Cas9;	stp4-6-8-9-10-11; HEXOKINASE1 (HXK1)	Plant cell	Glucose Uptake via STP Transporters Inhibits in Vitro Pollen Tube Growth in a HEXOKINASE1-Dependent Manner in Arabidopsis thaliana.	2018	30(9):2057-2081	[Rottmann T et al.]	Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany.	30120167	10.1105/tpc.18.00356	Pollen tube growth requires a high amount of metabolic energy and precise targeting toward the ovules. Sugars, especially glucose, can serve as nutrients and as signaling molecules. Unexpectedly, in vitro assays revealed an inhibitory effect of glucose on pollen tube elongation, contradicting the hypothesis that monosaccharide uptake is a source of nutrition for growing pollen tubes. Measurements with Forster resonance energy transfer-based nanosensors revealed that glucose is taken up into pollen tubes and that the intracellular concentration is in the low micromolar range. Pollen tubes of stp4-6-8-9-10-11 sextuple knockout plants generated by crossings and CRISPR/Cas9 showed only a weak response to glucose, indicating that glucose uptake into pollen tubes is mediated mainly by these six monosaccharide transporters of the SUGAR TRANSPORT PROTEIN (STP) family. Analyses of HEXOKINASE1 (HXK1) showed a strong expression of this gene in pollen. Together with the glucose insensitivity and altered semi-in vivo growth rate of pollen tubes from hxk1 knockout lines, this strongly suggests that glucose is an important signaling molecule for pollen tubes, is taken up by STPs, and detected by HXK1. Equimolar amounts of fructose abolish the inhibitory effect of glucose indicating that only an excess of glucose is interpreted as a signal. This provides a possible model for the discrimination of signaling	Arabidopsis
386	plant	Arabidopsis thaliana	CRISPR;Cas9;TALENs;	MUN (MERISTEM UNSTRUCTURED)	Plant journal	MUN (MERISTEM UNSTRUCTURED), encoding a SPC24 homolog of NDC80 kinetochore complex, affects development through cell division in Arabidopsis thaliana.	2018	93(6):977-991	[Shin J et al.]	Seoul National University, Seoul, Korea.	29356153	10.1111/tpj.13823	Kinetochore, a protein super-complex on the centromere of chromosomes, mediates chromosome segregation during cell division by providing attachment sites for spindle microtubules. The NDC80 complex, composed of four proteins, NDC80, NUF2, SPC24 and SPC25, is localized at the outer kinetochore and connects spindle fibers to the kinetochore. Although it is conserved across species, functional studies of this complex are rare in Arabidopsis. Here, we characterize a recessive mutant, meristem unstructured-1 (mun-1), exhibiting an abnormal phenotype with unstructured shoot apical meristem caused by ectopic expression of the WUSCHEL gene in unexpected tissues. mun-1 is a weak allele because of the insertion of T-DNA in the promoter region of the SPC24 homolog. The mutant exhibits stunted growth, embryo arrest, DNA aneuploidy, and defects in chromosome segregation with a low cell division rate. Null mutants of MUN from TALEN and CRISPR/Cas9-mediated mutagenesis showed zygotic embryonic lethality similar to nuf2-1; however, the null mutations were fully transmissible via pollen and ovules. Interactions among the components of the NDC80 complex were confirmed in a yeast two-hybrid assay and in planta co-immunoprecipitation. MUN is co-localized at the centromere with HTR12/CENH3, which is a centromere-specific histone variant, but MUN is not required to recruit HTR12/CENH3 to the kinetochore. Our results support that MUN is a functional homolog of SPC24 in Arabidopsis, which is required for proper cell division. In addition, we report the ectopic generations of stem cell niches by the malfunction of	Arabidopsis
387	plant	Arabidopsis thaliana	CRISPR;Cas9;	nucleotide binding site leucine-rich repeat genes	Plant methods	An efficient CRISPR vector toolbox for engineering large deletions in Arabidopsis thaliana.	2018	14:65	[Wu R et al.]	Max Planck Institute for Developmental Biology, Tubingen, Germany.	30083222	10.1186/s13007-018-0330-7	Background: Our knowledge of natural genetic variation is increasing at an extremely rapid pace, affording an opportunity to come to a much richer understanding of how effects of specific genes are dependent on the genetic background. To achieve a systematic understanding of such GxG interactions, it is desirable to develop genome editing tools that can be rapidly deployed across many different genetic varieties. Results: We present an efficient CRISPR/Cas9 toolbox of super module (SM) vectors. These vectors are based on a previously described fluorescence protein marker expressed in seeds allowing identification of transgene-free mutants. We have used this vector series to delete genomic regions ranging from 1.7 to 13 kb in different natural accessions of the wild plant Arabidopsis thaliana. Based on results from 53 pairs of sgRNAs targeting individual nucleotide binding site leucine-rich repeat (NLR) genes, we provide a comprehensive overview of obtaining heritable deletions. Conclusions: The SM series of CRISPR/Cas9 vectors enables the rapid generation of transgene-free, genome edited plants for a diversity of functional studies.	Arabidopsis

388	plant	Arabidopsis thaliana	CRISPR;	20-kD endonuclease (atm20)	Plant physiology	The Mitochondrial Endonuclease M20 Participates in the Down-Regulation of Mitochondrial DNA in Pollen Cells.	2018	178(4):1537-1550	[Ma F et al.]	Peking University, Beijing, China.	30301773	10.1104/pp.18.00754	Maintaining the appropriate number of mitochondrial DNA (mtDNA) molecules is crucial for supporting mitochondrial metabolism and function in both plant and animal cells. For example, a substantial decrease in mtDNA levels occurs as a key part of pollen development. The molecular mechanisms regulating mtDNA copy number are largely unclear, particularly with regard to those that reduce mtDNA levels. Here, we identified and purified a , M20, from maize (Zea mays) pollen mitochondria. We found M20 to be an His-Asn-His/Asn (H-N-H/N) nuclease that degrades linear and circular DNA in the presence of Mg(2+) or Mn(2+) Arabidopsis (Arabidopsis thaliana) AtM20, which shared high sequence similarity with maize M20, localized to the mitochondria, had a similar H-N-H/N structure, and degraded both linear and circular DNA. AtM20 transcript levels increased during pollen development, in parallel with a rapid reduction in mtDNA. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 genome-editing techniques were used to generate knockout lines of AtM20 (atm20), which exhibited a significant delay in the reduction in mtDNA levels in pollen vegetative cells but normal mtDNA levels in somatic cells. The delayed reduction in pollen mtDNA levels was rescued by the transgenic expression of AtM20 in atm20 plants. This study thus uncovers an endonucleolytic DNase in plant mitochondria and its crucial role in reducing mtDNA levels, pointing to the complex	Arabidopsis
389	plant	Arabidopsis thaliana	CRISPR;	helper component proteinase silencing suppressor (HC-Pro); GFP target 2 (GFP-T2)	Viruses	Engineering RNA Virus Interference via the CRISPR/Cas13 Machinery in Arabidopsis.	2018	10(12)	[Aman R et al.]	King Abdullah University of Science and Technology, Thuwal, Saudi Arabia.	30572690	10.3390/v10120732	Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems are key immune mechanisms helping prokaryotic species fend off RNA and DNA viruses. CRISPR/Cas9 has broad applications in basic research and biotechnology and has been widely used across eukaryotic species for genome engineering and functional analysis of genes. The recently developed CRISPR/Cas13 systems target RNA rather than DNA and thus offer new potential for transcriptome engineering and combatting RNA viruses. Here, we used CRISPR/LshCas13a to stably engineer Arabidopsis thaliana for interference against the RNA genome of Turnip mosaic virus (TuMV). Our data demonstrate that CRISPR RNAs (crRNAs) guiding Cas13a to the sequences encoding helper component proteinase silencing suppressor (HC-Pro) or GFP target 2 (GFP-T2) provide better interference compared to crRNAs targeting other regions of the TuMV RNA genome. This work demonstrates the exciting potential of CRISPR/Cas13 to be used as an antiviral strategy to obstruct RNA viruses, and encourages the search for more robust and effective Cas13 variants or	Arabidopsis
390	plant	Arabidopsis; citrus plants	CRISPR;Cas9;		Plant journal	Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress.	2018	93(2):377-386	[LeBlanc C et al.]	Yale University, New Haven, CT, USA.	29161464	10.1111/tpj.13782	The CRISPR/Cas9 system has greatly improved our ability to engineer targeted mutations in eukaryotic genomes. While CRISPR/Cas9 appears to work universally, the efficiency of targeted mutagenesis and the adverse generation of off-target mutations vary greatly between different organisms. In this study, we report that Arabidopsis plants subjected to heat stress at 37 degrees C show much higher frequencies of CRISPR-induced mutations compared to plants grown continuously at the standard temperature (22 degrees C). Using quantitative assays relying on green fluorescent protein (GFP) reporter genes, we found that targeted mutagenesis by CRISPR/Cas9 in Arabidopsis is increased by approximately 5-fold in somatic tissues and up to 100-fold in the germline upon heat treatment. This effect of temperature on the mutation rate is not limited to Arabidopsis, as we observed a similar increase in targeted mutations by CRISPR/Cas9 in Citrus plants exposed to heat stress at 37 degrees C. In vitro assays demonstrate that Cas9 from Streptococcus pyogenes (SpCas9) is more active in creating double-stranded DNA breaks at 37 degrees C than at 22 degrees C, thus indicating a potential contributing mechanism for the in vivo effect of temperature on CRISPR/Cas9. This study reveals the importance of temperature in modulating SpCas9 activity in eukaryotes, and provides a simple method to increase on-target	arabidopsis;
391	plant	Arabidopsis; Nicotiana benthamiana	CRISPR;Cas9;		Genome biology	Conferring DNA virus resistance with high specificity in plants using virus-inducible genome-editing system.	2018	19(1):197	[Ji X et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.	30442181	10.1186/s13059-018-1580-4	The CRISPR/Cas9 system has recently been engineered to confer resistance to geminiviruses in plants. However, we show here that the usefulness of this antiviral strategy is undermined by off-target effects identified by deep sequencing in Arabidopsis. We construct two virus-inducible CRISPR/Cas9 vectors that efficiently inhibit beet severe curly top virus (BSCTV) accumulation in both transient assays (Nicotiana benthamiana) and transgenic lines (Arabidopsis). Deep sequencing detects no off-target effect in candidate sites of the transgenic Arabidopsis. This kind of virus-inducible genome-editing system should be widely applicable for generating virus-	Arabidopsis;
392	plant	Arabidopsis; Nicotiana benthamiana	CRISPR;Cas9;		Virus research	Pea early-browning virus-mediated genome editing via the CRISPR/Cas9 system in Nicotiana benthamiana and Arabidopsis.	2018	244:333-337	[Ali Z et al.]	King Abdullah University of Science and Technology, Thuwal, Saudi Arabia.	29051052	10.1016/j.virusres.2017.10.009	The clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated (Cas9) system has enabled efficient genome engineering in diverse plant species. However, delivery of genome engineering reagents, such as the single guide RNA (sgRNA), into plant cells remains challenging. Here, we report the engineering of Tobacco rattle virus (TRV) and Pea early browning virus (PEBV) to deliver one or multiple sgRNAs into Nicotiana benthamiana and Arabidopsis thaliana (Col-0) plants that overexpress a nuclear localization signal containing Cas9. Our data showed that TRV and PEBV can deliver sgRNAs into inoculated and systemic leaves, and this resulted in mutagenesis of the targeted genomic loci. Moreover, in N. benthamiana, PEBV-based sgRNA delivery resulted in more targeted mutations than TRV-based delivery. Our data indicate that TRV and PEBV can facilitate plant genome engineering and can be used to produce targeted mutations for functional analysis and other biotechnological applications across diverse plant species. Key message: Delivery of genome engineering reagents into plant cells is challenging and inefficient and this limit the applications of this technology in many plant species. RNA viruses such as TRV and PEBV provide an efficient tool to systemically deliver sgRNAs for targeted genome	Arabidopsis;

393	plant	banana (Cavendish cultivar "Williams")	CRISPR;Cas9;	phytoene desaturase (PDS)	Transgenic research	Gene editing the phytoene desaturase alleles of Cavendish banana using CRISPR/Cas9.	2018	27(5):451-460	[Naim F et al.]	Queensland University of Technology, Brisbane, QLD, Australia.	29987710	10.1007/s11248-018-0083-0	Bananas are a staple food source and a major export commodity worldwide. The Cavendish dessert banana is a triploid AAA genome type and accounts for around 47% of global production. Being essentially sterile, genetic modification is perhaps the only pathway available to improve this cultivar. In this study, we used the CRISPR/Cas9 gene editing system to deliver a self-cleaving polycistronic guide RNA (gRNA) designed to target exon 1 of the Phytoene desaturase (PDS) gene in the Cavendish cultivar "Williams". Genotyping of 19 independent events showed a 100% PDS modification rate primarily in the form of insertions (1-105 nt) or deletions (1-55 nt) (indels) at the predicted cleavage site. Tri-allelic disruptive modifications were observed in 63% of plants and resulted in both albinism and dwarfing. Pale green (16%) and wildtype green (21%) phenotypes generally correlated with in-frame indels in at least one of the three PDS alleles. Editing efficiency was dependent on both target site selection and Cas9 abundance. This is the first report of a highly effective CRISPR/Cas9 modification system using a polycistronic gRNA in Cavendish banana. Such an editing platform will be of considerable utility for the development of disease resistance and novel agro-traits in this commercially important cultivar into the future.	banana
394	plant	banana (cv. Rasthali)	CRISPR;Cas9;	phytoene desaturase (RAS-PDS)	Functional & integrative genomics	CRISPR/Cas9-mediated efficient editing in phytoene desaturase (PDS) demonstrates precise manipulation in banana cv. Rasthali genome.	2018	18(1):89-99	[Kaur N et al.]	National Agri-Food Biotechnology Institute (NABI), Ministry of Science and Technology (Government of India), Mohali, Punjab, India.	29188477	10.1007/s10142-017-0577-5	The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) has been reported for precise genome modification in many plants. In the current study, we demonstrate a successful mutation in phytoene desaturase (RAS-PDS) of banana cv. Rasthali using the CRISPR/Cas9 system. Two PDS genes were isolated from Rasthali (RAS-PDS1 and RAS-PDS2), and their protein sequence analysis confirmed that both PDS comprises conserved motifs for enzyme activity. Phylogenetic analysis of RAS-PDS1 and RAS-PDS2 revealed a close evolutionary relationship with other monocot species. The tissue-specific expression profile of RAS-PDS1 and RAS-PDS2 in Rasthali suggested differential regulation of the genes. A single 19-bp guide RNA (gRNA) was designed to target the conserved region of these two RAS-PDS and transformed with Cas9 in embryogenic cell suspension (ECS) cultures of cv. Rasthali. Complete albino and variegated phenotype were observed among regenerated plantlets. DNA sequencing of 13 plants confirmed the indels with 59% mutation frequency in RAS-PDS, suggesting activation of the non-homologous end-joining (NHEJ) pathway. The majority of mutations were either insertion (1-5) or deletion (1-4) of nucleotides near to protospacer adjacent motif (PAM). These mutations have created stop codons in RAS-PDS sequences which suggest premature termination of RAS-PDS protein synthesis. The decreased chlorophyll and total carotenoid contents were detected in mutant lines that revealed the functional disruption of both RAS-PDS genes. Our results demonstrate that	banana
395	plant	barley	Cas9;	cytokinin dehydrogenase (HvCKX1)	Frontiers in plant science	Modification of Barley Plant Productivity Through Regulation of Cytokinin Content by Reverse-Genetics Approaches.	2018	9:1676	[Holubova K et al.]	Palacky University, Olomouc, Czech Republic.	30542354	10.3389/fpls.2018.01676	Barley is one of the most important cereals, which is used for breweries, animal and human feeds. Genetic manipulation of plant hormone cytokinins may influence several physiological processes, besides others stress tolerance, root formation and crop yield. In planta, endogenous cytokinin status is finely regulated by the enzyme cytokinin dehydrogenase (EC 1.5.99.12; CKX), that irreversible degrades the side chain of adenine-derived isoprenoid cytokinins. Increasing grain yield by mean of manipulation of endogenous cytokinin content was assayed by the silencing of the HvCKX1 gene. Moreover, to elucidate the putative role of HvCKX1 gene on grain production, knocked-out Hvckx1 mutant plants were generated using the RNA-guided Cas9 system. Homozygote transgenic plants with silenced HvCKX1 gene and azygous knock-out Hvckx1 mutants have been selected and analyzed. Both reduced expression of HvCKX1 gene and CKX activity were measured in different stages of barley grain development. Phenotyping of the transgenic lines revealed reduced root growth, however, plants produced more tillers and grains than azygous wild-type controls and the total yield was increased up to 15 per cent. Although plant productivity was increased, total grain biomass was decreased to 80% of WT grains. RNA-seq analysis of knock-down transgenic lines revealed that several important macronutrient transporters were downregulated in the stage of massive starch accumulation. It suggests that local accumulation of cytokinins negatively affected nutrients flow resulting in reduced grain biomass. Obtained results confirmed the key role of HvCKX1	barley
396	plant	barley	Cas9;	Microrchidia protein (HvMORC1)	Plant biotechnology journal	Further analysis of barley MORC1 using a highly efficient RNA-guided Cas9 gene-editing system.	2018	16(11):1892-1903	[Kumar N et al.]	Justus-Liebig University Giessen, Giessen, Germany.	29577542	10.1111/pbi.12924	Microrchidia (MORC) proteins comprise a family of proteins that have been identified in prokaryotes and eukaryotes. They are defined by two hallmark domains: a GHKL-type ATPase and an S5-fold. In plants, MORC proteins were first discovered in a genetic screen for Arabidopsis thaliana mutants compromised for resistance to a viral pathogen. Subsequent studies expanded their role in plant immunity and revealed their involvement in gene silencing and genome stabilization. Little is known about the role of MORC proteins of cereals, especially because knockout (KO) mutants were not available and assessment of loss of function relied only on RNAi strategies, which were arguable, given that MORC proteins in itself are influencing gene silencing. Here, we used a Streptococcus pyogenes Cas9 (SpCas9)-mediated KO strategy to functionally study HvMORC1, one of the current seven MORC members of barley. Using a novel barley RNA Pol III-dependent U3 small nuclear RNA (snRNA) promoter to drive expression of the synthetic single guide RNA (sgRNA), we achieved a very high mutation frequency in HvMORC1. High frequencies of mutations were detectable by target sequencing in the callus, the T0 generation (77%) and T1 generation (70%-100%), which constitutes an important improvement of the gene-editing technology in cereals. Corroborating and extending earlier findings, SpCas9-edited hvmorc1-KO barley, in clear contrast to Arabidopsis atmorc1 mutants, had a distinct phenotype of increased disease resistance to fungal pathogens, while morc1 mutants of either plant showed de-repressed expression of transposable elements (TEs), substantiating that plant MORC proteins contribute to genome stabilization in monocotyledonous and	barley

397	plant	barley (Hordeum vulgare L.)	agroinfiltration; CRISPR;Cas9;	cytokinin oxidase/dehydrogenase (HvCKX1; HvCKX3); Nud	Plant methods	A simple and efficient CRISPR/Cas9 platform for induction of single and multiple, heritable mutations in barley (Hordeum vulgare L.).	2018	14:111	[Gasparis S et al.]	Plant Breeding and Acclimatization Institute – National Research Institute, Blonie, Poland.	30568723	10.1186/s13007-018-0382-8	Background: Genome editing of monocot plants can be accomplished by using the components of the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR associated Cas9) technology specifically optimized for these types of plants. Here, we present the development of RNA-guided Cas9 system for simplex and multiplex genome editing in barley. Results: We developed a set of customizable RNA-guided Cas9 binary vectors and sgRNA modules for simplex and multiplex editing in barley. To facilitate the design of RNA-guided Cas9 constructs, the pBract derived binary vectors were adapted to Gateway cloning and only one restriction enzyme was required for construction of the sgRNA. We designed a synthetic, codon optimized Cas9 gene containing the N terminal SV40 nuclear localization signal and the UBQ10 Arabidopsis 1st intron. Two different sgRNAs were constructed for simplex editing and one polycistronic tRNA-gRNA construct (PTG) for multiplex editing using an endogenous tRNA processing system. The RNA-guided Cas9 constructs were validated in transgenic barley plants produced by Agrobacterium-mediated transformation. The highest mutation rate was observed in simplex editing of the cytokinin oxidase/dehydrogenase HvCKX1 gene, where mutations at the hvckx1 locus were detected in 88% of the screened T0 plants. We also proved the efficacy of the PTG construct in the multiplex editing of two CKX genes by obtaining 9 plants (21% of all edited plants) with mutations induced in both HvCKX1 and HvCKX3. Analysis of the T1 lines revealed that mutations in the HvCKX1 gene were transmitted to the next generation of plants. Among 220 screened T1 plants we identified 85 heterozygous and 28 homozygous mutants, most of them bearing frameshift mutations in the HvCKX1 gene. We also observed independent segregation of mutations and the Cas9-sgRNA T-DNA insert in several T1 plants. Moreover, the knockout mutations of the Nud gene generated phenotype mutants with naked grains, and the phenotypic changes were identifiable in T0 plants. Conclusions: We demonstrated the effectiveness of an optimized RNA-guided Cas9 system that can be used for generating homozygous knockout mutants in the progeny of transgenic barely plants. This is also the first	barley
398	plant	Brassica carinata	CRISPR;Cas9;	P-specific reacting genes; four genes that reacted specifically to Pi starvation including fasciclin-like arabinogalactan protein 1; inorganic phosphate transporter	Frontiers in plant science	Molecular Background of Pi Deficiency-Induced Root Hair Growth in Brassica carinata – A Fasciclin-Like Arabinogalactan Protein Is Involved.	2018	9:1372	[Kirchner TW et al.]	Leibniz Universitat Hannover, Hanover, Germany.	30283481	10.3389/fpls.2018.01372	Formation of longer root hairs under limiting phosphate (P) conditions can increase the inorganic P (Pi) uptake. Here, regulatory candidate genes for Pi deficiency-induced root hair growth were identified by comparison of massive analysis of cDNA ends (MACE) provided expression profiles of two Brassica carinata cultivars (cv.) differing in their root hair response to Pi deficiency: cv. Bale develops longer root hairs under Pi deficiency, but not cv. Bacho. A split-root experiment was conducted for the differentiation between locally and systemically regulated genes. Furthermore, plants were exposed to nitrogen and potassium deficiency to identify P-specific reacting genes. The latter were knocked out by CRISPR/Cas9 and the effect on the root hair length was determined. About 500 genes were differentially expressed under Pi deficiency in cv. Bale, while these genes did not respond to the low P supply in cv. Bacho. Thirty-three candidate genes with a potential regulatory role were selected and the transcriptional regulation of 30 genes was confirmed by quantitative PCR. Only five candidate genes seemed to be either exclusively regulated locally (two) or systemically (three), whereas 25 genes seemed to be involved in both local and systemic signaling pathways. Potassium deficiency affected neither the root hair length nor the expression of the 30 candidate genes. By contrast, both P and nitrogen deficiency increased the root hair length, and both affected the transcript levels in 26 cases. However, four genes reacted specifically to Pi starvation. These genes and, additionally, INORGANIC PHOSPHATE TRANSPORTER 1 (BcPHT1) were targeted by CRISPR/Cas9. However, even if the transcript levels of five of these genes were clearly decreased, FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 1 (BcFLA1) was the only gene whose downregulation reduced the root hair length in transgenic hairy roots under Pi-deficient conditions. To the best of our knowledge, this is the first study	Brassica
399	plant	Brassica napus	CRISPR;Cas9;	APETALA2 (BnAP2)	Frontiers in plant science	Defective APETALA2 Genes Lead to Sepal Modification in Brassica Crops.	2018	9:367	[Zhang Y et al.]	Hybrid Rapeseed Research Center of Shaanxi Province, Yangling, China.	29616073	10.3389/fpls.2018.00367	Many vegetable and oilseed crops belong to Brassica species. The seed production of these crops is hampered often by abnormal floral organs, especially under the conditions of abiotic conditions. However, the molecular reasons for these abnormal floral organs remains poorly understood. Here, we report a novel pistil-like flower mutant of B. rapa. In the flower of this mutant, the four sepals are modified to one merged carpel that look like a ring in the sepal positions, enveloping some abnormal stamens and a pistil, and resulting in poor seed production. This novel mutant is named sepal-carpel modification (scm). DNA sequencing showed that the BrAP2a gene, the ortholog of Arabidopsis APETALA2 (AP2) that specifies sepal identity, losses the function of in scm mutant due to a 119-bp repeated sequence insertion that resulted in an early transcription termination. BrAP2b, the paralog of BrAP2a featured two single-nucleotide substitutions that cause a single amino acid substitution in the highly conserved acidic serine-rich transcriptional activation domain. Each of the two BrAP2 genes rescues the sepal defective phenotype of the ap2-5 mutant of Arabidopsis. Furthermore, the knockout mutation of the corresponding BnAP2 genes of oilseed rape (B. napus) by CRISPR/Cas9-mediated genome editing system resulted in scm-like phenotype. These results suggest that BrAP2 gene plays a key role in sepal modification. Our finding provides an insight into molecular mechanism underlying morphological modification of floral organs and is useful for genetic manipulation of	brassica

400	plant	Brassica napus	CRISPR;Cas9;	BnWRKY11; BnWRKY70	International journal of molecular sciences	CRISPR/Cas9-Mediated Multiplex Genome Editing of the BnWRKY11 and BnWRKY70 Genes in Brassica napus L.	2018	19(9)	[Sun Q et al.]	Yangzhou University, Yangzhou, China.	30208656	10.3390/ijms19092716	Targeted genome editing is a desirable means of basic science and crop improvement. The clustered, regularly interspaced, palindromic repeat (CRISPR)/Cas9 (CRISPR-associated 9) system is currently the simplest and most commonly used system in targeted genomic editing in plants. Single and multiplex genome editing in plants can be achieved under this system. In Arabidopsis, AtWRKY11 and AtWRKY70 genes were involved in JA- and SA-induced resistance to pathogens, in rapeseed (Brassica napus L.), BnWRKY11 and BnWRKY70 genes were found to be differently expressed after inoculated with the pathogenic fungus, Sclerotinia sclerotiorum (Lib.) de Bary. In this study, two Cas9/sgRNA constructs targeting two copies of BnWRKY11 and four copies of BnWRKY70 were designed to generate BnWRKY11 and BnWRKY70 mutants respectively. As a result, twenty-two BnWRKY11 and eight BnWRKY70 independent transformants (T(0)) were obtained, with the mutation ratios of 54.5% (12/22) and 50% (4/8) in BnWRKY11 and BnWRKY70 transformants respectively. Eight and two plants with two copies of mutated BnWRKY11 and BnWRKY70 were obtained respectively. In T(1) generation of each plant examined, new mutations on target genes were detected with high efficiency. The vast majority of BnWRKY70 mutants showed editing in three copies of BnWRKY70 in examined T(1) plants. BnWRKY70 mutants exhibited enhanced resistance to Sclerotinia, while BnWRKY11 mutants showed no significant difference in Sclerotinia resistance when compared to non-transgenic plants. In addition, plants that overexpressed BnWRKY70 showed increased sensitivity when compared to non-transgenic plants. Altogether, our results demonstrated that BnWRKY70 may function as a regulating factor to negatively control the Sclerotinia resistance and CRISPR/Cas9 system could be used to generate germplasm in B. napus with high	brassica
401	plant	Brassica napus	CRISPR;Cas9;	CLAVATA	Plant biotechnology journal	Precise editing of CLAVATA genes in Brassica napus L. regulates multilocular silique development.	2018	16(7):1322-1335	[Yang Y et al.]	Huazhong Agricultural University, Wuhan, China.	29250878	10.1111/pbi.12872	Multilocular silique is a desirable agricultural trait with great potential for the development of high-yield varieties of Brassica. To date, no spontaneous or induced multilocular mutants have been reported in Brassica napus, which likely reflects its allotetraploid nature and the extremely low probability of the simultaneous random mutagenesis of multiple gene copies with functional redundancy. Here, we present evidence for the efficient knockout of rapeseed homologues of CLAVATA3 (CLV3) for a secreted peptide and its related receptors CLV1 and CLV2 in the CLV signalling pathway using the CRISPR/Cas9 system and achieved stable transmission of the mutations across three generations. Each BnCLV gene has two copies located in two subgenomes. The multilocular phenotype can be recovered only in knockout mutations of both copies of each BnCLV gene, illustrating that the simultaneous alteration of multiple gene copies by CRISPR/Cas9 mutagenesis has great potential in generating agronomically important mutations in rapeseed. The mutagenesis efficiency varied widely from 0% to 48.65% in T0 with different single-guide RNAs (sgRNAs), indicating that the appropriate selection of the sgRNA is important for effectively generating indels in rapeseed. The double mutation of BnCLV3 produced more leaves and multilocular siliques with a significantly higher number of seeds per silique and a higher seed weight than the wild-type and single mutant plants, potentially contributing to increased seed production. We also assessed the efficiency of the horizontal transfer of Cas9/gRNA cassettes by pollination. Our findings reveal the potential for plant	brassica
402	plant	Brassica napus	CRISPR;Cas9;	homologues of the Arabidopsis histone 3 lysine 36 (H3K36) methyltransferase SDG8 (BnaSDG8.A; BnaSDG8.C)	Plant journal	Histone lysine methyltransferases BnaSDG8.A and BnaSDG8.C are involved in the floral transition in Brassica napus.	2018		[Jiang L et al.]	Hunan Agricultural University, Changsha, China.	29797624	10.1111/tpj.13978	Although increasing experimental evidence demonstrates that histone methylations play important roles in Arabidopsis plant growth and development, little information is available regarding Brassica napus. In this study, we characterized two genes encoding homologues of the Arabidopsis histone 3 lysine 36 (H3K36) methyltransferase SDG8, namely, BnaSDG8.A and BnaSDG8.C. Although no duplication of SDG8 homologous genes had been previously reported to occur during the evolution of any sequenced species, a domain-duplication was uncovered in BnaSDG8.C. This duplication led to the identification of a previously unknown NNH domain in the SDG8 homologues, providing a useful reference for future studies and revealing the finer mechanism of SDG8 function. One NNH domain is present in BnaSDG8.A, while two adjacent NNH domains are present in BnaSDG8.C. Reverse transcriptase-quantitative polymerase chain reaction analysis revealed similar patterns but with varied levels of expression of BnaSDG8.A/C in different plant organs/tissues. To directly investigate their function, BnaSDG8.A/C cDNA was ectopically expressed to complement the Arabidopsis mutant. We observed that the expression of either BnaSDG8.A or BnaSDG8.C could rescue the Arabidopsis sdg8 mutant to the wild-type phenotype. Using RNAi and CRISPR/Cas9-mediated gene editing, we obtained BnaSDG8.A/C knockdown and knockout mutants with the early flowering phenotype as compared with the control. Further analysis of two types of the mutants revealed that BnaSDG8.A/C are required for H3K36 m2/3 deposition and prevent the floral transition of B. napus by directly enhancing the H3K36 m2/3 levels at the BnaFLC chromatin loci. This observation on the floral transition by epigenetic modification in B. napus provides useful information	brassica
403	plant	Brassica napus	CRISPR;Cas9;	fatty acid desaturase 2	Plant physiology and biochemistry	CRISPR/Cas9-mediated genome editing of the fatty acid desaturase 2 gene in Brassica napus.	2018	131:63-69	[Okuzaki A et al.]	Tamagawa University,Machida, Tokyo, Japan.	29753601	10.1016/j.plaphy.2018.04.025	The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9-mediated genome editing system has been widely applied as a powerful tool for modifying preferable endogenous genes. This system is highly expected to be further applied for the breeding of various agronomically important plant species. Here we report the modification of a fatty acid desaturase 2 gene (FAD2), which encodes an enzyme that catalyzes the desaturation of oleic acid, in Brassica napus cv. Westar using the CRISPR/Cas9 system. Two guide RNAs were designed for BnaA.FAD2.a (FAD2_Aa). Of 22 regenerated shoots with FAD2_Aa editing vectors, three contained mutant alleles. Further analysis revealed that two of three mature plants (Aa1#13 and Aa2#2) contained the mutant alleles. The mutant fad2_Aa allele had a 4-bp deletion, which was inherited by backcross progenies (BC1) in the Aa1#13 line. Furthermore, plants with the fad2_Aa allele without transgenes were selected from the BC1 progenies and plants homozygous for fad2_Aa were then produced by self-crossing these BC1 progenies (BC1S1). Fatty acid composition analysis of their seeds revealed a statistically significant increase in the content of oleic acid compared with that in wild-type seeds. These results showed that the application of the CRISPR/Cas9 system is useful to produce desirable mutant plants with an agronomically suitable	brassica

404	plant	Brassica oleracea; Brassica rapa	CRISPR;Cas9;	FRI and PDS genes	Frontiers in plant science	DNA-Free Genome Editing of Brassica oleracea and B. rapa Protoplasts Using CRISPR-Cas9 Ribonucleoprotein Complexes.	2018	9:1594	[Murovec J et al.]	University of Ljubljana, Ljubljana, Slovenia.	30455712	10.3389/fpls.2018.01594	The CRISPR/Cas9 genome editing system has already proved its efficiency, versatility and simplicity in numerous applications in human, animal, microbe and plant cells. Together with the vast amount of genome and transcriptome databases available, it represents an enormous potential for plant breeding and research. Although most changes produced with CRISPR/Cas9 do not differ from naturally occurring mutations, the use of transgenesis during varietal development can still trigger GMO legislation in countries that rely on process-based regulation. Moreover, stable integration of DNA coding for genome-editing tools into plant genomes can result in insertional mutagenesis, while its prolonged expression can cause mutations in off-target sites. These pitfalls can be avoided with the delivery of preassembled ribonucleoprotein complexes (RNPs) composed of purified recombinant enzyme Cas9 and in vitro-transcribed or synthesized sgRNA. We therefore aimed to develop a DNA-free protocol for site-directed mutagenesis of three species of the genus Brassica (B. oleracea, B. napus, and B. rapa) with the use of RNPs. We chose cabbage, rapeseed and Chinese cabbage as species representatives and introduced RNPs into their protoplasts with PEG 4000. Four sgRNAs targeting two endogenous genes (the FRI and PDS genes, two sgRNAs per gene) were introduced into all three species. No mutations were detected after transfection of rapeseed protoplasts, while we obtained mutation frequencies of 0.09 to 2.25% and 1.15 to 24.51% in cabbage and Chinese cabbage, respectively. In both species, a positive correlation was displayed between the amount (7.5, 15, 30, and 60 mug) of Cas9 enzyme and sgRNA introduced and mutation frequency. Nucleotide changes (insertions and deletions) were detected 24 h after transfection and did not differ 72 h after transfection. They were species-, gene- and locus-dependent. In summary, we demonstrated the suitability of RNP transfection into B. oleracea and B. rapa protoplasts for high-efficiency indel induction of two endogenous genes. Due to the relatively high mutation frequencies detected (up to 24.51%), this study paves the	Brassica
405	plant	Brassica rapa	RdDM;		Plant journal	Maternal components of RNA-directed DNA methylation are required for seed development in Brassica rapa.	2018	94(4):575-582	[Grover JW et al.]	University of Arizona, Tucson, AZ, USA.	29569777	10.1111/tpj.13910	Small RNAs trigger repressive DNA methylation at thousands of transposable elements in a process called RNA-directed DNA methylation (RdDM). The molecular mechanism of RdDM is well characterized in Arabidopsis, yet the biological function remains unclear, as loss of RdDM in Arabidopsis causes no overt defects, even after generations of inbreeding. It is known that 24 nucleotide Pol IV-dependent siRNAs, the hallmark of RdDM, are abundant in flowers and developing seeds, indicating that RdDM might be important during reproduction. Here we show that, unlike Arabidopsis, mutations in the Pol IV-dependent small RNA pathway cause severe and specific reproductive defects in Brassica rapa. High rates of abortion occur when seeds have RdDM mutant mothers, but not when they have mutant fathers. Although abortion occurs after fertilization, RdDM function is required in maternal somatic tissue, not in the female gametophyte or the developing zygote, suggesting that siRNAs from the maternal soma might function in filial tissues. We propose that recently outbreeding species such as B. rapa are key to understanding the role of RdDM during plant	brassica
406	plant	rapeseed	CRISPR;Cas9;	BnSPL3-A5; BnSPL3-A4; BnSPL3-C3; BnSPL3-C4; BnSPL3-Cnn	Frontiers in plant science	An Efficient CRISPR/Cas9 Platform for Rapidly Generating Simultaneous Mutagenesis of Multiple Gene Homoeologs in Allotetraploid Oilseed Rape.	2018	9:442	[Li C et al.]	Oil Crops Research Institute of Chinese Academy of Agricultural Sciences,Wuhan, China.	29731757	10.3389/fpls.2018.00442	With the rapid development of sequence specific nucleases (SSNs) for genome targeting, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) is now considered the most promising method for functional genetic researches, as well as genetic improvement in crop plants. However, the gene redundancy feature within the allotetraploid rapeseed genome is one of the major obstacles for simultaneous modification of different homologs in the first generation. In addition, large scale screening to identify mutated transgenic plants is very time-and labor-consuming using the conventional restriction enzyme-based approaches. In this study, a streamlined rapeseed CRISPR-Cas9 genome editing platform was developed through synthesizing a premade U6-26 driven sgRNA expression cassette and optimizing polyacrylamide gel electrophoresis (PAGE)-based screening approach. In our experiment, a sgRNA was constructed to target five rapeseed SPL3 homologous gene copies, BnSPL3-A5/BnSPL3-A4/BnSPL3-C3/BnSPL3-C4/BnSPL3-Cnn. High-throughput sequencing analysis demonstrated that the editing frequency of CRISPR/Cas9-induced mutagenesis ranged from 96.8 to 100.0% in plants with obvious heteroduplexed PAGE bands, otherwise this proportion was only 0.00-60.8%. Consistent with those molecular analyses, Bnspl3 mutants exhibited developmental delay phenotype in the first generation. In summary, our data suggest that this set of CRISPR/Cas9 platform is qualified for rapidly generating and	brassica
407	plant	rapeseed (Brassica napus L.)	CRISPR;Cas9;	BnA10.LMI1	TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik	Promoter variations in a homeobox gene, BnA10.LMI1, determine lobed leaves in rapeseed (Brassica napus L.).	2018	131(12):2699-2708	[Hu L et al.]	Huazhong Agricultural University, Wuhan, China.	30219987	10.1007/s00122-018-3184-5	KEY MESSAGE: BnA10.LMI1 positively regulates the development of leaf lobes in Brassica napus, and cis-regulatory divergences cause the different allele effects. Leaf shape is an important agronomic trait, and large variations in this trait exist within the Brassica germplasm. The lobed leaf is a unique morphological characteristic for Brassica improvement. Nevertheless, the molecular basis of leaf lobing in Brassica is poorly understood. Here, we show that an incompletely dominant locus, BnLLA10, is responsible for the lobed-leaf shape in rapeseed. A LATE MERISTEM IDENTITY1 (LMI1)-like gene (BnA10.LMI1) encoding an HD-Zip I transcription factor is the causal gene underlying the BnLLA10 locus. Sequence analysis of parental alleles revealed no sequence variations in the coding sequences, whereas abundant variations were identified in the regulatory region. Consistent with this finding, the expression levels of BnLMI1 were substantially elevated in the lobed-leaf parent compared with its near-isogenic line. The knockout mutations of BnA10.LMI1 gene were induced using the CRISPR/Cas9 system in both HY (the lobed-leaf parent) and J9707 (serrated leaf) genetic backgrounds. BnA10.LMI1 null mutations in the HY background were sufficient to produce unlobed leaves, whereas null mutations in the J9707 background showed no obvious changes in leaf shape compared with the control. Collectively, our results indicate that BnA10.LMI1 positively regulates the development of leaf lobes in B. napus, with cis-regulatory divergences causing the different allelic effects, providing new insights into the molecular mechanism of leaf lobe formation in Brassica crops.	brassica

408	plant	Camelina sativa	CRISPR;Cas9;	Fatty Acid Elongase1 (FAE1)	Plant physiology and biochemistry	Mutagenesis of the FAE1 genes significantly changes fatty acid composition in seeds of Camelina sativa.	2018	123:1–7	[Ozseyhan ME et al.]	Montana State University, Bozeman, MT, USA.	29216494	10.1016/j.plaphy.2017.11.021	Camelina sativa is a re-emerging low-input oilseed crop that has great potentials. It is necessary to ameliorate camelina oils for optimized fatty acid composition that can meet different application requirements. Camelina seed contains significant amounts of C20-C24 very long-chain fatty acids (VLCFAs) that may not be desirable. We demonstrated that these VLCFAs can be effectively reduced by deactivating the Fatty Acid Elongase1 (FAE1) in camelina. The allohexaploid camelina contains three alleles of FAE1 genes. Ethyl methanesulfonate (EMS) induced mutation at the FAE1-B gene caused over 60% reduction of VLCFAs in seed. Homozygous knockout mutants were successfully created in a single generation by simultaneously targeting three FAE1 alleles using the CRISPR technology with an egg cell-specific Cas9 expression. VLCFAs were reduced to less than 2% of total fatty acids compared to over 22% in the wild type, and the C18 unsaturated fatty acids were concomitantly increased. The fae1 mutants were indistinguishable from wild type in seed physiology and plant growth. This study demonstrated that the CRISPR/Cas9 technology can be effectively applied to the polyploid crop camelina to rapidly obtain desired traits such as optimal fatty acid composition in its seed oil. Knocking out FAE1 also provides a means to increase the levels of oleic acid or alpha-linolenic acid in camelina oils that are desirable for	camelina sat
409	plant	carrot	agroinfiltration; CRISPR;Cas9;	flavanone-3-hydroxylase (F3H)	Plant cell reports	Efficient CRISPR/Cas9-based genome editing in carrot cells.	2018	37(4):575–586	[Klimek-Chodacka M et al.]	University of Agriculture in Krakow, Krakow, Poland.	29332168	10.1007/s00299-018-2252-2	KEY MESSAGE: The first report presenting successful and efficient carrot genome editing using CRISPR/Cas9 system. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas9) is a powerful genome editing tool that has been widely adopted in model organisms recently, but has not been used in carrot-a model species for in vitro culture studies and an important health-promoting crop grown worldwide. In this study, for the first time, we report application of the CRISPR/Cas9 system for efficient targeted mutagenesis of the carrot genome. Multiplexing CRISPR/Cas9 vectors expressing two single-guide RNA (gRNAs) targeting the carrot flavanone-3-hydroxylase (F3H) gene were tested for blockage of the anthocyanin biosynthesis in a model purple-colored callus using Agrobacterium-mediated genetic transformation. This approach allowed fast and visual comparison of three codon-optimized Cas9 genes and revealed that the most efficient one in generating F3H mutants was the Arabidopsis codon-optimized AteCas9 gene with up to 90% efficiency. Knockout of F3H gene resulted in the discoloration of calli, validating the functional role of this gene in the anthocyanin biosynthesis in carrot as well as providing a visual marker for screening successfully edited events. Most resulting mutations were small Indels, but long chromosome fragment deletions of 116-119 nt were also generated with simultaneous cleavage mediated by two gRNAs. The results demonstrate successful site-directed mutagenesis in carrot with CRISPR/Cas9 and the usefulness of a model callus culture to validate genome editing systems. Given that the carrot genome has been sequenced recently, our timely study sheds light on the promising application of genome editing tools for boosting basic and translational	carrot
410	plant	cassava	CRISPR;Cas9;	PROTEIN TARGETING TO STARCH (PTST1); GRANULE BOUND STARCH SYNTHASE (GBSS)	Science advances	Accelerated ex situ breeding of GBSS- and PTST1-edited cassava for modified starch.	2018	4(9):eaat6086	[Bull SE et al.]	ETH Zurich, Zurich, Switzerland.	30191180	10.1126/sciadv.aat6086	Crop diversification required to meet demands for food security and industrial use is often challenged by breeding time and amenability of varieties to genome modification. Cassava is one such crop. Grown for its large starch-rich storage roots, it serves as a staple food and a commodity in the multibillion-dollar starch industry. Starch is composed of the glucose polymers amylopectin and amylose, with the latter strongly influencing the physicochemical properties of starch during cooking and processing. We demonstrate that CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9)-mediated targeted mutagenesis of two genes involved in amylose biosynthesis, PROTEIN TARGETING TO STARCH (PTST1) or GRANULE BOUND STARCH SYNTHASE (GBSS), can reduce or eliminate amylose content in root starch. Integration of the Arabidopsis FLOWERING LOCUS T gene in the genome-editing cassette allowed us to accelerate flowering-an event seldom seen under glasshouse conditions. Germinated seeds yielded S1, a transgene-free progeny that inherited edited genes. This attractive new plant breeding technique for modified cassava could be extended to other crops to provide a suite of novel varieties with	cassava
411	plant	Catharansus roseus	agroinfiltration	tryptophan decarboxylase; strictosidine synthase gene	Protoplasma	Genetic engineering approach using early Vinca alkaloid biosynthesis genes led to increased tryptamine and terpenoid indole alkaloids biosynthesis in differentiating cultures of Catharanthus roseus.	2018	255(1):425–435	[Sharma A et al.]	Central Institute of Medicinal and Aromatic Plants (CIMAP), Council of Scientific and Industrial Research, Lucknow, India.	28808798	10.1007/s00709-017-1151-7	Catharanthus roseus today occupies the central position in ongoing metabolic engineering efforts in medicinal plants. The entire multi-step biogenetic pathway of its very expensive anticancerous alkaloids vinblastine and vincristine is fairly very well dissected at biochemical and gene levels except the pathway steps leading to biosynthesis of monomeric alkaloid catharanthine and tabersonine. In order to enhance the plant-based productivity of these pharma molecules for the drug industry, cell and tissue cultures of C. roseus are being increasingly tested to provide their alternate production platforms. However, a rigid developmental regulation and involvement of different cell, tissues, and organelles in the synthesis of these alkaloids have restricted the utility of these cultures. Therefore, the present study was carried out with pushing the terpenoid indole alkaloid pathway metabolic flux towards dimeric alkaloids vinblastine and vincristine production by over-expressing the two upstream pathway genes tryptophan decarboxylase and strictosidine synthase at two different levels of cellular organization viz. callus and leaf tissues. The transformation experiments were carried out using Agrobacterium tumefaciens LBA1119 strain having tryptophan decarboxylase and strictosidine synthase gene cassette. The callus transformation reported a maximum of 0.027% dry wt vindoline and 0.053% dry wt catharanthine production, whereas, the transiently transformed leaves reported a maximum of 0.30%	Catharanthu

412	plant	Catharanthus roseus	Agroinfiltration;	bifunctional geranyl(geranyl) diphosphate synthase [G(G)PPS] and geraniol synthase (GES)	Frontiers in plant science	Terpene Moiety Enhancement by Overexpression of Geranyl(geranyl) Diphosphate Synthase and Geraniol Synthase Elevates Monomeric and Dimeric Monoterpene Indole Alkaloids in Transgenic Catharanthus roseus.	2018	9:942	[Kumar SR et al.]	CSIR-Central Institute of Medicinal and Aromatic Plants, Bengaluru, India.	30034406	10.3389/fpls.2018.00942	Catharanthus roseus is the sole source of two of the most important anticancer monoterpene indole alkaloids (MIAs), vinblastine and vincristine and their precursors, vindoline and catharanthine. The MIAs are produced from the condensation of precursors derived from indole and terpene secoiridoid pathways. It has been previously reported that the terpene moiety limits MIA biosynthesis in C. roseus. Here, to overcome this limitation and enhance MIAs levels in C. roseus, bifunctional geranyl(geranyl) diphosphate synthase [G(G)PPS] and geraniol synthase (GES) that provide precursors for early steps of terpene moiety (secologanin) formation, were overexpressed transiently by agroinfiltration and stably by Agrobacterium-mediated transformation. Both transient and stable overexpression of G(G)PPS and co-expression of G(G)PPS+GES significantly enhanced the accumulation of secologanin, which in turn elevated the levels of monomeric MIAs. In addition, transgenic C. roseus plants exhibited increased levels of root alkaloid ajmalicine. The dimeric alkaloid vinblastine was enhanced only in G(G)PPS but not in G(G)PPS+GES transgenic lines that correlated with transcript levels of peroxidase-1 (PRX1) involved in coupling of vindoline and catharanthine into 3',4'-anhydrovinblastine, the immediate precursor of vinblastine. Moreover, first generation (T1) lines exhibited comparable transcript and metabolite levels to that of T0 lines. In addition, transgenic lines displayed normal growth similar to wild-type plants indicating that the bifunctional G(G)PPS enhanced flux toward both primary and secondary metabolism. These results revealed that improved availability of early precursors for terpene moiety biosynthesis enhanced production of MIAs in C. roseus at the whole plant level. This is the first report	Catharanthu
413	plant	Catharanthus roseus	CRISPR;Cas9;	receptor-like kinase 1-like (MEDOS1 to 4)	Scientific reports	Multiplex mutagenesis of four clustered CrRLK1L with CRISPR/Cas9 exposes their growth regulatory roles in response to metal ions.	2018	8(1):12182	[Richter J et al.]	University of Natural Resources and Life Sciences (BOKU), Vienna, Austria.	30111865	10.1038/s41598-018-30711-3	Resolving functions of closely linked genes is challenging or nearly impossible with classical genetic tools. Four members of the Catharanthus roseus receptor-like kinase 1-like (CrRLK1L) family are clustered on Arabidopsis chromosome five. To resolve the potentially redundant functions of this subclass of CrRLK1Ls named MEDOS1 to 4 (MDS1 to 4), we generated a single CRISPR/Cas9 transformation vector using a Golden Gate based cloning system to target all four genes simultaneously. We introduce single mutations within and deletions between MDS genes as well as knock-outs of the whole 11 kb gene cluster. The large MDS cluster deletion was inherited in up to 25% of plants lacking the CRISPR/Cas9 construct in the T2 generation. In contrast to described phenotypes of already characterized CrRLK1L mutants, quadruple mds knock-outs were fully fertile, developed normal root hairs and trichomes and responded to pharmacological inhibition of cellulose biosynthesis similar to wildtype. Recently, we demonstrated the role of four CrRLK1L in growth adaptation to metal ion stress. Here we show the involvement of MDS genes in response to Ni(2+) during hypocotyl elongation and to Cd(2+) and Zn(2+) during root growth. Our finding supports the model of an organ specific network of positively and negatively acting	Catharanthu
414	plant	Chinese kale (Brassica oleracea var. alboglabra)	agroinfiltration; CRISPR;Cas9;	BaPDS1; BaPDS2	Scientific reports	CRISPR/Cas9-mediated mutagenesis of homologous genes in Chinese kale.	2018	8(1):16786	[Sun B et al.]	Sichuan Agricultural University, Chengdu, China.	30429497	10.1038/s41598-018-34884-9	The clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR/Cas9) system has developed into a powerful gene-editing tool that has been successfully applied to various plant species. However, studies on the application of the CRISPR/Cas9 system to cultivated Brassica vegetables are limited. Here, we reported CRISPR/Cas9-mediated genome editing in Chinese kale (Brassica oleracea var. alboglabra) for the first time. A stretch of homologous genes, namely BaPDS1 and BaPDS2, was selected as the target site. Several stable transgenic lines with different types of mutations were generated via Agrobacterium-mediated transformation, including BaPDS1 and BaPDS2 double mutations and BaPDS1 or BaPDS2 single mutations. The overall mutation rate reached 76.47%, and these mutations involved nucleotide changes of fewer than 10 bp. The clear albino phenotype was observed in all of the mutants, including one that harbored a mutation within an intron region, thereby indicating the importance of the intron. Cleavage in Chinese kale using CRISPR/Cas9 was biased towards AT-rich sequences. Furthermore, no off-target events were observed. Functional differences between BaPDS1 and BaPDS2 were also assessed in terms of the phenotypes of the respective mutants. In combination, these findings showed that CRISPR/Cas9-mediated targeted mutagenesis can simultaneously and efficiently modify homologous gene copies of Chinese kale and provide a convenient approach for studying gene function and improving the yield and	chinese kale
415	plant	coffee (Coffea arabica L.)	Agroinfiltration;	mgfp5; uidA; cry10a	3 Biotech	A simple and efficient agroinfiltration method in coffee leaves (Coffea arabica L.): assessment of factors affecting transgene expression.	2018	8(11):471	[Vargas-Guevara C et al.]	Universidad de Costa Rica, San Jose, Costa Rica.	30456005	10.1007/s13205-018-1495-5	The establishment of a simple, rapid and efficient transient expression system is a necessary tool for the functional validation of candidate genes in coffee biotechnology. The effects of Agrobacterium strain, age of the donor plant, infiltration method, and infiltration medium on transgene expression in detached coffee leaves were evaluated. Regarding the effect of Agrobacterium strain, the expression of uidA was higher in GV3101-treated coffee disks than in LBA4404 and ATHV-treated samples. On the other hand, transient expression of uidA was significantly higher in leaf disks from young plants (6-weeks-old) (13.1 +/- 1.4%) than in mature tissue (12-weeks-old) (1.6 +/- 1.2%). Transient uidA expression was higher in detached coffee leaf disks from young plants infiltrated with one injection of 15 microL of Agrobacterium strain GV3101::1303 suspended in MS salts supplemented with 30 g/L sucrose, 1.9 g/L MES and 200 uM AS with subsequent sanding of the abaxial epidermis. Using the optimized protocol, expression of the uidA gene was observed 6, 24 and 48 h and 5 weeks after bacterial injection. DNA was extracted from coffee disks with positive GUS expression and specific mgfp5 and uidA fragments were amplified 5 weeks post-agroinfiltration. On the other hand, using the optimized protocol, a specific cry10Aa (500 bp) fragment was amplified in the agro-infiltrated coffee leaf disks 5 weeks post-agroinfiltration with the plasmid pB427-35S-cry10Aa. Moreover, the expression of the gene cry10Aa in two infiltrated coffee leaf disks was verified by RT-PCR and an expected 500 bp fragment	coffee

416	plant	cotton	CRISPR;Cas9;	Gh14-3-3d	Frontiers in plant science	Simultaneous Editing of Two Copies of Gh14-3-3d Confers Enhanced Transgene-Clean Plant Defense Against Verticillium dahliae in Allotetraploid Upland Cotton.	2018	9:842	[Zhang Z et al.]	Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.	30013582	10.3389/fpls.2018.00842	Gossypium hirsutum is an allotetraploid species, meaning that mutants that are difficult to be generated by classical approaches due to gene redundancy. The CRISPR/Cas9 genome editing system is a robust and highly efficient tool for generating target gene mutants, by which the genes of interest may be functionally dissected and applied through genotype-to-phenotype approaches. In this study, the CRISPR/Cas9 genome editing system was developed in G. hirsutum through editing the Gh14-3-3d gene. In T0 transgenic plants, lots of insertions and deletions (indels) in Gh14-3-3d at the expected target site were detected in the allotetraploid cotton At or Dt subgenomes. The results of the PCR, T7EI digestion and sequencing analyses showed that the indels in Gh14-3-3d gene can be stably transmitted to the next generation. Additionally, the indels in the At and Dt subgenomes were segregated in the T1 transgenic plants following Mendelian law, independing on the T-DNA segregation. Two homozygous Gh14-3-3d-edited plants free of T-DNA were chosen by PCR and sequencing assays in the T1 plants, which were called transgene-clean editing plants and were designated ce1 and ce2 in the T2 lines showed higher resistance to Verticillium dahliae infestation compared to the wild-type plants. Thus, the two transgene-clean edited lines can be used as a germplasm to breed disease-resistant cotton cultivars, possibly avoiding	cotton
417	plant	cotton	CRISPR;Cas9;	alanine-rich protein (ALARP)	International journal of molecular sciences	Highly Efficient Targeted Gene Editing in Upland Cotton Using the CRISPR/Cas9 System.	2018	19(10)	[Zhu S et al.]	Shihezi University, Shihezi, Xinjiang, China.	30275376	10.3390/ijms19103000	The clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) gene editing system has been shown to be able to induce highly efficient mutagenesis in the targeted DNA of many plants, including cotton, and has become an important tool for investigation of gene function and crop improvement. Here, we developed a simple and easy to operate CRISPR/Cas9 system and demonstrated its high editing efficiency in cotton by targeting-ALARP, a gene encoding alanine-rich protein that is preferentially expressed in cotton fibers. Based on sequence analysis of the target site in the 10 transgenic cottons containing CRISPR/Cas9, we found that the mutation frequencies of GhALARP-A and GhALARP-D target sites were 71.4(-)100% and 92.9(-)100%, respectively. The most common editing event was deletion, but deletion together with large insertion was also observed. Mosaic mutation editing events were detected in most transgenic plants. No off-target mutation event was detected in any the 15 predicted sites analyzed. This study provided mutants for further study of the function of GhALARP in cotton fiber development. Our results further demonstrated the feasibility of use of CRISPR/Cas9 as a targeted mutagenesis tool in cotton, and provided an efficient tool for targeted	cotton
418	plant	cotton	CRISPR;Cas9;	Discosoma red fluorescent protein2(DsRed2); GhCLA1	Plant biotechnology journal	High efficient multisites genome editing in allotetraploid cotton (Gossypium hirsutum) using CRISPR/Cas9 system.	2018	16(1):137-150	[Wang P et al.]	Huazhong Agricultural University, Wuhan, Hubei, China.	28499063	10.1111/pbi.12755	Gossypium hirsutum is an allotetraploid with a complex genome. Most genes have multiple copies that belong to At and Dt subgenomes. Sequence similarity is also very high between gene homologues. To efficiently achieve site/gene-specific mutation is quite needed. Due to its high efficiency and robustness, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system has exerted broad site-specific genome editing from prokaryotes to eukaryotes. In this study, we utilized a CRISPR/Cas9 system to generate two sgRNAs in a single vector to conduct multiple sites genome editing in allotetraploid cotton. An exogenously transformed gene Discosoma red fluorescent protein2(DsRed2) and an endogenous gene GhCLA1 were chosen as targets. The DsRed2-edited plants in T0 generation reverted its traits to wild type, with vanished red fluorescence the whole plants. Besides, the mutated phenotype and genotype were inherited to their T1 progenies. For the endogenous gene GhCLA1, 75% of regenerated plants exhibited albino phenotype with obvious nucleotides and DNA fragments deletion. The efficiency of gene editing at each target site is 66.7-100%. The mutation genotype was checked for both genes with Sanger sequencing. Barcode-based high-throughput sequencing, which could be highly efficient for genotyping to a population of mutants, was conducted in GhCLA1-edited T0 plants and it matched well with Sanger sequencing results. No off-target editing was detected at the potential off-target sites. These results prove that the CRISPR/Cas9	cotton
419	plant	cotton	CRISPR;Cas9;		Plant methods	Optimization of CRISPR/Cas9 genome editing in cotton by improved sgRNA expression.	2018	14:85	[Long L et al.]	Henan University, Kaifeng, Henan, China.	30305839	10.1186/s13007-018-0353-0	Background: When developing CRISPR/Cas9 systems for crops, it is crucial to invest time characterizing the genome editing efficiency of the CRISPR/Cas9 cassettes, especially if the transformation system is difficult or time-consuming. Cotton is an important crop for the production of fiber, oil, and biofuel. However, the cotton stable transformation is usually performed using Agrobacterium tumefaciens taking between 8 and 12 months to generate T0 plants. Furthermore, cotton is a heterotetraploid and targeted mutagenesis is considered to be difficult as many genes are duplicated in this complex genome. The application of CRISPR/Cas9 in cotton is severely hampered by the long and technically challenging genetic transformation process, making it imperative to maximize its efficiency. Results: In this study, we provide a new system to evaluate and validate the efficiency of CRISPR/Cas9 cassettes in cotton using a transient expression system. By using this system, we could select the most effective CRISPR/Cas9 cassettes before the stable transformation. We have also optimized the existing cotton CRISPR/Cas9 system to achieve vastly improved mutagenesis efficiency by incorporating an endogenous GhU6 promoter that increases sgRNA expression levels over the Arabidopsis AtU6-29 promoter. The 300 bp GhU6.3 promoter was cloned and validated using the transient expression system. When sgRNAs were expressed under the control of the GhU6.3 promoter in CRISPR/Cas9 cassettes, expression levels were 6-7 times higher than those provided by the AtU6-29 promoter and CRISPR/Cas9-mediated mutation efficiency was improved 4-6 times. Conclusions: This study provides essential improvements to maximize CRISPR/Cas9-mediated mutation efficiency by reducing risk and workload for the application of	cotton

420	plant	grape	CRISPR;Cas9;	VvWRKY52 transcription factor gene	Plant biotechnology journal	CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation.	2018	16(4):844-855	[Wang X et al.]	Northwest A&F University, Yangling, Shaanxi, China.	28905515	10.1111/pbi.12832	The clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR/Cas9) system is a powerful tool for editing plant genomes. Efficient genome editing of grape (Vitis vinifera) suspension cells using the type II CRISPR/Cas9 system has been demonstrated; however, it has not been established whether this system can be applied to get biallelic mutations in the first generation of grape. In this current study, we designed four guide RNAs for the VvWRKY52 transcription factor gene for using with the CRISPR/Cas9 system, and obtained transgenic plants via Agrobacterium-mediated transformation, using somatic embryos of the Thompson Seedless cultivar. Analysis of the first-generation transgenic plants verified 22 mutant plants of the 72 T-DNA-inserted plants. Of these, 15 lines carried biallelic mutations and seven were heterozygous. A range of RNA-guided editing events, including large deletions, were found in the mutant plants, while smaller deletions comprised the majority of the detected mutations. Sequencing of potential off-target sites for all four targets revealed no off-target events. In addition, knockout of VvWRKY52 in grape increased the resistance to Botrytis cinerea. We conclude that the CRISPR/Cas9 system allows precise genome editing in the first generation of grape and represents a useful tool for gene functional analysis and grape molecular breeding.	grape
421	plant	groundcherry (Physalis pruinosa)	CRISPR;Cas9;	orthologues of tomato domestication and improvement genes that control plant architecture, flower production and fruit size	Nature plants	Rapid improvement of domestication traits in an orphan crop by genome editing.	2018	4(10):766-770	[Lemmon ZH et al.]	Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.	30287957	10.1038/s41477-018-0259-x	Genome editing holds great promise for increasing crop productivity, and there is particular interest in advancing breeding in orphan crops, which are often burdened by undesirable characteristics resembling wild relatives. We developed genomic resources and efficient transformation in the orphan Solanaceae crop 'groundcherry' (Physalis pruinosa) and used clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein-9 nuclease (Cas9) (CRISPR-Cas9) to mutate orthologues of tomato domestication and improvement genes that control plant architecture, flower production and fruit size, thereby improving these major productivity traits. Thus, translating knowledge from model crops enables rapid creation of targeted allelic diversity and novel breeding germplasm in distantly related orphan	groundcherry
422	plant	Ipomoea nil	CRISPR;Cas9;	carotenoid cleavage dioxygenase 4 (InCCD4)	Transgenic research	Alteration of flower colour in Ipomoea nil through CRISPR/Cas9-mediated mutagenesis of carotenoid cleavage dioxygenase 4.	2018	27(1):25-38	[Watanabe K et al.]	University of Tsukuba, Tsukuba, Ibaraki, Japan.	29247330	10.1007/s11248-017-0051-0	Japanese morning glory, Ipomoea nil, exhibits a variety of flower colours, except yellow, reflecting the accumulation of only trace amounts of carotenoids in the petals. In a previous study, we attributed this effect to the low expression levels of carotenogenic genes in the petals, but there may be other contributing factors. In the present study, we investigated the possible involvement of carotenoid cleavage dioxygenase (CCD), which cleaves specific double bonds of the polyene chains of carotenoids, in the regulation of carotenoid accumulation in the petals of I. nil. Using bioinformatics analysis, seven InCCD genes were identified in the I. nil genome. Sequencing and expression analyses indicated potential involvement of InCCD4 in carotenoid degradation in the petals. Successful knockout of InCCD4 using the CRISPR/Cas9 system in the white-flowered cultivar I. nil cv. AK77 caused the white petals to turn pale yellow. The total amount of carotenoids in the petals of ccd4 plants was increased 20-fold relative to non-transgenic plants. This result indicates that in the petals of I. nil, not only low carotenogenic gene expression but also carotenoid degradation leads	Ipomoea nil
423	plant	Japanese morning glory	CRISPR;Cas9;	EPHEMERAL1 (EPH1)	Plant physiology and biochemistry	CRISPR/Cas9-mediated mutagenesis of the EPHEMERAL1 locus that regulates petal senescence in Japanese morning glory.	2018	131:53-57	[Shibuya K et al.]	Institute of Vegetable and Floriculture Science, NARO, Tsukuba, Japan.	29739710	10.1016/j.plaphy.2018.04.036	Flower longevity is one of the most important traits in ornamental plants. In Japanese morning glory (Ipomoea nil), EPHEMERAL1 (EPH1), a NAC transcription factor, is reportedly a key regulator of petal senescence. CRISPR/Cas9-mediated targeted mutagenesis is a powerful tool for crop breeding as well as for biological research. Here we report the application of CRISPR/Cas9 technology to targeted mutagenesis of the EPH1 gene in I. nil. Three regions within the EPH1 gene were simultaneously targeted by a single binary vector containing three single-guide RNA cassettes. We selected eight T0 transgenic plants containing the transferred DNA (T-DNA). Cleaved amplified polymorphic sequence (CAPS) analysis revealed that mutations occurred at single or multiple target sites in all eight plants. These plants harbored various mutations consisting of single base insertions and/or deletions of a single or more than two bases at the target sites. Several mutations generated at target sites were inherited in the T1 progeny with or without T-DNA insertions. Mutant plants in the T1 generations exhibited a clear delay in petal senescence. These results confirm that CRISPR/Cas9 technology can efficiently induce mutations in a target I. nil gene and that EPH1 plays a crucial role in the regulation of petal senescence. The eph1 mutants obtained in this study will be a useful tool for the elucidation of regulatory mechanisms in petal	Japanese mo
424	plant	Jatropha curcas	CRISPR;Cas9;	cytochrome P450 monooxygenase, family 735, subfamily A (JcCYP735A)	PeerJ	Identification and expression analysis of cytokinin metabolic genes IPTs, CYP735A and CKXs in the biofuel plant Jatropha curcas.	2018	6:e4812	[Cai L et al.]	Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan, China.	29785355	10.7717/peerj.4812	The seed oil of Jatropha curcas is considered a potential bioenergy source that could replace fossil fuels. However, the seed yield of Jatropha is low and has yet to be improved. We previously reported that exogenous cytokinin treatment increased the seed yield of Jatropha. Cytokinin levels are directly regulated by isopentenyl transferase (IPT), cytochrome P450 monooxygenase, family 735, subfamily A (CYP735A), and cytokinin oxidase/dehydrogenase (CKX). In this study, we cloned six IPT genes, one JcCYP735A gene, and seven JcCKX genes. The expression patterns of these 14 genes in various organs were determined using real-time quantitative PCR. JcIPT1 was primarily expressed in roots and seeds, JcIPT2 was expressed in roots, apical meristems, and mature leaves, JcIPT3 was expressed in stems and mature leaves, JcIPT5 was expressed in roots and mature leaves, JcIPT6 was expressed in seeds at 10 days after pollination, and JcIPT9 was expressed in mature leaves. JcCYP735A was mainly expressed in roots, flower buds, and seeds. The seven JcCKX genes also showed different expression patterns in different organs of Jatropha. In addition, CK levels were detected in flower buds and seeds at different stages of development. The concentration of N(6)-(Delta(2)-isopentenyl)-adenine (iP), iP-riboside, and trans-zeatin (tZ) increased with flower development, and the concentration of iP decreased with seed development, while that of tZ increased. We further analyzed the function of JcCYP735A using the CRISPR-Cas9 system, and found that the concentrations of tZ and tZ-riboside decreased significantly in the Jccyp735a mutants, which showed severely retarded growth. These findings will be helpful for further studies of the functions of cytokinin metabolic genes and	Jatropha cu

425	plant	kiwifruit	CRISPR;Cas9;	phytoene desaturase gene (AcPDS)	Plant biotechnology journal	Optimized paired-sgRNA/Cas9 cloning and expression cassette triggers high-efficiency multiplex genome editing in kiwifruit.	2018	16(8):1424-1433	[Wang Z et al.]	South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, Guangdong, China.	29331077	10.1111/pbi.12884	Kiwifruit is an important fruit crop; however, technologies for its functional genomic and molecular improvement are limited. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system has been successfully applied to genetic improvement in many crops, but its editing capability is variable depending on the different combinations of the synthetic guide RNA (sgRNA) and Cas9 protein expression devices. Optimizing conditions for its use within a particular species is therefore needed to achieve highly efficient genome editing. In this study, we developed a new cloning strategy for generating paired-sgRNA/Cas9 vectors containing four sgRNAs targeting the kiwifruit phytoene desaturase gene (AcPDS). Comparing to the previous method of paired-sgRNA cloning, our strategy only requires the synthesis of two gRNA-containing primers which largely reduces the cost. We further compared efficiencies of paired-sgRNA/Cas9 vectors containing different sgRNA expression devices, including both the polycistronic tRNA-sgRNA cassette (PTG) and the traditional CRISPR expression cassette. We found the mutagenesis frequency of the PTG/Cas9 system was 10-fold higher than that of the CRISPR/Cas9 system, coinciding with the relative expressions of sgRNAs in two different expression cassettes. In particular, we identified large chromosomal fragment deletions induced by the paired-sgRNAs of the PTG/Cas9 system. Finally, as expected, we found both systems can successfully induce the albino phenotype of kiwifruit plantlets regenerated from the G418-resistance callus lines. We conclude that the PTG/Cas9 system is a more powerful system than the traditional CRISPR/Cas9 system for kiwifruit genome editing, which provides valuable clues for optimizing CRISPR/Cas9 editing system in	kiwifruit
426	plant	lettuce (Lactuca sativa)	CRISPR;Cas9;	9-cis-EPOXYCAROTENOID DIOXYGENASE4 (LsNCED4)	G3	High-Resolution Analysis of the Efficiency, Heritability, and Editing Outcomes of CRISPR/Cas9-Induced Modifications of NCED4 in Lettuce (Lactuca sativa).	2018	8(5):1513-1521	[Bertier LD et al.]	University of California, Davis, CA, USA.	29511025	10.1534/g3.117.300396	CRISPR/Cas9 is a transformative tool for making targeted genetic alterations. In plants, high mutation efficiencies have been reported in primary transformants. However, many of the mutations analyzed were somatic and therefore not heritable. To provide more insights into the efficiency of creating stable homozygous mutants using CRISPR/Cas9, we targeted LsNCED4 (9-cis-EPOXYCAROTENOID DIOXYGENASE4), a gene conditioning thermoinhibition of seed germination in lettuce. Three constructs, each capable of expressing Cas9 and a single gRNA targeting different sites in LsNCED4, were stably transformed into lettuce (Lactuca sativa) cvs. Salinas and Cobham Green. Analysis of 47 primary transformants (T1) and 368 T2 plants by deep amplicon sequencing revealed that 57% of T1 plants contained events at the target site: 28% of plants had germline mutations in one allele indicative of an early editing event (mono-allelic), 8% of plants had germline mutations in both alleles indicative of two early editing events (bi-allelic), and the remaining 21% of plants had multiple low frequency mutations indicative of late events (chimeric plants). Editing efficiency was similar in both genotypes, while the different gRNAs varied in efficiency. Amplicon sequencing of 20 T1 and more than 100 T2 plants for each of the three gRNAs showed that repair outcomes were not random, but reproducible and characteristic for each gRNA. Knockouts of NCED4 resulted in large increases in the maximum temperature for seed germination, with seeds of both cultivars capable of germinating >70% at 37 degrees . Knockouts of NCED4 provide a whole-plant selectable phenotype that has minimal pleiotropic consequences. Targeting NCED4 in a co-editing strategy could therefore be used to enrich for germline-edited events simply by germinating seeds at	lettuce
427	plant	lettuce; Nicotiana benthamiana; tomato; eggplant; hot pepper; melon; orchid	Agroinfiltration;	GFP	Scientific reports	Improvement of the transient expression system for production of recombinant proteins in plants.	2018	8(1):4755	[Yamamoto T et al.]	University of Tsukuba, Tsukuba, Japan.	29555968	10.1038/s41598-018-23024-y	An efficient and high yielding expression system is required to produce recombinant proteins. Furthermore, the transient expression system can be used to identify the localization of proteins in plant cells. In this study, we demonstrated that combination of a geminiviral replication and a double terminator dramatically enhanced the transient protein expression level in plants. The GFP protein was expressed transiently in lettuce, Nicotiana benthamiana, tomatoes, eggplants, hot peppers, melons, and orchids with agroinfiltration. Compared to a single terminator, a double terminator enhanced the expression level. A heat shock protein terminator combined with an extensin terminator resulted in the highest protein expression. Transiently expressed GFP was confirmed by immunoblot analysis with anti-GFP antibodies. Quantitative analysis revealed that the geminiviral vector with a double terminator resulted in the expression of at least 3.7 mg/g fresh weight of GFP in Nicotiana benthamiana, approximately 2-fold that of the geminiviral vector with a single terminator. These results indicated that combination of the geminiviral replication and a double terminator is a useful tool for transient	lettuce; nico
428	plant	Lotus japonicus	CRISPR;	C3HC4-type RING finger protein (LjCZF1)	Journal of integrative plant biology	A C3HC4-type RING finger protein regulates rhizobial infection and nodule organogenesis in Lotus japonicus.	2018	60(9):878-896	[Cai K et al.]	Huazhong Agricultural University, Wuhan, China.	30047576	10.1111/jipb.12703	During the establishment of rhizobia-legume symbiosis, the cytokinin receptor LHK1 (Lotus Histidine Kinase 1) is essential for nodule formation. However, the mechanism by which cytokinin signaling regulates symbiosis remains largely unknown. In this study, an LHK1-interacting protein, LjCZF1, was identified and further characterized. LjCZF1 is a C3HC4-type RING finger protein that is highly conserved in plants. LjCZF1 specifically interacted with LHK1 in yeast two-hybrid, in vitro pull-down and co-immunoprecipitation assays conducted in tobacco. Phosphomimetic mutation of the potential threonine (T167D) phosphorylation site enhanced the interaction between LjCZF1 and LHK1, whereas phosphorylation mutation (T167A) eliminated this interaction. Transcript abundance of LjCZF1 was up-regulated significantly after inoculation with rhizobia. The LORE1 insertion mutant and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9-mediated knockout mutant Lotus japonicus plants demonstrated significantly reduced number of infection threads and nodules. In contrast, plants over-expressing LjCZF1 exhibited increased numbers of infection threads and nodules. Collectively, these data support the notion that LjCZF1 is a positive regulator of symbiotic nodulation, possibly	Lotus japoni

429	plant	maize	agroinfiltration; CRISPR;Cas9;	MS8	Frontiers in plant science	Generation of Transgene-Free Maize Male Sterile Lines Using the CRISPR/Cas9 System.	2018	9:1180	[Chen R et al.]	Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China.	30245698	10.3389/fpls.2018.01180	Male sterility (MS) provides a useful breeding tool to harness hybrid vigor for hybrid seed production. It is necessary to generate new male sterile mutant lines for the development of hybrid seed production technology. The CRISPR/Cas9 technology is well suited for targeting genomes to generate male sterile mutants. In this study, we artificially synthesized Streptococcus pyogenes Cas9 gene with biased codons of maize. A CRISPR/Cas9 vector targeting the MS8 gene of maize was constructed and transformed into maize using an Agrobacterium-mediated method, and eight T0 independent transgenic lines were generated. Sequencing results showed that MS8 genes in these T0 transgenic lines were not mutated. However, we detected mutations in the MS8 gene in F1 and F2 progenies of the transgenic line H17. A potential off-target site sequence which had a single nucleotide that was different from the target was also mutated in the F2 progeny of the transgenic line H17. Mutation in the MS8 gene and the male sterile phenotype could be stably inherited by the next generation in a Mendelian fashion. Transgene-free ms8 male sterile plants were obtained by screening the F2 generation of male sterile plants, and the MS phenotype could be	maize
430	plant	maize	RdDM;		G3	Subtle Perturbations of the Maize Methylome Reveal Genes and Transposons Silenced by Chromomethylase or RNA-Directed DNA Methylation Pathways.	2018	8(6):1921-1932	[Anderson SN et al.]	University of Minnesota, St. Paul, MN, USA.	29618467	10.1534/g3.118.200284	DNA methylation is a chromatin modification that can provide epigenetic regulation of gene and transposon expression. Plants utilize several pathways to establish and maintain DNA methylation in specific sequence contexts. The chromomethylase (CMT) genes maintain CHG (where H = A, C or T) methylation. The RNA-directed DNA methylation (RdDM) pathway is important for CHH methylation. Transcriptome analysis was performed in a collection of Zea mays lines carrying mutant alleles for CMT or RdDM-associated genes. While the majority of the transcriptome was not affected, we identified sets of genes and transposon families sensitive to context-specific decreases in DNA methylation in mutant lines. Many of the genes that are up-regulated in CMT mutant lines have high levels of CHG methylation, while genes that are differentially expressed in RdDM mutants are enriched for having nearby mCHH islands, implicating context-specific DNA methylation in the regulation of expression for a small number of genes. Many genes regulated by CMTs exhibit natural variation for DNA methylation and transcript abundance in a panel of diverse inbred lines. Transposon families with differential expression in the mutant genotypes show few defining features, though several families up-regulated in RdDM mutants show enriched expression in endosperm tissue, highlighting the potential importance for this pathway during reproduction. Taken together, our findings suggest that while the number of genes and transposon families whose expression is reproducibly affected by mild perturbations in context-specific methylation is small, there are distinct patterns for	maize
431	plant	maize	ODM;	non functional Green Fluorescent Protein	Journal of plant research	Relaxed chromatin induced by histone deacetylase inhibitors improves the oligonucleotide-directed gene editing in plant cells.	2018	131(1):179-189	[Tiricz H et al.]	Institute of Plant Biology, Hungarian Academy of Sciences, Szeged, Hungary.	28836127	10.1007/s10265-017-0975-8	Improving efficiency of oligonucleotide-directed mutagenesis (ODM) is a prerequisite for wide application of this gene-editing approach in plant science and breeding. Here we have tested histone deacetylase inhibitor treatments for induction of relaxed chromatin and for increasing the efficiency of ODM in cultured maize cells. For phenotypic assay we produced transgenic maize cell lines expressing the non-functional Green Fluorescent Protein (mGFP) gene carrying a TAG stop codon. These transgenic cells were bombarded with corrective oligonucleotide as editing reagent to recover GFP expression. Repair of green fluorescent protein function was monitored by confocal fluorescence microscopy and flow cytometry was used for quantification of correction events. Sequencing PCR fragments of the GFP gene from corrected cells indicated a nucleotide exchange in the stop codon (TAG) from T to G nucleotide that resulted in the restoration of GFP function. We show that pretreatment of maize cells with sodium butyrate (5-10 mM) and nicotinamide (1-5 mM) as known inhibitors of histone deacetylases can cause elevated chromatin sensitivity to DNase I that was visualized in agarose gels and confirmed by the reduced presence of intact PCR template for the inserted exogenous mGFP gene. Maize cells with more relaxed chromatin could serve as an improved recipient for targeted nucleotide exchange as indicated by an average of 2.67- to 3.62-fold increase in GFP-positive cells. Our results stimulate further studies on the role of the condition of the recipient cells in ODM and testing the application of chromatin modifying agents, in other, programmable	maize
432	plant	maize	CRISPR;Cas9;	dmc1 gene promoter combined with the U3 promoter in 3 loci	Plant biotechnology journal	High-efficiency genome editing using a dmc1 promoter-controlled CRISPR/Cas9 system in maize.	2018	16(11):1848-1857	[Feng C et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.	29569825	10.1111/pbi.12920	Previous studies revealed that the promoters for driving both Cas9 and sgRNAs are quite important for efficient genome editing by CRISPR/Cas9 in plants. Here, we report our results of targeted genome editing using the maize dmc1 gene promoter combined with the U3 promoter for Cas9 and sgRNA, respectively. Three loci in the maize genome were selected for targeting. The T0 plants regenerated were highly efficiently edited at the target sites with homozygous or bi-allelic mutants accounting for about 66%. The mutations in T0 plants could be stably transmitted to the T1 generation, and new mutations could be generated in gametes or zygotes. Whole-genome resequencing indicated that no off-target mutations could be detected in the predicted loci with sequence similarity to the targeted site. Our results show that the dmc1 promoter-controlled (DPC) CRISPR/Cas9 system is highly efficient in maize and provide further evidence that the optimization of the promoters used for the CRISPR/Cas9 system is important for enhancing the efficiency of targeted genome editing in plants. The evolutionary conservation of the dmc1 gene suggests its potential for use in other	maize
433	plant	maize	CRISPR;Cas9;	bZIP-type transcription factor zmbzip22	Plant cell	The ZmbZIP22 Transcription Factor Regulates 27-kD gamma-Zein Gene Transcription during Maize Endosperm Development.	2018	30(10):2402-2424	[Li C et al.]	China Agricultural University, Beijing, China.	30242039	10.1105/tpc.18.00422	Zeins are the most abundant storage proteins in maize (Zea mays) kernels, thereby affecting the nutritional quality and texture of this crop. 27-kD gamma-zein is highly expressed and plays a crucial role in protein body formation. Several transcription factors (TFs) (O2, PBF1, OHP1, and OHP2) regulate the expression of the 27-kD gamma-zein gene, but the complexity of its transcriptional regulation is not fully understood. Here, using probe affinity purification and mass spectrometry analysis, we identified ZmbZIP22, a TF that binds to the 27-kD gamma-zein promoter. ZmbZIP22 is a bZIP-type TF that is specifically expressed in endosperm. ZmbZIP22 bound directly to the ACAGCTCA box in the 27-kD gamma-zein promoter and activated its expression in wild tobacco (Nicotiana benthamiana) cells. 27-kD gamma-zein gene expression was significantly reduced in CRISPR/Cas9-generated zmbzip22 mutants. ChIP-seq (chromatin immunoprecipitation coupled to high-throughput sequencing) confirmed that ZmbZIP22 binds to the 27-kD gamma-zein promoter in vivo and identified additional direct targets of ZmbZIP22. ZmbZIP22 can interact with PBF1, OHP1, and OHP2, but not O2. Transactivation assays using various combinations of these TFs revealed multiple interaction modes for the transcriptional activity of the 27-kD gamma-zein promoter. Therefore, ZmbZIP22 regulates 27-kD gamma-zein gene	maize

434	plant	maize	CRISPR;Cas9;	hnRNP-like glycine-rich RNA binding protein (ZmGRP1)	Plant cell	Genome-Wide Association Analyses Reveal the Importance of Alternative Splicing in Diversifying Gene Function and Regulating Phenotypic Variation in Maize.	2018	30(7):1404-1423	[Chen Q et al.]	China Agricultural University, Beijing, China.	29967286	10.1105/tpc.18.00109	Alternative splicing (AS) enhances transcriptome diversity and plays important roles in regulating plant processes. Although widespread natural variation in AS has been observed in plants, how AS is regulated and contribute to phenotypic variation is poorly understood. Here, we report a population-level transcriptome assembly and genome-wide association study to identify splicing quantitative trait loci (sQTLs) in developing maize (Zea mays) kernels from 368 inbred lines. We detected 19,554 unique sQTLs for 6570 genes. Most sQTLs showed small isoform usage changes without involving major isoform switching between genotypes. The sQTL-affected isoforms tend to display distinct protein functions. We demonstrate that nonsense-mediated mRNA decay, microRNA-mediated regulation, and small interfering peptide-mediated peptide interference are frequently involved in sQTL regulation. The natural variation in AS and overall mRNA level appears to be independently regulated with different cis-sequences preferentially used. We identified 214 putative trans-acting splicing regulators, among which ZmGRP1, encoding an hnRNP-like glycine-rich RNA binding protein, regulates the largest trans-cluster. Knockout of ZmGRP1 by CRISPR/Cas9 altered splicing of numerous downstream genes. We found that 739 sQTLs colocalized with previous marker-trait associations, most of which occurred without changes in overall mRNA level. Our findings uncover the importance of AS in diversifying gene function and	maize
435	plant	maize	CRISPR;Cas9;	maize heterotrimeric G protein alpha subunit COMPACT PLANT2 (CT2); eXtra Large GTP-binding proteins (XLGs)	PLoS genetics	Role of heterotrimeric Galpha proteins in maize development and enhancement of agronomic traits.	2018	14(4):e1007374	[Wu Q et al.]	Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.	29708966	10.1371/journal.pgen.1007374	Plant shoot systems derive from the shoot apical meristems (SAMs), pools of stems cells that are regulated by a feedback between the WUSCHEL (WUS) homeobox protein and CLAVATA (CLV) peptides and receptors. The maize heterotrimeric G protein alpha subunit COMPACT PLANT2 (CT2) functions with CLV receptors to regulate meristem development. In addition to the sole canonical Galpha CT2, maize also contains three eXtra Large GTP-binding proteins (XLGs), which have a domain with homology to Galpha as well as additional domains. By either forcing CT2 to be constitutively active, or by depleting XLGs using CRISPR-Cas9, here we show that both CT2 and XLGs play important roles in maize meristem regulation, and their manipulation improved agronomic traits. For example, we show that expression of a constitutively active CT2 resulted in higher spikelet density and kernel row number, larger ear inflorescence meristems (IMs) and more upright leaves, all beneficial traits selected during maize improvement. Our findings suggest that both the canonical Galpha, CT2 and the non-canonical XLGs play important roles in maize meristem regulation and further demonstrate that weak alleles of plant stem cell regulatory genes have the capacity to	maize
436	plant	maize	CRISPR;Cas9;	CCT transcription factor (ZmCCT9)	Proceedings of the National Academy of Sciences of the United States of America	ZmCCT9 enhances maize adaptation to higher latitudes.	2018	115(2):E334-E341	[Huang C et al.]	China Agricultural University, Beijing, China.	29279404	10.1073/pnas.1718058115	From its tropical origin in southwestern Mexico, maize spread over a wide latitudinal cline in the Americas. This feat defies the rule that crops are inhibited from spreading easily across latitudes. How the widespread latitudinal adaptation of maize was accomplished is largely unknown. Through positional cloning and association mapping, we resolved a flowering-time quantitative trait locus to a Harbinger-like transposable element positioned 57 kb upstream of a CCT transcription factor (ZmCCT9). The Harbinger-like element acts in cis to repress ZmCCT9 expression to promote flowering under long days. Knockout of ZmCCT9 by CRISPR/Cas9 causes early flowering under long days. ZmCCT9 is diurnally regulated and negatively regulates the expression of the florigen ZCN8, thereby resulting in late flowering under long days. Population genetics analyses revealed that the Harbinger-like transposon insertion at ZmCCT9 and the CACTA-like transposon insertion at another CCT paralog, ZmCCT10, arose sequentially following domestication and were targeted by selection for maize adaptation to higher latitudes. Our findings help explain how the dynamic maize genome with abundant transposon activity enabled maize to adapt over 90 degrees of latitude	maize
437	plant	maize	CRISPR;Cas9;	male sterility33 (ZmMs33)	TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik	Map-based cloning and characterization of Zea mays male sterility33 (ZmMs33) gene, encoding a glycerol-3-phosphate acyltransferase.	2018	131(6):1363-1378	[Xie K et al.]	University of Science and Technology Beijing, Beijing, China.	29546443	10.1007/s00122-018-3083-9	KEY MESSAGE: Map-based cloning of maize ms33 gene showed that ZmMs33 encodes a sn-2 glycerol-3-phosphate acyltransferase, the ortholog of rice OsGPAT3, and it is essential for male fertility in maize. Genetic male sterility has been widely studied for its biological significance and commercial value in hybrid seed production. Although many male-sterile mutants have been identified in maize (Zea mays L.), it is likely that most genes that cause male sterility are unknown. Here, we report a recessive genetic male-sterile mutant, male sterility33 (ms33), which displays small, pale yellow anthers, and complete male sterility. Using a map-based cloning approach, maize GRMZM2G070304 was identified as the ms33 gene (ZmMs33). ZmMs33 encodes a novel sn-2 glycerol-3-phosphate acyltransferase (GPAT) in maize. A functional complementation experiment showed that GRMZM2G070304 can rescue the male-sterile phenotype of the ms33-6029 mutant. GRMZM2G070304 was further confirmed to be the ms33 gene via targeted knockouts induced by the clustered regularly interspersed short palindromic repeats (CRISPR)/Cas9 system. ZmMs33 is preferentially expressed in the immature anther from the quartet to early-vacuolate microspore stages and in root tissues at the fifth leaf growth stage. Phylogenetic analysis indicated that ZmMs33 and OsGPAT3 are evolutionarily conserved for anther and pollen development in monocot species. This study reveals that the monocot-specific GPAT3 protein plays an important role in male fertility in maize, and ZmMs33	maize
438	plant	Marchantia polymorpha	CRISPR;Cas9;	ortholog of the METHYLTRANSFERASE 1 (MpMET)	Plant & cell physiology	Loss of CG Methylation in Marchantia polymorpha Causes Disorganization of Cell Division and Reveals Unique DNA Methylation Regulatory Mechanisms of Non-CG Methylation.	2018	59(12):2421-2431	[Ikeda Y et al.]	Okayama University, Kurashiki, Japan.	30102384	10.1093/pcp/pcy161	DNA methylation is an epigenetic mark that ensures silencing of transposable elements (TEs) and affects gene expression in many organisms. The function of different DNA methylation regulatory pathways has been largely characterized in the model plant Arabidopsis thaliana. However, far less is known about DNA methylation regulation and functions in basal land plants. Here we focus on the liverwort Marchantia polymorpha, an emerging model species that represents a basal lineage of land plants. We identified MpMET, the M. polymorpha ortholog of the METHYLTRANSFERASE 1 (MET1) gene required for maintenance of methylation at CG sites in angiosperms. We generated Mpmet mutants using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein9) system, which showed a significant loss of CG methylation and severe morphological changes and developmental defects. The mutants developed many adventitious shoot-like structures, suggesting that MpMET is required for maintaining differentiated cellular identities in the gametophyte. Even though numerous TEs were up-regulated, non-CG methylation was generally highly increased at TEs in the Mpmet mutants. Closer inspection of CHG methylation revealed features unique to M. polymorpha. Methylation of CCG sites in M. polymorpha does not depend on MET1, unlike in A. thaliana and Physcomitrella patens. Our results highlight the diversity of non-CG methylation regulatory mechanisms in plants.	Marchantia p

439	plant	Marchantia polymorpha	CRISPR;Cas9;	UV RESISTANCE LOCUS8 (UVR8)	Plant journal	UVR8-mediated induction of flavonoid biosynthesis for UVB tolerance is conserved between the liverwort Marchantia polymorpha and flowering plants.	2018	96(3):503-517	[Clayton WA et al.]	New Zealand Institute for Plant & Food Research Limited, Palmerston North, New Zealand.	30044520	10.1111/tpj.14044	Damaging UVB radiation is a major abiotic stress facing land plants. In angiosperms the UV RESISTANCE LOCUS8 (UVR8) photoreceptor coordinates UVB responses, including inducing biosynthesis of protective flavonoids. We characterised the UVB responses of Marchantia polymorpha (marchantia), the model species for the liverwort group of basal plants. Physiological, chemical and transcriptomic analyses were conducted on wild-type marchantia exposed to three different UVB regimes. CRISPR/Cas9 was used to obtain plant lines with mutations for components of the UVB signal pathway or the flavonoid biosynthetic pathway, and transgenics overexpressing the marchantia UVR8 sequence were generated. The mutant and transgenic lines were analysed for changes in flavonoid content, their response to UVB exposure, and transcript abundance of a set of 48 genes that included components of the UVB response pathway characterised for angiosperms. The marchantia UVB response included many components in common with Arabidopsis, including production of UVB-absorbing flavonoids, the central activator role of ELONGATED HYPOCOTYL5 (HY5), and negative feedback regulation by REPRESSOR OF UV-B PHOTOMORPHOGENESIS1 (RUP1). Notable differences included the greater importance of CHALCONE ISOMERASE-LIKE (CHIL). Mutants disrupted in the response pathway (hy5) or flavonoid production (chalcone isomerase, chil) were more easily damaged by UVB. Mutants (rup1) or transgenics (35S:MpMYB14) with increased flavonoid content had increased UVB tolerance. The results suggest that UVR8-mediated flavonoid induction is a UVB tolerance character conserved across land	Marchantia p
440	plant	Marchantia polymorpha	CRISPR;Cas9;	2 loci	PloS one	Efficient CRISPR/Cas9-based genome editing and its application to conditional genetic analysis in Marchantia polymorpha.	2018	13(10):e0205117	[Sugano SS et al.]	Ritsumeikan University, Kusatsu, Shiga, Japan.	30379827	10.1371/journal.pone.0205117	Marchantia polymorpha is one of the model species of basal land plants. Although CRISPR/Cas9-based genome editing has already been demonstrated for this plant, the efficiency was too low to apply to functional analysis. In this study, we show the establishment of CRISPR/Cas9 genome editing vectors with high efficiency for both construction and genome editing. Codon optimization of Cas9 to Arabidopsis achieved over 70% genome editing efficiency at two loci tested. Systematic assessment revealed that guide sequences of 17 nt or shorter dramatically decreased this efficiency. We also demonstrated that a combinatorial use of this system and a floxed complementation construct enabled conditional analysis of a nearly essential gene. This study reports that simple, rapid, and efficient genome editing is feasible with the	marchantia p
441	plant	Medicago sativa	CRISPR;Cas9;	squamosa promoter binding protein like 9 (SPL9)	Planta	Gene editing by CRISPR/Cas9 in the obligatory outcrossing Medicago sativa.	2018	247(4):1043-1050	[Gao R et al.]	Agriculture and Agri-Food Canada, London, ON, Canada.	29492697	10.1007/s00425-018-2866-1	MAIN CONCLUSION: The CRISPR/Cas9 technique was successfully used to edit the genome of the obligatory outcrossing plant species Medicago sativa L. (alfalfa). RNA-guided genome engineering using Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/Cas9 technology enables a variety of applications in plants. Successful application and validation of the CRISPR technique in a multiplex genome, such as that of M. sativa (alfalfa) will ultimately lead to major advances in the improvement of this crop. We used CRISPR/Cas9 technique to mutate squamosa promoter binding protein like 9 (SPL9) gene in alfalfa. Because of the complex features of the alfalfa genome, we first used droplet digital PCR (ddPCR) for high-throughput screening of large populations of CRISPR-modified plants. Based on the results of genome editing rates obtained from the ddPCR screening, plants with relatively high rates were subjected to further analysis by restriction enzyme digestion/PCR amplification analyses. PCR products encompassing the respective small guided RNA target locus were then sub-cloned and sequenced to verify genome editing. In summary, we successfully applied the CRISPR/Cas9 technique to edit the SPL9 gene in a multiplex genome, providing some insights into opportunities to apply this technology in future alfalfa breeding. The overall efficiency in the polyploid alfalfa genome was lower compared to other less-complex plant genomes. Further refinement of the CRISPR technology system will thus be required for more efficient genome	Medicago sa
442	plant	papaya	agroinfiltration; CRISPR;Cas9;	extracellular cystatin-like cysteine protease inhibitor (PpalEPIC8)	Molecular plant-microbe interactions	A Phytophthora palmivora Extracellular Cystatin-Like Protease Inhibitor Targets Papain to Contribute to Virulence on Papaya.	2018	31(3):363-373	[Gumtow R et al.]	University of Hawaii at Manoa, Honolulu, USA.	29068239	10.1094/MPMI-06-17-0131-FI	Papaya fruits, stems, and leaves are rich in papain, a cysteine protease that has been shown to mediate plant defense against pathogens and insects. Yet the oomycete Phytophthora palmivora is a destructive pathogen that infects all parts of papaya plants, suggesting that it has evolved cysteine protease inhibitors to inhibit papain to enable successful infection. Out of five putative extracellular cystatin-like cysteine protease inhibitors (PpalEPICs) from P. palmivora transcriptomic sequence data, PpalEPIC8 appeared to be unique to P. palmivora and was highly induced during infection of papaya. Purified recombinant PpalEPIC8 strongly inhibited papain enzyme activity, suggesting that it is a functional cysteine protease inhibitor. Homozygous PpalEPIC8 mutants were generated using CRISPR/Cas9-mediated gene editing via Agrobacterium-mediated transformation (AMT). Increased papain sensitivity of in-vitro growth and reduced pathogenicity during infection of papaya fruits were observed for the mutants compared with the wild-type strain, suggesting that PpalEPIC8, indeed, plays a role in P. palmivora virulence by inhibiting papain. This study provided genetic evidence demonstrating that plant-pathogenic oomycetes secrete cystatins as important weapons to invade plants. It also established an effective gene-editing system for P. palmivora by the combined use of CRISPR/Cas9 and AMT, which is	papaya
443	plant	Parasopnia andersonii	agroinfiltration; CRISPR;Cas9;	PanHK4; PanEIN2; PanNSP1; PanNSP2	Frontiers in plant science	CRISPR/Cas9-Mediated Mutagenesis of Four Putative Symbiosis Genes of the Tropical Tree Parasponia andersonii Reveals Novel Phenotypes.	2018	9:284	[van Zeijl A et al.]	Wageningen University & Research, Wageningen, Netherlands.	29559988	10.3389/fpls.2018.00284	Parasponia represents five fast-growing tropical tree species in the Cannabaceae and is the only plant lineage besides legumes that can establish nitrogen-fixing nodules with rhizobium. Comparative analyses between legumes and Parasponia allows identification of conserved genetic networks controlling this symbiosis. However, such studies are hampered due to the absence of powerful reverse genetic tools for Parasponia. Here, we present a fast and efficient protocol for Agrobacterium tumefaciens-mediated transformation and CRISPR/Cas9 mutagenesis of Parasponia andersonii. Using this protocol, knockout mutants are obtained within 3 months. Due to efficient micro-propagation, bi-allelic mutants can be studied in the T0 generation, allowing phenotypic evaluation within 6 months after transformation. We mutated four genes – PanHK4, PanEIN2, PanNSP1, and PanNSP2 – that control cytokinin, ethylene, or strigolactone hormonal networks and that in legumes commit essential symbiotic functions. Knockout mutants in Panhk4 and Panein2 displayed developmental phenotypes, namely reduced procambium activity in Panhk4 and disturbed sex differentiation in Panein2 mutants. The symbiotic phenotypes of Panhk4 and Panein2 mutant lines differ from those in legumes. In contrast, PanNSP1 and PanNSP2 are essential for nodule formation, a phenotype similar as reported for legumes. This indicates a conserved role for these GRAS-type transcriptional regulators in rhizobium	Parasponia a

444	plant	peanut (Arachis hypogaea L.)	TALENs;	fatty acid desaturase 2	Plant molecular biology	TALEN-mediated targeted mutagenesis of fatty acid desaturase 2 (FAD2) in peanut (Arachis hypogaea L.) promotes the accumulation of oleic acid.	2018	97(1-2):177-185	[Wen S et al.]	Crops Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China.	29700675	10.1007/s11103-018-0731-z	KEY MESSAGE: A first creation of high oleic acid peanut varieties by using transcription activator-like effecter nucleases (TALENs) mediated targeted mutagenesis of Fatty Acid Desaturase 2 (FAD2). Transcription activator like effector nucleases (TALENs), which allow the precise editing of DNA, have already been developed and applied for genome engineering in diverse organisms. However, they are scarcely used in higher plant study and crop improvement, especially in allopolyploid plants. In the present study, we aimed to create targeted mutagenesis by TALENs in peanut. Targeted mutations in the conserved coding sequence of Arachis hypogaea fatty acid desaturase 2 (AhFAD2) were created by TALENs. Genetic stability of AhFAD2 mutations was identified by DNA sequencing in up to 9.52 and 4.11% of the regeneration plants at two different targeted sites, respectively. Mutation frequencies among AhFAD2 mutant lines were significantly correlated to oleic acid accumulation. Genetically, stable individuals of positive mutant lines displayed a 0.5-2 fold increase in the oleic acid content compared with non-transgenic controls. This finding suggested that TALEN-mediated targeted mutagenesis could increase the oleic acid content in edible peanut oil. Furthermore, this was the first report on peanut genome editing	peanut
445	plant	Petunia inflata	CRISPR;Cas9;	S-Locus F-Box Protein (S2 - SLF1)	Plant cell	S-Locus F-Box Proteins Are Solely Responsible for S-RNase-Based Self-Incompatibility of Petunia Pollen.	2018	30(12):2959-2972	[Sun L et al.]	Pennsylvania State University, University Park, PA, USA.	30377238	10.1105/tpc.18.00615	Self-incompatibility (SI) in Petunia is regulated by a polymorphic S-locus. For each S-haplotype, the S-locus contains a pistil-specific S-RNase gene and multiple pollen-specific S-locus F-box (SLF) genes. Both gain-of-function and loss-of-function experiments have shown that S-RNase alone regulates pistil specificity in SI. Gain-of-function experiments on SLF genes suggest that the entire suite of encoded proteins constitute the pollen specificity determinant. However, clear-cut loss-of-function experiments must be performed to determine if SLF proteins are essential for SI of pollen. Here, we used CRISPR/Cas9 to generate two frame-shift indel alleles of S2 -SLF1 (SLF1 of S2 -haplotype) in S2S3 plants of P. inflata and examined the effect on the SI behavior of S2 pollen. In the absence of a functional S2-SLF1, S2 pollen was either rejected by or remained compatible with pistils carrying one of eight normally compatible S-haplotypes. All results are consistent with interaction relationships between the 17 SLF proteins of S2 -haplotype and these eight S-RNases that had been determined by gain-of-function experiments performed previously or in this work. Our loss-of-function results provide definitive evidence that SLF proteins are solely responsible for SI of pollen, and they reveal their diverse and complex interaction relationships with S-RNases to maintain SI while ensuring cross-compatibility.	Petunia infla
446	plant	Petunia inflata	CRISPR;Cas9;	PiSSK1	Plant reproduction	CRISPR/Cas9-mediated knockout of PiSSK1 reveals essential role of S-locus F-box protein-containing SCF complexes in recognition of non-self S-RNases during cross-compatible pollination in self-incompatible Petunia inflata.	2018	31(2):129-143	[Sun L et al.]	Pennsylvania State University, University Park, PA, USA.	29192328	10.1007/s00497-017-0314-1	KEY MESSAGE: Function of Petunia PiSSK1. Self-incompatibility (SI), an inbreeding-preventing mechanism, is regulated in Petunia inflata by the polymorphic S-locus, which houses multiple pollen-specific S-locus F-box (SLF) genes and a single pistil-specific S-RNase gene. S 2-haplotype and S 3-haplotype possess the same 17 polymorphic SLF genes (named SLF1 to SLF17), and each SLF protein produced in pollen is assembled into an SCF (Skp1-Cullin1-F-box) E3 ubiquitin ligase complex. A complete suite of SLF proteins is thought to collectively interact with all non-self S-RNases to mediate their ubiquitination and degradation by the 26S proteasome, allowing cross-compatible pollination. For each SCF(SLF) complex, the Cullin1 subunit (named PiCUL1-P) and Skp1 subunit (named PiSSK1), like the F-box protein subunits (SLFs), are pollen-specific, raising the possibility that they also evolved specifically to function in SI. Here we used CRISPR/Cas9-meditated genome editing to generate frame-shift indel mutations in PiSSK1 and examined the SI behavior of a T 0 plant (S 2 S 3) with biallelic mutations in the pollen genome and two progeny plants (S 2 S 2) each homozygous for one of the indel alleles and not carrying the Cas9-containing T-DNA. Their pollen was completely incompatible with pistils of seven otherwise-compatible S-genotypes, but fully compatible with pistils of an S 3 S 3 transgenic plant in which production of S3-RNase was completely suppressed by an antisense S 3-RNase gene, and with pistils of immature flower buds, which produce little S-RNase. These results suggest that PiSSK1 specifically functions in SI and support the	Petunia infla
447	plant	Physcomitrella patens	CRISPR;Cas9;	SPR2	Cell structure and function	SPIRAL2 Stabilises Endoplasmic Microtubule Minus Ends in the Moss Physcomitrella patens.	2018	43(1):53-60	[Leong SY et al.]	Nagoya University, Japan.	29445053	10.1247/csf.18001	Stabilisation of minus ends of microtubules (MTs) is critical for organising MT networks in land plant cells, in which all MTs are nucleated independent of centrosomes. Recently, Arabidopsis SPIRAL2 (SPR2) protein was shown to localise to plus and minus ends of cortical MTs, and increase stability of both ends. Here, we report molecular and functional characterisation of SPR2 of the basal land plant, the moss Physcomitrella patens. In protonemal cells of P. patens, where non-cortical, endoplasmic MT network is organised, we observed SPR2 at minus ends, but not plus ends, of endoplasmic MTs and likely also of phragmoplast MTs. Minus end decoration was reconstituted in vitro using purified SPR2, suggesting that moss SPR2 is a minus end-specific binding protein (-TIP). We generated a loss-of-function mutant of SPR2, in which frameshift-causing deletions/insertions were introduced into all four paralogous SPR2 genes by means of CRISPR/Cas9. Protonemal cells of the mutant showed instability of endoplasmic MT minus ends. These results indicate that moss SPR2 is a MT minus end stabilising factor.Key words: acentrosomal microtubule network, microtubule minus end, P. patens,	Physcomitre

448	plant	poplar	CRISPR;Cas9;	floral meristem identity gene, LEAFY (LFY); two poplar orthologs of the floral organ identity gene AGAMOUS (AG)	Frontiers in plant science	Variation in Mutation Spectra Among CRISPR/Cas9 Mutagenized Poplars.	2018	9:594	[Elorriaga E et al.]	Oregon State University, Corvallis, OR, USA.	29868058	10.3389/fpls.2018.00594	In an effort to produce reliably contained transgenic trees, we used the CRISPR/Cas9 system to alter three genes expected to be required for normal flowering in poplar (genus Populus). We designed synthetic guide RNAs (sgRNAs) to target the poplar homolog of the floral meristem identity gene, LEAFY (LFY), and the two poplar orthologs of the floral organ identity gene AGAMOUS (AG). We generated 557 transgenic events with sgRNA(s) and the Cas9 transgene and 49 events with Cas9 but no sgRNA, and analyzed all events by Sanger Sequencing of both alleles. Out of the 684 amplicons from events with sgRNAs, 474 had mutations in both alleles (77.5%). We sequenced both AG paralogs for 71 events in INRA clone 717-1B4 and 22 events in INRA clone 353-53, and found that 67 (94.4%) and 21 (95.5%) were double locus knockouts. Due partly to a single nucleotide polymorphism (SNP) present in the target region, one sgRNA targeting the AG paralogs was found to be completely inactive by itself (0%) but showed some activity in generating deletions when used in a construct with a second sgRNA (10.3-24.5%). Small insertion/deletion (indel) mutations were prevalent among mutated alleles of events with only one sgRNA (ranging from 94.3 to 99.1%), while large deletions were prevalent among alleles with two active sgRNAs (mean proportion of mutated alleles was 22.6% for small indels vs. 77.4% for large indels). For both LFY and AG, each individual sgRNA-gene combination had a unique mutation spectrum ($p < 0.001$). An AG-sgRNA construct with two sgRNAs had similar mutation spectra among two poplar clones ($p > 0.05$), however, a LFY-sgRNA construct with a single sgRNA gave significantly different mutation spectra among the same two clones ($p < 0.001$). The 49 empty vector control events had no mutations in either allele, and 310 potential "off-target" sequences also had no mutations in 58 transgenic events studied. CRISPR/Cas9 is a very powerful and precise system for generating loss-of-function mutations in poplars, and should be effective for generating reliably infertile trees that may promote regulatory, market, or public acceptance of	Populus
449	plant	poplar (Populus spp.)	CRISPR;Cas9;	JMJ25	Plant journal	Histone H3K9 demethylase JMJ25 epigenetically modulates anthocyanin biosynthesis in poplar.	2018	96(6):1121-1136	[Fan D et al.]	Southwest University, Chongqing, China.	30218582	10.1111/tpj.14092	Anthocyanins are involved in several aspects of development and defence in poplar (Populus spp.). Although, over the past decades, significant progress has been made in uncovering these anthocyanin biosynthetic and regulatory mechanisms, the fundamental understanding of the epigenetic regulation in this pathway is still largely unclear. Here, we isolated a histone H3K9 demethylase gene JMJ25 from Populus and characterized its role in anthocyanin biosynthesis by genetic and biochemical approaches. JMJ25 was induced by continuous dark treatment. Overexpression of JMJ25 led to downregulated expression of anthocyanin biosynthetic genes in transgenic poplar, resulting in a significant reduction in anthocyanin content. ChIP-qPCR assays showed that JMJ25 could directly associate with MYB182 chromatin and dynamically demethylate at H3K9me2. Furthermore, JMJ25 also affected the DNA methylation levels of MYB182. By contrast, knockout of JMJ25 by CRISPR/Cas9 resulted in ectopic anthocyanin accumulation under dark condition and increased expression of anthocyanin biosynthetic genes. Our results support a model in which JMJ25 directly affects MYB182 expression by altering the histone methylation status of its chromatin and DNA methylation, resulting in repression of anthocyanin accumulation. This study uncovered an epigenetic mechanism that modulates	Populus
450	plant	Populus	CRISPR;Cas9;	BRANCHED1-1; BRANCHED2-1 candidate genes	Tree physiology	CRISPR/Cas9-mediated knockout of Populus BRANCHED1 and BRANCHED2 orthologs reveals a major function in bud outgrowth control.	2018	38(10):1588-1597	[Muhr M et al.]	Georg-August-University, Gottingen, Germany.	30265349	10.1093/treephys/tpy088	The TCP-type transcription factors BRANCHED1 and BRANCHED2 shape plant architecture by suppressing bud outgrowth, with BRANCHED2 only playing a minor role in Arabidopsis. Here, we investigated the function of orthologs of these genes in the model tree Populus. We used CRISPR/Cas9 to generate loss-of-function mutants of previously identified Populus BRANCHED1-1 and BRANCHED2-1 candidate genes. BRANCHED1-1 mutants exhibited strongly enhanced bud outgrowth. BRANCHED2-1 mutants had an extreme bud outgrowth phenotype and possessed two ectopic leaves at each node. While BRANCHED1 function is conserved in poplar, BRANCHED2, in contrast to its Arabidopsis counterpart, plays an even more critical role in bud outgrowth regulation. In addition, we identified a new, not yet reported association of	Populus
451	plant	Populus tomentosa	CRISPR;Cas9;	cytochrome P450 protein (PtoDWF4)	Tree physiology	Molecular cloning and characterization of a brassinosteriod biosynthesis-related gene PtoDWF4 from Populus tomentosa.	2018	38(9):1424-1436	[Shen Y et al.]	Southwest University, Chongqing, China.	29579304	10.1093/treephys/tpy027	Brassinosteroids (BRs) as steroid hormones play an important role in plant growth and development. However, little is known about how BRs affect secondary wall biosynthesis in woody plants. In this study, we cloned and characterized PtoDWF4, a homologus gene of Arabidopsis DWF4 encoding a cytochrome P450 protein, from Populus tomentosa. qRT-PCR analysis showed that PtoDWF4 was highly expressed in stems, especially in xylem. Overexpression of PtoDWF4 (PtoDWF4-OE) in poplar promoted growth rate and biomass yield, increased area and cell layers of xylem. Transgenic plants showed a significant increase in plant height and stem diameter compared with the wild type. In contrast, the CRISPR/Cas9-generated mutation of PtoDWF4 (PtoDWF4-KO) resulted in significantly decreased biomass production in transgenic plants. Further studies revealed that constitutive expression of PtoDWF4 up-regulated the expression of secondary cell wall (SCW) biosynthesis-related genes, whereas knock-out of PtoDWF4 down-regulated their expression. Quantitative analysis of cell wall components showed a significant increase in PtoDWF4-OE lines but a reduction in PtoDWF4-KO lines compared with wild-type plants. Taken together, our results indicate that PtoDWF4 plays a positive role in improving growth rate and	Populus
452	plant	potato	Cas9;	coilin; phytoene desaturase	Doklady. Biochemistry and biophysics	Guide RNA Design for CRISPR/Cas9-Mediated Potato Genome Editing.	2018	479(1):90-94	[Khromov AV et al.]	OOO Doka Gene Technologies, Moscow oblast, Russia.	29779105	10.1134/S1607672918020084	The activity of the pool of sgRNA molecules designed for different regions of potato coilin and phytoene desaturase genes was compared in vitro. Due to the presence of nucleotides unpaired with DNA, sgRNA is able not only to inhibit but also to stimulate the activity of the Cas9-sgRNA complex in vitro. Although the first six nucleotides located in the DNA substrate proximally to the PAM site at the 3' end are the binding sites for cas9, they had no significant effect on the activity of the Cas9-sgRNA	potato
453	plant	potato	CRISPR;Cas9;	S-RNase	Nature plants	Generation of self-compatible diploid potato by knockout of S-RNase.	2018	4(9):651-654	[Ye M et al.]	Yunnan Normal University, Kunming, China.	30104651	10.1038/s41477-018-0218-6	Re-domestication of potato into an inbred line-based diploid crop propagated by seed represents a promising alternative to traditional clonal propagation of tetraploid potato, but self-incompatibility has hindered the development of inbred lines. To address this problem, we created self-compatible diploid potatoes by knocking out the self-incompatibility gene S-RNase using the CRISPR-Cas9 system. This strategy opens new avenues for diploid potato breeding and will also be useful for studying other self-	potato

454	plant	potato	CRISPR;Cas9;	steroid 16alpha-hydroxylase (St16DOX)	Plant physiology and biochemistry	Generation of alpha-solanine-free hairy roots of potato by CRISPR/Cas9 mediated genome editing of the St16DOX gene.	2018	131:70-77	[Nakayasu M et al.]	Kobe University, Kobe, Hyogo, Japan.	29735370	10.1016/j.plaphy.2018.04.026	Potato (Solanum tuberosum) is a major food crop, while the most tissues of potato accumulates steroidal glycoalkaloids (SGAs) alpha-solanine and alpha-chaconine. Since SGAs confer a bitter taste on human and show the toxicity against various organisms, reducing the SGA content in the tubers is requisite for potato breeding. However, generation of SGA-free potato has not been achieved yet, although silencing of several SGA biosynthetic genes led a decrease in SGAs. Here, we show that the knockout of St16DOX encoding a steroid 16alpha-hydroxylase in SGA biosynthesis causes the complete abolition of the SGA accumulation in potato hairy roots. Nine candidate guide RNA (gRNA) target sequences were selected from St16DOX by in silico analysis, and the two or three gRNAs were introduced into a CRISPR/Cas9 vector designated as pMgP237-2A-GFP that can express multiplex gRNAs based on the pre-tRNA processing system. To establish rapid screening of the candidate gRNAs that can efficiently mutate the St16DOX gene, we used a potato hairy root culture system for the introduction of the pMgP237 vectors. Among the transgenic hairy roots, two independent lines showed no detectable SGAs but accumulated the glycosides of 22,26-dihydroxycholesterol, which is the substrate of St16DOX. Analysis of the two lines with sequencing exhibited the mutated sequences of St16DOX with no wild-type sequences. Thus, generation of SGA-free hairy roots of tetraploid potato was achieved by the combination of the hairy root culture and the pMgP237-2A-GFP vector. This experimental system is useful to evaluate the efficacy of candidate gRNA target	potato
455	plant	potato	CRISPR;Cas9;	granule-bound starch synthase I	Scientific reports	Establishment of a modified CRISPR/Cas9 system with increased mutagenesis frequency using the translational enhancer dMac3 and multiple guide RNAs in potato.	2018	8(1):13753	[Kusano H et al.]	Tokyo University of Science, Tokyo, Japan.	30214055	10.1038/s41598-018-32049-2	CRISPR/Cas9 is a programmable nuclease composed of the Cas9 protein and a guide RNA (gRNA) molecule. To create a mutant potato, a powerful genome-editing system was required because potato has a tetraploid genome. The translational enhancer dMac3, consisting of a portion of the OsMac3 mRNA 5'-untranslated region, greatly enhanced the production of the protein encoded in the downstream ORF. To enrich the amount of Cas9, we applied the dMac3 translational enhancer to the Cas9 expression system with multiple gRNA genes. CRISPR/Cas9 systems targeting the potato granule-bound starch synthase I (GBSSI) gene examined the frequency of mutant alleles in transgenic potato plants. The efficiency of the targeted mutagenesis strongly increased when the dMac3-installed Cas9 was used. In this case, the ratio of transformants containing four mutant alleles reached approximately 25% when estimated by CAPS analysis. The mutants that exhibited targeted mutagenesis in the GBSSI gene showed characteristics of low amylose starch in their tubers. This result suggests that our system may facilitate genome-editing events in polyploid plants.	potato
456	plant	potato (Solanum tuberosum)	CRISPR;Cas9;	granule bound starch synthase (GBSS)	Physiologia plantarum	Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery.	2018	164(4):378-384	[Andersson M et al.]	Swedish University of Agricultural Sciences, Alnarp, Sweden.	29572864	10.1111/ppl.12731	Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein-9 (CRISPR-Cas9) can be used as an efficient tool for genome editing in potato (Solanum tuberosum). From both a scientific and a regulatory perspective, it is beneficial if integration of DNA in the potato genome is avoided. We have implemented a DNA-free genome editing method, using delivery of CRISPR-Cas9 ribonucleoproteins (RNPs) to potato protoplasts, by targeting the gene encoding a granule bound starch synthase (GBSS, EC 2.4.1.242). The RNP method was directly implemented using previously developed protoplast isolation, transfection and regeneration protocols without further adjustments. Cas9 protein was preassembled with RNA produced either synthetically or by in vitro transcription. RNP with synthetically produced RNA (cr-RNP) induced mutations, i.e. indels, at a frequency of up to 9%, with all mutated lines being transgene-free. A mutagenesis frequency of 25% of all regenerated shoots was found when using RNP with in vitro transcriptionally produced RNA (IVT-RNP). However, more than 80% of the shoots with confirmed mutations had unintended inserts in the cut site, which was in the same range as when using DNA delivery. The inserts originated both from DNA template remnants from the in vitro transcription, and from chromosomal potato DNA. In 2-3% of the regenerated shoots from the RNP-experiments, mutations were induced in all four alleles resulting in a complete knockout	potato (Sola
457	plant	radish	Agroinfiltration;	staphylococcal enteroxin B	Journal of immunology research	Leaf-Encapsulated Vaccines: Agroinfiltration and Transient Expression of the Antigen Staphylococcal Endotoxin B in Radish Leaves.	2018	2018:3710961	[Liu PF et al.]	University of California, San Diego, CA, USA.	29577048	10.1155/2018/3710961	Transgene introgression is a major concern associated with transgenic plant-based vaccines. Agroinfiltration can be used to selectively transform nonreproductive organs and avoid introgression. Here, we introduce a new vaccine modality in which Staphylococcal enterotoxin B (SEB) genes are agroinfiltrated into radishes (Raphanw sativus L.), resulting in transient expression and accumulation of SEB in planta. This approach can simultaneously express multiple antigens in a single leaf. Furthermore, the potential of high-throughput vaccine production was demonstrated by simultaneously agroinfiltrating multiple radish leaves using a multichannel pipette. The expression of SEB was detectable in two leaf cell types (epidermal and guard cells) in agroinfiltrated leaves. ICR mice intranasally immunized with homogenized leaves agroinfiltrated with SEB elicited detectable antibody to SEB and displayed protection against SEB-induced interferon-gamma (IFN-gamma) production. The concept of encapsulating antigens in leaves rather than purifying them for immunization may	radish
458	plant	rice	CRISPR;Cas9;	DEP1	BMC biology	Genome sequencing of rice subspecies and genetic analysis of recombinant lines reveals regional yield- and quality-associated loci.	2018	16(1):102	[Li X et al.]	Shenyang Agricultural University, Shenyang, China.	30227868	10.1186/s12915-018-0572-x	BACKGROUND: Two of the most widely cultivated rice strains are Oryza sativa indica and O. sativa japonica, and understanding the genetic basis of their agronomic traits is of importance for crop production. These two species are highly distinct in terms of geographical distribution and morphological traits. However, the relationship among genetic background, ecological conditions, and agronomic traits is unclear. RESULTS: In this study, we performed the de novo assembly of a high-quality genome of SN265, a cultivar that is extensively cultivated as a backbone japonica parent in northern China, using single-molecule sequencing. Recombinant inbred lines (RILs) derived from a cross between SN265 and R99 (indica) were re-sequenced and cultivated in three distinct ecological conditions. We identify 79 QTLs related to 15 agronomic traits. We found that several genes underwent functional alterations when the ecological conditions were changed, and some alleles exhibited contracted responses to different genetic backgrounds. We validated the involvement of one candidate gene, DEP1, in determining panicle length, using CRISPR/Cas9 gene editing. CONCLUSIONS: This study provides information on the suitable environmental conditions, and genetic background, for functional genes in rice breeding. Moreover, the public availability of the reference genome of northern japonica SN265 provides a valuable resource for	rice

459	plant	rice	CRISPR;Cas9;	osmotic stress/ABA-activated protein kinase 1 (SAPK1); SAPK2	BMC plant biology	The sucrose non-fermenting-1-related protein kinases SAPK1 and SAPK2 function collaboratively as positive regulators of salt stress tolerance in rice.	2018	18(1):203	[Lou D et al.]	Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan, China.	30236054	10.1186/s12870-018-1408-0	BACKGROUND: The sucrose non-fermenting-1-related protein kinase 2 family (SnRK2s) unifies different abiotic stress signals in plants. To date, the functions of two rice SnRK2s, osmotic stress/ABA-activated protein kinase 1 (SAPK1) and SAPK2, have been unknown. We investigated their roles in response to salt stress by generating loss-of-function lines using the CRISPR/Cas9 system and by overexpressing these proteins in transgenic rice plants. RESULTS: Expression profiling revealed that SAPK1 and SAPK2 expression were strongly induced by drought, NaCl, and PEG treatment, but not by ABA. SAPK2 expression was highest in the leaves, followed by the roots, whereas SAPK1 was highest expressed in roots followed by leaves. Both proteins were localized to the nucleus and the cytoplasm. Under salt stress, sapk1, sapk2 and, in particular, sapk1/2 mutants, exhibited reduced germination rates, more severe growth inhibition, more distinct chlorosis, reduced chlorophyll contents, and reduced survival rates in comparison with the wild-type plants. In contrast, SAPK1- and SAPK2-overexpression lines had increased germination rates and reduced sensitivities to salt; including mild reductions in growth inhibition, reduced chlorosis, increased chlorophyll contents and improved survival rates in comparison with the wild-type plants. These results suggest that SAPK1 and SAPK2 may function collaboratively as positive regulators of salt stress tolerance at the germination and seedling stages. We also found that SAPK1 and SAPK2 affected the osmotic potential following salt stress by promoting the generation of osmotically active metabolites such as proline. SAPK1 and SAPK2 also improved reactive oxygen species (ROS) detoxification following salt stress by promoting the generation of ROS scavengers such as ascorbic acid, and by increasing the expression levels of proteins such as superoxide dismutase (SOD) and catalase (CAT). SAPK1 and SAPK2 may function collaboratively in reducing Na(+) toxicity by affecting the Na(+) distribution between roots and shoots, Na(+) exclusion from the cytoplasm, and Na(+) sequestration into the vacuoles. These effects may be facilitated through the expression of Na(+)-and K(+)-homeostasis-related genes. CONCLUSION: SAPK1 and SAPK2 may function collaboratively as positive regulators of salt stress tolerance at the germination and	rice
460	plant	rice	CRISPR;Cas9;	CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7)	BMC plant biology	Engineering plant architecture via CRISPR/Cas9-mediated alteration of strigolactone biosynthesis.	2018	18(1):174	[Butt H et al.]	King Abdullah University of Science and Technology, Thuwal, Saudi Arabia.	30157762	10.1186/s12870-018-1387-1	BACKGROUND: Precision plant genome engineering holds much promise for targeted improvement of crop traits via unprecedented single-base level control over the genetic material. Strigolactones (SLs) are a key determinant of plant architecture, known for their role in inhibiting shoot branching (tillering). RESULTS: We used CRISPR/Cas9 in rice (Oryza sativa) for targeted disruption of CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7), which controls a key step in SL biosynthesis. The ccd7 mutants exhibited a striking increase in tillering, combined with a reduced height, which could be rescued by application of the synthetic SL analog GR24. Striga germination assays and liquid chromatography-mass spectrometry analysis showed that root exudates of ccd7 mutants were also SL deficient. CONCLUSIONS: Taken together, our results show the potential and feasibility of the use of the CRISPR/Cas9 system for targeted engineering of plant architecture and for elucidating the molecular	rice
461	plant	rice	CRISPR;Cas9;	G2-like transcription factor, OsPHL3	BMC plant biology	Identification of a G2-like transcription factor, OsPHL3, functions as a negative regulator of flowering in rice by co-expression and reverse genetic analysis.	2018	18(1):157	[Zeng L et al.]	Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China.	30081823	10.1186/s12870-018-1382-6	BACKGROUND: Flowering time is a key trait for regional adaption and seed production in rice (Oryza sativa L.). Forward and reverse genetic studies have characterized a number of flowering-time genes. However, co-expression analysis has not been used to identify the flowering-time genes. RESULTS: We predicted a G2-like family transcription factor, OsPHL3, by co-expression networks analysis with photoperiodic flowering pathway genes. OsPHL3 contains a MYB-CC domain, and was localized in the nucleus with transcriptional activation potential. OsPHL3 was mainly expressed in the leaves and exhibited a circadian rhythmic expression pattern. Rice lines overexpressing OsPHL3 showed a delayed flowering time in the genetic background of TP309 under both long-day (Beijing) and short-day (Hainan) conditions. By contrast, the knockout rice lines of OsPHL3 by CRISPR/Cas9 technology promoted flowering time regardless of genetic backgrounds (i.e. Nipponbare and TP309) or day length. Further analysis indicated that OsPHL3 delayed flowering time by down-regulating the expression of Hd3a and RFT1 through promoting Hd1 under long-day conditions (LDs), or suppressing Ehd1/Hd1 under short-day conditions (SDs). CONCLUSIONS: Our results suggested that co-expression analysis is a useful strategy for identifying novel	rice
462	plant	rice	CRISPR;Cas9;	acetolactate synthase (ALS)	Data in brief	Herbicide tolerance-assisted multiplex targeted nucleotide substitution in rice.	2018	20:1325-1331	[Shimatani Z et al.]	Kobe University, Kobe, Hyogo, Japan.	30246111	10.1016/j.dib.2018.08.124	Acetolactate synthase (ALS) catalyzes the initial step in the biosynthesis of branched-chain amino acids, and is highly conserved from bacteria to higher plants. ALS is encoded by a single copy gene in rice genome and is a target enzyme of several classes of herbicides. Although ALS mutations conferring herbicide-resistance property to plants are well documented, effect of Imazamox (IMZ) on rice and the mutations in ALS correlated with IMZ tolerance were unclear. In this article, the effect of IMZ on rice calli and seedlings in tissue culture conditions were evaluated. Also, the ALSA96V mutation was confirmed to improve IMZ tolerance of rice calli. Based on these results, ALS-assisted multiplex targeted base editing in rice was demonstrated in combination with Target-AID, a CRISPR/Cas9-cytidine deaminase fusion system [1].	rice
463	plant	rice	CRISPR;Cas9;	ERECTA genes (OsER1; OsER2)	Frontiers in plant science	Phylogenetic and CRISPR/Cas9 Studies in Deciphering the Evolutionary Trajectory and Phenotypic Impacts of Rice ERECTA Genes.	2018	9:473	[Zhang Y et al.]	Nanjing University, Nanjing, China.	29692796	10.3389/fpls.2018.00473	The ERECTA family genes (ERfs) have been found to play diverse functions in Arabidopsis, including controlling cell proliferation and cell growth, regulating stomata patterning, and responding to various stresses. This wide range of functions has rendered them as a potential candidate for crop improvement. However, information on their functional roles, particularly their morphological impact, in crop genomes, such as rice, is limited. Here, through evolutionary prediction, we first depict the evolutionary trajectory of the ER family, and show that the ER family is actually highly conserved across different species, suggesting that most of their functions may also be observed in other plant species. We then take advantage of the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats-associated nuclease 9) system to assess their morphological impact on one of the most important crops, rice. Loss-of-function mutants of OsER1 and OsER2 display shortened plant stature and reduced panicle size, suggesting they possibly also functioned in regulating cell proliferation and cell growth in rice. In addition to functions similar to that in Arabidopsis, we also find clues that rice ERfs may play unique functional roles. The OsER2 displayed more severe phenotypic changes than OsER1, indicating putative differentiation in their functions. The OsERL might be of essential in its function, and the proper function of all three rice ER genes might be dependent of their genetic background. Future investigations relating to these functions are key to exploiting ERfs in crop	rice

464	plant	rice	CRISPR;Cas9;	OsABA2	Frontiers in plant science	Mutation in Rice Abscisic Acid2 Results in Cell Death, Enhanced Disease-Resistance, Altered Seed Dormancy and Development.	2018	9:405	[Liao Y et al.]	Sichuan Agricultural University, Sichuan, China.	29643863	10.3389/fpls.2018.00405	Lesion mimic mutants display spontaneous cell death, and thus are valuable for understanding the molecular mechanism of cell death and disease resistance. Although a lot of such mutants have been characterized in rice, the relationship between lesion formation and abscisic acid (ABA) synthesis pathway is not reported. In the present study, we identified a rice mutant, lesion mimic mutant 9150 (lmm9150), exhibiting spontaneous cell death, pre-harvest sprouting, enhanced growth, and resistance to rice bacterial and blast diseases. Cell death in the mutant was accompanied with excessive accumulation of H2O2. Enhanced disease resistance was associated with cell death and upregulation of defense-related genes. Map-based cloning identified a G-to-A point mutation resulting in a D-to-N substitution at the amino acid position 110 of OsABA2 (LOC_Os03g59610) in lmm9150. Knock-out of OsABA2 through CRISPR/Cas9 led to phenotypes similar to those of lmm9150. Consistent with the function of OsABA2 in ABA biosynthesis, ABA level in the lmm9150 mutant was significantly reduced. Moreover, exogenous application of ABA could rescue all the mutant phenotypes of lmm9150. Taken together, our data linked ABA deficiency to cell death	rice
465	plant	rice	CRISPR;Cas9;Cpf1;		Genome biology	A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice.	2018	19(1):84	[Tang X et al.]	University of Electronic Science and Technology of China, Chengdu, China.	29973285	10.1186/s13059-018-1458-5	BACKGROUND: Targeting specificity has been a barrier to applying genome editing systems in functional genomics, precise medicine and plant breeding. In plants, only limited studies have used whole-genome sequencing (WGS) to test off-target effects of Cas9. The cause of numerous discovered mutations is still controversial. Furthermore, WGS-based off-target analysis of Cpf1 (Cas12a) has not been reported in any higher organism to date. RESULTS: We conduct a WGS analysis of 34 plants edited by Cas9 and 15 plants edited by Cpf1 in T0 and T1 generations along with 20 diverse control plants in rice. The sequencing depths range from 45x to 105x with read mapping rates above 96%. Our results clearly show that most mutations in edited plants are created by the tissue culture process, which causes approximately 102 to 148 single nucleotide variations (SNVs) and approximately 32 to 83 insertions/deletions (indels) per plant. Among 12 Cas9 single guide RNAs (sgRNAs) and three Cpf1 CRISPR RNAs (crRNAs) assessed by WGS, only one Cas9 sgRNA resulted in off-target mutations in T0 lines at sites predicted by computer programs. Moreover, we cannot find evidence for bona fide off-target mutations due to continued expression of Cas9 or Cpf1 with guide RNAs in T1 generation. CONCLUSIONS: Our comprehensive and rigorous analysis of WGS data across multiple sample types suggests both Cas9 and Cpf1 nucleases are very specific in generating targeted DNA modifications and off-targeting	rice
466	plant	rice	CRISPR;Cas9;	asparagine synthetase 1 (OsASN1)	International journal of molecular sciences	OsASN1 Plays a Critical Role in Asparagine-Dependent Rice Development.	2018	20(1)	[Luo L et al.]	Nanjing Agricultural University, Nanjing, China.	30602689	10.3390/ijms20010130	Asparagine is one of the important amino acids for long-distance transport of nitrogen (N) in plants. However, little is known about the effect of asparagine on plant development, especially in crops. Here, a new T-DNA insertion mutant, asparagine synthetase 1 (asn1), was isolated and showed a different plant height, root length, and tiller number compared with wild type (WT). In asn1, the amount of asparagine decreased sharply while the total nitrogen (N) absorption was not influenced. In later stages, asn1 showed reduced tiller number, which resulted in suppressed tiller bud outgrowth. The relative expression of many genes involved in the asparagine metabolic pathways declined in accordance with the decreased amino acid concentration. The CRISPR/Cas9 mutant lines of OsASN1 showed similar phenotype with asn1. These results suggest that OsASN1 is involved in the regulation of rice development and is	rice
467	plant	rice	CRISPR;Cas9;	glycerol-3-phosphate acyltransferase (OsGPAT3)	International journal of molecular sciences	OsGPAT3 Plays a Critical Role in Anther Wall Programmed Cell Death and Pollen Development in Rice.	2018	19(12)	[Sun L et al.]	China National Rice Research Institute, Hangzhou, China.	30545137	10.3390/ijms19124017	In flowering plants, ideal male reproductive development requires the systematic coordination of various processes, in which timely differentiation and degradation of the anther wall, especially the tapetum, is essential for both pollen formation and anther dehiscence. Here, we show that OsGPAT3, a conserved glycerol-3-phosphate acyltransferase gene, plays a critical role in regulating anther wall degradation and pollen exine formation. The gpat3-2 mutant had defective synthesis of Ubisch bodies, delayed programmed cell death (PCD) of the inner three anther layers, and abnormal degradation of micropores/pollen grains, resulting in failure of pollen maturation and complete male sterility. Complementation and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) experiments demonstrated that OsGPAT3 is responsible for the male sterility phenotype. Furthermore, the expression level of tapetal PCD-related and nutrient metabolism-related genes changed significantly in the gpat3-2 anthers. Based on these genetic and cytological analyses, OsGPAT3 is proposed to coordinate the differentiation and degradation of the anther wall and pollen grains in addition to regulating lipid biosynthesis. This study provides insights for understanding the function of GPATs in regulating rice male reproductive development, and also lays a theoretical basis for hybrid rice breeding.	rice
468	plant	rice	CRISPR;Cas9;	White Belly 1 (WB1)	International journal of molecular sciences	WB1, a Regulator of Endosperm Development in Rice, Is Identified by a Modified MutMap Method.	2018	19(8)	[Wang H et al.]	China National Rice Research Institute, Hangzhou, Zhejiang, China.	30042352	10.3390/ijms19082159	Abnormally developed endosperm strongly affects rice (Oryza sativa) appearance quality and grain weight. Endosperm formation is a complex process, and although many enzymes and related regulators have been identified, many other related factors remain largely unknown. Here, we report the isolation and characterization of a recessive mutation of White Belly 1 (WB1), which regulates rice endosperm development, using a modified MutMap method in the rice mutant wb1. The wb1 mutant develops a white-belly endosperm and abnormal starch granules in the inner portion of white grains. Representative of the white-belly phenotype, grains of wb1 showed a higher grain chalkiness rate and degree and a lower 1000-grain weight (decreased by ~34%), in comparison with that of Wild Type (WT). The contents of amylose and amylopectin in wb1 significantly decreased, and its physical properties were also altered. We adopted the modified MutMap method to identify 2.52 Mb candidate regions with a high specificity, where we detected 275 SNPs in chromosome 4. Finally, we identified 19 SNPs at 12 candidate genes. Transcript levels analysis of all candidate genes showed that WB1 (Os04t0413500), encoding a cell-wall invertase, was the most probable cause of white-belly endosperm phenotype. Switching off WB1 with the CRISPR/cas9 system in Japonica cv. Nipponbare demonstrates that WB1 regulates endosperm development and that different mutations of WB1 disrupt its biological function. All of these results taken together suggest that the wb1 mutant is controlled by the mutation of WB1, and that the modified MutMap method is feasible to identify	rice

469	plant	rice	CRISPR;Cas9;	ERF domain protein FZP	Journal of experimental botany	FZP determines grain size and sterile lemma fate in rice.	2018	69(20):4853-4866	[Ren D et al.]	China National Rice Research Institute, Hangzhou, China.	30032251	10.1093/jxb/ery264	In grass, the spikelet is a unique inflorescence structure that directly determines grain yield. Despite a great deal of research, the molecular mechanisms behind spikelet development are not fully understood. In the study, FZP encodes an ERF domain protein, and functions in grain size and sterile lemma identity. Mutation of FZP causes smaller grains and degenerated sterile lemmas. The small fzp-12 grains were caused by a reduction in cell number and size in the hulls. Interestingly, the sterile lemma underwent a homeotic transformation into a rudimentary glume in the fzp-12 and fzp-13 mutants, whereas the sterile lemma underwent a homeotic transformation into a lemma in FZP over-expressing plants, suggesting that FZP specifically determines the sterile lemma identity. We confirmed the function of FZP by complementation, CRISPR-Cas9 gene editing, and cytological and molecular tests. Additionally, FZP interacts specifically with the GCC-box and DRE motifs, and may be involved in regulation of the downstream genes. Our results revealed that FZP plays a vital role in the regulation of grain size, and first provides clear evidence in support of the hypothesis that the lemma, rudimentary glume, and sterile lemma are homologous organs.	rice
470	plant	rice	CRISPR;Cpf1;	ALS	Journal of experimental botany	Synthesis-dependent repair of Cpf1-induced double strand DNA breaks enables targeted gene replacement in rice.	2018	69(20):4715-4721	[Li S et al.]	Institute of Crop Sciences (ICS), Chinese Academy of Agricultural Sciences (CAAS), Beijing, China.	29955893	10.1093/jxb/ery245	The recently developed CRISPR (clustered regularly interspaced short palindromic repeats)/Cpf1 system expands the range of genome editing and is emerging as an alternative powerful tool for both plant functional genomics and crop improvement. Cpf1-CRISPR RNA (crRNA) produces double strand DNA breaks (DSBs) with long 5'-protruding ends, which may facilitate the pairing and insertion of repair templates through homology-directed repair (HDR) for targeted gene replacement and introduction of the desired DNA elements at specific gene loci for crop improvement. However, the potential mechanism underlying HDR of DSBs generated by Cpf1-crRNA remains to be investigated, and the inherent low efficiency of HDR and poor availability of exogenous donor DNA as repair templates strongly impede the use of HDR for precise genome editing in crop plants. Here, we provide evidence of synthesis-dependent repair of Cpf1-induced DSBs, which enables us precisely to replace the wild-type ALS gene with the intended mutant version that carries two discrete point mutations conferring herbicide resistance to rice plants. Our observation that the donor repair template (DRT) with only the left homologous arm is sufficient for precise targeted allele replacement offers a better understanding of the mechanism underlying HDR in plants, and greatly simplifies the design of DRTs for precision genome editing in	rice
471	plant	rice	CRISPR;Cas9;	an important subunit of the exocyst complex (OsSEC3A)	Journal of experimental botany	Disruption of OsSEC3A increases the content of salicylic acid and induces plant defense responses in rice.	2018	69(5):1051-1064	[Ma J et al.]	Peking University, Beijing, China.	29300985	10.1093/jxb/erx458	The exocyst, an evolutionarily conserved octameric protein complex involved in exocytosis, has been reported to be involved in diverse aspects of morphogenesis in Arabidopsis. However, the molecular functions of such exocytotic molecules in rice are poorly understood. Here, we examined the molecular function of OsSEC3A, an important subunit of the exocyst complex in rice. The OsSEC3A gene is expressed in various organs, and OsSEC3A has the potential ability to participate in the exocyst complex by interacting with several other exocyst subunits. Disruption of OsSEC3A by CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) caused dwarf stature and a lesion-mimic phenotype. The Ossec3a mutant exhibited enhanced defense responses, as shown by up-regulated transcript levels of pathogenesis- and salicylic acid synthesis-related genes, increased levels of salicylic acid, and enhanced resistance to the fungal pathogen Magnaporthe oryzae. Subcellular localization analysis demonstrated that OsSEC3A has a punctate distribution with the plasma membrane. In addition, OsSEC3A interacted with rice SNAP25-type t-SNARE protein OsSNAP32, which is involved in rice blast resistance, via the C-terminus and bound to phosphatidylinositol lipids, particularly phosphatidylinositol-3-phosphate, through its N-terminus. These findings uncover the	rice
472	plant	rice	CRISPR;Cas9;Cpf1;		Journal of integrative plant biology	Multiplex gene editing in rice with simplified CRISPR-Cpf1 and CRISPR-Cas9 systems.	2018	60(8):626-631	[Wang M et al.]	Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.	29762900	10.1111/jipb.12667	We developed simplified single transcriptional unit (SSTU) CRISPR systems for multiplex gene editing in rice using FnCpf1, LbCpf1 or Cas9, in which the nuclease and its crRNA array are co-expressed from a single Pol II promoter, without any additional processing machinery. Our SSTU systems are easy to construct and effective in mediating multiplex genome editing.	rice
473	plant	rice	CRISPR;Cas9;	NRT1.1B	Journal of integrative plant biology	Efficient allelic replacement in rice by gene editing: A case study of the NRT1.1B gene.	2018	60(7):536-540	[Li J et al.]	Institute of Crop Sciences (ICS), Chinese Academy of Agricultural Sciences (CAAS), Beijing, China.	29575650	10.1111/jipb.12650	Precise replacement of an existing allele in commercial cultivars with an elite allele is a major goal in crop breeding. A single nucleotide polymorphism in the NRT1.1B gene between japonica and indica rice is responsible for the improved nitrogen use efficiency in indica rice. Herein, we precisely replaced the japonica NRT1.1B allele with the indica allele, in just one generation, using CRISPR/Cas9 gene-editing technology. No additional selective pressure was needed to enrich the precise replacement events. This work demonstrates the feasibility of replacing any genes with elite alleles within one generation, greatly expanding our ability to improve agriculturally important traits.	rice
474	plant	rice	CRISPR;Cas9;	Waxy	Journal of integrative plant biology	Generation of new glutinous rice by CRISPR/Cas9-targeted mutagenesis of the Waxy gene in elite rice varieties.	2018	60(5):369-375	[Zhang J et al.]	Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.	29210506	10.1111/jipb.12620	In rice, amylose content (AC) is controlled by a single dominant Waxy gene. We used Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9) to introduce a loss-of-function mutation into the Waxy gene in two widely cultivated elite japonica varieties. Our results show that mutations in the Waxy gene reduce AC and convert the rice into glutinous ones without affecting other desirable agronomic traits, offering an effective and easy strategy to improve glutinosity in elite varieties. Importantly, we successfully removed the transgenes from the progeny. Our study provides an example of generating improved crops with potential for commercialization, by editing a gene of interest directly in elite crop	rice

475	plant	rice	CRISPR;	FRUCTOKINASE-LIKE PROTEIN (OsFLN1)	Journal of integrative plant biology	FRUCTOKINASE-LIKE PROTEIN 1 interacts with TRXz to regulate chloroplast development in rice.	2018	60(2):94-111	[He L et al.]	China National Rice Research Institute, Hangzhou, China.	29319227	10.1111/jipb.12631	Chloroplast genes are transcribed by the plastid-encoded RNA polymerase (PEP) or nucleus-encoded RNA polymerase. FRUCTOKINASE-LIKE PROTEINS (FLNs) are phosphofructokinase-B (PfkB)-type carbohydrate kinases that act as part of the PEP complex; however, the molecular mechanisms underlying FLN activity in rice remain elusive. Previously, we identified and characterized a heat-stress sensitive albino (hsa1) mutant in rice. Map-based cloning revealed that HSA1 encodes a putative OsFLN2. Here, we further demonstrated that knockdown or knockout of the OsFLN1, a close homolog of HSA1/OsFLN2, considerably inhibits chloroplast biogenesis and the fln1 knockout mutants, created by clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associate protein 9, exhibit severe albino phenotype and seedling lethality. Moreover, OsFLN1 localizes to the chloroplast. Yeast two-hybrid, pull-down and bimolecular fluorescence complementation experiments revealed that OsFLN1 and HSA1/OsFLN2 interact with THIOREDOXINZ (OsTRXz) to regulate chloroplast development. In agreement with this, knockout of OsTRXz resulted in a similar albino and seedling lethality phenotype to that of the fln1 mutants. Quantitative reverse transcription polymerase chain reaction and immunoblot analysis revealed that the transcription and translation of PEP-dependent genes were strongly inhibited in fln1 and trxz mutants, indicating that loss of OsFLN1, HSA1/OsFLN2, or OsTRXz function perturbs the stability of the transcriptionally active chromosome complex and PEP activity. These results show that OsFLN1 and HSA1/OsFLN2 contribute to	rice
476	plant	rice	CRISPR;Cas9;		Journal of integrative plant biology	QTL editing confers opposing yield performance in different rice varieties.	2018	60(2):89-93	[Shen L et al.]	Yangzhou University, Yangzhou, China.	27628577	10.1111/jipb.12501	Grain yield is one of the most important and complex trait for genetic improvement in crops; it is known to be controlled by a number of genes known as quantitative trait loci (QTLs). In the past decade, many yield-contributing QTLs have been identified in crops. However, it remains unclear whether those QTLs confer the same yield performance in different genetic backgrounds. Here, we performed CRISPR/Cas9-mediated QTL editing in five widely-cultivated rice varieties and revealed that the same QTL can have diverse, even opposing, effects on grain yield in different genetic	rice
477	plant	rice	CRISPR;	six genes encoding enzymes of acyl-ACP-consuming pathways including PlsX	Metabolic engineering	Diversion of the long-chain acyl-ACP pool in Synechocystis to fatty alcohols through CRISPRi repression of the essential phosphate acyltransferase PlsX.	2018	45:59-66	[Kaczmarzyk D et al.]	KTH - Royal Institute of Technology, Stockholm, Sweden.	29199103	10.1016/j.ymben.2017.11.014	Fatty alcohol production in Synechocystis sp. PCC 6803 was achieved through heterologous expression of the fatty acyl-CoA/ACP reductase Maqu2220 from the bacteria Marinobacter aquaeolei VT8 and the fatty acyl-ACP reductase DPW from the rice Oryza sativa. These platform strains became models for testing multiplex CRISPR-interference (CRISPRi) metabolic engineering strategies to both improve fatty alcohol production and to study membrane homeostasis. CRISPRi allowed partial repression of up to six genes simultaneously, each encoding enzymes of acyl-ACP-consuming pathways. We identified the essential phosphate acyltransferase enzyme PlsX (slr1510) as a key node in C18 fatty acyl-ACP consumption, repression of slr1510 increased octadecanol productivity threefold over the base strain and gave the highest specific titers reported for this host, 10.3mgg(-1) DCW. PlsX catalyzes the first committed step of phosphatidic acid synthesis, and has not been characterized in Synechocystis previously. We found that accumulation of fatty alcohols impaired growth, altered the membrane composition, and caused a build-up of reactive oxygen species.	rice
478	plant	rice	CRISPR;Cas9;	WUSCHEL homeobox2 (WOX2)-like (WOX2L)	Molecular plant	Rice Interploidy Crosses Disrupt Epigenetic Regulation, Gene Expression, and Seed Development.	2018	11(2):300-314	[Wang L et al.]	Nanjing Agricultural University, Nanjing, China.	29269023	10.1016/j.molp.2017.12.006	Seed development in angiosperms requires a 2:1 maternal-to-paternal genome ratio (2m:1p) in the endosperm. When the ratio is disrupted, the seed development is impaired. Rice interploidy crosses result in endosperm failures, but the underlying molecular mechanisms remain unclear. Here, we report that the defective endosperm in rice interploidy crosses was associated with nonadditive expression of small RNAs and protein-coding genes. Interestingly, 24-nt small interfering RNAs were enriched in the 5' and 3' flanking sequences of nonadditively expressed genes in the interploidy crosses and were negatively associated with the expression of imprinted genes. Furthermore, some PRC2 family genes and DNA methylation-related genes including OsMET1b and OsCMT3a were upregulated in the 2x4 cross (pollinating a diploid "mother" with a tetraploid "father") but repressed in the reciprocal cross. These different epigenetic effects could lead to precocious or delayed cellularization during endosperm development. Notably, many endosperm-preferred genes, including starch metabolic and storage protein genes during grain filling, were found to be associated with DNA methylation or H3K27me3, which are repressed in both 2x4 and 4x2 crosses. WUSCHEL homeobox2 (WOX2)-like (WOX2L), an endosperm-preferred gene, was expressed specifically in the rice endosperm, in contrast to WOX2 expression in the Arabidopsis embryo. Disruption of WOX2L in transgenic rice by CRISPR/Cas9-mediated gene editing blocked starch and protein accumulation, resulting in seed abortion. In addition to gene repression, disrupting epigenetic process in the interploidy crosses also induced expression of stress-responsive genes. Thus, maintaining the 2m:1p genome ratio in the endosperm is essential for normal grain development in rice.	rice
479	plant	rice	CRISPR;Cas9;	protein with four Armadillo repeats (Ptr)	Nature communications	The rice blast resistance gene Ptr encodes an atypical protein required for broad-spectrum disease resistance.	2018	9(1):2039	[Zhao H et al.]	USDA ARS Dale Bumpers National Rice Research Center, Stuttgart, AR, USA.	29795191	10.1038/s41467-018-04369-4	Plant resistance genes typically encode proteins with nucleotide binding site-leucine rich repeat (NLR) domains. Here we show that Ptr is an atypical resistance gene encoding a protein with four Armadillo repeats. Ptr is required for broad-spectrum blast resistance mediated by the NLR R gene Pi-ta and by the associated R gene Pi-ta2. Ptr is expressed constitutively and encodes two isoforms that are mainly localized in the cytoplasm. A two base pair deletion within the Ptr coding region in the fast neutron-generated mutant line M2354 creates a truncated protein, resulting in susceptibility to M. oryzae. Targeted mutation of Ptr in a resistant cultivar using CRISPR/Cas9 leads to blast susceptibility, further confirming its resistance function. The cloning of Ptr may aid in the development of broad spectrum blast resistant rice.	rice

480	plant	rice	RdDM;	calnexin (CNX); protein disulphide isomerase (PDIL1-1); luminal binding protein (BiP1); endoplasmic reticulum stress-inducible gene (OsbZIP50); genes with seed-specific expression (alpha-globulin (Glb-1); glutelin-B4 (GluB4))	Plant biotechnology journal	Transgene-independent heredity of RdDM-mediated transcriptional gene silencing of endogenous genes in rice.	2018	16(12):2007-2015	[Wakasa Y et al.]	Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Japan.	29704881	10.1111/pbi.12934	To induce transcriptional gene silencing (TGS) of endogenous genes of rice (Oryza sativa L.), we expressed double-strand RNA of each promoter region and thus induced RNA-directed DNA methylation (RdDM). We targeted constitutively expressed genes encoding calnexin (CNX), protein disulphide isomerase (PDIL1-1) and luminal binding protein (BiP1); an endoplasmic reticulum stress-inducible gene (OsbZIP50); and genes with seed-specific expression encoding alpha-globulin (Glb-1) and glutelin-B4 (GluB4). TGS of four genes was obtained with high efficiency (CNX, 66.7% of regenerated plants; OsBiP1, 67.4%; OsbZIP50, 63.4%; GluB4, 66.1%), whereas the efficiency was lower for PDIL1-1 (33.3%) and Glb-1 TGS lines (10.5%). The heredity of TGS, methylation levels of promoter regions and specificity of silencing of the target gene were investigated in some of the TGS lines. In progeny of CNX and OsbZIP50 TGS lines, suppression of the target genes was preserved (except in the endosperm) even after the removal of trigger genes (T-DNA) by segregation. TGS of CNX was reverted by demethylation treatment, and a significant difference in CG and CHG methylation levels in the -1 to -250 bp region of the CNX promoter was detected between the TGS and revertant lines, suggesting that TGS is closely related to the methylation levels of promoter. TGS exhibited specific suppression towards the target gene compared with post-transcriptional gene silencing when GluB4 gene from glutelin multigene family was targeted. Based on these results, future perspectives and problems to be solved in the	rice
481	plant	rice	CRISPR;Cas9;	initiation factor 4 gamma gene (eIF4G)	Plant biotechnology journal	Novel alleles of rice eIF4G generated by CRISPR/Cas9-targeted mutagenesis confer resistance to Rice tungro spherical virus.	2018	16(11):1918-1927	[Macovei A et al.]	International Rice Research Institute (IRRI), Metro Manila, Philippines.	29604159	10.1111/pbi.12927	Rice tungro disease (RTD) is a serious constraint in rice production across tropical Asia. RTD is caused by the interaction between Rice tungro spherical virus (RTSV) and Rice tungro bacilliform virus. RTSV resistance found in traditional cultivars has contributed to a reduction in the incidence of RTD in the field. Natural RTSV resistance is a recessive trait controlled by the translation initiation factor 4 gamma gene (eIF4G). The Y(1059) V(1060) V(1061) residues of eIF4G are known to be associated with the reactions to RTSV. To develop new sources of resistance to RTD, mutations in eIF4G were generated using the CRISPR/Cas9 system in the RTSV-susceptible variety IR64, widely grown across tropical Asia. The mutation rates ranged from 36.0% to 86.6%, depending on the target site, and the mutations were successfully transmitted to the next generations. Among various mutated eIF4G alleles examined, only those resulting in in-frame mutations in SVLFPNLAGKS residues (mainly NL), adjacent to the YVV residues, conferred resistance. Furthermore, our data suggest that eIF4G is essential for normal development, as alleles resulting in truncated eIF4G could not be maintained in homozygous state. The final products with RTSV resistance and enhanced yield under glasshouse conditions were found to no longer contain the Cas9 sequence. Hence, the RTSV-resistant plants with the novel eIF4G alleles represent a	rice
482	plant	rice	CRISPR;	rice amino acid transporter (OsAAP3)	Plant biotechnology journal	Blocking amino acid transporter OsAAP3 improves grain yield by promoting outgrowth buds and increasing tiller number in rice.	2018	16(10):1710-1722	[Lu K et al.]	Wuhan Institute of Bioengineering, Wuhan, China.	29479779	10.1111/pbi.12907	Amino acid transporters (AATs) play indispensable roles in nutrient allocation during plant development. In this study, we demonstrated that inhibiting expression of the rice amino acid transporter OsAAP3 increased grain yield due to a formation of larger numbers of tillers as a result of increased bud outgrowth. Elevated expression of OsAAP3 in transgenic plants resulted in significantly higher amino acid concentrations of Lys, Arg, His, Asp, Ala, Gln, Gly, Thr and Tyr, and inhibited bud outgrowth and rice tillering. However, RNAi of OsAAP3 decreased significantly Arg, Lys, Asp and Thr concentrations to a small extent, and thus promoted bud outgrowth, increased significantly tiller numbers and effective panicle numbers per plant, and further enhanced significantly grain yield and nitrogen use efficiency (NUE). The promoter sequences of OsAAP3 showed some divergence between Japonica and Indica rice, and expression of the gene was higher in Japonica, which produced fewer tillers than Indica. We generated knockout lines of OsAAP3 on Japonica ZH11 and KY131 using CRISPR technology and found that grain yield could be increased significantly. These results suggest that manipulation of OsAAP3 expression could be used to increase	rice
483	plant	rice	CRISPR;Cas9;	sites containing NGA PAM	Plant biotechnology journal	Increasing the efficiency of CRISPR-Cas9-VQR precise genome editing in rice.	2018	16(1):292-297	[Hu X et al.]	China National Rice Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China.	28605576	10.1111/pbi.12771	Clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9) is a revolutionary technology that enables efficient genomic modification in many organisms. Currently, the wide use of Streptococcus pyogenes Cas9 (SpCas9) primarily recognizes sites harbouring a canonical NGG protospacer adjacent motif (PAM). The newly developed VQR (D1135V/R1335Q/T1337R) variant of Cas9 has been shown to cleave sites containing NGA PAM in rice, which greatly expanded the range of genome editing. However, the low editing efficiency of the VQR variant remains, which limits its wide application in genome editing. In this study, by modifying the single guide RNA (sgRNA) structure and strong endogenous promoters, we significantly increased the editing efficiency of the VQR variant. The modified CRISPR-Cas9-VQR system provides a robust toolbox for multiplex genome editing at	rice
484	plant	rice	CRISPR;Cas9;	receptor-like cytoplasmic kinase LOC_Os03g24930 (OsBBS1)	Plant cell reports	A guanine insert in OsBBS1 leads to early leaf senescence and salt stress sensitivity in rice (Oryza sativa L.).	2018	37(6):933-946	[Zeng DD et al.]	Zhejiang University, Hangzhou, China.	29572657	10.1007/s00299-018-2280-y	KEY MESSAGE: A rice receptor-like kinase gene OSBBS1/OsRLCK109 was identified; this gene played vital roles in leaf senescence and the salt stress response. Early leaf senescence can cause negative effects on rice yield, but the underlying molecular regulation is not fully understood. bilateral blade senescence 1 (bbs1), an early leaf senescence mutant with a premature senescence phenotype that occurs mainly performing at the leaf margins, was isolated from a rice mutant population generated by ethylmethane sulfonate (EMS) treatment. The mutant showed premature leaf senescence beginning at the tillering stage and exhibited severe symptoms at the late grain-filling stage. bbs1 showed accelerated dark-induced leaf senescence. The OsBBS1 gene was cloned by a map-based cloning strategy, and a guanine (G) insertion was found in the first exon of LOC_Os03g24930. This gene encodes a receptor-like cytoplasmic kinase and was named OsRLCK109 in a previous study. Transgenic LOC_Os03g24930 knockout plants generated by a CRISPR/Cas9 strategy exhibited similar early leaf senescence phenotypes as did the bbs1 mutant, which confirmed that LOC_Os03g24930 was the OsBBS1 gene. OsBBS1/OsRLCK109 was expressed in all detected tissues and was predominantly expressed in the main vein region of mature leaves. The expression of OsBBS1 could be greatly induced by salt stress, and the bbs1 mutant exhibited hypersensitivity to salt stress. In conclusion, this is the first identification of OsRLCKs participating in leaf senescence and playing critical roles in	rice

485	plant	rice	CRISPR;Cas9;	plant-specific type III polyketide synthase (OsPKS2)	Plant cell reports	OsPKS2 is required for rice male fertility by participating in pollen wall formation.	2018	37(5):759-773	[Zou T et al.]	Sichuan Agricultural University, Chengdu, China.	29411094	10.1007/s00299-018-2265-x	KEY MESSAGE: OsPKS2, the rice orthologous gene of Arabidopsis PKSB/LAP5, encodes a polyketide synthase that is involved in pollen wall formation in rice. In flowering plants, the pollen wall protects male gametes from various environmental stresses and pathogen attacks, as well as promotes pollen germination. The biosynthesis of sporopollenin in tapetal cell is critical for pollen wall formation. Recently, progress has been made in understanding sporopollenin metabolism during pollen wall development in Arabidopsis. However, little is known about the molecular mechanism that underlies the sporopollenin synthesis in pollen wall formation in rice (Oryza sativa). In this study, we identified that a point mutation in OsPKS2, a plant-specific type III polyketide synthase gene, caused male sterility in rice by affecting the normal progress of pollen wall formation. Two other allelic mutants of OsPKS2 were generated using the CRISPR/Cas9 system and are also completely male sterile. This result thus further confirmed that OsPKS2 controls rice male fertility. We also showed that OsPKS2 is an orthologous gene of Arabidopsis PKSB/LAP5 and has a tapetum-specific expression pattern. In addition, its product localizes in the endoplasmic reticulum. Results suggested that OsPKS2 is critical for pollen wall formation, and plays	rice
486	plant	rice	CRISPR;Cas9;	p-Coumaroyl ester 3-hydroxylase (C3'H)	Plant journal	Downregulation of p-COUMAROYL ESTER 3-HYDROXYLASE in rice leads to altered cell wall structures and improves biomass saccharification.	2018		[Takeda Y et al.]	Kyoto University, Uji, Kyoto, Japan.	29890017	10.1111/tpj.13988	p-Coumaroyl ester 3-hydroxylase (C3'H) is a key enzyme involved in the biosynthesis of lignin, a phenylpropanoid polymer that is the major constituent of secondary cell walls in vascular plants. Although the crucial role of C3'H in lignification and its manipulation to upgrade lignocellulose have been investigated in eudicots, limited information is available in monocotyledonous grass species, despite their potential as biomass feedstocks. Here we address the pronounced impacts of C3'H deficiency on the structure and properties of grass cell walls. C3'H-knockdown lines generated via RNA interference (RNAi)-mediated gene silencing, with about 0.5% of the residual expression levels, reached maturity and set seeds. In contrast, C3'H-knockout rice mutants generated via CRISPR/Cas9-mediated mutagenesis were severely dwarfed and sterile. Cell wall analysis of the mature C3'H-knockdown RNAi lines revealed that their lignins were largely enriched in p-hydroxyphenyl (H) units while being substantially reduced in the normally dominant guaiacyl (G) and syringyl (S) units. Interestingly, however, the enrichment of H units was limited to within the non-acylated lignin units, with grass-specific gamma-p-coumaroylated lignin units remaining apparently unchanged. Suppression of C3'H also resulted in relative augmentation in tricin residues in lignin as well as a substantial reduction in wall cross-linking ferulates. Collectively, our data demonstrate that C3'H expression is an important determinant not only of lignin content and composition but also of the degree of cell wall cross-	rice
487	plant	rice	CRISPR;Cas9;	fatty acid desaturase 2 (OsFAD2-1)	Plant physiology and biochemistry	Production of high oleic/low linoleic rice by genome editing.	2018	131:58-62	[Abe K et al.]	Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan.	29735369	10.1016/j.plaphy.2018.04.033	Rice bran oil (RBO) contains many valuable healthy constituents, including oleic acid. Improvement of the fatty acid composition in RBO, including an increase in the content of oleic acid, which helps suppress lifestyle disease, would increase health benefits. The enzyme fatty acid desaturase 2 (FAD2) catalyzes the conversion of oleic acid to linoleic acid in plants, and FAD2 mutants exhibit altered oleic and linoleic acid content in many crops. There are three functional FAD2 genes in the genome of rice (Oryza sativa L.), and, of these, expression of the OsFAD2-1 gene is highest in rice seeds. In order to produce high oleic/low linoleic RBO, we attempted to disrupt the OsFAD2-1 gene by CRISPR/Cas9-mediated targeted mutagenesis. We succeeded in the production of homozygous OsFAD2-1 knockout rice plants. The content of oleic acid increased to more than twice that of wild type, and, surprisingly, linoleic acid, a catabolite of oleic acid by FAD2, decreased dramatically to undetectable levels in fad2-1 mutant brown rice seeds. In this study, by genome editing based on genome information, we succeeded in the production of rice whose fatty acid composition is greatly improved. We suggest that CRISPR/Cas9-mediated mutagenesis of a major gene that shows dominant expression in the target tissue could be a powerful tool to improve target traits in a tissue-specific manner.	rice
488	plant	rice	CRISPR;Cas9;	POLYKETIDE SYNTHASE 1 (OsPKS1)	Plant science	OsPKS1 is required for sexine layer formation, which shows functional conservation between rice and Arabidopsis.	2018	277:145-154	[Shi QS et al.]	Shanghai Normal University, Shanghai, China.	30466580	10.1016/j.plantsci.2018.08.009	The sporopollenin precursors, as a general constituent of sexine, are synthesized in the tapetum and deposited on the pollen surface after transportation and processing. The polyketide synthase condenses the acyl-CoA into a hydroxyalkyl alpha-pyrone, which is predicted to be a component of the sporopollenin precursors. In this study, we found that the rice POLYKETIDE SYNTHASE 1 (OsPKS1) was the orthologue of Arabidopsis POLYKETIDE SYNTHASE A/LESS ADHESIVE POLLEN 6 (PKSA/LAP6) through sequence alignment. The OsPKS1 knockout mutants obtained by Crispr-Cas9-mediated editing exhibited a complete male sterile phenotype. Cytological observations revealed that abnormal bacula deposition and ubisch body structures for sexine formation led to pollen rupture in ospks1. The expression analysis showed that the OsPKS1 was highly expressed in tapetal cells and anther locules from stage 9 to stage 11 during anther development in rice. Subcellular localization demonstrated that the OsPKS1 protein was preferentially localized to the ER. The genomic sequence of OsPKS1 driven by the PKSA/LAP6 promoter restored the sexine pattern of Arabidopsis pksa/lap6. These results indicated that OsPKS1 is required for sexine layer formation in rice and functionally conserved in the sporopollenin synthesis	rice

489	plant	rice	CRISPR;Cas9;	heat-sensitive albino1 (hsa1)	Plant science	The newly identified heat-stress sensitive albino 1 gene affects chloroplast development in rice.	2018	267:168-179	[Qiu Z et al.]	China National Rice Research Institute, Hangzhou, China.	29362095	10.1016/j.plantsci.2017.11.015	High temperature, a major abiotic stress, significantly affects the yield and quality of crops in many parts of the world. Components of the photosynthetic apparatus are highly susceptible to thermal damage. Although the responses to acute heat stress have been studied intensively, the mechanisms that regulate chloroplast development under heat stress remain obscure, especially in crop plants. Here, we cloned and characterized the gene responsible for the heat-sensitive albino1 (hsa1) mutation in rice (Oryza sativa). The hsa1 mutant harbors a recessive mutation in a gene encoding fructokinase-like protein2 (FLN2); the mutation causes a premature stop codon and results in a severe albino phenotype, with defects in early chloroplast development. The color of hsa1 mutant plants gradually changed from albino to green at later stages of development at various temperatures and chloroplast biogenesis was strongly delayed at high temperature (32 degrees C). HSA1 expression was strongly reduced in hsa1 plants compared to wild type (WT). HSA1 localizes to the chloroplast and regulates chloroplast development. An HSA1 deletion mutant induced by CRISPR/Cas9 was heat sensitive but had a faster greening phenotype than the original hsa1 allele at all temperatures. RNA and protein levels of plastid-encoded RNA polymerase-dependent plastid genes were markedly reduced in hsa1 plants compared to WT. These results demonstrated that HSA1 plays important roles in chloroplast development at early stages, and functions in protecting chloroplasts under heat stress at later stages	rice
490	plant	rice	TALENs;	translational enhancer dMac3; iPromotor	PloS one	Establishment of a conditional TALEN system using the translational enhancer dMac3 and an inducible promoter activated by glucocorticoid treatment to increase the frequency of targeted mutagenesis in plants.	2018	13(12):e0208959	[Onodera H et al.]	Tokyo University of Science, Tokyo, Japan.	30586438	10.1371/journal.pone.0208959	Transcription activator-like effector nuclease (TALEN) is an artificial nuclease that causes DNA cleavage at the target site and induces few off-target reactions because of its high sequence specificity. Powerful and variable tools using TALENs can be used in practical applications and may facilitate the molecular breeding of many plant species. We have developed a convenient construction system for a plant TALEN vector named the Emerald Gateway TALEN system. In this study, we added new properties to this system, which led to an increase in the efficiency of targeted mutagenesis. Rice dMac3 is a translational enhancer that highly increases the efficiency of translation of the downstream ORF. We inserted dMac3 into the 5' untranslated region of the TALEN gene. In the cultured rice cells to which the TALEN gene was introduced, the frequency of targeted mutagenesis was highly increased compared with those altered using the conventional system. Next, the promoter for the TALEN gene was replaced with iPromoter, and its expression was stringently controlled by a GVG transcription factor that was activated in the presence of glucocorticoid. This conditional expression system worked effectively and led to a higher frequency of targeted mutagenesis than that by the constitutive expression system, while no mutagenesis was detected without glucocorticoid treatment. These results suggest	rice
491	plant	rice	CRISPR;Cas9		Proceedings of the National Academy of Sciences of the United States of America	Identifying a large number of high-yield genes in rice by pedigree analysis, whole-genome sequencing, and CRISPR-Cas9 gene knockout.	2018	115(32):E7559-E7567	[Huang J et al.]	Nanjing University, Nanjing, China.	30037991	10.1073/pnas.1806110115	Repeated artificial selection of a complex trait facilitates the identification of genes underlying the trait, especially if multiple selected descendant lines are available. Here we developed a pedigree-based approach to identify genes underlying the Green Revolution (GR) phenotype. From a pedigree analysis, we selected 30 cultivars including the "miracle rice" IR8, a GR landmark, its ancestors and descendants, and also other related cultivars for identifying high-yield genes. Through sequencing of these genomes, we identified 28 ancestral chromosomal blocks that were maintained in all the high-yield cultivars under study. In these blocks, we identified six genes of known function, including the GR gene sd1, and 123 loci with genes of unknown function. We randomly selected 57 genes from the 123 loci for knockout or knockdown studies and found that a high proportion of these genes are essential or have phenotypic effects related to rice production. Notably, knockout lines have significant changes in plant height ($P < 0.003$), a key GR trait, compared with wild-type lines. Some gene knockouts or knockdowns were especially interesting. For example, knockout of Os10g0555100, a putative glucosyltransferase gene, showed both reduced growth and altered panicle architecture. In addition, we found that in some retained chromosome blocks several GR-related genes were clustered, although they have unrelated sequences, suggesting clustering of genes with similar functions. In conclusion, we have identified many high-yield genes in rice. Our method provides a	rice
492	plant	rice	CRISPR;Cas9;	group I (PYL1-PYL6; PYL12) and group II (PYL7-PYL11; PYL13) pyrabactin resistance 1-like (PYL) genes	Proceedings of the National Academy of Sciences of the United States of America	Mutations in a subfamily of abscisic acid receptor genes promote rice growth and productivity.	2018	115(23):6058-6063	[Miao C et al.]	Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.	29784797	10.1073/pnas.1804774115	Abscisic acid (ABA) is a key phytohormone that controls plant growth and stress responses. It is sensed by the pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/regulatory components of the ABA receptor (RCAR) family of proteins. Here, we utilized CRISPR/Cas9 technology to edit group I (PYL1-PYL6 and PYL12) and group II (PYL7-PYL11 and PYL13) PYL genes in rice. Characterization of the combinatorial mutants suggested that genes in group I have more important roles in stomatal movement, seed dormancy, and growth regulation than those in group II. Among all of the single pyl mutants, only pyl1 and pyl12 exhibited significant defects in seed dormancy. Interestingly, high-order group I mutants, but not any group II mutants, displayed enhanced growth. Among group I mutants, pyl1/4/6 exhibited the best growth and improved grain productivity in natural paddy field conditions, while maintaining nearly normal seed dormancy. Our results suggest that a subfamily of rice PYLs has evolved to have particularly important roles in regulating plant growth and reveal a	rice

493	plant	rice	CRISPR;Cas9;	NAC family transcription factor (OsSND2)	Rice	OsSND2, a NAC family transcription factor, is involved in secondary cell wall biosynthesis through regulating MYBs expression in rice.	2018	11(1):36	[Ye Y et al.]	Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei, Anhui, China.	29855737	10.1186/s12284-018-0228-z	BACKGROUND: As one of the most important staple food crops, rice produces huge agronomic biomass residues that contain lots of secondary cell walls (SCWs) comprising cellulose, hemicelluloses and lignin. The transcriptional regulation mechanism underlying SCWs biosynthesis remains elusive. RESULTS: In this study, we isolated a NAC family transcription factor (TF), OsSND2 through yeast one-hybrid screening using the secondary wall NAC-binding element (SNBE) on the promoter region of OsMYB61 which is known transcription factor for regulation of SCWs biosynthesis as bait. We used an electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation analysis (ChIP) to further confirm that OsSND2 can directly bind to the promoter of OsMYB61 both in vitro and in vivo. OsSND2, a close homolog of AtSND2, is localized in the nucleus and has transcriptional activation activity. Expression pattern analysis indicated that OsSND2 was mainly expressed in internodes and panicles. Overexpression of OsSND2 resulted in rolled leaf, increased cellulose content and up-regulated expression of SCWs related genes. The knockout of OsSND2 using CRISPR/Cas9 system decreased cellulose content and down-regulated the expression of SCWs related genes. Furthermore, OsSND2 can also directly bind to the promoters of other MYB family TFs by transactivation analysis in yeast cells and rice protoplasts. Altogether, our findings suggest that OsSND2 may function as a master regulator to mediate SCWs biosynthesis. CONCLUSION: OsSND2 was identified as a positive regulator of cellulose biosynthesis in rice. An increase in the expression level of this gene can improve the SCWs cellulose content. Therefore, the study of the	rice
494	plant	rice	CRISPR;Cas9;	gene encoding glycerophosphoryl diester phosphodiesterase	Rice	Genome-wide analyses of late pollen-preferred genes conserved in various rice cultivars and functional identification of a gene involved in the key processes of late pollen development.	2018	11(1):28	[Moon S et al.]	Kyung Hee University, Yongin, Korea.	29687350	10.1186/s12284-018-0219-0	BACKGROUND: Understanding late pollen development, including the maturation and pollination process, is a key component in maintaining crop yields. Transcriptome data obtained through microarray or RNA-seq technologies can provide useful insight into those developmental processes. Six series of microarray data from a public transcriptome database, the Gene Expression Omnibus of the National Center for Biotechnology Information, are related to anther and pollen development. RESULTS: We performed a systematic and functional study across the rice genome of genes that are preferentially expressed in the late stages of pollen development, including maturation and germination. By comparing the transcriptomes of sporophytes and male gametes over time, we identified 627 late pollen-preferred genes that are conserved among japonica and indica rice cultivars. Functional classification analysis with a MapMan tool kit revealed a significant association between cell wall organization/metabolism and mature pollen grains. Comparative analysis of rice and Arabidopsis demonstrated that genes involved in cell wall modifications and the metabolism of major carbohydrates are unique to rice. We used the GUS reporter system to monitor the expression of eight of those genes. In addition, we evaluated the significance of our candidate genes, using T-DNA insertional mutant population and the CRISPR/Cas9 system. Mutants from T-DNA insertion and CRISPR/Cas9 systems of a rice gene encoding glycerophosphoryl diester phosphodiesterase are defective in their male gamete transfer. CONCLUSION: Through the global analyses of the late pollen-preferred genes from rice, we found several biological features of these genes. First, biological process related to cell wall organization and modification is over-represented in these genes to support rapid tube growth. Second, comparative analysis of late pollen preferred genes between rice and Arabidopsis provide a significant insight on the evolutional disparateness in cell wall biogenesis and storage reserves of pollen. In addition, these candidates might be useful targets for future examinations of late pollen development, and will be a valuable resource for accelerating the understanding of molecular mechanisms for pollen	rice
495	plant	rice	CRISPR;Cas9;	Ghd7.1	TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik	Genetic dissection and validation of candidate genes for flag leaf size in rice (Oryza sativa L.).	2018	131(4):801-815	[Tang X et al.]	National Key Laboratory of Crop Genetic Improvement, Wuhan, China.	29218376	10.1007/s00122-017-3036-8	KEY MESSAGE: Two major loci with functional candidate genes were identified and validated affecting flag leaf size, which offer desirable genes to improve leaf architecture and photosynthetic capacity in rice. Leaf size is a major determinant of plant architecture and yield potential in crops. However, the genetic and molecular mechanisms regulating leaf size remain largely elusive. In this study, quantitative trait loci (QTLs) for flag leaf length and flag leaf width in rice were detected with high-density single nucleotide polymorphism genotyping of a chromosomal segment substitution line (CSSL) population, in which each line carries one or a few chromosomal segments from the japonica cultivar Nipponbare in a common background of the indica variety Zhenshan 97. In total, 14 QTLs for flag leaf length and nine QTLs for flag leaf width were identified in the CSSL population. Among them, qFW4-2 for flag leaf width was mapped to a 37-kb interval, with the most likely candidate gene being the previously characterized NAL1. Another major QTL for both flag leaf width and length was delimited by substitution mapping to a small region of 13.5 kb that contains a single gene, Ghd7.1. Mutants of Ghd7.1 generated using CRISPR/CAS9 approach showed reduced leaf size. Allelic variation analyses also validated Ghd7.1 as a functional candidate gene for leaf size, photosynthetic capacity and other yield-related traits. These results provide useful genetic information for the improvement of leaf size	rice
496	plant	rice	CRISPR;Cas9;	cytosolic glucose-1-phosphate adenylyl transferase large subunit (OsAPL2)	Transgenic research	CRISPR/Cas9-induced monoallelic mutations in the cytosolic AGPase large subunit gene APL2 induce the ectopic expression of APL2 and the corresponding small subunit gene APS2b in rice leaves.	2018	27(5):423-439	[Perez L et al.]	University of Lleida-Agrotecnio Center, Lleida, Spain.	30099722	10.1007/s11248-018-0089-7	The first committed step in the endosperm starch biosynthetic pathway is catalyzed by the cytosolic glucose-1-phosphate adenylyl transferase (AGPase) comprising large and small subunits encoded by the OsAPL2 and OsAPS2b genes, respectively. OsAPL2 is expressed solely in the endosperm so we hypothesized that mutating this gene would block starch biosynthesis in the endosperm without affecting the leaves. We used CRISPR/Cas9 to create two heterozygous mutants, one with a severely truncated and nonfunctional AGPase and the other with a C-terminal structural modification causing a partial loss of activity. Unexpectedly, we observed starch depletion in the leaves of both mutants and a corresponding increase in the level of soluble sugars. This reflected the unanticipated expression of both OsAPL2 and OsAPS2b in the leaves, generating a complete ectopic AGPase in the leaf cytosol, and a corresponding decrease in the expression of the plastidial small subunit OsAPS2a that was only partially complemented by an increase in the expression of OsAPS1. The new cytosolic AGPase was not sufficient to compensate for the loss of plastidial AGPase, most likely because there is no wider starch biosynthesis pathway in the leaf cytosol and because pathway intermediates are not shuttled between the two compartments.	rice

497	plant	rice	CRISPR;Cas9;	narrow leaf 1; glossy1	Yi chuan = Hereditas	[Cas9 protein variant VQR recognizes NGAC protospacer adjacent motif in rice].	2018	40(12):1112-1119	[Xin GW et al.]	Shanxi Agricultural University, Taigu, China.	30559100	10.16288/j.yczz.18-126	Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system is the third-generation genome editing tools that was developed and widely used in recent years. However, Streptococcus pyogenes Cas9 (SpCas9) in this system could only recognize NGG PAM (protospacer adjacent motif), which largely restricts the range of genome editing. The VQR (D1135V/R1335Q/T1337R) variant of SpCas9 could recognize NGAA, NGAG and NGAT PAMs in rice. However, whether VQR variant could recognize NGAC PAM remains unclear. In this study, three low editing efficiency sites of the VQR variant, NAL1-Q1, NAL1-Q2 and LPA1-Q, were selected for genome editing using the improved CRISPR/VQR system. The improved CRISPR/VQR system effectively edited these target sites, and the gene editing efficiency was 9.75%, 43.90% and 29.26% respectively. To ensure the recognition of NGAC PAM by the improved CRISPR/VQR system, two NGAC PAM containing sites (NAL-C and GL1-C) in the NARROW LEAF 1 (NAL1) for leaf length and GLOSSY1 (GL1) genes for wax biosynthesis were selected for genome editing in rice in this study, and 57 transgenic plants were obtained. The PCR amplification and sequencing results showed that 27 plants (47.36%) had mutation in the NAL1-C site, 44 plants (77.19%) had mutation in the GL1 gene, and 26 plants (45.61%) had mutation in the NAL-C and GL1-C sites. Further analysis revealed that there were four types of mutations caused by the CRISPR/VQR system, respectively for the hybrid mutation, biallelic mutation, chimeric mutation and homozygous mutations. Among them, heterozygous mutation and biallelic mutation were dominant changes. These results indicated that the improved CRISPR/VQR system could efficiently edit the NGAC PAM sites of the rice and produce abundant mutant types.	rice
498	plant	rice; wheat	CRISPR;Cas9;	an endogenous gene	Genome biology	Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion.	2018	19(1):59	[Li C et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.	29807545	10.1186/s13059-018-1443-z	Nucleotide base editors in plants have been limited to conversion of cytosine to thymine. Here, we describe a new plant adenine base editor based on an evolved tRNA adenosine deaminase fused to the nickase CRISPR/Cas9, enabling A*T to G*C conversion at frequencies up to 7.5% in protoplasts and 59.1% in regenerated rice and wheat plants. An endogenous gene is also successfully modified through introducing a gain-of-function point mutation to directly produce an herbicide-tolerant rice plant. With this new adenine base editing system, it is now possible to precisely edit all base pairs, thus expanding the toolset for precise editing in plants.	rice; wheat
499	plant	rose (Rosa hybrida)	Agroinfiltration;	different sets of flower color genes	Physiology and molecular biology of plants	Agroinfiltration: a rapid and reliable method to select suitable rose cultivars for blue flower production.	2018	24(3):503-511	[Zeinipour M et al.]	Agricultural Biotechnology Research Institute of Iran (ABRII), Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.	29692557	10.1007/s12298-018-0516-5	Rose cultivars with blue flower color are among the most attractive breeding targets in floriculture. However, they are difficult to produce due to the low efficiency of transformation systems, interactive effects of hosts and vectors, and lengthy processes. In this study, agroinfiltration-mediated transient expression was investigated as a tool to assess the function of flower color genes and to determine appropriate host cultivars for stable transformation in Rosa hybrida. To induce delphinidin accumulation and consequently to produce blue hue, the petals of 30 rose cultivars were infiltrated with three different expression vectors namely pBIH-35S-CcF3'5'H, pBIH-35S-Del2 and pBIH-35S-Del8, harbouring different sets of flower color genes. The results obtained showed that the ectopic expression of the genes was only detected in three cultivars with dark pink petals (i.e. 'Purple power', 'High & Mora' and 'Marina') after 6-8 days. The high performance liquid chromatography analyses confirmed delphinidin accumulation in the infiltrated petals caused by transient expression of CcF3'5'H gene. Moreover, there were significant differences in the amounts of delphinidin among the three cultivars infiltrated with the three different expression vectors. More specifically, the highest delphinidin content was detected in the cultivar 'Purple power' (4.67 microg g(-1) FW), infiltrated with the pBIH-35S-Del2 vector. The expression of CcF3'5'H gene in the infiltrated petals was also confirmed by real time PCR. In conclusion and based on the findings of the present study, the agroinfiltration could be regarded as a reliable method to identify suitable rose cultivars	rose (Rosa h
500	plant	Salvia miltiorrhiza	CRISPR;Cas9;	rosmarinic acid synthase gene (SmRAS)	Phytochemistry	CRISPR/Cas9-mediated efficient targeted mutagenesis of RAS in Salvia miltiorrhiza.	2018	148:63-70	[Zhou Z et al.]	Second Military Medical University, Shanghai, China.	29421512	10.1016/j.phytochem.2018.01.015	The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-associated) system is a powerful genome editing tool that has been used in many species. In this study, we focused on the phenolic acid metabolic pathway in the traditional Chinese medicinal herb Salvia miltiorrhiza, using the CRISPR/Cas9 system to edit the rosmarinic acid synthase gene (SmRAS) in the water-soluble phenolic acid biosynthetic pathway. The single guide RNA (sgRNA) was designed to precisely edit the most important SmRAS gene, which was selected from 11 family members through a bioinformatics analysis. The sequencing results showed that the genomes of 50% of the transgenic regenerated hairy roots had been successfully edited. Five biallelic mutants, two heterozygous mutants and one homozygous mutant were obtained from 16 independent transgenic hairy root lines when the sgRNA was driven by the Arabidopsis U6 promoter, while no mutants were obtained from 13 independent transgenic hairy root lines when the sgRNA was driven by the rice U3 promoter. Subsequently, expression and metabolomics analysis showed that the contents of phenolic acids, including rosmarinic acid (RA) and lithospermic acid B, and the RAS expression level were decreased in the successfully edited hairy root lines, particularly in the homozygous mutants. In addition, the level of the RA precursor 3,4-dihydroxyphenyllactic acid clearly increased. These results indicated that the CRISPR/Cas9 system can be utilized to identify important genes in a gene family with the assistance of bioinformatics analysis and that this new technology is an efficient and specific tool for genome editing in S. miltiorrhiza. This new system presents a promising potential method to regulate plant metabolic networks and improve the	Salvia miltior
501	plant	Salvia miltiorrhiza	CRISPR;Cas9;	SmPAL1	Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica	[Directing construction of CRISPR/Cas9 vector of SmPAL1 in Salvia miltiorrhiza by target efficiency detection in vitro].	2018	43(21):4226-4230	[Qiu JR et al.]	Shandong Agricultural University, Taian, China.	30583622	10.19540/j.cnki.cjcmm.20180726.007	To construct CRISPR/Cas9 vectors for the editing of SmPAL1 in the phenylpropane metabolic pathway of Salvia miltiorrhiza, CIRSPR/Cas9 target sites of SmPAL1 were designed by online software. Its target efficiencies were detected in vitro by enzyme digestion and sequences with highly efficiency were constructed into CRISPR/Cas9 vectors. Three possible CRISPR target sequences (SmPAL1-g1, SmPAL1-g2, SmPAL1-g3) were designed and the enzyme digestion efficiencies were 53.3%, 76.6% and 10.0%. SmPAL1-g1 and SmPAL1-g2 were constructed into vector VK005-03 named as VK005-03-g1 and VK005-03-g2. The results of sequencing showed that the two CRISPR/Cas target sequences were all constructed into VK005-03. Here we first laid the foundation for the study of SmPAL1 and provided an effective strategy for the	Salvia miltior

502	plant	Setaria viridis	CRISPR;Cas9;	GFP	Plant signaling & behavior	Particle bombardment - mediated gene transfer and GFP transient expression in Seteria viridis.	2018	13(4):e1441657	[Mookkan M et al.]	Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.	29621423	10.1080/15592324.2018.1441657	Setaria viridis is one of the most important model grasses in studying monocot plant biology. Transient gene expression study is a very important tool in plant biotechnology, functional genomics, and CRISPR-Cas9 genome editing technology via particle bombardment. In this study, a particle bombardment-mediated protocol was developed to introduce DNA into Setaria viridis in vitro leaf explants. In addition, physical and biological parameters, such as helium pressure, distance from stopping screen to the target tissues, DNA concentration, and number of bombardments, were tested and optimized. Optimum concentration of transient GFP expression was achieved using 1.5 ug plasmid DNA with 0.6 mm gold particles and 6 cm bombardment distance, using 1,100 psi. Doubling the bombardment instances provides the maximum number of foci of transient GFP expression. This simple protocol will be helpful for	Setaria viridi
503	plant	sorghum	agroinfiltration; CRISPR;Cas9;		Plant biotechnology journal	Developing a flexible, high-efficiency Agrobacterium-mediated sorghum transformation system with broad application.	2018	16(7):1388-1395	[Che P et al.]	DuPont Pioneer, Johnston, IA, USA.	29327444	10.1111/pbi.12879	Sorghum is the fifth most widely planted cereal crop in the world and is commonly cultivated in arid and semi-arid regions such as Africa. Despite its importance as a food source, sorghum genetic improvement through transgenic approaches has been limited because of an inefficient transformation system. Here, we report a ternary vector (also known as cohabitating vector) system using a recently described pVIR accessory plasmid that facilitates efficient Agrobacterium-mediated transformation of sorghum. We report regeneration frequencies ranging from 6% to 29% in Tx430 using different selectable markers and single copy, backbone free 'quality events' ranging from 45% to 66% of the total events produced. Furthermore, we successfully applied this ternary system to develop transformation protocols for popular but recalcitrant African varieties including Macia, Malisor 84-7 and Tegemeo. In addition, we report the use of this technology to develop the first stable CRISPR/Cas9-mediated gene knockouts in	sorghum
504	plant	sorghum (Sorghum bicolor)	CRISPR;Cas9;	alpha-kafirins (k1C)	Plant physiology	Editing of an Alpha-Kafirin Gene Family Increases, Digestibility and Protein Quality in Sorghum.	2018	177(4):1425-1438	[Li A et al.]	University of Nebraska, Lincoln, NE, USA.	29925584	10.1104/pp.18.00200	Kafirins are the major storage proteins in sorghum (Sorghum bicolor) grains and form protein bodies with poor digestibility. Since kafirins are devoid of the essential amino acid lysine, they also impart poor protein quality to the kernel. The alpha-kafirins, which make up most of the total kafirins, are largely encoded by the k1C family of highly similar genes. We used a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing approach to target the k1C genes to create variants with reduced kafirin levels and improved protein quality and digestibility. A single guide RNA was designed to introduce mutations in a conserved region encoding the endoplasmic reticulum signal peptide of alpha-kafirins. Sequencing of kafirin PCR products revealed extensive edits in 25 of 26 events in one or multiple k1C family members. T1 and T2 seeds showed reduced alpha-kafirin levels, and selected T2 events showed significantly increased grain protein digestibility and lysine content. Thus, a single consensus single guide RNA carrying target sequence mismatches is sufficient for extensive editing of all k1C genes. The resulting quality improvements can be deployed rapidly for breeding and the generation of transgene-	sorghum
505	plant	soybean	CRISPR;agroinfiltration;	GmMYB118	BMC plant biology	Identification and characterization of GmMYB118 responses to drought and salt stress.	2018	18(1):320	[Du YT et al.]	Institute of Crop Sciences, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China.	30509166	10.1186/s12870-018-1551-7	BACKGROUND: Abiotic stress severely influences plant growth and development. MYB transcription factors (TFs), which compose one of the largest TF families, play an important role in abiotic stress responses. RESULT: We identified 139 soybean MYB-related genes; these genes were divided into six groups based on their conserved domain and were distributed among 20 chromosomes (Chrs). Quantitative real-time PCR (qRT-PCR) indicated that GmMYB118 highly responsive to drought, salt and high temperature stress; thus, this gene was selected for further analysis. Subcellular localization revealed that the GmMYB118 protein located in the nucleus. Ectopic expression (EX) of GmMYB118 increased tolerance to drought and salt stress and regulated the expression of several stress-associated genes in transgenic Arabidopsis plants. Similarly, GmMYB118-overexpressing (OE) soybean plants generated via Agrobacterium rhizogenes (A. rhizogenes)-mediated transformation of the hairy roots showed improved drought and salt tolerance. Furthermore, compared with the control (CK) plants, the clustered, regularly interspaced, short palindromic repeat (CRISPR)-transformed plants exhibited reduced drought and salt tolerance. The contents of proline and chlorophyll in the OE plants were significantly greater than those in the CK plants, whose contents were greater than those in the CRISPR plants under drought and salt stress conditions. In contrast, the reactive oxygen species (ROS) and malondialdehyde (MDA) contents were significantly lower in the OE plants than in the CK plants, whose contents were lower than those in the CRISPR plants under stress conditions. CONCLUSIONS: These results indicated that GmMYB118 could improve tolerance to drought and salt stress by promoting expression of stress-associated genes and regulating osmotic and oxidizing substances to maintain cell homeostasis.	soybean
506	plant	soybean	CRISPR;Cas9;	GmFT2a; GmFT5a	International journal of molecular sciences	CRISPR/Cas9-Mediated Deletion of Large Genomic Fragments in Soybean.	2018	19(12)	[Cai Y et al.]	Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China.	30513774	10.3390/ijms19123835	At present, the application of CRISPR/Cas9 in soybean (Glycine max (L.) Merr.) has been mainly focused on knocking out target genes, and most site-directed mutagenesis has occurred at single cleavage sites and resulted in short deletions and/or insertions. However, the use of multiple guide RNAs for complex genome editing, especially the deletion of large DNA fragments in soybean, has not been systematically explored. In this study, we employed CRISPR/Cas9 technology to specifically induce targeted deletions of DNA fragments in GmFT2a (Glyma16g26660) and GmFT5a (Glyma16g04830) in soybean using a dual-sgRNA/Cas9 design. We achieved a deletion frequency of 15.6% for target fragments ranging from 599 to 1618 bp in GmFT2a. We also achieved deletion frequencies of 12.1% for target fragments exceeding 4.5 kb in GmFT2a and 15.8% for target fragments ranging from 1069 to 1161 bp in GmFT5a. In addition, we demonstrated that these CRISPR/Cas9-induced large fragment deletions can be inherited. The T2 'transgene-free' homozygous ft2a mutants with a 1618 bp deletion exhibited the late-flowering phenotype. In this study, we developed an efficient system for deleting large fragments in soybean using CRISPR/Cas9; this system could benefit future research on gene function and improve agriculture via chromosome	soybean

507	plant	soybean	agroinfiltration; CRISPR;Cas9;	GmFT2a	Plant biotechnology journal	CRISPR/Cas9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean.	2018	16(1):176-185	[Cai Y et al.]	Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China.	28509421	10.1111/pbi.12758	Flowering is an indication of the transition from vegetative growth to reproductive growth and has considerable effects on the life cycle of soya bean (Glycine max). In this study, we employed the CRISPR/Cas9 system to specifically induce targeted mutagenesis of GmFT2a, an integrator in the photoperiod flowering pathway in soya bean. The soya bean cultivar Jack was transformed with three sgRNA/Cas9 vectors targeting different sites of endogenous GmFT2a via Agrobacterium tumefaciens-mediated transformation. Site-directed mutations were observed at all targeted sites by DNA sequencing analysis. T1-generation soya bean plants homozygous for null alleles of GmFT2a frameshift mutated by a 1-bp insertion or short deletion exhibited late flowering under natural conditions (summer) in Beijing, China (N39 degrees 58', E116 degrees 20'). We also found that the targeted mutagenesis was stably heritable in the following T2 generation, and the homozygous GmFT2a mutants exhibited late flowering under both long-day and short-day conditions. We identified some 'transgene-clean' soya bean plants that were homozygous for null alleles of endogenous GmFT2a and without any transgenic element from the T1 and T2 generations. These 'transgene-clean' mutants of GmFT2a may provide materials for more in-depth research of GmFT2a functions and the molecular mechanism of	soybean
508	plant	soybean	agroinfiltration; CRISPR;Cas9;	GmPPD1; GmPPD2	Plant cell reports	Simultaneous site-directed mutagenesis of duplicated loci in soybean using a single guide RNA.	2018	37(3):553-563	[Kanazashi Y et al.]	Hokkaido University, Sapporo, Hokkaido, Japan.	29333573	10.1007/s00299-018-2251-3	KEY MESSAGE: Using a gRNA and Agrobacterium-mediated transformation, we performed simultaneous site-directed mutagenesis of two GmPPD loci in soybean. Mutations in GmPPD loci were confirmed in at least 33% of T2 seeds. The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated endonuclease 9 (Cas9) system is a powerful tool for site-directed mutagenesis in crops. Using a single guide RNA (gRNA) and Agrobacterium-mediated transformation, we performed simultaneous site-directed mutagenesis of two homoeologous loci in soybean (Glycine max), GmPPD1 and GmPPD2, which encode the orthologs of Arabidopsis thaliana PEAPOD (PPD). Most of the T1 plants had heterozygous and/or chimeric mutations for the targeted loci. The sequencing analysis of T1 and T2 generations indicates that putative mutation induced in the T0 plant is transmitted to the T1 generation. The inheritable mutation induced in the T1 plant was also detected. This result indicates that continuous induction of mutations during T1 plant development increases the occurrence of mutations in germ cells, which ensures the transmission of mutations to the next generation. Simultaneous site-directed mutagenesis in both GmPPD loci was confirmed in at least 33% of T2 seeds examined. Approximately 19% of double mutants did not contain the Cas9/gRNA expression construct. Double mutants with frameshift mutations in both GmPPD1 and GmPPD2 had dome-shaped trifoliate leaves, extremely twisted pods, and produced few seeds. Taken together, our data indicate that continuous induction of mutations in the whole plant and advancing generations of transgenic plants enable efficient simultaneous	soybean
509	plant	soybean	agroinfiltration; CRISPR;Cas9;	sucrose non-fermenting related protein kinase (SmSnRK1.1; GmSnRK1.2)	Yi chuan = Hereditas	[Preliminary analysis of the role of GmSnRK1.1 and GmSnRK1.2 in the ABA and alkaline stress response of the soybean using the CRISPR/Cas9-based gene double-knockout system].	2018	40(6):496-507	[Li HQ et al.]	Northeast Agricultural University, Harbin, China.	29959122	10.16288/j.yczz.17-424	Sucrose non-fermenting related protein kinases (SnRKs) are a ubiquitous Ser/Thr protein kinase in the plant kingdom. These kinases play important roles in plant growth, development, metabolism and resistance to environmental stresses. The soybean (Glycine max L.) genome has four SnRK1 genes, of which GmSnRK1.1 and GmSnRK1.2 are predominant and participate in multiple stress response pathways. To dissect the mechanism of the role of GmSnRK1.1 and GmSnRK1.2 proteins in response to ABA and alkaline stresses, we constructed a dual-gRNA CRISPR vector to specifically knock out GmSnRK1.1 and GmSnRK1.2. The resultant constructs were transformed into soybean cotyledon nodes to induce hairy roots by agrobacteria (Agrobacterium rhizogenes). The soybean hairy roots obtained were genotyped, and the results showed that GmSnRK1.1 and GmSnRK1.2 were efficiently doubly knocked out in 48.6% hairy roots. We also generated control hairy roots that over-expressed GmSnRK1. The materials were treated with 25 mumol/L ABA for 15 days and the results showed that the growths of wild-type and GmSnRK1 over-expressed roots were significantly inhibited than GmSnRK1.1 GmSnRK1.2 double-knockout roots, as the controls displayed less root lengths and fresh weights. However, after treating with 50 mmol/L NaHCO3 for 15 days, we found that the growths of GmSnRK1.1 GmSnRK1.2 double-knockout roots were significantly inhibited than the wild-type and GmSnRK1 over-expressed control roots, as the knockout groups contained less root lengths and fresh weights. These results implied that the GmSnRK1.1 GmSnRK1.2 double knockout mitigated hairy root sensitivity to ABA and resistance to alkaline stress. Taken together, we established the CRISPR/Cas9 system to perform gene double knockout in the soybean and by using this technique, we determined the roles of GmSnRK1.1 and	soybean
510	plant	soybean (Glycine max); Medicago truncatula	CRISPR;Cas9;TALENs;	soybean Double-stranded RNA-binding2 (GmDrb2a and GmDrb2b); M. truncatula Hua enhancer1 (MtHen1); soybean Dicer-like3 gene and GmHen1a; combining Gmdcl1a, Gmdcl1b and Gmdcl4b mutants with the Gmdrb2ab double mutant	Plant biotechnology journal	CRISPR/Cas9 and TALENs generate heritable mutations for genes involved in small RNA processing of Glycine max and Medicago truncatula.	2018	16(6):1125-1137	[Curtin SJ et al.]	University of Minnesota, St. Paul, MN, USA.	29087011	10.1111/pbi.12857	Processing of double-stranded RNA precursors into small RNAs is an essential regulator of gene expression in plant development and stress response. Small RNA processing requires the combined activity of a functionally diverse group of molecular components. However, in most of the plant species, there are insufficient mutant resources to functionally characterize each encoding gene. Here, mutations in loci encoding protein machinery involved in small RNA processing in soya bean and Medicago truncatula were generated using the CRISPR/Cas9 and TAL-effector nuclease (TALEN) mutagenesis platforms. An efficient CRISPR/Cas9 reagent was used to create a bi-allelic double mutant for the two soya bean paralogous Double-stranded RNA-binding2 (GmDrb2a and GmDrb2b) genes. These mutations, along with a CRISPR/Cas9-generated mutation of the M. truncatula Hua enhancer1 (MtHen1) gene, were determined to be germ-line transmissible. Furthermore, TALENs were used to generate a mutation within the soya bean Dicer-like2 gene. CRISPR/Cas9 mutagenesis of the soya bean Dicer-like3 gene and the GmHen1a gene was observed in the T0 generation, but these mutations failed to transmit to the T1 generation. The irregular transmission of induced mutations and the corresponding transgenes was investigated by whole-genome sequencing to reveal a spectrum of non-germ-line-targeted mutations and multiple transgene insertion events. Finally, a suite of combinatorial mutant plants were generated by combining the previously reported Gmdcl1a, Gmdcl1b and Gmdcl4b mutants with the Gmdrb2ab double mutant. Altogether, this study demonstrates the synergistic use of different genome engineering platforms to	soybean (Gly

511	plant	wild strawberry (Fragaria vesca)	CRISPR;Cas9;	auxin biosynthesis gene TAA1 and auxin response factor 8 (ARF8)	Plant biotechnology journal	Efficient genome editing of wild strawberry genes, vector development and validation.	2018	16(11):1868–1877	[Zhou J et al.]	University of Maryland, College Park, MD, USA.	29577545	10.1111/pbi.12922	The clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 system is an effective genome editing tool for plant and animal genomes. However, there are still few reports on the successful application of CRISPR–Cas9 to horticultural plants, especially with regard to germ–line transmission of targeted mutations. Here, we report high–efficiency genome editing in the wild strawberry Fragaria vesca and its successful application to mutate the auxin biosynthesis gene TAA1 and auxin response factor 8 (ARF8). In our CRISPR system, the Arabidopsis U6 promoter AtU6–26 and the wild strawberry U6 promoter FveU6–2 were each used to drive the expression of sgRNA, and both promoters were shown to lead to high–efficiency genome editing in strawberry. To test germ–line transmission of the edited mutations and new mutations induced in the next generation, the progeny of the primary (T0) transgenic plants carrying the CRISPR construct was analysed. New mutations were detected in the progeny plants at a high efficiency, including large deletions between the two PAM sites. Further, T1 plants harbouring arf8 homozygous knockout mutations grew considerably faster than wild–type plants. The results indicate that our CRISPR vectors can be used to edit the wild strawberry genome at a high efficiency and that both sgRNA design and appropriate U6 promoters contribute to the success of genomic editing. Our results open up exciting opportunities for engineering strawberry and	strawberry
512	plant	sugarcane	TALENs;	caffeic acid O–methyltransferase (COMT)	Plant biotechnology journal	TALEN–mediated targeted mutagenesis of more than 100 COMT copies/alleles in highly polyploid sugarcane improves saccharification efficiency without compromising biomass yield.	2018	16(4):856–866	[Kannan B et al.]	University of Florida, Gainesville, FL, USA.	28905511	10.1111/pbi.12833	Sugarcane is the world's most efficient feedstock for commercial production of bioethanol due to its superior biomass production and accumulation of sucrose in stems. Integrating first– and second–generation ethanol conversion processes will enhance the biofuel yield per unit area by utilizing both sucrose and cell wall–bound sugars for fermentation. RNAi suppression of the lignin biosynthetic gene caffeic acid O–methyltransferase (COMT) has been demonstrated to improve bioethanol production from lignocellulosic biomass. Genome editing has been used in a number of crops for creation of loss of function phenotypes but is very challenging in sugarcane due to its highly polyploid genome. In this study, a conserved region of COMT was targeted with a single–transcription activator–like effector nuclease (TALEN) pair for multi–allelic mutagenesis to modify lignin biosynthesis in sugarcane. Field–grown TALEN–mediated COMT mutants showed up to 19.7% lignin reduction and significantly decreased syringyl to guaiacyl (S/G) ratio resulting in an up to 43.8% improved saccharification efficiency. Biomass production of COMT mutant lines with superior saccharification efficiency did not differ significantly from the original cultivar under replicated field conditions. Sanger sequencing of cloned COMT amplicons (1351–1657 bp) revealed co–editing of 107 of the 109 unique COMT copies/alleles in vegetative progeny of line CB6 using a single TALEN pair. Line CB6 combined altered cell wall composition and drastically improved saccharification efficiency with good agronomic performance. These findings confirm the feasibility of co–mutagenesis of a very large number of target alleles/copies for	sugarcane
513	plant	switchgrass (Panicum virgatum L.)	CRISPR;Cas9;	teosinte branched 1a and 1b; phosphoglycerate mutase	Plant biotechnology journal	Targeted mutagenesis in tetraploid switchgrass (Panicum virgatum L.) using CRISPR/Cas9.	2018	16(2):381–393	[Liu Y et al.]	Iowa State University, Ames, IA, USA.	28640964	10.1111/pbi.12778	The CRISPR/Cas9 system has become a powerful tool for targeted mutagenesis. Switchgrass (Panicum virgatum L.) is a high yielding perennial grass species that has been designated as a model biomass crop by the U.S. Department of Energy. The self–infertility and high ploidy level make it difficult to study gene function or improve germplasm. To overcome these constraints, we explored the feasibility of using CRISPR/Cas9 for targeted mutagenesis in a tetraploid cultivar 'Alamo' switchgrass. We first developed a transient assay by which a non–functional green–fluorescent protein gene containing a 1–bp frameshift insertion in its 5' coding region was successfully mutated by a Cas9/sgRNA complex resulting in its restored function. Agrobacterium–mediated stable transformation of embryogenic calli derived from mature caryopses averaged a 3.0% transformation efficiency targeting the genes of teosinte branched 1(tb1)a and b and phosphoglycerate mutase (PGM). With a single construct containing two sgRNAs targeting different regions of tb1a and tb1b genes, primary transformants (T0) containing CRISPR/Cas9–induced mutations were obtained at frequencies of 95.5% (tb1a) and 11% (tb1b), respectively, with T0 mutants exhibiting increased tiller production. Meanwhile, a mutation frequency of 13.7% was obtained for the PGM gene with a CRISPR/Cas9 construct containing a single sgRNA. Among the PGM T0 mutants, six are heterozygous and one is homozygous for a 1–bp deletion in the target region with no apparent phenotypical alterations. We show that CRISPR/Cas9 system can generate targeted mutagenesis effectively and obtain targeted homozygous mutants in T0 generation in switchgrass, circumventing the need of inbreeding.	switchgrass
514	plant	Theobroma cacao	agroinfiltration; CRISPR;Cas9;	Non–Expressor of Pathogenesis–Related 3 (TcNPR3)	Frontiers in plant science	Transient Expression of CRISPR/Cas9 Machinery Targeting TcNPR3 Enhances Defense Response in Theobroma cacao.	2018	9:268	[Fister AS et al.]	Pennsylvania State University, University Park, PA, USA.	29552023	10.3389/fpls.2018.00268	Theobroma cacao, the source of cocoa, suffers significant losses to a variety of pathogens resulting in reduced incomes for millions of farmers in developing countries. Development of disease resistant cacao varieties is an essential strategy to combat this threat, but is limited by sources of genetic resistance and the slow generation time of this tropical tree crop. In this study, we present the first application of genome editing technology in cacao, using Agrobacterium–mediated transient transformation to introduce CRISPR/Cas9 components into cacao leaves and cotyledon cells. As a first proof of concept, we targeted the cacao Non–Expressor of Pathogenesis–Related 3 (TcNPR3) gene, a suppressor of the defense response. After demonstrating activity of designed single–guide RNAs (sgRNA) in vitro, we used Agrobacterium to introduce a CRISPR/Cas9 system into leaf tissue, and identified the presence of deletions in 27% of TcNPR3 copies in the treated tissues. The edited tissue exhibited an increased resistance to infection with the cacao pathogen Phytophthora tropicalis and elevated expression of downstream defense genes. Analysis of off–target mutagenesis in sequences similar to sgRNA target sites using high–throughput sequencing did not reveal mutations above background sequencing error rates. These results confirm the function of NPR3 as a repressor of the cacao immune system and demonstrate the application of CRISPR/Cas9 as a powerful functional genomics tool for cacao. Several stably transformed and genome edited somatic embryos were obtained via Agrobacterium–mediated transformation, and ongoing work will test the effectiveness	Theobroma c

515	plant	Nicotiana benthamiana	Agroinfiltration;	Arabidopsis thaliana genes encoding homogentisate phytyltransferase (HPT) and tocopherol cyclase (TC)	3 Biotech	Rapid enhancement of alpha-tocopherol content in Nicotiana benthamiana by transient expression of Arabidopsis thaliana Tocopherol cyclase and Homogentisate phytyl transferase genes.	2018	8(12):485	[Sathish S et al.]	Bharathiar University, Coimbatore, India.	30498659	10.1007/s13205-018-1496-4	Agrobacterium-mediated transient gene expression have become a method of choice over stable plant genetic transformation. Tocopherols are a family of vitamin E compounds, which are categorized along with tocotrienols occurring naturally in vegetable oils, nuts and leafy green vegetables. This is the first report involving AtTC and AtHPT transient expression in Nicotiana benthamiana and this system can be used efficiently for large scale production of vitamin E. Agroinfiltration studies were carried out in N.benthamiana for the expression of Arabidopsis thaliana (At) genes encoding homogentisate phytyltransferase (HPT) and tocopherol cyclase (TC) individually and in combination (HPT + TC). The transgene presence was analyzed by reverse transcription PCR, which showed the presence of both the vitamin E biosynthetic pathway genes. The gene expression analysis was carried out by (reverse transcription quantitative real-time polymerase chain reaction) RT-qPCR and alpha-tocopherol content was quantified using high performance liquid chromatography (HPLC). The relative gene expression analysis by RT-qPCR confirmed an increased expression pattern where TC + HPT combination recorded the highest of 231 fold, followed by TC gene with 186 fold, whereas the HPT gene recorded 178 fold. The alpha-tocopherol content in leaves expressing HPT, TC, and HPT + TC was increased by 4.2, 5.9 and 11.3 fold, respectively, as compared to the control. These results indicate that the transient expression of HPT and TC genes has enhanced the vitamin E levels and stable expression of both A. thaliana genes could be an efficient strategy to enhance	tobacco
516	plant	Nicotiana benthamiana	CRISPR;		Genome biology	RNA virus interference via CRISPR/Cas13a system in plants.	2018	19(1):1	[Aman R et al.]	King Abdullah University of Science and Technology, Thuwal, Saudi Arabia.	29301551	10.1186/s13059-017-1381-1	BACKGROUND: CRISPR/Cas systems confer immunity against invading nucleic acids and phages in bacteria and archaea. CRISPR/Cas13a (known previously as C2c2) is a class 2 type VI-A ribonuclease capable of targeting and cleaving single-stranded RNA (ssRNA) molecules of the phage genome. Here, we employ CRISPR/Cas13a to engineer interference with an RNA virus, Turnip Mosaic Virus (TuMV), in plants. RESULTS: CRISPR/Cas13a produces interference against green fluorescent protein (GFP)-expressing TuMV in transient assays and stable overexpression lines of Nicotiana benthamiana. CRISPR RNA (crRNAs) targeting the HC-Pro and GFP sequences exhibit better interference than those targeting other regions such as coat protein (CP) sequence. Cas13a can also process pre-crRNAs into functional crRNAs. CONCLUSIONS: Our data indicate that CRISPR/Cas13a can be used for engineering interference against RNA viruses, providing a potential novel mechanism for RNA-guided immunity against RNA viruses and for other RNA manipulations in plants.	tobacco
517	plant	Nicotiana benthamiana	Agroinfiltration;	Fc-fused capillary morphogenesis protein 2 (CMG2-Fc) containing one N-glycosylation site on the Fc domain	International journal of molecular sciences	Glycoform Modification of Secreted Recombinant Glycoproteins through Kifunensine Addition during Transient Vacuum Agroinfiltration.	2018	19(3)	[Xiong Y et al.]	University of California, Davis, CA, USA.	29562594	10.3390/ijms19030890	Kifunensine, a potent and selective inhibitor of class I alpha-mannosidases, prevents alpha-mannosidases I from trimming mannose residues on glycoproteins, thus resulting in oligomannose-type glycans. We report for the first time that through one-time vacuum infiltration of kifunensine in plant tissue, N-linked glycosylation of a recombinant protein transiently produced in whole-plants shifted completely from complex-type to oligomannose-type. Fc-fused capillary morphogenesis protein 2 (CMG2-Fc) containing one N-glycosylation site on the Fc domain, produced in Nicotiana benthamiana whole plants, served as a model protein. The CMG2-Fc fusion protein was produced transiently through vacuum agroinfiltration, with and without kifunensine at a concentration of 5.4 microM in the agroinfiltration suspension. The CMG2-Fc N-glycan profile was determined using LC-MS/MS with a targeted dynamic multiple reaction monitoring (MRM) method. The CMG2-Fc expression level in the infiltrated plant tissue and the percentage of oligomannose-type N-glycans for kifunensine treated plants was 874 mg/kg leaf fresh weight (FW) and 98.2%, respectively, compared to 717 mg/kg leaf FW and 2.3% for untreated plants. Oligomannose glycans are amenable to in vitro enzymatic modification to produce more human-like N-glycan structures that are preferred for the production of HIV-1 viral vaccine and certain monoclonal antibodies. This method allows glycan modifications using a bioprocessing approach without compromising protein yield or modification of the primary sequence, and could be expanded to other small molecule inhibitors of glycan-processing enzymes. For recombinant protein targeted for secretion, kifunensine treatment allows collection of glycoform-modified target protein from apoplast wash fluid (AWF) with minimal plant-specific complex N-glycan at higher	tobacco
518	plant	Nicotiana benthamiana	Agroinfiltration;	recombinant gp51	Journal of virological methods	Transient expression of a bovine leukemia virus envelope glycoprotein in plants by a recombinant TBSV vector.	2018	255:1-7	[Zhumabek AT et al.]	National Center for Biotechnology (NCB), 1Astana, Kazakhstan.	29410083	10.1016/j.jviromet.2018.01.016	Plants offer a unique combination of advantages for the production of valuable recombinant proteins in a relatively short time. For instance, a variety of diagnostic tests have been developed that use recombinant antigens expressed in plants. The envelope glycoprotein gp51 encoded by Bovine leukemia virus (BLV) is one of the essential subunits for viral infectivity. It was indicated that the recombinant gp51 (rgp51) of BLV small es, Cyrillican be used as an synthetic alternative antigen useful in the diagnosis of BLV infection in cattle. Here we evaluate the potential for using a viral vector based on the genome of Tomato bushy stunt virus (TBSV) for the efficient expression of BLV envelope glycoprotein rgp51 in Nicotiana benthamiana plants. The codon-optimized gene encoding rgp51 was synthesized by the de novo DNA synthesis to replace the GFP gene in the TBSV-derived viral vector that was then delivered into 4-5 week old N. benthamiana plants by agroinfiltration. Expression of recombinant his-tagged rgp51 was verified by protein extraction followed by western blot procedures, and by purification using Ni(2+)-affinity chromatography. The molecular weight of this plant-expressed rgp51 ranged from 43 to 55kDa and it was shown to be glycosylated. Important for potential use in diagnostic tests, purified rgp51 specifically reacted with BLV infected bovine sera while no reaction was observed with the negative serum	tobacco

519	plant	Nicotiana benthamiana	CRISPR;Cas9;	chloride channel (CLC-Nt1)	New phytologist	CLC-Nt1 affects Potato Virus Y infection via regulation of endoplasmic reticulum luminal Ph.	2018	220(2):539-552	[Sun H et al.]	Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, China.	30022473	10.1111/nph.15310	Chloride channel (CLC) proteins are important anion transporters conserved in organisms ranging from bacteria and yeast to plants and animals. According to sequence comparison, some plant CLCs are predicted to function as Cl(-) /H(+) antiporters, but not Cl(-) channels. However, no direct evidence was provided to verify the role of these plant CLCs in regulating the pH of the intracellular compartment. We identified tobacco CLC-Nt1 interacting with the Potato virus Y (PVY) 6K2 protein. To investigate its physiological function, homologous genes of CLC-Nt1 in Nicotiana benthamiana were knocked out using the CRISPR/Cas9 system. Complementation experiments were subsequently performed by expression of wild-type or point-mutated CLC-Nt1 in knockout mutants. The data presented herein demonstrate that CLC-Nt1 is localized at endoplasmic reticulum (ER). Using a pH-sensitive fluorescent protein (pHluorin), we found that loss of CLC-Nt1 function resulted in a decreased ER luminal pH. Secreted GFP (secGFP) was retained mostly in ER in knockout mutants, indicating that CLC-Nt1 is also involved in protein secretion. PVY infection induced a rise in ER luminal pH, which was dependent on functional CLC-Nt1. By contrast, loss of CLC-Nt1 function inhibited PVY intracellular replication and systemic infection. We propose that PVY alters ER luminal pH for infection in a CLC-Nt1-dependent manner.	tobacco
520	plant	Nicotiana benthamiana	Agroinfiltration;	split-GFP	Plant journal	Detection of membrane protein-protein interaction in planta based on dual-intein-coupled tripartite split-GFP association.	2018	94(3):426-438	[Liu TY et al.]	National Tsing Hua University, Hsinchu, Taiwan.	29451720	10.1111/tpj.13874	Despite the great interest in identifying protein-protein interactions (PPIs) in biological systems, only a few attempts have been made at large-scale PPI screening in planta. Unlike biochemical assays, bimolecular fluorescence complementation allows visualization of transient and weak PPIs in vivo at subcellular resolution. However, when the non-fluorescent fragments are highly expressed, spontaneous and irreversible self-assembly of the split halves can easily generate false positives. The recently developed tripartite split-GFP system was shown to be a reliable PPI reporter in mammalian and yeast cells. In this study, we adapted this methodology, in combination with the beta-estradiol-inducible expression cassette, for the detection of membrane PPIs in planta. Using a transient expression assay by agroinfiltration of Nicotiana benthamiana leaves, we demonstrate the utility of the tripartite split-GFP association in plant cells and affirm that the tripartite split-GFP system yields no spurious background signal even with abundant fusion proteins readily accessible to the compartments of interaction. By validating a few of the Arabidopsis PPIs, including the membrane PPIs implicated in phosphate homeostasis, we proved the fidelity of this assay for detection of PPIs in various cellular compartments in planta. Moreover, the technique combining the tripartite split-GFP association and dual-intein-mediated cleavage of polyprotein precursor is feasible in stably transformed Arabidopsis plants. Our results provide a proof-of-concept implementation of the tripartite split-GFP	tobacco
521	plant	Nicotiana benthamiana	Agroinfiltration;	human Granulocyte-Colony Stimulating Factor	Plant methods	Transient co-expression with three O-glycosylation enzymes allows production of GalNAc-O-glycosylated Granulocyte-Colony Stimulating Factor in N. benthamiana.	2018	14:98	[Ramirez-Alanis IA et al.]	Tecnologico de Monterrey, Monterrey, NL, Mexico.	30410568	10.1186/s13007-018-0363-y	Background: Expression of economically relevant proteins in alternative expression platforms, especially plant expression platforms, has gained significant interest in recent years. A special interest in working with plants as bioreactors for the production of pharmaceutical proteins is related to low production costs, product safety and quality. Among the different properties that plants can also offer for the production of recombinant proteins, protein glycosylation is crucial since it may have an impact on pharmaceutical functionality and/or stability. Results: The pharmaceutical glycoprotein human Granulocyte-Colony Stimulating Factor was transiently expressed in Nicotiana benthamiana plants and subjected to mammalian-specific mucin-type O-glycosylation by co-expressing the pharmaceutical protein together with the glycosylation machinery responsible for such post-translational modification. Conclusions: The pharmaceutical glycoprotein human Granulocyte-Colony Stimulating Factor can be expressed in N. benthamiana plants via agroinfiltration with its native mammalian-specific mucin-type	tobacco
522	plant	Nicotiana benthamiana	Agroinfiltration;	GUS (beta-glucuronidase)	Plant methods	Improving agroinfiltration-based transient gene expression in Nicotiana benthamiana.	2018	14:71	[Norkunas K et al.]	Queensland University of Technology, Brisbane, QLD, Australia.	30159002	10.1186/s13007-018-0343-2	Background: Agroinfiltration is a simple and effective method of delivering transgenes into plant cells for the rapid production of recombinant proteins and has become the preferred transient expression platform to manufacture biologics in plants. Despite its popularity, few studies have sought to improve the efficiency of agroinfiltration to further increase protein yields. This study aimed to increase agroinfiltration-based transient gene expression in Nicotiana benthamiana by improving all levels of transgenesis. Results: Using the benchmark pEAQ-HT deconstructed virus vector system and the GUS reporter enzyme, physical, chemical, and molecular features were independently assessed for their ability to enhance Agrobacterium-mediated transformation and improve protein production capacities. Optimal Agrobacterium strain, cell culture density and co-cultivation time for maximal transient GUS (beta-glucuronidase) expression were established. The effects of chemical additives in the liquid infiltration media were investigated and acetosyringone (500 muM), the antioxidant lipoic acid (5 muM), and a surfactant Pluronic F-68 (0.002%) were all shown to significantly increase transgene expression. Gene products known to suppress post-transcriptional gene silencing, activate cell cycle progression and confer stress tolerance were also assessed by co-expression. A simple 37 degrees C heat shock to plants, 1-2 days post infiltration, was shown to dramatically increase GUS reporter levels. By combining the most effective features, a dual vector delivery system was developed that provided approximately 3.5-fold higher levels of absolute GUS protein compared to the pEAQ-HT platform. Conclusions: In this paper, different strategies were assessed and optimised with the aim of increasing plant-made protein capacities in Nicotiana benthamiana using agroinfiltration. Chemical additives, heat shock and the co-expression of genes known to suppress stress and gene silencing or stimulate cell cycle progression were all proven to increase agroinfiltration-based transient gene expression. By combining the most effective of these elements a novel expression	tobacco

523	plant	Nicotiana benthamiana	Agroinfiltration;	51-nt unstructured region (USR)	PLoS pathogens	RNA virus evasion of nonsense-mediated decay.	2018	14(11):e1007459	[May JP et al.]	University of Maryland-College Park, College Park, MD, USA.	30452463	10.1371/journal.ppat.1007459	Nonsense-mediated decay (NMD) is a host RNA control pathway that removes aberrant transcripts with long 3' untranslated regions (UTRs) due to premature termination codons (PTCs) that arise through mutation or defective splicing. To maximize coding potential, RNA viruses often contain internally located stop codons that should also be prime targets for NMD. Using an agroinfiltration-based NMD assay in Nicotiana benthamiana, we identified two segments conferring NMD-resistance in the carmovirus Turnip crinkle virus (TCV) genome. The ribosome readthrough structure just downstream of the TCV p28 termination codon stabilized an NMD-sensitive reporter as did a frameshifting element from umbravirus Pea enation mosaic virus. In addition, a 51-nt unstructured region (USR) at the beginning of the TCV 3' UTR increased NMD-resistance 3-fold when inserted into an unrelated NMD-sensitive 3' UTR. Several additional carmovirus 3' UTRs also conferred varying levels of NMD resistance depending on the construct despite no sequence similarity in the analogous region. Instead, these regions displayed a marked lack of RNA structure immediately following the NMD-targeted stop codon. NMD-resistance was only slightly reduced by conversion of 19 pyrimidines in the USR to purines, but resistance was abolished when a 2-nt mutation was introduced downstream of the USR that substantially increased the secondary structure in the USR through formation of a stable hairpin. The same 2-nt mutation also enhanced the NMD susceptibility of a subgenomic RNA expressed independently of the genomic RNA. The conserved lack of RNA structure among most carmoviruses at the 5' end of their 3' UTR could serve to enhance subgenomic RNA stability, which would increase expression of the encoded capsid protein that also functions as the RNA silencing suppressor. These results demonstrate that the TCV genome has features that are inherently NMD-resistant and these strategies could be	tobacco
524	plant	Nicotiana benthamiana; Arabidopsis	CRISPR;Cas9;	FnCas9 and sgRNA specific for the cucumber mosaic virus (CMV) or tobacco mosaic virus (TMV)	Plant biotechnology journal	Establishing RNA virus resistance in plants by harnessing CRISPR immune system.	2018	16(8):1415-1423	[Zhang T et al.]	South China Agricultural University, Guangzhou, Guangdong, China.	29327438	10.1111/pbi.12881	Recently, CRISPR-Cas (clustered, regularly interspaced short palindromic repeats-CRISPR-associated proteins) system has been used to produce plants resistant to DNA virus infections. However, there is no RNA virus control method in plants that uses CRISPR-Cas system to target the viral genome directly. Here, we reprogrammed the CRISPR-Cas9 system from Francisella novicida to confer molecular immunity against RNA viruses in Nicotiana benthamiana and Arabidopsis plants. Plants expressing FnCas9 and sgRNA specific for the cucumber mosaic virus (CMV) or tobacco mosaic virus (TMV) exhibited significantly attenuated virus infection symptoms and reduced viral RNA accumulation. Furthermore, in the transgenic virus-targeting plants, the resistance was inheritable and the progenies showed significantly less virus accumulation. These data reveal that the CRISPR/Cas9 system can be used to produce plant that stable resistant to RNA viruses, thereby broadening the use of such	tobacco
525	plant	Nicotiana benthamiana; tomato	CRISPR;Cas9;		Plant signaling & behavior	Engineering resistance against Tomato yellow leaf curl virus via the CRISPR/Cas9 system in tomato.	2018	13(10):e1525996	[Tashkandi M et al.]	King Abdullah University of Science and Technology , Thuwal , Saudi Arabia.	30289378	10.1080/15592324.2018.1525996	CRISPR/Cas systems confer molecular immunity against phages and conjugative plasmids in prokaryotes. Recently, CRISPR/Cas9 systems have been used to confer interference against eukaryotic viruses. Here, we engineered Nicotiana benthamiana and tomato (Solanum lycopersicum) plants with the CRISPR/Cas9 system to confer immunity against the Tomato yellow leaf curl virus (TYLCV). Targeting the TYLCV genome with Cas9-single guide RNA at the sequences encoding the coat protein (CP) or replicase (Rep) resulted in efficient virus interference, as evidenced by low accumulation of the TYLCV DNA genome in the transgenic plants. The CRISPR/Cas9-based immunity remained active across multiple generations in the N. benthamiana and tomato plants. Together, our results confirmed the efficiency of the CRISPR/Cas9 system for stable engineering of TYLCV resistance in N. benthamiana and tomato, and opens the possibilities of engineering virus resistance against single and multiple	tobacco
526	plant	Nicotiana occidentalis	Agroinfiltration;	GFLV-F13 protein 2A(HP) fused to an enhanced green fluorescent protein (EGFP) tag	Molecular plant pathology	The 50 distal amino acids of the 2A(HP) homing protein of Grapevine fanleaf virus elicit a hypersensitive reaction on Nicotiana occidentalis.	2018	19(3):731-743	[Martin IR et al.]	Universite de Strasbourg, INRA, Colmar, France.	28387986	10.1111/mpp.12558	Avirulence factors are critical for the arm's race between a virus and its host in determining incompatible reactions. The response of plants to viruses from the genus Nepovirus in the family Secoviridae, including Grapevine fanleaf virus (GFLV), is well characterized, although the nature and characteristics of the viral avirulence factor remain elusive. By using infectious clones of GFLV strains F13 and GHu in a reverse genetics approach with wild-type, assortant and chimeric viruses, the determinant of necrotic lesions caused by GFLV-F13 on inoculated leaves of Nicotiana occidentalis was mapped to the RNA2-encoded protein 2A(HP) , particularly to its 50 C-terminal amino acids. The necrotic response showed hallmark characteristics of a genuine hypersensitive reaction, such as the accumulation of phytoalexins, reactive oxygen species, pathogenesis-related protein 1c and hypersensitivity-related (hsr) 203J transcripts. Transient expression of the GFLV-F13 protein 2A(HP) fused to an enhanced green fluorescent protein (EGFP) tag in N. occidentalis by agroinfiltration was sufficient to elicit a hypersensitive reaction. In addition, the GFLV-F13 avirulence factor, when introduced in GFLV-GHu, which causes a compatible reaction on N. occidentalis, elicited necrosis and partially restricted the virus. This is the first identification of a nepovirus avirulence factor that is responsible for a hypersensitive	tobacco
527	plant	Nicotiana tabacum	Cas9;	NtNAC080	Frontiers in plant science	NAC Family Transcription Factors in Tobacco and Their Potential Role in Regulating Leaf Senescence.	2018	9:1900	[Li W et al.]	Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, China.	30622549	10.3389/fpls.2018.01900	The NAC family is one of the largest families of plant-specific transcription factors (TFs) and NAC proteins play important regulatory roles in a variety of developmental and stress response processes in plants. Members of the NAC family TFs have been shown to be important regulators of leaf senescence in a number of plant species. Here we report the identification of the NAC family in tobacco (Nicotiana tabacum) and characterization of the potential role of some of the tobacco NAC TFs in regulating leaf senescence. A total of 154 NAC genes (NtNACs) were identified and clustered together with the Arabidopsis NAC family into fifteen groups (a-o). Transcriptome data analysis followed by qRT-PCR validation showed that the majority of the senescence-up-regulated NtNACs fall into subgroups NAC-b and f. A number of known senescence regulators from Arabidopsis also belong to these two subgroups. Among these senescence-up-regulated NtNACs, NtNAC080, a close homolog of AtNAP, is a positive regulator of leaf senescence. Overexpression of NtNAC080 caused early senescence in Arabidopsis leaves and NtNAC080 mutation induced by Cas9/gRNA in tobacco led to	tobacco

528	plant	Nicotiana tabacum	CRISPR;Cas9;		Plant biotechnology journal	Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration.	2018	16(7):1295-1310	[Lin CS et al.]	Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan.	29230929	10.1111/pbi.12870	Plant protoplasts are useful for assessing the efficiency of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) mutagenesis. We improved the process of protoplast isolation and transfection of several plant species. We also developed a method to isolate and regenerate single mutagenized Nicotianna tabacum protoplasts into mature plants. Following transfection of protoplasts with constructs encoding Cas9 and sgRNAs, target gene DNA could be amplified for further analysis to determine mutagenesis efficiency. We investigated N. tabacum protoplasts and derived regenerated plants for targeted mutagenesis of the phytoene desaturase (NtPDS) gene. Genotyping of albino regenerants indicated that all four NtPDS alleles were mutated in amphidiploid tobacco, and no Cas9 DNA could be	tobacco
529	plant	tobacco	Agroinfiltration;	green fluorescent protein (GFP)	Biochemical and biophysical research communications	Expression of T7-based constructs in tobacco cells.	2018	499(2):196-201	[Sheen H et al.]	York University, Toronto, Ontario, Canada.	29555475	10.1016/j.bbrc.2018.03.123	Bacteriophage T7 promoter and RNA polymerase (T7-Pol) are widely used for recombinant protein expression in bacteria. In plants, there exists conflicting results regarding the efficacy of protein expression from T7-Pol-derived mRNAs. To reconcile these contradictory observations, the expression of green fluorescent protein (GFP) from T7 constructs was evaluated in tobacco protoplasts. T7 constructs transcribed by a nuclearly targeted T7-Pol did not express GFP in plant protoplasts, however T7-Pol lacking a nuclear targeting signal was able to translate cytosolically transcribed mRNAs, but only if the messages contained a viral translation enhancer. GFP expression was further evaluated at the plant level by using agroinfiltration-mediated transient expression system. Unlike for cytosolic expression, nuclear T7 transcripts containing a viral translation enhancer element did not express GFP, and modifications designed to stabilize and facilitate export of T7 transcripts to the cytosol did not improve the expression. We conclude that expression of nuclear T7 constructs is not feasible in tobacco cells, but cytosolic transcription provides an alternative means to	tobacco
530	plant	tobacco	agroinfiltration; CRISPR;Cas9;	phytoene desaturase (PDS)	Horticulture research	A method for the production and expedient screening of CRISPR/Cas9-mediated non-transgenic mutant plants.	2018	5:13	[Chen L et al.]	University of Connecticut, Storrs, CT, USA.	29531752	10.1038/s41438-018-0023-4	Developing CRISPR/Cas9-mediated non-transgenic mutants in asexually propagated perennial crop plants is challenging but highly desirable. Here, we report a highly useful method using an Agrobacterium-mediated transient CRISPR/Cas9 gene expression system to create non-transgenic mutant plants without the need for sexual segregation. We have also developed a rapid, cost-effective, and high-throughput mutant screening protocol based on Illumina sequencing followed by high-resolution melting (HRM) analysis. Using tetraploid tobacco as a model species and the phytoene desaturase (PDS) gene as a target, we successfully created and expediently identified mutant plants, which were verified as tetra-allelic mutants. We produced pds mutant shoots at a rate of 47.5% from tobacco leaf explants, without the use of antibiotic selection. Among these pds plants, 17.2% were confirmed to be non-transgenic, for an overall non-transgenic mutation rate of 8.2%. Our method is reliable and effective in creating non-transgenic mutant plants without the need to segregate out transgenes through sexual reproduction. This method should be applicable to many economically important, heterozygous, perennial crop species that are more difficult to regenerate.	tobacco
531	plant	tobacco	CRISPR;Cas9;	carotenoid cleavage dioxygenase (CCD) genes (NtCCD8A; NtCCD8B)	International journal of molecular sciences	CRISPR/Cas9-Mediated Mutagenesis of Carotenoid Cleavage Dioxygenase 8 (CCD8) in Tobacco Affects Shoot and Root Architecture.	2018	19(4)	[Gao J et al.]	Southwest University, Chongqing, China.	29614837	10.3390/ijms19041062	Strigolactones (SLs) are a class of phytohormones that regulate plant architecture. Carotenoid cleavage dioxygenase (CCD) genes are involved in the biosynthesis of SLs and are identified and characterized in many plants. However, the function of CCD genes in tobacco remains poorly understood. In this study, two closely related genes NtCCD8A and NtCCD8B were cloned from tobacco (Nicotiana tabacum L.). The two NtCCD8 genes are orthologues of the tomato (Solanum lycopersicum) carotenoid cleavage dioxygenase 8 (SlCCD8) gene. NtCCD8A and NtCCD8B were primarily expressed in tobacco roots, but low expression levels of these genes were detected in all plant tissues, and their transcript levels significantly increased in response to phosphate limitation. NtCCD8A and NtCCD8B mutations were introduced into tobacco using the CRISPR/Cas9 system and transgenic tobacco lines for both ntccd8 mutant alleles were identified. The ntccd8a and ntccd8b mutant alleles were inactivated by a deletion of three nucleotides and insertion of one nucleotide, respectively, both of which led to the production of premature stop codons. The ntccd8 mutants had increased shoot branching, reduced plant height, increased number of leaves and nodes, and reduced total plant biomass compared to wild-type plants; however, the root-to-shoot ratio was unchanged. In addition, mutant lines had shorter primary roots and more of lateral roots than wild type. These results suggest that NtCCD8 genes are	tobacco
532	plant	tobacco	Agroinfiltration;	prunasin hydrolases	Plant physiology and biochemistry	beta-Glucosidase activity in almond seeds.	2018	126:163-172	[Del Cueto J et al.]	CEBAS-CSIC, Murcia, Spain.	29524803	10.1016/j.plaphy.2017.12.028	Almond bitterness is the most important trait for breeding programs since bitter-kernelled seedlings are usually discarded. Amygdalin and its precursor prunasin are hydrolyzed by specific enzymes called beta-glucosidases. In order to better understand the genetic control of almond bitterness, some studies have shown differences in the location of prunasin hydrolases (PH, the beta-glucosidase that degrades prunasin) in sweet and bitter genotypes. The aim of this work was to isolate and characterize different PHs in sweet- and bitter-kernelled almonds to determine whether differences in their genomic or protein sequences are responsible for the sweet or bitter taste of their seeds. RNA was extracted from the tegument, nucellus and cotyledon of one sweet (Lauranne) and two bitter (D05-187 and S3067) almond genotypes throughout fruit ripening. Sequences of nine positive Phs were then obtained from all of the genotypes by RT-PCR and cloning. These clones, from mid ripening stage, were expressed in a heterologous system in tobacco plants by agroinfiltration. The PH activity was detected using the Feigl-Anger method and quantifying the hydrogen cyanide released with prunasin as substrate. Furthermore, beta-glucosidase activity was detected by Fast Blue BB salt and Umbelliferyl method. Differences at the sequence level (SNPs) and in the activity assays were detected, although no	tobacco

533	plant	tobacco	Agroinfiltration;	strawberry linalool/nerolidol synthase 1	Plant science	Nerolidol production in agroinfiltrated tobacco: Impact of protein stability and membrane targeting of strawberry (Fragraria ananassa) NEROLIDOL SYNTHASE1.	2018	267:112-123	[Andrade P et al.]	Center for Research in Agricultural Genomics, (CRAG) (CSIC-IRTA-UAB-UB), Barcelona, Spain.	29362090	10.1016/j.plantsci.2017.11.013	The sesquiterpene alcohol nerolidol, synthesized from farnesyl diphosphate (FDP), mediates plant-insect interactions across multiple trophic levels with major implications for pest management in agriculture. We compared nerolidol engineering strategies in tobacco using agroinfiltration to transiently express strawberry (Fragraria ananassa) linalool/nerolidol synthase (FaNES1) either at the endoplasmic reticulum (ER) or in the cytosol as a soluble protein. Using solid phase microextraction and gas chromatography-mass spectrometry (SPME-GCMS), we have determined that FaNES1 directed to the ER via fusion to the transmembrane domain of squalene synthase or hydroxymethylglutaryl - CoA reductase displayed significant improvements in terms of transcript levels, protein accumulation, and volatile production when compared to its cytosolic form. However, the highest levels of nerolidol production were observed when FaNES1 was fused to GFP and expressed in the cytosol. This SPME-GCMS method afforded a limit of detection and quantification of 1.54 and 5.13pg, respectively. Nerolidol production levels, which ranged from 0.5 to 3.0mug/g F.W., correlated more strongly to the accumulation of recombinant protein than transcript level, the former being highest in FaNES-GFP transfected plants. These results indicate that while the ER may represent an enriched source of FDP that can be exploited in metabolic engineering, protein accumulation is a better predictor of sesquiterpene production.	tobacco
534	plant	tomato	CRISPR;Cas9;		Frontiers in plant science	Efficient Multiplex Genome Editing Induces Precise, and Self-Ligated Type Mutations in Tomato Plants.	2018	9:916	[Hashimoto R et al.]	Tokushima University, Tokushima, Japan.	30018630	10.3389/fpls.2018.00916	Several expression systems for multiple guide RNA (gRNAs) have been developed in the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) system to induce multiple-gene modifications in plants. Here, we evaluated mutation efficiencies in the tomato genome using multiplex CRISPR/Cas9 vectors consisting of various Cas9 expression promoters with multiple gRNA expression combinations. In transgenic tomato calli induced with these vectors, mutation patterns varied depending on the promoters used to express Cas9. By using the tomato ELONGATION FACTOR-1alpha (SlEF1alpha) promoter to drive Cas9, occurrence of various types of mutations with high efficiency was detected in the tomato genome. Furthermore, sequence analysis showed that the majority of mutations using the multiplex system with the SlEF1alpha promoter corresponded to specific mutation pattern of deletions produced by self-ligation at two target sites of CRISPR/Cas9 with low mosaic mutations. These results suggest that optimizing the Cas9 expression promoter used in CRISPR/Cas9-mediated mutation improves multiplex genome editing, and could be used effectively to disrupt functional domains	tomato
535	plant	tomato	agroinfiltration; CRISPR;Cas9;	5 genes	Frontiers in plant science	Lycopene Is Enriched in Tomato Fruit by CRISPR/Cas9-Mediated Multiplex Genome Editing.	2018	9:559	[Li X et al.]	China Agricultural University, Beijing, China.	29755497	10.3389/fpls.2018.00559	Numerous studies have been focusing on breeding tomato plants with enhanced lycopene accumulation, considering its positive effects of fruits on the visual and functional properties. In this study, we used a bidirectional strategy: promoting the biosynthesis of lycopene, while inhibiting the conversion from lycopene to beta- and alpha-carotene. The accumulation of lycopene was promoted by knocking down some genes associated with the carotenoid metabolic pathway. Finally, five genes were selected to be edited in genome by CRISPR/Cas9 system using Agrobacterium tumefaciens-mediated transformation. Our findings indicated that CRISPR/Cas9 is a site-specific genome editing technology that allows highly efficient target mutagenesis in multiple genes of interest. Surprisingly, the lycopene content in tomato fruit subjected to genome editing was successfully increased to about 5.1-fold. The homozygous mutations were stably transmitted to subsequent generations. Taken together, our results suggest that CRISPR/Cas9 system can be used for significantly improving lycopene content in tomato fruit with advantages such as high efficiency,	tomato
536	plant	tomato	CRISPR;Cas9;	NAC transcription factor NOR-like1	Horticulture research	A NAC transcription factor, NOR-like1, is a new positive regulator of tomato fruit ripening.	2018	5:75	[Gao Y et al.]	China Agricultural University, Beijing, China.	30588320	10.1038/s41438-018-0111-5	Ripening of the model fruit tomato (Solanum lycopersicum) is controlled by a transcription factor network including NAC (NAM, ATAF1/2, and CUC2) domain proteins such as No-ripening (NOR), SlNAC1, and SlNAC4, but very little is known about the NAC targets or how they regulate ripening. Here, we conducted a systematic search of fruit-expressed NAC genes and showed that silencing NOR-like1 (Solyc07g063420) using virus-induced gene silencing (VIGS) inhibited specific aspects of ripening. Ripening initiation was delayed by 14 days when NOR-like1 function was inactivated by CRISPR/Cas9 and fruits showed obviously reduced ethylene production, retarded softening and chlorophyll loss, and reduced lycopene accumulation. RNA-sequencing profiling and gene promoter analysis suggested that genes involved in ethylene biosynthesis (SlACS2, SlACS4), color formation (SlGgpps2, SlSGR1), and cell wall metabolism (SlPG2a, SlPL, SlCEL2, and SlEXP1) are direct targets of NOR-like1. Electrophoretic mobility shift assays (EMSA), chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR), and dual-luciferase reporter assay (DLR) confirmed that NOR-like1 bound to the promoters of these genes both in vitro and in vivo, and activated their expression. Our findings demonstrate that NOR-like1 is a new positive	tomato
537	plant	tomato	CRISPR;Cas9;	sedoheptulose-1,7-bisphosphatase (SlSBPASE)	International journal of molecular sciences	Knockout of SlSBPASE Suppresses Carbon Assimilation and Alters Nitrogen Metabolism in Tomato Plants.	2018	19(12)	[Ding F et al.]	Northwest A&F University, Yangling, Shaanxi, China.	30558146	10.3390/ijms19124046	Sedoheptulose-1,7-bisphosphatase (SBPase) is an enzyme in the Calvin(-)Benson cycle and has been documented to be important in carbon assimilation, growth and stress tolerance in plants. However, information on the impact of SBPase on carbon assimilation and nitrogen metabolism in tomato plants (Solanum lycopersicum) is rather limited. In the present study, we investigated the role of SBPase in carbon assimilation and nitrogen metabolism in tomato plants by knocking out SBPase gene SlSBPASE using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing technology. Compared with wild-type plants, slsbpase mutant plants displayed severe growth retardation. Further analyses showed that knockout of SlSBPASE led to a substantial reduction in SBPase activity and as a consequence, ribulose-1,5-bisphosphate (RuBP) regeneration and carbon assimilation rate were dramatically inhibited in slsbpase mutant plants. It was further observed that much lower levels of sucrose and starch were accumulated in slsbpase mutant plants than their wild-type counterparts during the photoperiod. Intriguingly, mutation in SlSBPASE altered nitrogen metabolism as demonstrated by changes in levels of protein and amino acids and activities of nitrogen metabolic enzymes. Collectively, our data suggest that SlSBPASE is required for optimal growth, carbon assimilation and nitrogen	tomato

538	plant	tomato	CRISPR;Cas9;	sedoheptulose-1,7-bisphosphatase (SISBPASE)	International journal of molecular sciences	Sedoheptulose-1,7-Bisphosphatase is Involved in Methyl Jasmonate- and Dark-Induced Leaf Senescence in Tomato Plants.	2018	19(11)	[Ding F et al.]	Northwest A&F University, Yangling, Shaanxi, China.	30463360	10.3390/ijms19113673	Leaf senescence represents the final stage of leaf development and is regulated by diverse internal and environmental factors. Jasmonates (JAs) have been demonstrated to induce leaf senescence in several species; however, the mechanisms of JA-induced leaf senescence remain largely unknown in tomato plants (Solanum lycopersicum). In the present study, we tested the hypothesis that sedoheptulose-1,7-bisphosphatase (SBPase), an enzyme functioning in the photosynthetic carbon fixation in the Calvin(-)Benson cycle, was involved in methyl jasmonate (MeJA)- and dark-induced leaf senescence in tomato plants. We found that MeJA and dark induced senescence in detached tomato leaves and concomitantly downregulated the expression of SISBPASE and reduced SBPase activity. Furthermore, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9)-mediated mutagenesis of SlSBPASE led to senescence-associated characteristics in slsbpase mutant plants, including loss of chlorophyll, repressed photosynthesis, increased membrane ion leakage, and enhanced transcript abundance of senescence-associated genes. Collectively, our data suggest that repression of SBPase by MeJA and dark treatment plays a role in JA- and dark-induced leaf senescence.	tomato
539	plant	tomato	CRISPR;Cas9;	cis-regulatory regions or upstream open reading frames of genes associated with morphology, flower and fruit production, and ascorbic acid	Nature biotechnology	Domestication of wild tomato is accelerated by genome editing.	2018		[Li T et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.	30272676	10.1038/nbt.4273	Crop improvement by inbreeding often results in fitness penalties and loss of genetic diversity. We introduced desirable traits into four stress-tolerant wild-tomato accessions by using multiplex CRISPR-Cas9 editing of coding sequences, cis-regulatory regions or upstream open reading frames of genes associated with morphology, flower and fruit production, and ascorbic acid synthesis. Cas9-free progeny of edited plants had domesticated phenotypes yet retained parental disease resistance and salt tolerance.	tomato
540	plant	tomato	CRISPR;Cas9;	plant-specific group D mitogen-activated protein kinase (SIMPK20)	New phytologist	Evidence for a specific and critical role of mitogen-activated protein kinase 20 in uni-to-binucleate transition of microgametogenesis in tomato.	2018	219(1):176-194	[Chen L et al.]	Zhejiang University, Hangzhou, China.	29668051	10.1111/nph.15150	(MAPKs) regulate diverse aspects of plant growth. However, their potential role in reproductive development remains elusive. Here, we discovered an unique role of SIMPK20, a plant-specific group D MAPK, in pollen development in tomato. RNAi-mediated suppression of SIMPK20 or its knockout using CRISPR/Cas9 significantly reduced or completely abolished pollen viability, respectively, with no effects on maternal fertility. Cell biology and gene expression analyses established that SIMPK20 exerts its role specifically at the uni-to-binucleate transition during microgametogenesis. This assertion is based on the findings that the transgenic pollen was largely arrested at the binucleate stage with the appearance of subcellular abnormality at the middle uninucleate microspore stage; and SIMPK20 mRNA and SIMPK20-GUS signals were localized in the tetrads, uninuclear microspores and binuclear pollen grains but not in microspore mother cells or mature pollen grains. Transcriptomic and proteomic analyses revealed that knockout of SIMPK20 significantly reduced the expression of a large number of genes controlling sugar and auxin metabolism and signaling in anthers. Finally, protein-protein interaction assays identified SlMYB32 as a putative target protein of SIMPK20. We conclude that SIMPK20 specifically regulates post-meiotic pollen development through modulating sugar and	tomato
541	plant	tomato	CRISPR;	BRASSINAZOLE RESISTANT 1 (brz1)	Plant & cell physiology	BZR1 Transcription Factor Regulates Heat Stress Tolerance Through FERONIA Receptor-Like Kinase-Mediated Reactive Oxygen Species Signaling in Tomato.	2018	59(11):2239-2254	[Yin Y et al.]	Zhejiang University, Hangzhou, China.	30107607	10.1093/pcp/pcy146	BRASSINAZOLE RESISTANT 1 (BZR1), the critical regulator of brassinosteroid (BR) response, participates in various BR-mediated developmental processes. However, the roles of BZR1 in stress tolerance are less clear. Here, we found that BZR1-like protein in tomato controls BR response and is involved in thermotolerance by regulating the FERONIA (FER) homologs. The CRISPR-bzr1 mutant showed reduced growth and was not responsive to 24-epibrassinolide (EBR) with regard to the promotion of plant growth. Mutation in BZR1 impaired the induction of RESPIRATORY BURST OXIDASE HOMOLOG1 (RBOH1), production of H2O2 in the apoplast and heat tolerance. Exogenous H2O2 recovered the heat tolerance of the tomato bzr1 mutant. Overexpression of BZR1 enhanced the production of apoplastic H2O2 and heat stress responses. However, silencing of RBOH1 abolished the BZR1-mediated heat tolerance. Further analysis showed that BZR1 bound to the promoters of FERONIA2 (FER2) and FER3 and induced their expression. Silencing of FER2/3 suppressed BZR1-dependent BR signaling for the induction of RBOH1 transcripts, accumulation of apoplastic H2O2 and heat tolerance. These results indicate that BZR1 regulates heat stress responses in tomato through RBOH1-dependent reactive oxygen species (ROS) signaling, which is	tomato
542	plant	tomato	CRISPR;Cas9;	5 key genes in gamma-aminobutyric acid GABA production	Plant biotechnology journal	Multiplexed CRISPR/Cas9-mediated metabolic engineering of gamma-aminobutyric acid levels in Solanum lycopersicum.	2018	16(2):415-427	[Li R et al.]	China Agricultural University, Beijing, China.	28640983	10.1111/pbi.12781	In recent years, the type II CRISPR system has become a widely used and robust technique to implement site-directed mutagenesis in a variety of species including model and crop plants. However, few studies manipulated metabolic pathways in plants using the CRISPR system. Here, we introduced the pYLCRISPR/Cas9 system with one or two single-site guide RNAs to target the tomato phytoene desaturase gene. An obvious albino phenotype was observed in T0 regenerated plants, and more than 61% of the desired target sites were edited. Furthermore, we manipulated the gamma-aminobutyric acid (GABA) shunt in tomatoes using a multiplex pYLCRISPR/Cas9 system that targeted five key genes. Fifty-three genome-edited plants were obtained following single plant transformation, and these samples represented single to quadruple mutants. The GABA accumulation in both the leaves and fruits of genomically edited lines was significantly enhanced, and the GABA content in the leaves of quadruple mutants was 19-fold higher than that in wild-type plants. Our data demonstrate that the multiplex CRISPR/Cas9 system can be exploited to precisely edit tomato genomic sequences and effectively create multisite knockout mutations, which	tomato

543	plant	tomato	CRISPR;Cas9;	Slddm1a; Slddm1b	Plant cell	Redistribution of CHH Methylation and Small Interfering RNAs across the Genome of Tomato ddm1 Mutants.	2018	30(7):1628-1644	[Corem S et al.]	Institute of Plant Sciences, Agricultural Research Organization, Rishon LeZion, Israel.	29875274	10.1105/tpc.18.00167	In plants, cytosine methylation, an epigenetic mark critical for transposon silencing, is maintained over generations by key enzymes that directly methylate DNA and is facilitated by chromatin remodelers, like DECREASE IN DNA METHYLATION1 (DDM1). Short-interfering RNAs (siRNAs) also mediate transposon DNA methylation through a process called RNA-directed DNA methylation (RdDM). In tomato (Solanum lycopersicum), siRNAs are primarily mapped to gene-rich chromosome arms, and not to pericentromeric regions as in Arabidopsis thaliana Tomato encodes two DDM1 genes. To better understand their functions and interaction with the RdDM pathway, we targeted the corresponding genes via the CRISPR/Cas9 technology, resulting in the isolation of Slddm1a and Slddm1b knockout mutants. Unlike the single mutants, Slddm1a Slddm1b double mutant plants display pleiotropic vegetative and reproductive phenotypes, associated with severe hypomethylation of the heterochromatic transposons in both the CG and CHG methylation contexts. The methylation in the CHH context increased for some heterochromatic transposons and conversely decreased for others localized in euchromatin. We found that the number of heterochromatin-associated siRNAs, including RdDM-specific small RNAs, increased significantly, likely limiting the transcriptional reactivation of transposons in Slddm1a Slddm1b Taken together, we propose that the global production of siRNAs and the CHH methylation mediated by the RdDM pathway are restricted to chromosome arms in tomato. Our data suggest that both pathways are greatly enhanced in heterochromatin when DDM1 functions are lost, at the expense of silencing mechanisms normally	tomato
544	plant	tomato	CRISPR;Cas9;	SlMIR160; SlARF10/16/17	Plant journal	Tuning of SlARF10A dosage by sly-miR160a is critical for auxin-mediated compound leaf and flower development.	2018	96(4):855-868	[Damodharan S et al.]	University of California, Davis, Davis, CA, USA.	30144341	10.1111/tpj.14073	miR160 adjusts auxin-mediated development by post-transcriptional regulation of the auxin response factors ARF10/16/17. In tomato, knockdown of miR160 (sly-miR160) suggested that it is required for auxin-driven leaf blade outgrowth, but whether additional developmental events are adjusted by sly-miR160 is not clear. Here, the SlMIR160 genes and the genes of its SlARFs targets were edited by CRISPR/Cas9 resulting in the isolation of loss-of-function mutants. In addition, hypomorphic mutants that accumulate variable reduced levels of sly-miR160a were isolated. We found that the loss-of-function mutants in SlMIR160a (CR-slmir160a-6/7) produced only four wiry leaves, whereas the hypomorphic mutants developed leaves and flowers with graded developmental abnormalities. Phenotypic severity correlated with the upregulation of SlARF10A. Consistent with that, double mutants in SlMIR160a and SlARF10A restored leaf and flower development indicating that over-accumulation of SlARF10A underlay the developmental abnormalities exhibited in the CR-slmir160a mutants. Phenotype severity also correlated with the upregulation of the SHOOT MERISTEMLESS homolog Tomato Knotted 2, which in turn activated the transcription of the cytokinin biosynthesis genes SlIPT2 and SlIPT4. However, no change in Tomato Knotted 2 was detected in the absence of SlARF10A, suggesting that it is upregulated due to auxin signaling suppression by SlARF10A. Knockout of sly-miR160a-targeted SlARFs showed that whereas SlARF10A is indispensable for leaf blade outgrowth and floral organ patterning, the functions of SlARF16A and SlARF17 are redundant. Taken together our results suggest that sly-miR160a promotes blade outgrowth as well as leaf and leaflet initiation and floral organ development through the quantitative	tomato
545	plant	tomato	CRISPR;	caroteinoid isomerase; phytoene synthase 1	Plant journal	Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system.	2018	95(1):5-16	[Dahan-Meir T et al.]	Weizmann Institute of Science, Rehovot, Israel.	29668111	10.1111/tpj.13932	Current breeding relies mostly on random mutagenesis and recombination to generate novel genetic variation. However, targeted genome editing is becoming an increasingly important tool for precise plant breeding. Using the CRISPR-Cas system combined with the bean yellow dwarf virus rolling circle replicon, we optimized a method for targeted mutagenesis and gene replacement in tomato. The carotenoid isomerase (CRTISO) and phytoene synthase 1 (PSY1) genes from the carotenoid biosynthesis pathway were chosen as targets due to their easily detectable change of phenotype. We took advantage of the geminiviral replicon amplification as a means to provide a large amount of donor template for the repair of a CRISPR-Cas-induced DNA double-strand break (DSB) in the target gene, via homologous recombination (HR). Mutagenesis experiments, performed in the Micro-Tom variety, achieved precise modification of the CRTISO and PSY1 loci at an efficiency of up to 90%. In the gene targeting (GT) experiments, our target was a fast-neutron-induced crtiso allele that contained a 281-bp deletion. This deletion was repaired with the wild-type sequence through HR between the CRISPR-Cas-induced DSB in the crtiso target and the amplified donor in 25% of the plants transformed. This shows that efficient GT can be achieved in the absence of selection markers or reporters using a single and modular construct that is	tomato
546	plant	tomato	CRISPR;Cas9;	long non-coding RNA (lncRNA1459)	Plant journal	CRISPR/Cas9-mediated mutagenesis of lncRNA1459 alters tomato fruit ripening.	2018	94(3):513-524	[Li R et al.]	China Agricultural University, Beijing, China.	29446503	10.1111/tpj.13872	With the development of high-throughput sequencing, many long non-coding RNAs (lncRNAs) have been found to play important roles in diverse biological processes. However, the biological functions of most plant lncRNAs are still unknown. We have previously discovered a tomato ripening-related lncRNA, lncRNA1459. Here, we cloned the full-length lncRNA1459, giving two transcript isoforms. In addition, lncRNA1459 exhibited a specific location in the nucleus. Furthermore, in order to fully identify the function of lncRNA1459 in tomato ripening, loss-of-function mutants of lncRNA1459 were developed using clustered regularly interspaced short palindromic repeats (CRISPR)/-associated protein 9 (Cas9)-induced genome editing technology. Compared with wild-type fruits, the tomato ripening process was significantly repressed in lncRNA1459 mutants. Ethylene production and lycopene accumulation were largely repressed in lncRNA1459 mutants. Additionally, genes related to ethylene and carotenoid biosynthesis were distinctly downregulated in lncRNA1459 mutants compared with wild-type fruits. Moreover, expression of numerous ripening-related genes was changed significantly when lncRNA1459 was knocked out. Expression of potential tomato ripening-related lncRNAs was also specifically changed after knocking out lncRNA1459. Taken together, these results provide insight into the role of	tomato

547	plant	tomato	agroinfiltration; CRISPR;Cas9;	DFR; NptII	PloS one	The DFR locus: A smart landing pad for targeted transgene insertion in tomato.	2018	13(12):e0208395	[Danilo B et al.]	INRA PACA, UR 1052, Avignon, France.	30521567	10.1371/journal.pone.0208395	Targeted insertion of transgenes in plants is still challenging and requires further technical innovation. In the present study, we chose the tomato DFR gene involved in anthocyanin biosynthesis as a landing pad for targeted transgene insertion using CRISPR-Cas9 in a two-step strategy. First, a 1013 bp was deleted in the endogenous DFR gene. Hypocotyls and callus of in vitro regenerated plantlets homozygous for the deletion were green instead of the usual anthocyanin produced purple colour. Next, standard Agrobacterium-mediated transformation was used to target transgene insertion at the DFR landing pad in the dfr deletion line. The single binary vector carried two sgRNAs, a donor template containing two homology arms of 400 bp, the previously deleted DFR sequence, and a NptII expression cassette. Regenerating plantlets were screened for a purple-colour phenotype indicating that DFR function had been restored. Targeted insertions were identified in 1.29% of the transformed explants. Thus, we established an efficient method for selecting HDR-mediated transgene insertion using the CRISPR-Cas9 system in tomato. The visual screen used here facilitates selection of these rare gene targeting events, does not necessitate the systematic PCR screening of all the regenerating material and can be potentially	tomato
548	plant	tomato	CRISPR;Cas9;	Psy1; CrtR-b2	Transgenic research	CRISPR/Cas9 editing of carotenoid genes in tomato.	2018	27(4):367-378	[D'Ambrosio C et al.]	Agenzia Lucana per lo Sviluppo e l'Innovazione in Agricoltura, Metaponto, Italy.	29797189	10.1007/s11248-018-0079-9	CRISPR/Cas9 technology is rapidly spreading as genome editing system in crop breeding. The efficacy of CRISPR/Cas9 in tomato was tested on Psy1 and CrtR-b2, two key genes of carotenoid biosynthesis. Carotenoids are plant secondary metabolites that must be present in the diet of higher animals because they exert irreplaceable functions in important physiological processes. Psy1 and CrtR-b2 were chosen because their impairment is easily detectable as a change of fruit or flower color. Two CRISPR/Cas9 constructs were designed to target neighboring sequences on the first exon of each gene. Thirty-four out of forty-nine (69%) transformed plants showed the expected loss-of-function phenotypes due to the editing of both alleles of a locus. However, by including the seven plants edited only at one of the two homologs and showing a normal phenotype, the editing rate reaches the 84%. Although none chimeric phenotype was observed, the cloning of target region amplified fragments revealed that in the 40% of analyzed DNA samples were present more than two alleles. As concerning the type of mutation, it was possible to identify 34 new different alleles across the four transformation experiments. The sequence characterization of the CRISPR/Cas9-induced mutations showed that the most frequent repair errors were the insertion and the deletion of one base. The results of this study prove that the CRISPRCas9 system can be an efficient and quick method for the generation of useful mutations in tomato	tomato
549	plant	tomato (Solanum lycopersicum)	CRISPR;Cas9;	C-repeat binding factor (slcbf1)	Journal of agricultural and food chemistry	Reduction of Tomato-Plant Chilling Tolerance by CRISPR-Cas9-Mediated SlCBF1 Mutagenesis.	2018	66(34):9042-9051	[Li R et al.]	China Agricultural University , Beijing , China.	30096237	10.1021/acs.jafc.8b02177	Chilling stress is the main constraint in tomato (Solanum lycopersicum) production, as this is a chilling-sensitive horticultural crop. The highly conserved C-repeat binding factors (CBFs) are cold-response-system components found in many species. In this study, we generated slcbf1 mutants using the CRISPR-Cas9 system and investigated the role of SlCBF1 in tomato-plant chilling tolerances. The slcbf1 mutants exhibited more severe chilling-injury symptoms with higher electrolyte leakage and malondialdehyde levels than wild-type (WT) plants. Additionally, slcbf1 mutants showed lower proline and protein contents and higher hydrogen peroxide contents and activities of antioxidant enzymes than WT plants. Knockout of SlCBF1 significantly increased indole acetic acid contents but decreased methyl jasmonate, abscisic acid, and zeatin riboside contents. The reduced chilling tolerance of the slcbf1 mutants was further reflected by the down-regulation of CBF-related genes. These results contribute to a better understanding of the molecular basis underlying SlCBF1	tomato
550	plant	tomato (Solanum pennellii)	CRISPR;Cas9;	farnesyl pyrophosphate synthase 2	Plant journal	A farnesyl pyrophosphate synthase gene expressed in pollen functions in S-RNase-independent unilateral incompatibility.	2018	93(3):417-430	[Qin X et al.]	University of California, Davis, CA, USA.	29206320	10.1111/tpj.13796	Multiple independent and overlapping pollen rejection pathways contribute to unilateral interspecific incompatibility (UI). In crosses between tomato species, pollen rejection usually occurs when the female parent is self-incompatible (SI) and the male parent self-compatible (SC) (the 'SI x SC rule'). Additional, as yet unknown, UI mechanisms are independent of self-incompatibility and contribute to UI between SC species or populations. We identified a major quantitative trait locus on chromosome 10 (ui10.1) which affects pollen-side UI responses in crosses between cultivated tomato, Solanum lycopersicum, and Solanum pennelliiLA0716, both of which are SC and lack S-RNase, the pistil determinant of S-specificity in Solanaceae. Here we show that ui10.1 is a farnesyl pyrophosphate synthase gene (FPS2) expressed in pollen. Expression is about 18-fold higher in pollen of S. pennellii than in S. lycopersicum. Pollen with the hypomorphic S. lycopersicum allele is selectively eliminated on pistils of the F1 hybrid, leading to transmission ratio distortion in the F2 progeny. CRISPR/Cas9-generated knockout mutants (fps2) in S. pennelliiLA0716 are self-sterile due to pollen rejection, but mutant pollen is fully functional on pistils of S. lycopersicum. F2 progeny of S. lycopersicum x S. pennellii (fps2) show reversed transmission ratio distortion due to selective elimination of pollen bearing the knockout allele. Overexpression of FPS2 in S. lycopersicum pollen rescues the pollen elimination phenotype. FPS2-based pollen selectivity does not involve S-RNase and has not been previously linked to UI. Our results point to an entirely new mechanism of interspecific pollen rejection in plants.	tomato
551	plant	tomato (Solanum pimpinellifolium)	CRISPR;Cas9;	6 loci	Nature biotechnology	De novo domestication of wild tomato using genome editing.	2018		[Zsogon A et al.]	Universidade Federal de Vicosa, Vicosa, Brazil.	30272678	10.1038/nbt.4272	Breeding of crops over millennia for yield and productivity has led to reduced genetic diversity. As a result, beneficial traits of wild species, such as disease resistance and stress tolerance, have been lost. We devised a CRISPR-Cas9 genome engineering strategy to combine agronomically desirable traits with useful traits present in wild lines. We report that editing of six loci that are important for yield and productivity in present-day tomato crop lines enabled de novo domestication of wild Solanum pimpinellifolium. Engineered S. pimpinellifolium morphology was altered, together with the size, number and nutritional value of the fruits. Compared with the wild parent, our engineered lines have a threefold increase in fruit size and a tenfold increase in fruit number. Notably, fruit lycopene accumulation is improved by 500% compared with the widely cultivated S. lycopersicum. Our results pave the way for molecular breeding	tomato

552	plant	torenia (Torenia fournieri L.)	CRISPR;Cas9;	flavanone-3-hydroxylase	BMC plant biology	Application of the CRISPR/Cas9 system for modification of flower color in Torenia fournieri.	2018	18(1):331	[Nishihara M et al.]	Iwate Biotechnology Research Center, Kitakami, Iwate, Japan.	30518324	10.1186/s12870-018-1539-3	BACKGROUND: CRISPR/Cas9 technology is one of the most powerful and useful tools for genome editing in various living organisms. In higher plants, the system has been widely exploited not only for basic research, such as gene functional analysis, but also for applied research such as crop breeding. Although the CRISPR/Cas9 system has been used to induce mutations in genes involved in various plant developmental processes, few studies have been performed to modify the color of ornamental flowers. We therefore attempted to use this system to modify flower color in the model plant torenia (Torenia fournieri L.). RESULTS: We attempted to induce mutations in the torenia flavanone 3-hydroxylase (F3H) gene, which encodes a key enzyme involved in flavonoid biosynthesis. Application of the CRISPR/Cas9 system successfully generated pale blue (almost white) flowers at a high frequency (ca. 80% of regenerated lines) in transgenic torenia T0 plants. Sequence analysis of PCR amplicons by Sanger and next-generation sequencing revealed the occurrence of mutations such as base substitutions and insertions/deletions in the F3H target sequence, thus indicating that the obtained phenotype was induced by the targeted mutagenesis of the endogenous F3H gene. CONCLUSIONS: These results clearly demonstrate that flower color modification by genome editing with the CRISPR/Cas9 system is easily and efficiently achievable. Our findings further indicate that this system may be useful for future	torenia (Tore
553	plant	tragopogon	agroinfiltration; CRISPR;Cas9;	phytoene desaturase (TraPDS)	Molecular ecology resources	Application of CRISPR/Cas9 to Tragopogon (Asteraceae), an evolutionary model for the study of polyploidy.	2018	18(6):1427-1443	[Shan S et al.]	University of Florida, Gainesville, FL, USA.	30086204	10.1111/1755-0998.12935	Tragopogon (Asteraceae) is an excellent natural system for studies of recent polyploidy. Development of an efficient CRISPR/Cas9-based genome editing platform in Tragopogon will facilitate novel studies of the genetic consequences of polyploidy. Here, we report our initial results of developing CRISPR/Cas9 in Tragopogon. We have established a feasible tissue culture and transformation protocol for Tragopogon. Through protoplast transient assays, use of the TragCRISPR system (i.e. the CRISPR/Cas9 system adapted for Tragopogon) was capable of introducing site-specific mutations in Tragopogon protoplasts. Agrobacterium-mediated transformation with Cas9-sgRNA constructs targeting the phytoene desaturase gene (TraPDS) was implemented in this model polyploid system. Sequencing of PCR amplicons from the target regions indicated simultaneous mutations of two alleles and four alleles of TraPDS in albino shoots from Tragopogon porrifolius (2x) and Tragopogon mirus (4x), respectively. The average proportions of successfully transformed calli with the albino phenotype were 87% and 78% in the diploid and polyploid, respectively. This appears to be the first demonstration of CRISPR/Cas9-based genome editing in any naturally formed neopolyploid system. Although a more efficient tissue culture system should be developed in Tragopogon, application of a robust CRISPR/Cas9 system will permit unique studies of biased fractionation, the gene-balance hypothesis and cytonuclear interactions in polyploids. In addition, the CRISPR/Cas9 platform enables investigations of those genes involved in phenotypic changes in polyploids and will also facilitate novel functional biology studies in Asteraceae. Our workflow provides a guide for	tragopogon
554	plant	Tripterygium wilfordii	CRISPR;Cas9;	sesquiterpene cyclase	Biochemical journal	Eudesmane-type sesquiterpene diols directly synthesized by a sesquiterpene cyclase in Tripterygium wilfordii.	2018	475(17):2713-2725	[Tong YR et al.]	Shenyang Pharmaceutical University, Shenyang, China.	30049895	10.1042/BCJ20180353	Cryptomeridiol, a typical eudesmane diol, is the active principle component of the antispasmodic Proximol. Although it has been used for many years, the biosynthesis pathway of cryptomeridiol has remained blur. Among terpenoid natural products, terpenoid cyclases are responsible for cyclization and generation of hydrocarbon backbones. The cyclization is mediated by carbocationic cascades and ultimately terminated via deprotonation or nucleophilic capture. Isoprene precursors are, respectively, converted into hydrocarbons or hydroxylated backbones. A sesquiterpene cyclase in Tripterygium wilfordii (TwCS) was determined to directly catalyze (E,E)-farnesyl pyrophosphate (FPP) to unexpected eudesmane diols, primarily cryptomeridiol. The function of TwCS was characterized by a modular pathway engineering system in Saccharomyces cerevisiae The major product determined by NMR spectroscopy turned out to be cryptomeridiol. This unprecedented production was further investigated in vitro, which verified that TwCS can directly produce eudesmane diols from FPP. Some key residues for TwCS catalysis were screened depending on the molecular model of TwCS and mutagenesis studies. As cryptomeridiol showed a small amount of volatile and medicinal properties, the biosynthesis of cryptomeridiol was reconstructed in S. cerevisiae Optimized assays including modular pathway engineering and the CRISPR-cas9 system were successfully used to improve the yield of cryptomeridiol in the S. cerevisiae The best engineered strain TE9 (BY4741 erg9::Delta-200-176 rox1::mut/pYX212-IDI + TwCS/p424-tHMG1) ultimately produced 19.73 mg/l	Tripterygium
555	plant	common wheat (Triticum aestivum L.)	agroinfiltration; CRISPR;Cas9;	DA1	BMC plant biology	Targeted mutagenesis using the Agrobacterium tumefaciens-mediated CRISPR-Cas9 system in common wheat.	2018	18(1):302	[Zhang S et al.]	Crop Research Institute, Shandong Academy of Agricultural Sciences, Jinan, Shandong, China.	30477421	10.1186/s12870-018-1496-x	BACKGROUND: Recently, the CRISPR/Cas9 system has been widely used to precisely edit plant genomes. Due to the difficulty in Agrobacterium-mediated genetic transformation of wheat, the reported applications in CRISPR/Cas9 system were all based on the biolistic transformation. RESULTS: In the present study, we efficiently applied targeted mutagenesis in common wheat (Triticum aestivum L.) protoplasts and transgenic T0 plants using the CRISPR/Cas9 system delivered via Agrobacterium tumefaciens. Seven target sites in three genes (Pinb, waxy and DA1) were selected to construct individual expression vectors. The activities of the sgRNAs were evaluated by transforming the constructed vectors into wheat protoplasts. Mutations in the targets were detected by Illumina sequencing. Genome editing, including insertions or deletions at the target sites, was found in the wheat protoplast cells. The highest mutation efficiency was 6.8% in the DA1 gene. The CRISPR/Cas9 binary vector targeting the DA1 gene was then transformed into common wheat plants by Agrobacterium tumefaciens-mediated transformation, resulting in efficient target gene editing in the T0 generation. Thirteen mutant lines were generated, and the mutation efficiency was 54.17%. Mutations were found in the A and B genomes of the transgenic plants but not in the D genome. In addition, off-target mutations were not detected in regions that were highly homologous to the sgRNA sequences. CONCLUSIONS: Our results showed that our Agrobacterium-mediated CRISPR/Cas9 system can be used	wheat

556	plant	Triticum aestivum	ZFN;	acetohydroxyacid synthase	Plant biotechnology journal	Zinc finger nuclease-mediated precision genome editing of an endogenous gene in hexaploid bread wheat (Triticum aestivum) using a DNA repair template.	2018	16(12):2088-2101	[Ran Y et al.]	Genovo Biotechnology Co. Ltd, Tianjin, China.	29734518	10.1111/pbi.12941	Sequence-specific nucleases have been used to engineer targeted genome modifications in various plants. While targeted gene knockouts resulting in loss of function have been reported with relatively high rates of success, targeted gene editing using an exogenously supplied DNA repair template and site-specific transgene integration has been more challenging. Here, we report the first application of zinc finger nuclease (ZFN)-mediated, nonhomologous end-joining (NHEJ)-directed editing of a native gene in allohexaploid bread wheat to introduce, via a supplied DNA repair template, a specific single amino acid change into the coding sequence of acetohydroxyacid synthase (AHAS) to confer resistance to imidazolinone herbicides. We recovered edited wheat plants having the targeted amino acid modification in one or more AHAS homoalleles via direct selection for resistance to imazamox, an AHAS-inhibiting imidazolinone herbicide. Using a cotransformation strategy based on chemical selection for an exogenous marker, we achieved a 1.2% recovery rate of edited plants having the desired amino acid change and a 2.9% recovery of plants with targeted mutations at the AHAS locus resulting in a loss-of-function gene knockout. The latter results demonstrate a broadly applicable approach to introduce targeted modifications into native genes for nonselectable traits. All ZFN-mediated changes were faithfully	wheat
557	plant	wheat	CRISPR;Cas9;	TaGW2; TaLpx-1; TaMLO	CRISPR journal	Transgenerational CRISPR-Cas9 Activity Facilitates Multiplex Gene Editing in Allopolyploid Wheat.	2018	1(1):65-74	[Wang W et al.]	Kansas State University, Manhattan, KS, USA.	30627700	10.1089/crispr.2017.0010	The CRISPR-Cas9-based multiplexed gene editing (MGE) provides a powerful method to modify multiple genomic regions simultaneously controlling different agronomic traits in crops. We applied the MGE construct built by combining the tandemly arrayed tRNA-gRNA units to generate heritable mutations in the TaGW2, TaLpx-1, and TaMLO genes of hexaploid wheat. The knockout mutations generated by this construct in all three homoeologous copies of one of the target genes, TaGW2, resulted in a substantial increase in seed size and thousand grain weight. We showed that the non-modified gRNA targets in the early generation plants can be edited by CRISPR-Cas9 in the following generations. Our results demonstrate that transgenerational gene editing activity can serve as the source of novel variation in the progeny of CRISPR-Cas9-expressing plants and suggest that the Cas9-inducible trait transfer for crop improvement can be achieved by crossing the plants expressing the gene editing	wheat
558	plant	wheat	CRISPR;Cas9;	wheat dehydration responsive element binding protein 2 (TaDREB2); wheat ethylene responsive factor 3 (TaERF3)	Functional & integrative genomics	CRISPR/Cas9 genome editing in wheat.	2018	18(1):31-41	[Kim D et al.]	Montana State University, Bozeman, MT, USA.	28918562	10.1007/s10142-017-0572-x	Genome editing has been a long-term challenge for molecular biology research, particularly for plants possess complex genome. The recently discovered Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a versatile tool for genome editing which enables editing of multiple genes based on the guidance of small RNAs. Even though the efficiency of CRISPR/Cas9 system has been shown with several studies from diploid plants, its application remains a challenge for plants with polyploid and complex genome. Here, we applied CRISPR/Cas9 genome editing system in wheat protoplast to conduct the targeted editing of stress-responsive transcription factor genes, wheat dehydration responsive element binding protein 2 (TaDREB2) and wheat ethylene responsive factor 3 (TaERF3). Targeted genome editing of TaDREB2 and TaERF3 was achieved with transient expression of small guide RNA and Cas9 protein in wheat protoplast. The effectiveness of mutagenesis in wheat protoplast was confirmed with restriction enzyme digestion assay, T7 endonuclease assay, and sequencing. Furthermore, several off-target regions for designed sgRNAs were analyzed, and the specificity of genome editing was confirmed with amplicon sequencing. Overall results suggested that CRISPR/Cas9 genome editing system can easily be established on wheat protoplast and it has a huge potentiality for targeted manipulation of wheat genome for crop	wheat
559	plant	wheat	CRISPR/Cas9	alpha-gliadin genes	Plant biotechnology journal	Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9.	2018	16(4):902-910	[Sanchez-Leon S et al.]	Instituto de Agricultura Sostenible (IAS-CSIC), Cordoba, Spain.	28921815	10.1111/pbi.12837	Coeliac disease is an autoimmune disorder triggered in genetically predisposed individuals by the ingestion of gluten proteins from wheat, barley and rye. The alpha-gliadin gene family of wheat contains four highly stimulatory peptides, of which the 33-mer is the main immunodominant peptide in patients with coeliac. We designed two sgRNAs to target a conserved region adjacent to the coding sequence for the 33-mer in the alpha-gliadin genes. Twenty-one mutant lines were generated, all showing strong reduction in alpha-gliadins. Up to 35 different genes were mutated in one of the lines of the 45 different genes identified in the wild type, while immunoreactivity was reduced by 85%. Transgene-free lines were identified, and no off-target mutations have been detected in any of the potential targets. The low-gluten, transgene-free wheat lines described here could be used to produce low-gluten foodstuff and serve as source material to introgress this trait into elite wheat varieties.	wheat
560	plant	wheat	CRISPR;Cas9;	3 Male sterile 45 (Ms45) homeologs	Plant molecular biology	Concurrent modifications in the three homeologs of Ms45 gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (Triticum aestivum L.).	2018	97(4-5):371-383	[Singh M et al.]	DuPont Pioneer, Johnston, IA, USA.	29959585	10.1007/s11103-018-0749-2	KEY MESSAGE: Hexaploid bread wheat is not readily amenable to traditional mutagenesis approaches. In this study, we show efficient utilization of CRISPR-Cas system and Next Generation Sequencing for mutant analysis in wheat. Identification and manipulation of male fertility genes in hexaploid bread wheat is important for understanding the molecular basis of pollen development and to obtain novel sources of nuclear genetic male sterility (NGMS). The maize Male sterile 45 (Ms45) gene encodes a strictosidine synthase-like enzyme and has been shown to be required for male fertility. To investigate the role of Ms45 gene in wheat, mutations in the A, B and D homeologs were produced using CRISPR-Cas9. A variety of mutations in the three homeologs were recovered, including a plant from two different genotypes each with mutations in all three homeologs. Genetic analysis of the mutations demonstrated that all three wheat Ms45 homeologs contribute to male fertility and that triple homozygous mutants are required to abort pollen development and achieve male sterility. Further, it was demonstrated that a wild-type copy of Ms45 gene from rice was able to restore fertility to these wheat mutant plants. Taken together, these observations provide insights into the conservation of MS45 function in a polyploid species. Ms45 based NGMS can be potentially utilized for a Seed Production Technology (SPT)-like hybrid	wheat

561	plant	wheat	CRISPR;Cas9;	TaGASR7	Scientific reports	Biolistic-delivery-based transient CRISPR/Cas9 expression enables in planta genome editing in wheat.	2018	8(1):14422	[Hamada H et al.]	KANEKA CORPORATION, Takasago, Japan.	30258105	10.1038/s41598-018-32714-6	The current application of genome editing to crop plants is limited to cultivars that are amenable to in vitro culture and regeneration. Here, we report an in planta genome-editing which does not require callus culture and regeneration. Shoot apical meristems (SAMs) contain a subepidermal cell layer, L2, from which germ cells later develop during floral organogenesis. The biolistic delivery of gold particles coated with plasmids expressing CRISPR/Cas9 components designed to target TaGASR7 were bombarded into SAM-exposed embryos of imbibed seeds. Bombarded embryos showing transient GFP expression within SAM were selected and grown into adult plants. Mutations in the target gene were assessed in fifth-leaf tissue by cleaved amplified polymorphic sequence analysis. Eleven (5.2%) of the 210 bombarded plants carried mutant alleles, and the mutations of three (1.4%) of these were inherited in the next generation. Genotype analysis of T1 plants identified plants homozygous for the three homeologous genes, which were all derived from one T0 plant. These plants showed no detectable integration of the Cas9 and guide RNA genes, indicating that transient expression of CRISPR/Cas9 introduced the mutations. Together, our current method can be used to achieve in planta genome editing in wheat using CRISPR/Cas9 and suggests possible	wheat
562	plant	wheat	CRISPR;Cas9;TALENs;	DsRed; wheat genes (TaLox2; TaUbiL1)	Scientific reports	Targeted mutagenesis in wheat microspores using CRISPR/Cas9.	2018	8(1):6502	[Bhowmik P et al.]	National Research Council Canada, Saskatoon, SK, Canada.	29695804	10.1038/s41598-018-24690-8	CRISPR/Cas9 genome editing is a transformative technology that will facilitate the development of crops to meet future demands. However, application of gene editing is hindered by the long life cycle of many crop species and because desired genotypes generally require multiple generations to achieve. Single-celled microspores are haploid cells that can develop into double haploid plants and have been widely used as a breeding tool to generate homozygous plants within a generation. In this study, we combined the CRISPR/Cas9 system with microspore technology and developed an optimized haploid mutagenesis system to induce genetic modifications in the wheat genome. We investigated a number of factors that may affect the delivery of CRISPR/Cas9 reagents into microspores and found that electroporation of a minimum of 75,000 cells using 10-20 microg DNA and a pulsing voltage of 500 V is optimal for microspore transfection using the Neon transfection system. Using multiple Cas9 and sgRNA constructs, we present evidence for the seamless introduction of targeted modifications in an exogenous DsRed gene and two endogenous wheat genes, including TaLox2 and TaUbiL1. This study demonstrates the value and feasibility of combining microspore technology and CRISPR/Cas9-based gene editing for trait	wheat
563	plant	wheat	CRISPR;Cas9;	TaGW2	TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik	Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of TaGW2 homoeologues to grain size and weight in wheat.	2018	131(11):2463-2475	[Wang W et al.]	Kansas State University, Manhattan, KS, USA.	30136108	10.1007/s00122-018-3166-7	KEY MESSAGE: CRISPR-Cas9-based genome editing and EMS mutagenesis revealed inter-cultivar differences and additivity in the contribution of TaGW2 homoeologues to grain size and weight in wheat. The TaGW2 gene homoeologues have been reported to be negative regulators of grain size (GS) and thousand grain weight (TGW) in wheat. However, the contribution of each homoeologue to trait variation among different wheat cultivars is not well documented. We used the CRISPR-Cas9 system and TILLING to mutagenize each homoeologous gene copy in cultivars Bobwhite and Paragon, respectively. Plants carrying single-copy nonsense mutations in different genomes showed different levels of GS/TGW increase, with TGW increasing by an average of 5.5% (edited lines) and 5.3% (TILLING mutants). In any combination, the double homoeologue mutants showed higher phenotypic effects than the respective single-genome mutants. The double mutants had on average 12.1% (edited) and 10.5% (TILLING) higher TGW with respect to wild-type lines. The highest increase in GS and TGW was shown for triple mutants of both cultivars, with increases in 16.3% (edited) and 20.7% (TILLING) in TGW. The additive effects of the TaGW2 homoeologues were also demonstrated by the negative correlation between the functional gene copy number and GS/TGW in Bobwhite mutants and an F2 population. The highest single-genome increases in GS and TGW in Paragon and Bobwhite were obtained by mutations in the B and D genomes, respectively. These inter-cultivar differences in the phenotypic effects between the TaGW2 gene homoeologues coincide with inter-cultivar differences in the homoeologue expression levels. These results indicate that GS/TGW variation in wheat can be modulated by the dosage of homoeologous genes	wheat
564	plant	wheat	Agroinfiltration;	green fluorescent protein	Virology	Octapartite negative-sense RNA genome of High Plains wheat mosaic virus encodes two suppressors of RNA silencing.	2018	518:152-162	[Gupta AK et al.]	University of Nebraska-Lincoln, Lincoln, NE, USA.	29499560	10.1016/j.virol.2018.02.013	High Plains wheat mosaic virus (HPWMoV, genus Emaravirus; family Fimoviridae), transmitted by the wheat curl mite (Aceria tosichella Keifer), harbors a monocistronic octapartite single-stranded negative-sense RNA genome. In this study, putative proteins encoded by HPWMoV genomic RNAs 2-8 were screened for potential RNA silencing suppression activity by using a green fluorescent protein-based reporter agroinfiltration assay. We found that proteins encoded by RNAs 7 (P7) and 8 (P8) suppressed silencing induced by single- or double-stranded RNAs and efficiently suppressed the transitive pathway of RNA silencing. Additionally, a Wheat streak mosaic virus (WSMV, genus Tritimovirus; family Potyviridae) mutant lacking the suppressor of RNA silencing (DeltaP1) but having either P7 or P8 from HPWMoV restored cell-to-cell and long-distance movement in wheat, thus indicating that P7 or P8 rescued silencing suppressor-deficient WSMV. Furthermore, HPWMoV P7 and P8 substantially enhanced the pathogenicity of Potato virus X in Nicotiana benthamiana. Collectively, these data demonstrate that the octapartite genome of HPWMoV encodes	wheat
565	plant	wheat; rice; potato	Cas9;		Nature biotechnology	Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A.	2018		[Zong Y et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.	30272679	10.1038/nbt.4261	Base editors (BEs) have been used to create C-to-T substitutions in various organisms. However, editing with rat APOBEC1-based BE3 is limited to a 5-nt sequence editing window and is inefficient in GC contexts. Here, we show that a base editor fusion protein composed of Cas9 nickase and human APOBEC3A (A3A-PBE) converts cytidine to thymidine efficiently in wheat, rice and potato with a 17-nucleotide editing window at all examined sites, independent of sequence context.	wheat; rice; p

566	plant		Cas9;TALENs;		Biochemistry	Efficient and Orthogonal Transcription Regulation by Chemically Inducible Artificial Transcription Factors.	2018	57(45):6452-6459	[Nomura W et al.]	Tokyo Medical and Dental University, Tokyo, Japan.	30366497	10.1021/acs.biochem.8b00741	The DNA-binding specificity of genome editing tools can be applied to gene regulation. Recently, multiple artificial transcription factors (ATFs) were shown to synergistically and efficiently regulate gene expression. Chemically triggered protein associations are useful for functional regulation at specific timings. A combination of several inducible protein association systems could enable the regulation of multiple genes at different loci with independent timing. We applied the FKBP-rapamycin-FRB and GAI-gibberellin-GID systems for gene regulation using multiple TALEs and dCas9. By the combined use of currently available systems, reporter gene assays were performed; the results indicated that gene expression was regulated by rapamycin or gibberellin in the presence of the FRB or GAI effector domains, respectively. Furthermore, the activation of endogenous genes was differentially regulated by the system. This success suggests the usability of the chemically inducible multiple ATFs for the time-dependent regulation of multiple genes, such as the case for cellular phenomena that are dependent on the programmable timing of expression and the differential expression of	
567	plant		CRISPR;	vip1-2	Frontiers in plant science	VIP1 and Its Homologs Are Not Required for Agrobacterium-Mediated Transformation, but Play a Role in Botrytis and Salt Stress Responses.	2018	9:749	[Lapham R et al.]	Purdue University, West Lafayette, IN, USA.	29946325	10.3389/fpls.2018.00749	The bZIP transcription factor VIP1 interacts with the Agrobacterium virulence protein VirE2, but the role of VIP1 in Agrobacterium-mediated transformation remains controversial. Previously tested vip1-1 mutant plants produce a truncated protein containing the crucial bZIP DNA-binding domain. We generated the CRISPR/Cas mutant vip1-2 that lacks this domain. The transformation susceptibility of vip1-2 and wild-type plants is similar. Because of potential functional redundancy among VIP1 homologs, we tested transgenic lines expressing VIP1 fused to a SRDX repression domain. All VIP1-SRDX transgenic lines showed wild-type levels of transformation, indicating that neither VIP1 nor its homologs are required for Agrobacterium-mediated transformation. Because VIP1 is involved in innate immune response signaling, we tested the susceptibility of vip1 mutant and VIP1-SRDX plants to Pseudomonas syringae and Botrytis cinerea. vip1 mutant and VIP1-SRDX plants show increased susceptibility to B. cinerea but not to P. syringae infection, suggesting a role for VIP1 in B. cinerea, but not in P. syringae, defense signaling. B. cinerea susceptibility is dependent on abscisic acid (ABA) which is also important for abiotic stress responses. The germination of vip1 mutant and VIP1-SRDX seeds is sensitive to exogenous ABA, suggesting a role for VIP1 in response to ABA. vip1 mutant and VIP1-SRDX plants	
568	plant		CRISPR;Cas9;TALENs;		Molecular plant	Robust Transcriptional Activation in Plants Using Multiplexed CRISPR-Act2.0 and mTALE-Act Systems.	2018	11(2):245-256	[Lowder LG et al.]	East Carolina University, Greenville, NC, USA.	29197638	10.1016/j.molp.2017.11.010	User-friendly tools for robust transcriptional activation of endogenous genes are highly demanded in plants. We previously showed that a dCas9-VP64 system consisting of the deactivated CRISPR-associated protein 9 (dCas9) fused with four tandem repeats of the transcriptional activator VP16 (VP64) could be used for transcriptional activation of endogenous genes in plants. In this study, we developed a second generation of vector systems for enhanced transcriptional activation in plants. We tested multiple strategies for dCas9-based transcriptional activation, and found that simultaneous recruitment of VP64 by dCas9 and a modified guide RNA scaffold gRNA2.0 (designated CRISPR-Act2.0) yielded stronger transcriptional activation than the dCas9-VP64 system. Moreover, we developed a multiplex transcription activator-like effector activation (mTALE-Act) system for simultaneous activation of up to four genes in plants. Our results suggest that mTALE-Act is even more effective than CRISPR-Act2.0 in most cases tested. In addition, we explored tissue-specific gene activation using positive feedback loops. Interestingly, our study revealed that certain endogenous genes are more amenable than others to transcriptional activation, and tightly regulated genes may cause target gene silencing when perturbed by activation probes. Hence, these new tools could be used to investigate gene regulatory networks and their control mechanisms. Assembly of multiplex CRISPR-Act2.0 and mTALE-Act systems are both based on streamlined and PCR-independent Golden Gate and Gateway cloning strategies, which will facilitate transcriptional activation applications in both	
569	plant		CRISPR;Cas9;		Plant direct	CRISPR/Cas9-mediated resistance to cauliflower mosaic virus.	2018	2(3):e00047	[Liu H et al.]	Virginia Tech, Blacksburg, VA, USA.	31245713	10.1002/pld3.47	Viral diseases are a leading cause of worldwide yield losses in crop production. Breeding of resistance genes (R gene) into elite crop cultivars has been the standard and most cost-effective practice. However, R gene-mediated resistance is limited by the available R genes within genetic resources and in many cases, by strain specificity. Therefore, it is important to generate new and broad-spectrum antiviral strategies. The CRISPR-Cas9 (clustered regularly interspaced palindromic repeat, CRISPR-associated) editing system has been employed to confer resistance to human viruses and several plant single-stranded DNA geminiviruses, pointing out the possible application of the CRISPR-Cas9 system for virus control. Here, we demonstrate that strong viral resistance to cauliflower mosaic virus (CaMV), a pararetrovirus with a double-stranded DNA genome, can be achieved through Cas9-mediated multiplex targeting of the viral coat protein sequence. We further show that small interfering RNAs (siRNA) are produced and mostly map to the 3' end of single-guide RNAs (sgRNA), although very low levels of siRNAs map to the spacer region as well. However, these siRNAs are not responsible for the inhibited CaMV infection because there is no resistance if Cas9 is not present. We have also observed edited viruses in systematically infected leaves in some transgenic plants, with short deletions or insertions consistent with Cas9-induced DNA breaks at the sgRNA target sites in coat protein coding sequence. These edited coat proteins, in most cases, led to earlier translation stop and thus, nonfunctional coat proteins. We also recovered wild-type CP sequence in these infected transgenic plants, suggesting these edited viral genomes were packaged by wild-type coat proteins. Our data demonstrate that the CRISPR-Cas9 system can be	

570	plant		CRISPR;	PPO	Scientific reports	True gene-targeting events by CRISPR/Cas-induced DSB repair of the PPO locus with an ectopically integrated repair template.	2018	8(1):3338	[de Pater S et al.]	Leiden University, Leiden, The Netherlands.	29463822	10.1038/s41598-018-21697-z	In recent years, several tools have become available for improved gene-targeting (GT) in plants. DNA breaks at specific sites activate local DNA repair and recombination, including recombination with ectopic sequences leading to GT. Large-scale transformation with the repair template can be avoided by pre-insertion of the repair template in the genome and liberation by sequence-specific nucleases (in planta GT procedure). Here, we tested whether release of the repair template was required for GT. Plants were transformed with constructs encoding a CRISPR/Cas nuclease with a recognition site in the endogenous PPO gene and a repair template harboring a 5' truncated PPO gene with two amino acid substitutions rendering the enzyme insensitive to the herbicide butafenacil. Selection resulted in so-called true GT events, repaired via homologous recombination at both ends of the gene and transmitted to the next generation. As the template was surrounded by geminiviral LIR sequences, we also tested whether replication of the template could be induced by crossing-in an integrated geminivirus REP gene. However, we could not find evidence for repair template replication by REP and we obtained similar numbers of GT events in these plants. Thus, GT is possible without any further processing of the pre-inserted repair	
571	plant	apple; pear	CRISPR;Cas9;	phytoene desaturase; terminal flower 1	Frontiers in plant science	Efficient Targeted Mutagenesis in Apple and First Time Edition of Pear Using the CRISPR-Cas9 System.	2019	10:40	[Charrier A et al.]	INRA, Agrocampus-Ouest, Universite d'Angers, Beaucouze, France.	30787936	10.3389/fpls.2019.00040	Targeted genome engineering has emerged as an alternative to classical plant breeding and transgenic methods to improve crop plants. Among other methods (zinc finger nucleases or TAL effector nucleases) the CRISPR-Cas system proved to be the most effective, convenient and least expensive method. In this study, we optimized the conditions of application of this system on apple and explored its feasibility on pear. As a proof of concept, we chose to knock-out the Phytoene Desaturase (PDS) and Terminal Flower 1 (TFL1) genes. To improve the edition efficiency, two different single guide RNAs (gRNAs) were associated to the Cas9 nuclease for each target gene. These gRNAs were placed under the control of the U3 and U6 apple promoters. Characteristic albino phenotype was obtained for 85% of the apple transgenic lines targeted in MdPDS gene. Early flowering was observed in 93% of the apple transgenic lines targeted in MdTFL1.1 gene and 9% of the pear transgenic lines targeted in PcTFL1.1. Sequencing of the target zones in apple and pear CRISPR-PDS and CRISPR-TFL1.1 transgenic lines showed that the two gRNAs induced mutations but at variable frequencies. In most cases, Cas9 nuclease cut the DNA in the twenty targeted base pairs near the protospacer adjacent motif and insertions were more frequent than deletions or substitutions. The most frequent edition profile of PDS as well as TFL1.1 genes was chimeric biallelic. Analysis of a sample of potential off-target sequences of the CRISPR-TFL1.1 construct indicated the absence of edition in cases of three mismatches. In addition, transient transformation with the CRISPR-PDS construct produced two T-DNA free edited apple lines. Our overall results indicate that, despite the frequent occurrence of chimerism, the CRISPR-Cas 9 system is a powerful and	apple; pear
572	plant	Arabidopsis	CRISPR;Cas9;	hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase (HCT); Golgi-localized nucleotide sugar transporter2 (GONST2)	Biotechnology for biofuels	A screening method to identify efficient sgRNAs in Arabidopsis, used in conjunction with cell-specific lignin reduction.	2019	12:130	[Liang Y et al.]	Joint BioEnergy Institute, Emeryville, CA USA.	31143243	10.1186/s13068-019-1467-y	Background: Single guide RNA (sgRNA) selection is important for the efficiency of CRISPR/Cas9-mediated genome editing. However, in plants, the rules governing selection are not well established. Results: We developed a facile transient assay to screen sgRNA efficiency. We then used it to test top-performing bioinformatically predicted sgRNAs for two different Arabidopsis genes. In our assay, these sgRNAs had vastly different editing efficiencies, and these efficiencies were replicated in stably transformed Arabidopsis lines. One of the genes, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase (HCT), is an essential gene, required for lignin biosynthesis. Previously, HCT function has been studied using gene silencing. Here, to avoid the negative growth effects that are due to the loss of HCT activity in xylem vessels, we used a fiber-specific promoter to drive CAS9 expression. Two independent transgenic lines showed the expected lignin decrease. Successful editing was confirmed via the observation of mutations at the HCT target loci, as well as an approximately 90% decrease in HCT activity. Histochemical analysis and a normal growth phenotype support the fiber-specific knockout of HCT. For the targeting of the second gene, Golgi-localized nucleotide sugar transporter2 (GONST2), a highly efficient sgRNA drastically increased the rate of germline editing in T1 generation. Conclusions: This screening method is widely applicable, and the selection and use of efficient sgRNAs will accelerate the process of expanding germplasm for both molecular breeding and research. In addition, this, to the best of our knowledge, is the first application of constrained genome editing to obtain chimeric plants of essential genes.	Arabidopsis
573	plant	Arabidopsis	CRISPR;Cas9;	PDX1.2	BMC plant biology	Clarification of the dispensability of PDX1.2 for Arabidopsis viability using CRISPR/Cas9.	2019	19(1):464	[Dell'Aglio E et al.]	University of Geneva, Geneva, Switzerland.	31684863	10.1186/s12870-019-2071-9	BACKGROUND: PDX1.2 has recently been shown to be a regulator of vitamin B6 biosynthesis in plants and is implicated in biotic and abiotic stress resistance. PDX1.2 expression is strongly and rapidly induced by heat stress. Interestingly, PDX1.2 is restricted to eudicota, wherein it behaves as a non-catalytic pseudoenzyme and is suggested to provide an adaptive advantage to this clade. A first report on an Arabidopsis insertion mutant claims that PDX1.2 is indispensable for viability, being essential for embryogenesis. However, a later study using an independent insertion allele suggests that knockout mutants of pdx1.2 are viable. Therefore, the essentiality of PDX1.2 for Arabidopsis viability is a matter of debate. Given the important implications of PDX1.2 in stress responses, it is imperative to clarify if it is essential for plant viability. RESULTS: We have studied the previously reported insertion alleles of PDX1.2, one of which is claimed to be essential for embryogenesis (pdx1.2-1), whereas the other is viable (pdx1.2-2). Our study shows that pdx1.2-1 carries multiple T-DNA insertions, but the T-DNA insertion in PDX1.2 is not responsible for the loss of embryogenesis. By contrast, the pdx1.2-2 allele is an overexpressor of PDX1.2 under standard growth conditions and not a null allele as previously reported. Nonetheless, upregulation of PDX1.2 expression under heat stress is impaired in this mutant line. In wild type Arabidopsis, studies of PDX1.2-YFP fusion proteins show that the protein is enhanced under heat stress conditions. To clarify if PDX1.2 is essential for Arabidopsis viability, we generated several independent mutant lines using the CRISPR-Cas9 gene editing technology. All of these lines are viable and behave similar to wild type under standard growth conditions. Reciprocal crosses of a subset of the CRISPR lines with pdx1.2-1 recovers viability of the latter line and demonstrates that knocking out the functionality of PDX1.2 does not impair embryogenesis. CONCLUSIONS: Gene editing reveals that PDX1.2 is dispensable for Arabidopsis viability and resolves conflicting	Arabidopsis

574	plant	Arabidopsis	RdDM;siRNA;	diverse RdDM proteins that are capable of targeting methylation and silencing in Arabidopsis when tethered to an artificial zinc finger (ZF-RdDM)?	Cell	Co-targeting RNA Polymerases IV and V Promotes Efficient De Novo DNA Methylation in Arabidopsis.	2019	176(5):1068-1082.e19	[Gallego-Bartolome J et al.]	University of California at Los Angeles, Los Angeles, CA, USA.	30739798	10.1016/j.cell.2019.01.029	The RNA-directed DNA methylation (RdDM) pathway in plants controls gene expression via cytosine DNA methylation. The ability to manipulate RdDM would shed light on the mechanisms and applications of DNA methylation to control gene expression. Here, we identified diverse RdDM proteins that are capable of targeting methylation and silencing in Arabidopsis when tethered to an artificial zinc finger (ZF-RdDM). We studied their order of action within the RdDM pathway by testing their ability to target methylation in different mutants. We also evaluated ectopic siRNA biogenesis, RNA polymerase V (Pol V) recruitment, targeted DNA methylation, and gene-expression changes at thousands of ZF-RdDM targets. We found that co-targeting both arms of the RdDM pathway, siRNA biogenesis and Pol V recruitment, dramatically enhanced targeted methylation. This work defines how RdDM components establish DNA methylation and enables new strategies for epigenetic gene regulation	Arabidopsis
575	plant	Arabidopsis	CRISPR;Cas9;	AP1; SVP; TFL1	Horticulture research	Targeted deletion of floral development genes in Arabidopsis with CRISPR/Cas9 using the RNA endoribonuclease Csy4 processing system.	2019	6:99	[Liu Y et al.]	Beijing Forestry University, Beijing, China.	31666960	10.1038/s41438-019-0179-6	The formation of flowers in higher plants is controlled by complex gene regulatory networks. The study of floral development in Arabidopsis is promoted and maintained by transposon-tagged mutant lines. In this study, we report a CRISPR/Cas9 genome-editing system based on RNA endoribonuclease Csy4 processing to induce high-efficiency and inheritable targeted deletion of transcription factors involved in floral development in Arabidopsis. Using AP1, SVP, and TFL1 as the target genes, multisite and multiple-gene mutations were achieved with a tandemly arrayed Csy4-sgRNA architecture to express multiplexed sgRNAs from a single transcript driven by the Pol Ⅱ promoter in transgenic lines. Targeted deletions of chromosomal fragments between the first exon and second exon in either one or three genes were generated by using a single binary vector. Interestingly, the efficiency of site-targeted deletion was comparable to that of indel mutation with the multiplexed sgRNAs. DNA sequencing analysis of RT-PCR products showed that targeted deletions of AP1 and TFL1 could lead to frameshift mutations and introduce premature stop codons to disrupt the open-reading frames of the target genes. In addition, no RT-PCR amplified product was acquired after SVP-targeted deletion. Furthermore, the targeted deletions resulted in abnormal floral development in the mutant lines compared to that of wild-type plants. AP1 and SVP mutations increased plant branching significantly, while TFL1 mutant plants displayed a change from indeterminate to determinate inflorescences. Thus, our results demonstrate that CRISPR/Cas9 with the RNA	Arabidopsis
576	plant	Arabidopsis	CRISPR;Cas9;	DPA4 (Development-Related PcG Target in the APEX4); SOD7 (Suppressor of da1-1)	International journal of molecular sciences	Genome Editing to Integrate Seed Size and Abiotic Stress Tolerance Traits in Arabidopsis Reveals a Role for DPA4 and SOD7 in the Regulation of Inflorescence Architecture.	2019	20(11)	[Chen S et al.]	Linyi University, Linyi, China.	31159296	10.3390/ijms20112695	Both seed size and abiotic stress tolerance are important agronomic traits in crops. In Arabidopsis, two closely related transcription repressors DPA4 (Development-Related PcG Target in the APEX4)/NGAL3 and SOD7 (Suppressor of da1-1)/NGAL2 (NGATHA-like protein) function redundantly to regulate seed size, which was increased in the dpa4 sod7 double mutants. Whereas ABA-induced transcription repressors (AITRs) are involved in the regulation of ABA signaling and abiotic stress tolerance, Arabidopsis aitr2 aitr5 aitr6 (aitr256) triple mutant showed enhanced tolerance to drought and salt. Here we performed CRISPR/Cas9 genome editing to disrupt DPA4 and SOD7 in aitr256 mutant, trying to integrate seed size and abiotic stress tolerance traits in Arabidopsis, and also to examine whether DPA4 and SOD7 may regulate other aspects of plant growth and development. Indeed, seed size was increased in the dpa4 sod7 aitr256 quintuple mutants, and enhanced tolerance to drought was observed in the mutants. In addition, we found that shoot branching was affected in the dpa4 sod7 aitr256 mutants. The mutant plants failed to produce secondary branches, and flowers/siliques were distributed irregularly on the main stems of the plants. Floral organ number and fertility were also affected in the dpa4 sod7 aitr256 mutant plants. To examine if these phenotypes were dependent on loss-of-function of AITRs, dpa4 sod7 double mutants were generated in Col wild type background, and we found that the dpa4 sod7 mutant plants showed a phenotype similar to the dpa4 sod7 aitr256 quintuple mutants. Taken together, our results indicate that the integration of seed size and abiotic stress tolerance traits by CRISPR/Cas9 editing was successful, and our results also revealed a role of DPA4 and SOD7 in the regulation of inflorescence	Arabidopsis
577	plant	Arabidopsis	CRISPR;Cas9;	wuschel-related homeobox 11-3; wox12-3	Journal of genetics and genomics = Yi chuan xue bao	Control of de novo root regeneration efficiency by developmental status of Arabidopsis leaf explants.	2019	46(3):133-140	[Pan J et al.]	Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China.	30928533	10.1016/j.jgg.2019.03.001	De novo root regeneration (DNRR) has wide applications in agriculture such as those related to cutting technology. Detached Arabidopsis thaliana leaf explants can regenerate adventitious roots without added hormones. The regenerative ability is highly dependent on the developmental status of the leaf. An immature leaf has a higher regenerative ability, while a mature leaf is difficult to regenerate. Using RNA-Seq analysis, we showed that the expression levels of many genes, including those in the auxin network, changed during leaf maturation. Particularly, the expression levels of many YUCCA (YUC) genes in the auxin biosynthesis pathway are responsive to leaf maturation. Overexpression of YUC1 in the yuc-1D dominant mutant rescued the rooting defects caused by leaf maturation. In addition, YUC4 expression levels were also affected by circadian rhythms. The regenerative ability was reduced in both immature and mature mutant leaf explants from the new wuschel-related homeobox 11-3 (wox11-3) and wox12-3 mutant alleles created by the CRISPR/Cas9 method. Overall, the transcriptome and genetic data, together with the auxin concentration analysis, indicate that the ability to upregulate auxin levels upon detachment may be reduced during leaf maturation. Thus, multiple developmental and environmental signals may converge to control auxin accumulation, which affects the efficiency of the	Arabidopsis

578	plant	Arabidopsis	CRISPR;Cas9;	NAC transcription factor (ANAC092)	Journal of integrative plant biology	Arabidopsis ANAC092 regulates auxin-mediated root development by binding to the ARF8 and PIN4 promoters.	2019	61(9):1015-1031	[Xi D et al.]	Fudan University, Shanghai, China.	30415491	10.1111/jipb.12735	Auxin is an important plant hormone that is essential for growth and development due to its effects on organogenesis, morphogenesis, tropisms, and apical dominance. The functional diversity of auxin highlights the importance of its biosynthesis, transport, and associated responses. In this study, we show that a NAC transcription factor, ANAC092 (also named AtNAC2 and ORESARA1), known to positively regulate leaf senescence and contribute to abiotic stress responses, also affects primary root development. Plants overexpressing ANAC092 had altered root meristem lengths and shorter primary roots compared with the wild-type control. Additionally, expression of the proANAC092::GUS was strongly induced by indole-3-acetic acid. Quantitative real-time RT-PCR (qRT-PCR) analysis revealed that the YUCCA2, PIN, and ARF expression levels were downregulated in ANAC092-overexpressing plants. Moreover, yeast one-hybrid and chromatin immunoprecipitation assays confirmed that ANAC092 binds to the promoters of AUXIN RESPONSE FACTOR 8 (ARF8) and PIN-FORMED 4 (PIN4). Furthermore, a dual-luciferase assay indicated that ANAC092 decreases ARF8 and PIN4 promoter activities. We also applied a CRISPR/Cas9 system to mutate ANAC092. The roots of three of the analyzed mutants were longer than normal. Collectively, our findings indicate that ANAC092 negatively affects root development	Arabidopsis
579	plant	Arabidopsis	CRISPR;Cas9;	green fluorescent protein	Molecular biology reports	CRISPR/Cas9-mediated gfp gene inactivation in Arabidopsis suspension cells.	2019	46(6):5735-5743	[Permyakova NV et al.]	Federal Research Center Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, Russia.	31392536	10.1007/s11033-019-05007-y	Targeted genome editing using CRISPR/Cas9 is a promising technology successfully verified in various plant species; however, it has hardly been used in plant cell suspension cultures. Here, we describe a successful knockout of a green fluorescent protein (gfp) reporter gene in Arabidopsis cell culture. We transformed seven transgenic suspension cell lines carrying one to three gfp gene copies with a binary vector containing genes coding for Cas9 and guide RNAs targeting the gfp gene. We detected the site-specific mutations by restriction analysis of a gfp amplicon. DNA sequencing of the PCR products confirmed high diversity of insertion-deletion mutations in the cell lines after the editing. We also analyzed gfp mRNA expression by real-time PCR and observed a decrease in gfp transcription after the target site modification. We can conclude that the CRISPR/Cas9 system can be successfully used for introducing site-specific mutations into the genome of cultured suspension cells of Arabidopsis.	Arabidopsis
580	plant	Arabidopsis	CRISPR;Cas9;	orosomucoid(orm) 1; orm2	Molecular plant	A Plant Immune Receptor Degraded by Selective Autophagy.	2019	12(1):113-123	[Yang F et al.]	University of Nebraska, Lincoln, NE, USA.	30508598	10.1016/j.molp.2018.11.011	Plants recycle non-activated immune receptors to maintain a functional immune system. The Arabidopsis immune receptor kinase FLAGELLIN-SENSING 2 (FLS2) recognizes bacterial flagellin. However, the molecular mechanisms by which non-activated FLS2 and other non-activated plant PRRs are recycled remain not well understood. Here, we provide evidence showing that Arabidopsis orosomucoid (ORM) proteins, which have been known to be negative regulators of sphingolipid biosynthesis, act as selective autophagy receptors to mediate the degradation of FLS2. Arabidopsis plants overexpressing ORM1 or ORM2 have undetectable or greatly diminished FLS2 accumulation, nearly lack FLS2 signaling, and are more susceptible to the bacterial pathogen Pseudomonas syringae. On the other hand, ORM1/2 RNAi plants and orm1 or orm2 mutants generated by the CRISPR/Cas9-mediated gene editing have increased FLS2 accumulation and enhanced FLS2 signaling, and are more resistant to P. syringae. ORM proteins interact with FLS2 and the autophagy-related protein ATG8. Interestingly, overexpression of ORM1 or ORM2 in autophagy-defective mutants showed FLS2 abundance that is comparable to that in wild-type plants. Moreover, FLS2 levels were not decreased in Arabidopsis plants overexpressing ORM1/2 derivatives that do not interact with ATG8. Taken together, these results suggest that selective autophagy functions in maintaining the homeostasis of a plant immune receptor and that beyond sphingolipid metabolic regulation ORM proteins can also act	Arabidopsis
581	plant	Arabidopsis	CRISPR;Cas9;	specific loci, including the FWA promoter, triggering a developmental phenotype, and the SUPERMAN promoter	Nature communications	Site-specific manipulation of Arabidopsis loci using CRISPR-Cas9 SunTag systems.	2019	10(1):729	[Papikian A et al.]	University of California, Los Angeles, CA, USA.	30760722	10.1038/s41467-019-08736-7	Understanding genomic functions requires site-specific manipulation of loci via efficient protein effector targeting systems. However, few approaches for targeted manipulation of the epigenome are available in plants. Here, we adapt the dCas9-SunTag system to engineer targeted gene activation and DNA methylation in Arabidopsis. We demonstrate that a dCas9-SunTag system utilizing the transcriptional activator VP64 drives robust and specific activation of several loci, including protein coding genes and transposable elements, in diverse chromatin contexts. In addition, we present a CRISPR-based methylation targeting system for plants, utilizing a SunTag system with the catalytic domain of the Nicotiana tabacum DRM methyltransferase, which efficiently targets DNA methylation to specific loci, including the FWA promoter, triggering a developmental phenotype, and the SUPERMAN promoter. These SunTag systems represent valuable tools for the site-specific manipulation of plant	Arabidopsis
582	plant	Arabidopsis	CRISPR;Cas9;	a nuclear locus that enhances sensitivity to the selection agent used for isolation of transplastomic events	Nature plants	High-efficiency generation of fertile transplastomic Arabidopsis plants.	2019	5(3):282-289	[Ruf S et al.]	Max-Planck-Institut fur Molekulare Pflanzenphysiologie, Potsdam, Germany.	30778165	10.1038/s41477-019-0359-2	The development of technologies for the stable genetic transformation of plastid (chloroplast) genomes has been a boon to both basic and applied research. However, extension of the transplastomic technology to major crops and model plants has proven extremely challenging, and the species range of plastid transformation is still very much limited in that most species currently remain recalcitrant to plastid genome engineering. Here, we report an efficient plastid transformation technology for the model plant Arabidopsis thaliana that relies on root-derived microcalli as a source tissue for biolistic transformation. The method produces fertile transplastomic plants at high frequency when combined with a clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9)-generated knockout allele of a nuclear locus that enhances sensitivity to the selection agent used for isolation of transplastomic events. Our work makes the model organism of plant biology amenable to routine engineering of the plastid genome, facilitates the combination of plastid engineering with the power of Arabidopsis nuclear genetics, and informs the future development of plastid transformation protocols for other recalcitrant species.	Arabidopsis

583	plant	Arabidopsis	CRISPR;Cas9;	N REQUIREMENT GENE 1 (NRG1A; NRG1B)	New phytologist	Differential regulation of TNL-mediated immune signaling by redundant helper CNLs.	2019	222(2):938-953	[Wu Z et al.]	University of British Columbia, Vancouver, BC, Canada.	30585636	10.1111/nph.15665	Higher plants utilize nucleotide-binding leucine-rich repeat domain proteins (NLRs) as intracellular immune receptors to recognize pathogen-derived effectors and trigger a robust defense. The Activated Disease Resistance 1 (ADR1) family of coiled-coil NLRs (CNLs) have evolved as helper NLRs that function downstream of many TIR-type sensor NLRs (TNLs). Close homologs of ADR1s form the N REQUIREMENT GENE 1 (NRG1) family in Arabidopsis, the function of which is unclear. Through CRISPR/Cas9 gene editing methods, we discovered that the tandemly repeated NRG1A and NRG1B are functionally redundant and operate downstream of TNLs with differential strengths. Interestingly, ADR1s and NRG1s function in two distinct parallel pathways contributing to TNL-specific immunity. Synergistic effects on basal and TNL-mediated defense were detected among ADR1s and NRG1s. An intact P-loop of NRG1s is not required for mediating signals from sensor TNLs, whereas auto-active NRG1A exhibits autoimmunity. Importantly, NRG1s localize to the cytosol and endomembrane network regardless of the presence of effectors, suggesting a cytosolic activation mechanism. Taken together, different sensor TNLs differentially use two groups of helper NLRs,	Arabidopsis
584	plant	Arabidopsis	CRISPR;Cas9;	eIF4E	Plant biotechnology journal	Mimicking natural polymorphism in eIF4E by CRISPR-Cas9 base editing is associated with resistance to potyviruses.	2019	17(9):1736-1750	[Bastet A et al.]	GAFL, INRA, Montfavet, France.	30784179	10.1111/pbi.13096	In many crop species, natural variation in eIF4E proteins confers resistance to potyviruses. Gene editing offers new opportunities to transfer genetic resistance to crops that seem to lack natural eIF4E alleles. However, because eIF4E are physiologically important proteins, any introduced modification for virus resistance must not bring adverse phenotype effects. In this study, we assessed the role of amino acid substitutions encoded by a Pisum sativum eIF4E virus-resistance allele (W69L, T80D S81D, S84A, G114R and N176K) by introducing them independently into the Arabidopsis thaliana eIF4E1 gene, a susceptibility factor to the Clover yellow vein virus (ClYVV). Results show that most mutations were sufficient to prevent ClYVV accumulation in plants without affecting plant growth. In addition, two of these engineered resistance alleles can be combined with a loss-of-function eIFiso4E to expand the resistance spectrum to other potyviruses. Finally, we use CRISPR-nCas9-cytidine deaminase technology to convert the Arabidopsis eIF4E1 susceptibility allele into a resistance allele by introducing the N176K mutation with a single-point mutation through C-to-G base editing to generate resistant plants. This study shows how combining knowledge on pathogen susceptibility factors with precise genome-editing technologies offers a feasible solution for engineering transgene-free genetic	Arabidopsis
585	plant	Arabidopsis	CRISPR;Cpf1;	three kinds of DNA ATPase complexes	Plant journal	In planta gene targeting can be enhanced by the use of CRISPR/Cas12a.	2019	100(5):1083-1094	[Wolter F et al.]	Karlsruhe Institute of Technology, Karlsruhe, Germany.	31381206	10.1111/tpj.14488	The controlled change of plant genomes by homologous recombination (HR) is still difficult to achieve. We previously developed the in planta gene targeting (ipGT) technology which depends on the simultaneous activation of the target locus by a double-strand break and the excision of the target vector. Whereas the use of SpCas9 resulted in low ipGT frequencies in Arabidopsis, we were recently able to improve the efficiency by using egg cell-specific expression of the potent but less broadly applicable SaCas9 nuclease. In this study, we now tested whether we could improve ipGT further, by either performing it in cells with enhanced intrachromosomal HR efficiencies or by the use of Cas12a, a different kind of CRISPR/Cas nuclease with an alternative cutting mechanism. We could show before that plants possess three kinds of DNA ATPase complexes, which all lead to instabilities of homologous genomic repeats if lost by mutation. As these proteins act in independent pathways, we tested ipGT in double mutants in which intrachromosomal HR is enhanced 20-80-fold. However, we were not able to obtain higher ipGT frequencies, indicating that mechanisms for gene targeting (GT) and chromosomal repeat-induced HR differ. However, using LbCas12a, the GT frequencies were higher than with SaCas9, despite a lower non-homologous end-joining (NHEJ) induction efficiency, demonstrating the particular suitability of Cas12a to induce HR. As SaCas9 has substantial restrictions due to its longer GC rich PAM sequence, the use of LbCas12a with its AT-rich PAM	Arabidopsis
586	plant	Arabidopsis	CRISPR;Cas9;	DRE2	PLoS genetics	Canonical cytosolic iron-sulfur cluster assembly and non-canonical functions of DRE2 in Arabidopsis.	2019	15(4):e1008094	[Wang X et al.]	Peking University, Beijing, China.	31034471	10.1371/journal.pgen.1008094	As a component of the Cytosolic Iron-sulfur cluster Assembly (CIA) pathway, DRE2 is essential in organisms from yeast to mammals. However, the roles of DRE2 remain incompletely understood largely due to the lack of viable dre2 mutants. In this study, we successfully created hypomorphic dre2 mutants using the CRISPR/Cas9 technology. Like other CIA pathway mutants, the dre2 mutants have accumulation of DNA lesions and show constitutive DNA damage response. In addition, the dre2 mutants exhibit DNA hypermethylation at hundreds of loci. The mutant forms of DRE2 in the dre2 mutants, which bear deletions in the linker region of DRE2, lost interaction with GRXS17 but have stronger interaction with NBP35, resulting in the CIA-related defects of dre2. Interestingly, we find that DRE2 is also involved in auxin response that may be independent of its CIA role. DRE2 localizes in both the cytoplasm and the nucleus and nuclear DRE2 associates with euchromatin. Furthermore, DRE2 directly associates with multiple auxin responsive genes and maintains their normal expression. Our study highlights the importance of the linker region of DRE2 in coordinating CIA-related protein interactions and identifies the canonical and non-canonical roles of DRE2 in maintaining genome stability, epigenomic patterns, and auxin response.	Arabidopsis
587	plant	Arabidopsis	CRISPR;Cas9;	AITR	PloS one	Integration of a FT expression cassette into CRISPR/Cas9 construct enables fast generation and easy identification of transgene-free mutants in Arabidopsis.	2019	14(9):e0218583	[Cheng Y et al.]	Northeast Normal University, Changchun, Jilin, China.	31545795	10.1371/journal.pone.0218583	The CRISPR/Cas9 genome editing technique has been widely used to generate transgene-free mutants in different plant species. Several different methods including fluorescence marker-assisted visual screen of transgene-free mutants and programmed self-elimination of CRISPR/Cas9 construct have been used to increase the efficiency of genome edited transgene-free mutant isolation, but the overall time length required to obtain transgene-free mutants has remained unchanged in these methods. We report here a method for fast generation and easy identification of transgene-free mutants in Arabidopsis. By generating and using a single FT expression cassette-containing CRISPR/Cas9 construct, we targeted two sites of the AITR1 gene. We obtained many early bolting plants in T1 generation, and found that about two thirds of these plants have detectable mutations. We then analyzed T2 generations of two representative lines of genome edited early bolting T1 plants, and identified plants without early bolting phenotype, i.e., transgene-free plants, for both lines. Further more, aitr1 homozygous mutants were successful obtained for both lines from these transgene-free plants. Taken together, these results suggest that the method described here enables fast generation, and at the mean time, easy identification of	Arabidopsis

588	plant	Arabidopsis	CRISPR;Cas9;		PloS one	Optimization of T-DNA architecture for Cas9-mediated mutagenesis in Arabidopsis.	2019	14(1):e0204778	[Castel B et al.]	Sainsbury Laboratory, Norwich, UK.	30625150	10.1371/journal.pone.0204778	Bacterial CRISPR systems have been widely adopted to create operator-specified site-specific nucleases. Such nuclease action commonly results in loss-of-function alleles, facilitating functional analysis of genes and gene families We conducted a systematic comparison of components and T-DNA architectures for CRISPR-mediated gene editing in Arabidopsis, testing multiple promoters, terminators, sgRNA backbones and Cas9 alleles. We identified a T-DNA architecture that usually results in stable (i.e. homozygous) mutations in the first generation after transformation. Notably, the transcription of sgRNA and Cas9 in head-to-head divergent orientation usually resulted in highly active lines. Our Arabidopsis data may prove useful for optimization	Arabidopsis
589	plant	Arabidopsis	CRISPR;Cas9;	abscisic acid (ABA)-responsive element binding protein 1 (AREB1)	Scientific reports	Improved drought stress tolerance in Arabidopsis by CRISPR/dCas9 fusion with a Histone AcetylTransferase.	2019	9(1):8080	[Roca Paixao JF et al.]	Embrapa Genetic Resources and Biotechnology, Brasilia, DF, Brazil	31147630	10.1038/s41598-019-44571-y	Drought episodes decrease plant growth and productivity, which in turn cause high economic losses. Plants naturally sense and respond to water stress by activating specific signalling pathways leading to physiological and developmental adaptations. Genetically engineering genes that belong to these pathways might improve the drought tolerance of plants. The abscisic acid (ABA)-responsive element binding protein 1/ABRE binding factor (AREB1/ABF2) is a key positive regulator of the drought stress response. We investigated whether the CRISPR activation (CRISPRa) system that targets AREB1 might contribute to improve drought stress tolerance in Arabidopsis. Arabidopsis histone acetyltransferase 1 (AtHAT1) promotes gene expression activation by switching chromatin to a relaxed state. Stable transgenic plants expressing chimeric dCas9(HAT) were first generated. Then, we showed that the CRISPRa dCas9(HAT) mechanism increased the promoter activity controlling the beta-glucuronidase (GUS) reporter gene. To activate the endogenous promoter of AREB1, the CRISPRa dCas9(HAT) system was set up, and resultant plants showed a dwarf phenotype. Our qRT-PCR experiments indicated that both AREB1 and RD29A, a gene positively regulated by AREB1, exhibited higher gene expression than the control plants. The plants generated here showed higher chlorophyll content and faster stomatal aperture under water deficit, in addition to a better survival rate after drought stress. Altogether, we report that CRISPRa dCas9(HAT) is a valuable biotechnological	Arabidopsis
590	plant	Arabidopsis thaliana	CRISPR;Cas9;	CBF2	American journal of botany	Genetic and physiological mechanisms of freezing tolerance in locally adapted populations of a winter annual.	2019		[Sanderson BJ et al.]	Purdue University, West Lafayette, IN, USA.	31762012	10.1002/ajb2.1385	PREMISE: Despite myriad examples of local adaptation, the phenotypes and genetic variants underlying such adaptive differentiation are seldom known. Recent work on freezing tolerance and local adaptation in ecotypes of Arabidopsis thaliana from Italy and Sweden provides an essential foundation for uncovering the genotype-phenotype-fitness map for an adaptive response to a key environmental stress. METHODS: We examined the consequences of a naturally occurring loss-of-function (LOF) mutation in an Italian allele of the gene that encodes the transcription factor CBF2, which underlies a major freezing-tolerance locus. We used four lines with a Swedish genetic background, each containing a LOF CBF2 allele. Two lines had introgression segments containing the Italian CBF2 allele, and two contained deletions created using CRISPR-Cas9. We used a growth chamber experiment to quantify freezing tolerance and gene expression before and after cold acclimation. RESULTS: Freezing tolerance was lower in the Italian (11%) compared to the Swedish (72%) ecotype, and all four experimental CBF2 LOF lines had reduced freezing tolerance compared to the Swedish ecotype. Differential expression analyses identified 10 genes for which all CBF2 LOF lines, and the IT ecotype had similar patterns of reduced cold responsive expression compared to the SW ecotype. CONCLUSIONS: We identified 10 genes that are at least partially regulated by CBF2 that may contribute to the differences in cold-acclimated freezing tolerance between the Italian and Swedish ecotypes. These results provide novel insight into the molecular and physiological mechanisms connecting a naturally	Arabidopsis
591	plant	Arabidopsis thaliana	CRISPR;Cas9;	SMALL AUXIN UP RNA SAUR41 subfamily genes (SAUR40; SAUR41; SAUR71; SAUR72)	Annals of botany	The SAUR41 subfamily of SMALL AUXIN UP RNA genes is abscisic acid-inducible to modulate cell expansion and salt tolerance in Arabidopsis thaliana seedlings.	2019		[Qiu T et al.]	Zhejiang University, Hangzhou, China.	31585004	10.1093/aob/mcz160	BACKGROUND AND AIMS: Most primary auxin response genes are classified into three families: AUX/IAAs, GH3s, and SAURs. Few studies have been conducted on Arabidopsis SAURs, possibly due to genetic redundancy among different subfamily members. Data mining on Arabidopsis transcriptional profiles indicates that the SAUR41 subfamily members of SMALL AUXIN UP RNA genes are, strikingly, induced by an inhibitory phytohormone, abscisic acid. We aimed to reveal the physiological roles of Arabidopsis SAUR41 subfamily genes containing SAUR40, SAUR41, SAUR71, and SAUR72. METHODS: Transcriptional responses of Arabidopsis SAUR41s to phytohormones were determined by quantitative real-time PCR. Knock out of SAUR41s was carried out with the CRISPR/Cas9 (Clustered Regulatory Interspaced Short Palindromic Repeats/CRISPR Associated Protein 9) genome editing technique. The saur41/40/71/72 quadruple mutants, the SAUR41 overexpression lines and the wild-type were subjected to ultra-structure observation, transcriptome analysis, and physiological characterization. KEY RESULTS: Transcription of Arabidopsis SAUR41 subfamily genes is activated by ABA but not by gibberellic acids and brassinosteroids. Quadruple mutations in saur41/40/71/72 led to reduced cell expansion/elongation in cotyledons and hypocotyls, opposite to the SAUR41 overexpression; however, irregular arrangement of cell size and shape was observed in both cases. The quadruple mutants had increased transcription of calcium homeostasis/signaling genes in seedling shoots, and the SAUR41 overexpression lines had decreased transcription of iron homeostasis genes in roots and increased ABA biosynthesis in shoots. Notably, both the quadruple mutants and the SAUR41 overexpression lines were hypersensitive to salt stress during seedling establishment, whereas specific expression of SAUR41 under the ABA-responsive RD29A (Responsive to Desiccation 29A) promoter in the quadruple mutants rescued the inhibitory effect of salt stress. CONCLUSIONS: The SAUR41 subfamily genes of Arabidopsis are ABA-inducible to modulate cell expansion, ion homeostasis and salt tolerance. Our work may provide new candidate genes for improvement of	Arabidopsis

592	plant	Arabidopsis thaliana	CRISPR;Cas9;	beta-ketoacyl-ACP synthase I (KASI)	Biotechnology for biofuels	Enhancing oil production in Arabidopsis through expression of a ketoacyl-ACP synthase domain of the PUFA synthase from Thraustochytrium.	2019	12:172	[Xie X et al.]	University of Saskatchewan, Saskatoon, SK, Canada.	31297160	10.1186/s13068-019-1514-8	Background: Plant seed oil is an important bioresource for human food and animal feed, as well as industrial bioproducts. Therefore, increasing oil content in seeds has been one of the primary targets in the breeding programs of oilseed crops. Thraustochytrium is a marine protist that can produce a high level of very long-chain polyunsaturated fatty acids (VLCPUFAs) using a PUFA synthase, a polyketide synthase-like fatty acid synthase with multiple catalytic domains. Our previous study showed that a KS domain from the synthase could complement an Escherichia coli mutant defective in beta-ketoacyl-ACP synthase I (FabB) and increase the total fatty acid production. In this study, this KS domain from the PUFA synthase was further functionally analyzed in Arabidopsis thaliana for the capacity of oil production. Results: The plastidial expression of the KS domain could complement the defective phenotypes of a KASI knockout mutant generated by CRISPR/Cas9. Seed-specific expression of the domain in wild-type Arabidopsis significantly increased seed weight and seed oil, and altered the unsaturation level of fatty acids in seeds, as well as promoted seed germination and early seedling growth. Conclusions: The condensation process of fatty acid biosynthesis in plants is a limiting step, and overexpression of the KS domain from a PUFA synthase of microbial origin offers a new strategy to increase oil production in	Arabidopsis
593	plant	Arabidopsis thaliana	CRISPR;	Col-0; BKN-1; BKN2	BMC plant biology	Investigations into a putative role for the novel BRASSIKIN pseudokinases in compatible pollen-stigma interactions in Arabidopsis thaliana.	2019	19(1):549	[Doucet J et al.]	University of Toronto, Toronto, Canada.	31829135	10.1186/s12870-019-2160-9	BACKGROUND: In the Brassicaceae, the early stages of compatible pollen-stigma interactions are tightly controlled with early checkpoints regulating pollen adhesion, hydration and germination, and pollen tube entry into the stigmatic surface. However, the early signalling events in the stigma which trigger these compatible interactions remain unknown. RESULTS: A set of stigma-expressed pseudokinase genes, termed BRASSIKINs (BKNs), were identified and found to be present in only core Brassicaceae genomes. In Arabidopsis thaliana Col-0, BKN1 displayed stigma-specific expression while the BKN2 gene was expressed in other tissues as well. CRISPR deletion mutations were generated for the two tandemly linked BKNs, and very mild hydration defects were observed for wild-type Col-0 pollen when placed on the bkn1/2 mutant stigmas. In further analyses, the predominant transcript for the stigma-specific BKN1 was found to have a premature stop codon in the Col-0 ecotype, but a survey of the 1001 Arabidopsis genomes uncovered three ecotypes that encoded a full-length BKN1 protein. Furthermore, phylogenetic analyses identified intact BKN1 orthologues in the closely related outcrossing Arabidopsis species, A. lyrata and A. halleri. Finally, the BKN pseudokinases were found to be plasma-membrane localized through the dual lipid modification of myristoylation and palmitoylation, and this localization would be consistent with a role in signaling complexes. CONCLUSION: In this study, we have characterized the novel Brassicaceae-specific family of BKN pseudokinase genes, and examined the function of BKN1 and BKN2 in the context of pollen-stigma interactions in A. thaliana Col-0. Additionally, premature stop codons were identified in the predicted stigma specific BKN1 gene in a number of the 1001 A. thaliana ecotype genomes, and this was in contrast to the out-crossing Arabidopsis species which carried intact copies of BKN1. Thus, understanding the function of BKN1 in other	Arabidopsis
594	plant	Arabidopsis thaliana	CRISPR;Cas9;	zerzaust homolog	G3	Asymmetric Redundancy of ZERZAUST and ZERZAUST HOMOLOG in Different Accessions of Arabidopsis thaliana.	2019	9(7):2245-2252	[Vaddepalli P et al.]	Wageningen University, Wageningen, the Netherlands.	31113822	10.1534/g3.119.400211	Divergence among duplicate genes is one of the important sources of evolutionary innovation. But, the contribution of duplicate divergence to variation in Arabidopsis accessions is sparsely known. Recently, we studied the role of a cell wall localized protein, ZERZAUST (ZET), in Landsberg erecta (Ler) accession, lack of which results in aberrant plant morphology. Here, we present the study of ZET in Columbia (Col) accession, which not only showed differential expression patterns in comparison to Ler, but also revealed its close homolog, ZERZAUST HOMOLOG (ZETH) Although, genetic analysis implied redundancy, expression analysis revealed divergence, with ZETH showing minimal expression in both Col and Ler In addition, ZETH shows relatively higher expression levels in Col compared to Ler Our data also reveal compensatory up-regulation of ZETH in Col, but not in Ler, implying it is perhaps dispensable in Ler However, a novel CRISPR/Cas9-induced zeth allele confirmed that ZETH has residual activity in Ler Finally, the synergistic interaction of the receptor-like kinase gene, ERECTA with ZET in ameliorating morphological defects suggests crucial role of modifiers on plant phenotype. The results provide genetic evidence for accession-specific differences in compensation mechanism and asymmetric gene contribution. Thus, our work reveals a novel example for how weakly expressed homologs contribute	Arabidopsis
595	plant	Arabidopsis thaliana	CRISPR;Cas9;	281 targets	International journal of molecular sciences	Comprehensive Analysis of CRISPR/Cas9-Mediated Mutagenesis in Arabidopsis thaliana by Genome-wide Sequencing.	2019	20(17)	[Xu W et al.]	China Agricultural University, Beijing, China.	31450868	10.3390/ijms20174125	The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system has been widely applied in functional genomics research and plant breeding. In contrast to the off-target studies of mammalian cells, there is little evidence for the common occurrence of off-target sites in plants and a great need exists for accurate detection of editing sites. Here, we summarized the precision of CRISPR/Cas9-mediated mutations for 281 targets and found that there is a preference for single nucleotide deletions/insertions and longer deletions starting from 40 nt upstream or ending at 30 nt downstream of the cleavage site, which suggested the candidate sequences for editing sites detection by whole-genome sequencing (WGS). We analyzed the on-/off-target sites of 6 CRISPR/Cas9-mediated Arabidopsis plants by the optimized method. The results showed that the on-target editing frequency ranged from 38.1% to 100%, and one off target at a frequency of 9.8%-97.3% cannot be prevented by increasing the specificity or reducing the expression level of the Cas9 enzyme. These results indicated that designing guide RNA with high specificity may be the preferred factor to avoid the off-target events, and it is necessary to predict or detect off-target sites by WGS-based methods for preventing	Arabidopsis

596	plant	Arabidopsis thaliana	CRISPR;Cas9;	immune-associated nucleotide-binding gene (ian9)	Molecular plant-microbe interactions	The IMMUNE-ASSOCIATED NUCLEOTIDE-BINDING 9 Protein Is a Regulator of Basal Immunity in Arabidopsis thaliana.	2019	32(1):65-75	[Wang Y et al.]	Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China.	29958083	10.1094/MPMI-03-18-0062-R	A robust regulation of plant immune responses requires a multitude of positive and negative regulators that act in concert. The immune-associated nucleotide-binding (IAN) gene family members are associated with immunity in different organisms, although no characterization of their function has been carried out to date in plants. In this work, we analyzed the expression patterns of IAN genes and found that IAN9 is repressed upon pathogen infection or treatment with immune elicitors. IAN9 encodes a plasma membrane-localized protein that genetically behaves as a negative regulator of immunity. A novel ian9 mutant generated by CRISPR/Cas9 shows increased resistance to Pseudomonas syringae, while transgenic plants overexpressing IAN9 show a slight increase in susceptibility. In vivo immunoprecipitation of IAN9-green fluorescent protein followed by mass spectrometry analysis revealed that IAN9 associates with a previously uncharacterized C3HC4-type RING-finger domain-containing protein that we named IAN9-associated protein 1 (IAP1), which also acts as a negative regulator of basal immunity. Interestingly, neither ian9 or iap1 mutant plants show any obvious developmental phenotype, suggesting that they display enhanced inducible immunity rather than constitutive immune responses. Because both IAN9 and IAP1 have orthologs in important crop species, they could be suitable targets to generate plants more resistant to diseases caused by bacterial pathogens without yield penalty.	Arabidopsis
597	plant	Arabidopsis thaliana	CRISPR;Cas9;	protospacer-adjacent motif	Plant & cell physiology	Developing Heritable Mutations in Arabidopsis thaliana Using a Modified CRISPR/Cas9 Toolkit Comprising PAM-Altered Cas9 Variants and gRNAs.	2019	60(10):2255-2262	[Yamamoto A et al.]	Kumamoto University, Kumamoto, Japan.	31198958	10.1093/pcp/pcz118	Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9), comprising an RNA-guided DNA endonuclease and a programmable guide RNA (gRNA), is currently recognized to be a powerful genome-editing tool and is widely used in biological science. Despite the usefulness of the system, a protospacer-adjacent motif (PAM) immediately downstream of the target sequence needs to be taken into account in the design of the gRNA, a requirement which limits the flexibility of the CRISPR-based genome-editing system. To overcome this limitation, a Cas9 isolated from Streptococcus pyogenes, namely SpCas9, engineered to develop several variants of Cas9 nuclease, has been generated. SpCas9 recognizes the NGG sequence as the PAM, whereas its variants are capable of interacting with different PAMs. Despite the potential advantage of the Cas9 variants, their functionalities have not previously been tested in the widely used model plant, Arabidopsis thaliana. Here, we developed a plant-specific vector series harboring SpCas9-VQR (NGAN or NGNG) or SpCas9-EQR (NGAG) and evaluated their functionalities. These modified Cas9 nucleases efficiently introduced mutations into the CLV3 and AS1 target genes using gRNAs that were compatible with atypical PAMs. Furthermore, the generated mutations were passed on to their offspring. This study illustrated the usefulness of the SpCas9 variants because the ability to generate heritable mutations will be of great benefit in molecular genetic analyses. A greater number of potential SpCas9-variant-recognition sites in these genes are predicted, compared with those of conventional SpCas9. These results demonstrated the usefulness of the SpCas9 variants for genome editing	Arabidopsis
598	plant	Arabidopsis thaliana	CRISPR;Cas9;	ELF3	Plant biotechnology	Monitoring single-cell bioluminescence of Arabidopsis leaves to quantitatively evaluate the efficiency of a transiently introduced CRISPR/Cas9 system targeting the circadian clock gene ELF3.	2019	36(3):187-193	[Kanesaka Y et al.]	Kyoto University, Japan.	31768121	10.5511/plantbiotechnology.19.0531a	The rapid assessment of gene function is crucial in biological research. The CRISPR/Cas9 system is widely used as a tool for targeted gene editing in many organisms including plants. Previously, we established a transient gene expression system for investigating cellular circadian rhythms in duckweed. In this system, circadian reporters and clock gene effectors-such as overexpressors, RNA interference (RNAi), and CRISPR/Cas9-were introduced into duckweed cells using a particle bombardment method. In the present study, we applied the CRISPR/Cas9 system at a single cell level to Arabidopsis thaliana, a model organism in plant biology. To evaluate the mutation induction efficiency of the system, we monitored single-cell bioluminescence after application of the CRISPR/Cas9 system targeting the ELF3 gene, which is essential for robust circadian rhythmicity. We evaluated the mutation induction efficiency by determining the proportion of cells with impaired circadian rhythms. Three single guide RNAs (sgRNAs) were designed, and the proportion of arrhythmic cells following their use ranged from 32 to 91%. A comparison of the mutation induction efficiencies of diploid and tetraploid Arabidopsis suggested that endoreduplication had a slight effect on efficiency. Taken together, our results demonstrate that the transiently introduced CRISPR/Cas9 system is useful for rapidly	Arabidopsis
599	plant	Arabidopsis thaliana	agroinfiltration; CRISPR;Cas9;		Plant biotechnology	pCYOs: Binary vectors for simple visible selection of transformants using an albino-cotyledon mutant in Arabidopsis thaliana.	2019	36(1):39-42	[Yamatani H et al.]	Hiroshima University, Higashi-Hiroshima, Japan.	31275047	10.5511/plantbiotechnology.18.1212a	Several selection markers for the screening of transformants have been developed; however, simple and reliable methods are generally preferred. We have developed a novel visible selection system for the identification of transformants in Arabidopsis thaliana that does not require any special reagent and/or equipment except using the albino-cotyledon mutant cyo1. In this system, the pCYO vector carrying the CYO1 genomic fragment as a selection marker is introduced into the cyo1 mutant. Transformation is performed by the Agrobacterium-mediated floral dip method and resultant T1 seeds are sown in soil. Seedlings with green cotyledons, not albino, are expected to be 'complemented' transformants with the transgene of interest. This system provides a very simple selection method that can be performed without any special equipment, reagent, sterile conditions, or UV illumination. We have constructed three vectors, (1) pCYO1, an empty vector; (2) pCYO2, an overexpression vector carrying CaMV35S promoter; and (3) pCYO3, a vector for genome editing, carrying the CRISPR/Cas9 cassette. Example transformation experiments using these vectors.	Arabidopsis
600	plant	Arabidopsis thaliana	CRISPR;		Plant cell	CRISPR-TSKO: A Technique for Efficient Mutagenesis in Specific Cell Types, Tissues, or Organs in Arabidopsis.	2019	31(12):2868-2887	[Decaestecker W et al.]	Ghent University, Ghent, Belgium.	31562216	10.1105/tpc.19.00454	Detailed functional analyses of many fundamentally important plant genes via conventional loss-of-function approaches are impeded by the severe pleiotropic phenotypes resulting from these losses. In particular, mutations in genes that are required for basic cellular functions and/or reproduction often interfere with the generation of homozygous mutant plants, precluding further functional studies. To overcome this limitation, we devised a clustered regularly interspaced short palindromic repeats (CRISPR)-based tissue-specific knockout system, CRISPR-TSKO, enabling the generation of somatic mutations in particular plant cell types, tissues, and organs. In Arabidopsis (Arabidopsis thaliana), CRISPR-TSKO mutations in essential genes caused well-defined, localized phenotypes in the root cap, stomatal lineage, or entire lateral roots. The modular cloning system developed in this study allows for the efficient selection, identification, and functional analysis of mutant lines directly in the first transgenic generation. The efficacy of CRISPR-TSKO opens avenues for discovering and analyzing gene functions in the spatial and temporal contexts of plant	Arabidopsis

601	plant	Arabidopsis thaliana	CRISPR;Cas9;	SPARTAN/ weak suppressor of smt3 (Wss1); WSS1A; TYROSYL-DNA PHOSPHODIESTERASE 1	Plant cell	The Protease WSS1A, the Endonuclease MUS81, and the Phosphodiesterase TDP1 Are Involved in Independent Pathways of DNA-protein Crosslink Repair in Plants.	2019	31(4):775-790	[Enderle J et al.]	Karlsruhe Institute of Technology, Karlsruhe, Germany.	30760561	10.1105/tpc.18.00824	DNA-protein crosslinks (DPCs) represent a severe threat to the genome integrity; however, the main mechanisms of DPC repair were only recently elucidated in humans and yeast. Here we define the pathways for DPC repair in plants. Using CRISPR/Cas9, we could show that only one of two homologs of the universal repair proteases SPARTAN/ weak suppressor of smt3 (Wss1), WSS1A, is essential for DPC repair in Arabidopsis (Arabidopsis thaliana). WSS1A defective lines exhibit developmental defects and are hypersensitive to camptothecin (CPT) and cis-platin. Interestingly, the CRISPR/Cas9 mutants of TYROSYL-DNA PHOSPHODIESTERASE 1 (TDP1) are insensitive to CPT, and only the wss1A tdp1 double mutant reveals a higher sensitivity than the wss1A single mutant. This indicates that TDP1 defines a minor backup pathway in the repair of DPCs. Moreover, we found that knock out of the endonuclease METHYL METHANESULFONATE AND UV SENSITIVE PROTEIN 81 (MUS81) results in a strong sensitivity to DPC-inducing agents. The fact that wss1A mus81 and tdp1 mus81 double mutants exhibit growth defects and an increase in dead cells in root meristems after CPT treatment demonstrates that there are three independent pathways for DPC repair in Arabidopsis. These pathways are defined by their different biochemical specificities, as main actors, the DNA endonuclease MUS81 and the	Arabidopsis
602	plant	Arabidopsis thaliana	CRISPR;	Protection of telomeres 1 (POT1c)	Plant cell reports	Recent emergence and extinction of the protection of telomeres 1c gene in Arabidopsis thaliana.	2019	38(9):1081-1097	[Kobayashi CR et al.]	Texas A&M University, College Station, TX, USA.	31134349	10.1007/s00299-019-02427-9	KEY MESSAGE: Duplicate POT1 genes must rapidly diverge or be inactivated. Protection of telomeres 1 (POT1) encodes a conserved telomere binding protein implicated in both chromosome end protection and telomere length maintenance. Most organisms harbor a single POT1 gene, but in the few lineages where the POT1 family has expanded, the duplicate genes have diversified. Arabidopsis thaliana bears three POT1-like loci, POT1a, POT1b and POT1c. POT1a retains the ancestral function of telomerase regulation, while POT1b is implicated in chromosome end protection. Here we examine the function and evolution of the third POT1 paralog, POT1c. POT1c is a new gene, unique to A. thaliana, and was derived from a duplication event involving the POT1a locus and a neighboring gene encoding ribosomal protein S17. The duplicate S17 locus (dS17) is highly conserved across A. thaliana accessions, while POT1c is highly divergent, harboring multiple deletions within the gene body and two transposable elements within the promoter. The POT1c locus is transcribed at very low to non-detectable levels under standard growth conditions. In addition, no discernable molecular or developmental defects are associated with plants bearing a CRISPR mutation in the POT1c locus. However, forced expression of POT1c leads to decreased telomerase enzyme activity and shortened telomeres. Evolutionary reconstruction indicates that transposons invaded the POT1c promoter soon after the locus was formed, permanently silencing the gene. Altogether, these findings argue that POT1 dosage is critically important for viability and duplicate gene copies are retained only	Arabidopsis
603	plant	Arabidopsis thaliana	Cas9;	TPS5	Plant cell reports	The trehalose-6-phosphate synthase TPS5 negatively regulates ABA signaling in Arabidopsis thaliana.	2019	38(8):869-882	[Tian L et al.]	Hunan Normal University, Changsha, Hunan, China.	30963238	10.1007/s00299-019-02408-y	KEY MESSAGE: The TPS5 negatively regulates ABA signaling by mediating ROS level and NR activity during seed germination and stomatal closure in Arabidopsis thaliana. Trehalose metabolism is important in plant growth and development and in abiotic stress response. Eleven TPS genes were identified in Arabidopsis, divided into Class I (TPS1-TPS4) and Class II (TPS5-TPS11). Although Class I has been shown to have TPS activity, the function of most members of Class II remains enigmatic. Here, we characterized the biological function of the trehalose-6-phosphate synthase TPS5 in ABA signaling in Arabidopsis. TPS5 expression was induced by ABA and abiotic stress, and expression in epidermal and guard cells was dramatically increased after ABA treatment. Loss-of-function analysis revealed that tps5 mutants (tps5-1 and tps5-cas9) are more sensitive to ABA during seed germination and ABA-mediated stomatal closure. Furthermore, the H2O2 level increased in the tps5-1 and tps5-cas9 mutants, which was consistent with the changes in the expression of RbohD and RbohF, key genes responsible for H2O2 production. Further, TPS5 knockout reduced the amounts of trehalose and other soluble carbohydrates as well as nitrate reductase (NR) activity. In vitro, trehalose and other soluble carbohydrates promoted NR activity, which was blocked by the tricarboxylic acid cycle inhibitor iodoacetic acid. Thus, this study identified that TPS5 functions as a negative regulator of ABA signaling and is involved	Arabidopsis
604	plant	Arabidopsis thaliana	CRISPR;Cas9;	plastidial phosphoglycerate kinase (pgkp1; pgkp2)	Plant journal	Pooled CRISPR/Cas9 reveals redundant roles of plastidial phosphoglycerate kinases in carbon fixation and metabolism.	2019	98(6):1078-1089	[Li R et al.]	Fujian Agriculture and Forestry University, Fuzhou, China.	30834637	10.1111/tpj.14303	Phosphoglycerate kinase (PGK) is a highly conserved reversible enzyme that participates in both glycolysis and photosynthesis. In Arabidopsis thaliana, one cytosolic PGK (PGKc) and two plastidial PGKs (PGKp) are known. It remains debatable whether the two PGKp isozymes are functionally redundant or specialized in plastidial carbon metabolism and fixation. Here, using a pooled clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) strategy, we found that plants with single mutations in pgkp1 or pgkp2 were not significantly affected, whereas a pgkp1pgkp2 double mutation was lethal due to retarded carbon fixation, suggesting that PGKp isozymes play redundant functional roles. Metabolomic analysis demonstrated that the sugar-deficient pgkp1pgkp2 double mutation was partially complemented by exogenous sugar, although respiration intermediates were not rescued. Chloroplast development was defective in pgkp1pgkp2, due to a deficiency in glycolysis-dependent galactoglycerolipid biosynthesis. Ectopic expression of a plastid targeting PGKc did not reverse the pgkp1pgkp2 double-mutant phenotypes. Therefore, PGKp1 and PGKp2 play redundant roles in carbon fixation and metabolism, whereas the molecular function of PGKc is more divergent. Our study demonstrated the functional	Arabidopsis

605	plant	Arabidopsis thaliana	Cas9;	KU70	Plant journal	Efficient induction of heritable inversions in plant genomes using the CRISPR/Cas system.	2019	98(4):577–589	[Schmidt C et al.]	Botanical Institute, Karlsruhe, Germany.	30900787	10.1111/tpj.14322	During the evolution of plant genomes, sequence inversions occurred repeatedly making the respective regions inaccessible for meiotic recombination and thus for breeding. Therefore, it is important to develop technologies that allow the induction of inversions within chromosomes in a directed and efficient manner. Using the Cas9 nuclease from Staphylococcus aureus (SaCas9), we were able to obtain scarless heritable inversions with high efficiency in the model plant Arabidopsis thaliana. Via deep sequencing, we defined the patterns of junction formation in wild-type and in the non-homologous end-joining (NHEJ) mutant ku70-1. Surprisingly, in plants deficient of KU70, inversion induction is enhanced, indicating that KU70 is required for tethering the local broken ends together during repair. However, in contrast to wild-type, most junctions are formed by microhomology-mediated NHEJ and thus are imperfect with mainly deletions, making this approach unsuitable for practical applications. Using egg-cell-specific expression of Cas9, we were able to induce heritable inversions at different genomic loci and at intervals between 3 and 18 kb, in the percentage range, in the T1 generation. By screening individual lines, inversion frequencies of up to the 10% range were found in T2. Most of these inversions had scarless junctions and were without any sequence change within the inverted region, making the technology attractive for use in crop plants. Applying our approach, it should be possible to reverse	Arabidopsis
606	plant	Arabidopsis thaliana	CRISPR;Cas9;	MICRORNA167 (MIR167)	Plant physiology	An Essential Role for miRNA167 in Maternal Control of Embryonic and Seed Development.	2019	180(1):453–464	[Yao X et al.]	Shanghai Normal University, Shanghai, China.	30867333	10.1104/pp.19.00127	Maternal cells play a critical role in ensuring the normal development of embryos, endosperms, and seeds. Mutations that disrupt the maternal control of embryogenesis and seed development are difficult to identify. Here, we completely deleted four MICRORNA167 (MIR167) genes in Arabidopsis (Arabidopsis thaliana) using a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 (Cas9) genome-editing technology. We found that plants with a deletion of MIR167A phenocopied plants overexpressing miRNA167-resistant versions of Auxin Response Factor6 (ARF6) or ARF8, two miRNA167 targets. Both the mir167a mutant and the ARF overexpression lines were defective in anther dehiscence and ovule development. Serendipitously, we found that the mir167a (female symbol) x wild type (male symbol) crosses failed to produce normal embryos and endosperms, despite the findings that embryos with either mir167a(+/-) or mir167a(-/-) genotypes developed normally when mir167a(+/-) plants were self-pollinated, revealing a central role of MIR167A in maternal control of seed development. The mir167a phenotype is 100% penetrant, providing a great genetic tool for studying the roles of miRNAs and auxin in maternal control. Moreover, we found that mir167a mutants flowered significantly later than wild-type plants, a phenotype that was not observed in the ARF overexpression lines. We show that the reproductive defects of mir167a mutants were suppressed by a decrease of activities of ARF6, ARF8, or both. Our results clearly demonstrate that MIR167A is the predominant MIR167 member in regulating Arabidopsis reproduction and that MIR167A acts as a maternal gene that functions largely through ARF6 and	Arabidopsis
607	plant	Arabidopsis thaliana	CRISPR;Cas9;	ABI5	Plant physiology	GOLDEN2-LIKE Transcription Factors Regulate WRKY40 Expression in Response to Abscisic Acid.	2019	179(4):1844–1860	[Ahmad R et al.]	Northeast Normal University, Changchun, China.	30723180	10.1104/pp.18.01466	Arabidopsis (Arabidopsis thaliana) GARP (Golden2, ARR-B, Psr1) family transcription factors, GOLDEN2-LIKE1 and -2 (GLK1/2), function in different biological processes; however, whether and how these transcription factors modulate the response to abscisic acid (ABA) remain unknown. In this study, we used a glk1 glk2 double mutant to examine the role of GLK1/2 in the ABA response. The glk1 glk2 double mutant displayed ABA-hypersensitive phenotypes during seed germination and seedling development and an osmotic stress-resistant phenotype during seedling development. Genome-wide RNA sequencing analysis of the glk1 glk2 double mutant revealed that GLK1/2 regulate several ABA-responsive genes, including WRKY40, in the presence of ABA. Chromatin immunoprecipitation and gel retardation assays showed that GLK1/2 directly associate with the WRKY40 promoter via the recognition of a consensus sequence. Additionally, RNA sequencing analysis of the glk1 glk2 double mutant and wrky40 single mutant revealed that GLK1/2 and WRKY40 control a common set of downstream target genes in response to ABA. Furthermore, results of a genetic interaction test showed that the glk1 glk2 wrky40 triple mutant displayed similar ABA hypersensitivity to the wrky40 single mutant and the glk1 glk2 double mutant, while the glk1 glk2 wrky40 abi5-c (ABI5 CRISPR/Cas9 mutant) quadruple mutant displayed similar ABA hyposensitivity to the abi5-7 single mutant. Based on these results, we propose that the GLK1/2-WRKY40 transcription module plays a negative regulatory	Arabidopsis
608	plant	Arabidopsis thaliana	CRISPR;	E6-like 1 (E6L1)	Plant reproduction	Identification of a role for an E6-like 1 gene in early pollen-stigma interactions in Arabidopsis thaliana.	2019	32(3):307–322	[Doucet J et al.]	University of Toronto, Toronto, Canada.	31069543	10.1007/s00497-019-00372-x	KEY MESSAGE: We describe a function for a novel Arabidopsis gene, E6-like 1 (E6L1), that was identified as a highly expressed gene in the stigma and plays a role in early post-pollination stages. In Arabidopsis, successful pollen-stigma interactions are dependent on rapid recognition of compatible pollen by the stigmatic papillae located on the surface of the pistil and the subsequent regulation of pollen hydration and germination, and followed by the growth of pollen tubes through the stigma surface. Here we have described the function of a novel gene, E6-like 1 (E6L1), that was identified through the analysis of transcriptome datasets, as one of highest expressed genes in the stigma, and furthermore, its expression was largely restricted to the stigma and trichomes. The first E6 gene was initially identified as a highly expressed gene during cotton fiber development, and related E6-like predicted proteins are found throughout the Angiosperms. To date, no orthologous genes have been assigned a biological function. Both the Arabidopsis E6L1 and cotton E6 proteins are predicted to be secreted, and this was confirmed using an E6L1:RFP fusion construct. To further investigate E6L1's function, one T-DNA and two independent CRISPR-generated mutants were analyzed for compatible pollen-stigma interactions, and pollen hydration, pollen adhesion, and seed set were mildly impaired for the e6l1 mutants. This work identifies E6L1 as a novel stigmatic factor that plays a role during the early post-	Arabidopsis

609	plant	Arabidopsis thaliana	CRISPR;Cas9;	flowering locus T; transcriptional activator VP64; H3K27 acetyltransferase p300; H3K9 methyltransferase KRYPTONITE	PloS one	CRISPR-based tools for targeted transcriptional and epigenetic regulation in plants.	2019	14(9):e0222778	[Lee JE et al.]	Umea University, Umea, Sweden.	31557222	10.1371/journal.pone.0222778	Programmable gene regulators that can modulate the activity of selected targets in trans are a useful tool for probing and manipulating gene function. CRISPR technology provides a convenient method for gene targeting that can also be adapted for multiplexing and other modifications to enable strong regulation by a range of different effectors. We generated a vector toolbox for CRISPR/dCas9-based targeted gene regulation in plants, modified with the previously described MS2 system to amplify the strength of regulation, and using Golden Gate-based cloning to enable rapid vector assembly with a high degree of flexibility in the choice of promoters, effectors and targets. We tested the system using the floral regulator FLOWERING LOCUS T (FT) as a target and a range of different effector domains including the transcriptional activator VP64, the H3K27 acetyltransferase p300 and the H3K9 methyltransferase KRYPTONITE. When transformed into Arabidopsis thaliana, several of the constructs caused altered flowering time phenotypes that were associated with changes in FT expression and/or epigenetic status, thus demonstrating the effectiveness of the system. The MS2-CRISPR/dCas9 system can be used to modulate transcriptional activity and epigenetic status of specific target genes in plants, and provides a versatile tool that can easily be used with different targets and types of regulation for a	Arabidopsis
610	plant	Arabidopsis; Nicotiana benthamiana	CRISPR;	nrg1; nrg2	New phytologist	Diverse NLR immune receptors activate defence via the RPW8-NLR NRG1.	2019	222(2):966-980	[Castel B et al.]	University of East Anglia, Norwich, UK.	30582759	10.1111/nph.15659	Most land plant genomes carry genes that encode RPW8-NLR Resistance (R) proteins. Angiosperms carry two RPW8-NLR subclasses: ADR1 and NRG1. ADR1s act as 'helper' NLRs for multiple TIR- and CC-NLR R proteins in Arabidopsis. In angiosperm families, NRG1 co-occurs with TIR-NLR Resistance (R) genes. We tested whether NRG1 is required for signalling of multiple TIR-NLRs. Using CRISPR mutagenesis, we obtained an nrg1a-nrg1b double mutant in two Arabidopsis accessions, and an nrg1 mutant in Nicotiana benthamiana. These mutants are compromised in signalling of all TIR-NLRs tested, including WRR4A, WRR4B, RPP1, RPP2, RPP4 and the pairs RRS1/RPS4, RRS1B/RPS4B, CHS1/SOC3 and CHS3/CSA1. In Arabidopsis, NRG1 is required for the hypersensitive cell death response (HR) and full oomycete resistance, but not for salicylic acid induction or bacterial resistance. By contrast, nrg1 loss of function does not compromise the CC-NLR R proteins RPS5 and MLA. RPM1 and RPS2 (CC-NLRs) function is slightly compromised in an nrg1 mutant. Thus, NRG1 is required for full TIR-NLR function and contributes to the signalling of some CC-NLRs. Some NRG1-dependent R proteins also signal partially via the NRG1 sister clade, ADR1. We propose that some NLRs signal via NRG1 only, some via ADR1 only and some via both or	Arabidopsis;
611	plant	Arabidopsis; rice	agroinfiltration; CRISPR;Cas9;		Current protocols in molecular biology	Targeted Transcriptional Activation in Plants Using a Potent Dead Cas9-Derived Synthetic Gene Activator.	2019	127(1):e89	[Li Z et al.]	Sun Yat-sen University, Guangzhou, China.	31237422	10.1002/cpmb.89	Genetic tools for specific perturbation of endogenous gene expression are highly desirable for interrogation of plant gene functions and improvement of crop traits. Synthetic transcriptional activators derived from the CRISPR/Cas9 system are emerging as powerful new tools for activating the endogenous expression of genes of interest in plants. These synthetic constructs, generated by tethering transcriptional activation domains to a nuclease-dead Cas9 (dCas9), can be directed to the promoters of endogenous target genes by single guide RNAs (sgRNAs) to activate transcription. Here, we provide a detailed protocol for targeted transcriptional activation in plants using a recently developed, highly potent dCas9 gene activator construct referred to as dCas9-TV. This protocol covers selection of sgRNA targets, construction of sgRNA expression cassettes, and screening for an optimal sgRNA using a protoplast-based promoter-luciferase assay. Finally, the dCas9-TV gene activator coupled with the optimal sgRNA is delivered into plants via Agrobacterium-mediated transformation, thereby enabling robust upregulation of target gene expression in transgenic	Arabidopsis;
612	plant	Arabidopsis; rice	Cas9;		Nature plants	Genome editing in plants by engineered CRISPR-Cas9 recognizing NG PAM.	2019	5(1):14-17	[Endo M et al.]	Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Japan.	30531939	10.1038/s41477-018-0321-8	Streptococcus pyogenes Cas9 (SpCas9) is widely used for genome editing and requires NGG as a protospacer adjacent motif (PAM). Here, we show that the engineered SpCas9 (SpCas9-NGv1) can efficiently mutagenize endogenous target sites with NG PAMs in the rice and Arabidopsis genomes. Furthermore, we demonstrate that the SpCas9-NGv1 nickase fused to cytidine deaminase mediates C-to-T substitutions near the 5' end of the target sequence.	Arabidopsis;
613	plant	Arabidopsis; rice	Cas9;	MTA; GL1-1; NAL1	New phytologist	Gene disruption through base editing-induced messenger RNA missplicing in plants.	2019	222(2):1139-1148	[Li Z et al.]	Sun Yat-sen University, Guangzhou, China.	30565255	10.1111/nph.15647	Gene knockout tools are highly desirable for basic and applied plant research. Here, we leverage the Cas9-derived cytosine base editor to introduce precise C-to-T mutations to disrupt the highly conserved intron donor site GT or acceptor site AG, thereby inducing messenger RNA (mRNA) missplicing and gene disruption. As proof of concept, we successfully obtained Arabidopsis null mutant of MTA gene in the T2 generation and rice double null mutant of GL1-1 and NAL1 genes in the T0 generation by this strategy. Elimination of the original intron donor site or acceptor site could trigger aberrant splicing at a new specific exonic site, but not at the closest GT or AG site, suggesting cryptic rules governing splice site recognition. The strategy presented expands the applications of base editing technologies in plants by providing a new means for gene inactivation without generating DNA double-strand breaks, and it can potentially serve as a useful tool for studying the biology of mRNA splicing.	Arabidopsis;
614	plant	Arabidopsis; rice; tomato	CRISPR;Cas9;	DsRED	Frontiers in plant science	Identification of Transgene-Free CRISPR-Edited Plants of Rice, Tomato, and Arabidopsis by Monitoring DsRED Fluorescence in Dry Seeds.	2019	10:1150	[Aliaga-Franco N et al.]	Instituto de Biologia Molecular y Celular de Plantas, Consejo Superior de Investigaciones Cientificas (CSIC)-Universidad Politecnica de Valencia, Valencia, Spain.	31620160	10.3389/fpls.2019.01150	Efficient elimination of the editing machinery remains a challenge in plant biotechnology after genome editing to minimize the probability of off-target mutations, but it is also important to deliver end users with edited plants free of foreign DNA. Using the modular cloning system Golden Braid, we have included a fluorescence-dependent transgene monitoring module to the genome-editing tool box. We have tested this approach in Solanum lycopersicum, Oryza sativa, and Arabidopsis thaliana. We demonstrate that DsRED fluorescence visualization works efficiently in dry seeds as marker for the detection of the transgene in the three species allowing an efficient method for selecting transgene-free dry seeds. In the first generation of DsRED-free CRISPR/Cas9 null segregants, we detected gene editing of selected targets including homozygous mutants for the plant species tested. We demonstrate that this strategy allows rapid selection of transgene-free homozygous edited crop plants in a single	Arabidopsis;

615	plant	Arabis alpina	CRISPR;Cas9;	floral integrator SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15 (SPL15	Science	A regulatory circuit conferring varied flowering response to cold in annual and perennial plants.	2019	363(6425):409-412	[Hyun Y et al.]	Max Planck Institute for Plant Breeding Research, Köln, Germany.	30679374	10.1126/science.aau8197	The reproductive strategies of plants are highly variable. Short-lived annuals flower abundantly soon after germination, whereas longer-lived perennials postpone and spatially restrict flowering. We used CRISPR/Cas9 and interspecies gene transfer to understand divergence in reproductive patterns between annual and perennial crucifers. We show that in perennial Arabis alpina, flowering in response to winter cold depends on the floral integrator SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15 (SPL15), whose activity is limited to older shoots and branches during cold exposure. In annuals, this regulatory system is conserved, but cold-induced flowering occurs in young shoots, without requirement for SPL15, through the photoperiodic pathway when plants return to warm. By reconstructing the annual response in perennials, we conclude that characteristic patterns of reproduction in annuals and perennials are conferred through variation in dependency on distinct flowering pathways acting in	Arabis alpina
616	plant	barley	agroinfiltration; CRISPR;Cas9;	Qsd1	Cell reports	Genome-Edited Triple-Recessive Mutation Alters Seed Dormancy in Wheat.	2019	28(5):1362-1369.e4	[Abe F et al.]	Institute of Crop Science, NARO, Tsukuba, Japan.	31365876	10.1016/j.celrep.2019.06.090	Common wheat has three sets of sub-genomes, making mutations difficult to observe, especially for traits controlled by recessive genes. Here, we produced hexaploid wheat lines with loss of function of homeoalleles of Qsd1, which controls seed dormancy in barley, by Agrobacterium-mediated CRISPR/Cas9. Of the eight transformed wheat events produced, three independent events carrying multiple mutations in wheat Qsd1 homeoalleles were obtained. Notably, one line had mutations in every homeoallele. We crossed this plant with wild-type cultivar Fielder to generate a transgene-free triple-recessive mutant, as revealed by Mendelian segregation. The mutant showed a significantly longer seed dormancy period than wild-type, which may result in reduced pre-harvest sprouting of grains on spikes. PCR, southern blotting, and whole-genome shotgun sequencing revealed that this segregant lacked transgenes in its genomic sequence. This technique serves as a model for trait improvement in wheat, particularly for genetically recessive traits, based on locus information from diploid barley.	barley
617	plant	barley	CRISPR;Cas9;	granule-bound starch synthase (Gbss1a); Protein Targeting to Starch 1 (ptst1)	Journal of experimental botany	Protein Targeting to Starch 1 is essential for starchy endosperm development in barley.	2019	70(2):485-496	[Zhong Y et al.]	Aarhus University, Slagelse, Denmark.	30407538	10.1093/jxb/ery398	Plant starch is the main energy contributor to the human diet. Its biosynthesis is catalyzed and regulated by co-ordinated actions of several enzymes. Recently, a factor termed Protein Targeting to Starch 1 (PTST1) was identified as being required for correct granule-bound starch synthase (GBSS) localization and demonstrated to be crucial for amylose synthesis in Arabidopsis. However, the function of its homologous protein in storage tissues (e.g. endosperm) is unknown. We identified a PTST1 homolog in barley and it was found to contain a crucial coiled-coil domain and carbohydrate-binding module. We demonstrated the interaction between PTST1 and GBSS1 by fluorescence resonance energy transfer (FRET) in barley endosperm. By tagging PTST1 with the fluorophore mCherry, we observed that it is localized in the stroma of barley endosperm amyloplasts. PTST1 overexpression in endosperm increased endogenous gbss1a gene expression and amylose content. Gbss1a and ptst1 mutants were generated using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-related protein 9 (Cas9)-based targeted mutagenesis. Homozygous gbss1a mutants showed a waxy phenotype. Grains of ptst1 mutants did not accumulate any starch. These grains dried out during the desiccation stage and were unable to germinate, suggesting that PTST1 is essential for development of starchy endosperm	barley
618	plant	barley (Hordeum vulgare L.)	Cas9;	HvCKX1; HvCKX3	Cells	Knockout of the HvCKX1 or HvCKX3 Gene in Barley (Hordeum vulgare L.) by RNA-Guided Cas9 Nuclease Affects the Regulation of Cytokinin Metabolism and Root Morphology.	2019	8(8)	[Gasparis S et al.]	Plant Breeding and Acclimatization Institute-National Research Institute, Blonie, Poland.	31357516	10.3390/cells8080782	Barley is among four of the most important cereal crops with respect to global production. Increasing barley yields to desired levels can be achieved by the genetic manipulation of cytokinin content. Cytokinins are plant hormones that regulate many developmental processes and have a strong influence on grain yield. Cytokinin homeostasis is regulated by members of several multigene families. CKX genes encode the cytokinin oxidase/dehydrogenase enzyme, which catalyzes the irreversible degradation of cytokinin. Several recent studies have demonstrated that the RNAi-based silencing of CKX genes leads to increased grain yields in some crop species. To assess the possibility of increasing the grain yield of barley by knocking out CKX genes, we used an RNA-guided Cas9 system to generate ckx1 and ckx3 mutant lines with knockout mutations in the HvCKX1 and HvCKX3 genes, respectively. Homozygous, transgene-free mutant lines were subsequently selected and analyzed. A significant decrease in CKX enzyme activity was observed in the spikes of the ckx1 lines, while in the ckx3 lines, the activity remained at a similar level to that in the control plants. Despite these differences, no changes in grain yield were observed in either mutant line. In turn, differences in CKX activity in the roots between the ckx1 and ckx3 mutants were reflected via root morphology. The decreased CKX activity in the ckx1 lines corresponded to greater root length, increased surface area, and greater numbers of root hairs, while the increased CKX activity in the ckx3 mutants gave the opposite results. RNA-seq analysis of the spike and root transcriptomes revealed an altered regulation of genes controlling cytokinin metabolism and signaling, as well as other genes that are important during seed development, such as those that encode nutrient transporters. The observed changes suggest that the knockout of a single CKX gene in	barley
619	plant	blueberry	graft	FLOWERING LOCUS T (FT)	Horticulture research	VcFT-induced mobile florigenic signals in transgenic and transgrafted blueberries.	2019	6:105	[Song GQ et al.]	Michigan State University, East Lansing, MI USA.	31645960	10.1038/s41438-019-0188-5	FLOWERING LOCUS T (FT) can promote early flowering in annual species, but such role has not been well demonstrated in woody species. We produced self and reciprocal grafts involving non-transgenic blueberry (NT) and transgenic blueberry (T) carrying a 35S-driven blueberry FT (VcFT-OX). We demonstrated that the transgenic VcFT-OX rootstock promoted flowering of non-transgenic blueberry scions in the NT (scion):T (rootstock) grafts. We further analyzed RNA-Seq profiles and six groups of phytohormones in both NT:T and NT:NT plants. We observed content changes of several hormone metabolites, in a descending order, in the transgenic NT:T, non-transgenic NT:T, and non-transgenic NT:NT leaves. By comparing differential expression transcripts (DETs) of these tissues in relative to their control, we found that the non-transgenic NT:T leaves had many DETs shared with the transgenic NT:T leaves, but very few with the transgenic NT:T roots. Interestingly, a number of these shared DETs belong to hormone pathway genes, concurring with the content changes of hormone metabolites in both transgenic and non-transgenic leaves of the NT:T plants. These results suggest that phytohormones induced by VcFT-OX in the transgenic leaves might serve as part of the signals that resulted in early flowering in	blueberry

620	plant	Brassica campestris	CRISPR;Cas9;	pectin methylesterase (BcPME37c)	Biochemical and biophysical research communications	BcPME37c is involved in pollen intine formation in Brassica campestris.	2019	517(1):63–68	[Xiong X et al.]	Zhejiang University, Hangzhou, China.	31320138	10.1016/j.bbrc.2019.07.009	Pollen wall development is one of the key processes of pollen development. Several pectin methylesterase (PME) genes participate in pollen germination and pollen tube growth. However, the relationship between PME genes and pollen intine formation remains unclear. In this study, we investigated the expression and subcellular localization of the PME gene BcPME37c in Brassica campestris. Furthermore, morphology and cytology methods were used to examine the phenotype of the CRISPR/Cas9 system–induced BcPME37c mutant. We found that BcPME37c is predominately expressed in mature stamen and located at the cell wall. BcPME37c mutation causes the abnormal thickening of the pollen intine of B. campestris. Our study indicated that BcPME37c is required for pollen intine formation in B. campestris.	Brassica
621	plant	Brassica campestris	CRISPR;Cas9;	Bra003491; Bra007665; Bra014410	Molecular genetics and genomics	Efficient genome editing of Brassica campestris based on the CRISPR/Cas9 system.	2019	294(5):1251–1261	[Xiong X et al.]	Zhejiang University, Hangzhou, China.	31129735	10.1007/s00438–019–01564–w	Conventional methods for gene function study in Brassica campestris have lots of drawbacks, which greatly hinder the identification of important genes' functions and molecular breeding. The clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR–associated protein 9 (CRISPR/Cas9) system is a versatile tool for genome editing that has been widely utilized in many plant species and has many advantages over conventional methods for gene function study. However, the application of CRISPR/Cas9 system in B. campestris remains unreported. The pectin–methylesterase genes Bra003491, Bra007665, and Bra014410 were selected as the targets of the CRISPR/Cas9 system. A single–targeting vector and a multitargeting vector were constructed. Different types of mutations were detected in T0 generation through Agrobacterium transformation. The mutation rate of the three designed sgRNA seeds varied from 20 to 56%. Although the majority of T0 mutants were chimeric, four homozygous mutants were identified. Transformation with the multitargeting vector generated one line with a large fragment deletion and one line with mutations in two target genes. Mutations in Bra003491 were stable and inherited by T1 and T2 generations. Nine mutants which did not contain T–DNA insertions were also obtained. No mutations were detected in predicted potential off–target sites. Our work demonstrated that CRISPR/Cas9 system is efficient on single and multiplex genome editing without off–targeting in B. campestris and that the mutations are stable and inheritable. Our results may greatly facilitate gene functional studies and the molecular	Brassica
622	plant	Brassica napus	CRISPR;Cas9;	M–locus protein kinase (BnaA3.MLPK; BnaC3.MLPK; BnaA4.MLPK; BnaC4.MLPK)	International journal of molecular sciences	Functional Analysis of M–Locus Protein Kinase Revealed a Novel Regulatory Mechanism of Self–Incompatibility in Brassica napus L.	2019	20(13)	[Chen F et al.]	Huazhong Agricultural University, Wuhan, China.	31284391	10.3390/ijms20133303	Self–incompatibility (SI) is a widespread mechanism in angiosperms that prevents inbreeding by rejecting self–pollen. However, the regulation of the SI response in Brassica napus is not well understood. Here, we report that the M–locus protein kinase (MLPK) BnaMLPKs, the functional homolog of BrMLPKs in Brassica rapa, controls SI in B. napus. We identified four paralogue MLPK genes in B. napus, including BnaA3.MLPK, BnaC3.MLPK, BnaA4.MLPK, and BnaC4.MLPK. Two transcripts of BnaA3.MLPK, BnaA3.MLPKf1 and BnaA3.MLPKf2, were generated by alternative splicing. Tissue expression pattern analysis demonstrated that BnaA3.MLPK, especially BnaA3.MLPKf2, is highly expressed in reproductive organs, particularly in stigmas. We subsequently created RNA–silencing lines and CRISPR/Cas9–induced quadruple mutants of BnaMLPKs in B. napus SI line S–70. Phenotypic analysis revealed that SI response is partially suppressed in RNA–silencing lines and is completely blocked in quadruple mutants. These results indicate the importance of BnaMLPKs in regulating the SI response of B. napus. We found that the expression of SI positive regulators S–locus receptor kinase (SRK) and Arm–Repeat Containing 1 (ARC1) are suppressed in bnmlpk mutant, whereas the self–compatibility (SC) element Glyoxalase I (GLO1) maintained a high expression level. Overall, our findings reveal a new regulatory mechanism of	brassica
623	plant	Brassica napus	CRISPR;Cas9;	BnaA9.WRKY47	Plant biotechnology journal	Transcription factor BnaA9.WRKY47 contributes to the adaptation of Brassica napus to low boron stress by up–regulating the boric acid channel gene BnaA3.NIP5;1.	2019		[Feng Y et al.]	Huazhong Agricultural University, Wuhan, China.	31705705	10.1111/pbi.13288	Boron (B) deficiency is one of the major causes of growth inhibition and yield reduction in Brassica napus (B. napus). However, the molecular mechanisms of low B adaptation in B. napus are largely unknown. Here, fifty–one BnaWRKY transcription factors were identified as responsive to B deficiency in B. napus, in which BnaAn.WRKY26, BnaA9.WRKY47, BnaA1.WKRY53 and BnaCn.WRKY57 were tested in yeast one–hybrid assays and showed strong binding activity with conserved sequences containing a W box in the promoters of the B transport–related genes BnaNIP5;1s and BnaBOR1s. Green fluorescent protein fused to the target protein demonstrated the nuclear localization of BnaA9.WRKY47. CRISPR/Cas9–mediated knockout lines of BnaA9.WRKY47 in B. napus had increased sensitivity to low B and lower contents of B than wild–type plants. In contrast, overexpression of BnaA9.WRKY47 enhanced the adaptation to low B with higher B contents in tissues than in wild–type plants. Consistent with the phenotypic response and B accumulation in these transgenic lines, the transcription activity of BnaA3.NIP5;1, a B efficiency candidate gene, was decreased in the knockout lines but was significantly increased in the overexpressing lines under low B conditions. Electrophoretic mobility shift assays, transient expression experiments in tobacco and in situ hybridizations showed that BnaA9.WRKY47 directly activated BnaA3.NIP5;1 expression through binding to the specific cis–element. Taken together, our findings support BnaWRKYs as new participants in response to low B, and BnaA9.WRKY47 contributes to the adaptation of B. napus to B deficiency through up–	brassica

624	plant	Brassica napus L.	CRISPR;Cas9;	lysophosphatidic acid acyltransferase (Bnlpat2; Bnlpat5)	Biotechnology for biofuels	Effective editing for lysophosphatidic acid acyltransferase 2/5 in allotetraploid rapeseed (Brassica napus L.) using CRISPR-Cas9 system.	2019	12:225	[Zhang K et al.]	Huazhong University of Science and Technology, Wuhan, China.	31548867	10.1186/s13068-019-1567-8	Background: Brassica napus is one of the most important oilseed crops, and can supply considerable amounts of edible oil as well as provide raw materials for the production of biodiesel in the biotechnology industry. Lysophosphatidic acid acyltransferase (LPAT), a key enzyme in the Kennedy pathway, catalyses fatty acid chains into 3-phosphoglycerate and promotes further production of oil in the form of triacylglycerol. However, because B. napus is an allotetraploid with two subgenomes, the precise genes which involved in oil production remain unclear due to the intractability of efficiently knocking out all copies with high genetic redundancy. Therefore, a robust gene editing technology is necessary for gene function analysis. Results: An efficient gene editing technology was developed for the allotetraploid plant B. napus using the CRISPR-Cas9 system. Previous studies showed poor results in either on-target or off-target activity in B. napus. In the present study, four single-gRNAs and two multi-gRNAs were deliberately designed from the conserved coding regions of BnLPAT2 which has seven homologous genes, and BnLPAT5, which has four homologous genes. The mutation frequency was found to range from 17 to 68%, while no mutation was observed in the putative off-target sites. The seeds of the Bnlpat2/Bnlpat5 mutant were wizened and showed enlarged oil bodies, disrupted distribution of protein bodies and increased accumulation of starch in mature seeds. The oil content decreased, with an average decrease of 32% for Bnlpat2 lines and 29% for Bnlpat5 lines in single-gRNA knockout lines, and a decline of 24% for Bnlpat2 mutant lines (i.e., g123) and 39% for Bnlpat2/Bnlpat5 double mutant lines (i.e., g134) in multi-gRNA knockout lines. Conclusions: Seven BnLPAT2 homologous genes and four BnLPAT5 homologous genes were cleaved completely using the CRISPR-Cas9 system, which indicated that it is effective for editing all homologous genes in allotetraploid rapeseed, despite the relatively low sequence identities of both gene families. The size of the oil bodies increased significantly while the oil content decreased, confirming that BnLPAT2 and BnLPAT5 play a role in oil biosynthesis. The present study lays a foundation for further	brassica
625	plant	Brassica napus L.	CRISPR;Cas9;	BnaMAX1 homologs	Plant biotechnology journal	Knockout of two BnaMAX1 homologs by CRISPR/Cas9-targeted mutagenesis improves plant architecture and increases yield in rapeseed (Brassica napus L.).	2019		[Zheng M et al.]	Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan, China.	31373135	10.1111/pbi.13228	Plant height and branch number are essential components of rapeseed plant architecture and are directly correlated with its yield. Presently, improvement of plant architecture is a major challenge in rapeseed breeding. In this study, we first verified that the two rapeseed BnaMAX1 genes had redundant functions resembling those of Arabidopsis MAX1, which regulates plant height and axillary bud outgrowth. Therefore, we designed two sgRNAs to edit these BnaMAX1 homologs using the CRISPR/Cas9 system. The T0 plants were edited very efficiently (56.30%-67.38%) at the BnaMAX1 target sites resulting in homozygous, heterozygous, bi-allelic and chimeric mutations. Transmission tests revealed that the mutations were passed on to the T1 and T2 progeny. We also obtained transgene-free lines created by the CRISPR/Cas9 editing, and no mutations were detected in potential off-target sites. Notably, simultaneous knockout of all four BnaMAX1 alleles resulted in semi-dwarf and increased branching phenotypes with more siliques, contributing to increased yield per plant relative to wild type. Therefore, these semi-dwarf and increased branching characteristics have the potential to help construct a rapeseed ideotype. Significantly, the editing resources obtained in our study provide desirable germplasm for further breeding of high yield in	brassica
626	plant	Brassica napus L.	CRISPR;Cas9;	indehiscent; alcatraz	TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik	CRISPR/Cas9-mediated genome editing reveals differences in the contribution of INDEHISCENT homologues to pod shatter resistance in Brassica napus L.	2019	132(7):2111-2123	[Zhai Y et al.]	Huazhong Agricultural University, Wuhan, China.	30980103	10.1007/s00122-019-03341-0	The INDEHISCENT (IND) and ALCATRAZ (ALC) gene homologues have been reported to be essential for dehiscence of fruits in Brassica species. But their functions for pod shatter resistance in Brassica napus, an important oil crops, are not well understood. Here, we assessed the functions of these two genes in rapeseed using CRISPR/Cas9 technology. The induced mutations were stably transmitted to successive generations, and a variety of homozygous mutants with loss-of-function alleles of the target genes were obtained for phenotyping. The results showed that the function of BnIND gene is essential for pod shatter and highly conserved in Brassica species, whereas the BnALC gene appears to have limited potential for rapeseed shatter resistance. The homoeologous copies of the BnIND gene have partially redundant roles in rapeseed pod shatter, with BnA03.IND exhibiting higher contributions than BnC03.IND. Analysis of data obtained from the gene expression and sequence variations of gene copies revealed that cis-regulatory divergences alter gene expression and underlie the functional differentiation of BnIND homologues. Collectively, our results generate valuable resources for rapeseed breeding programs, and more importantly provide a	brassica
627	plant	Brassica oleracea	CRISPR;Cas9;	phytoene desaturase gene (BoPDS); S-receptor kinase gene (BoSRK); male-sterility-associated gene (BoMS1)	Horticulture research	CRISPR/Cas9-mediated multiple gene editing in Brassica oleracea var. capitata using the endogenous tRNA-processing system.	2019	6:20	[Ma C et al.]	Southwest University, Chongqing, China.	30729010	10.1038/s41438-018-0107-1	Cabbage (Brassica oleracea var. capitata) is a biennial plant with strong self-incompatibility and an obligate requirement for prolonged vernalization by exposure to low temperatures to induce flowering. These characteristics significantly increase the difficulty of exploiting novel germplasm induced by physical or chemical mutagens. In this study, we report a CRISPR/Cas9 gene-editing system based on endogenous tRNA processing to induce high efficiency and inheritable mutagenesis in cabbage. Using the phytoene desaturase gene BoPDS, the S-receptor kinase gene BoSRK, and the male-sterility-associated gene BoMS1 as the target genes, multisite and multiple gene mutations were achieved using a construct with tandemly arrayed tRNA-sgRNA architecture to express multiple sgRNAs. The BoSRK3 gene mutation suppressed self-incompatibility completely, converting the self-incompatible line into a self-compatible line. In addition, the BoMS1 gene mutation produced a completely male-sterile mutant, which was highly cross compatible with its nonmutant isoline at the flowering stage as a result of a simultaneous BoSRK3 gene mutation, enabling the economic propagation of the male-sterile line through bee-mediated cross-pollination. Interestingly, higher site mutation efficiency was detected when a guide sequence was inserted into a location in the tandemly arrayed tRNA-sgRNA architecture that was distal from the upstream Pol III promoter. In addition, mutation sites were also detected in the paralogous genes of the BoPDS and BoSRK genes that had fully consistent sequences or base mismatches but beyond the "seed" region in the spacer sequence compared with the target sgRNAs. Collectively, our results demonstrate that the CRISPR/Cas9 system, coupled with an endogenous tRNA-processing system, is an efficient tool to	Brassica

628	plant	rapeseed (Brassica napus L.)	CRISPR;Cas9;	BnTT8	Plant biotechnology journal	Targeted mutagenesis of BnTT8 homologs controls yellow seed coat development for effective oil production in Brassica napus L.	2019		[Zhai Y et al.]	Huazhong Agricultural University, Wuhan, China.	31637846	10.1111/pbi.13281	Yellow seed is a desirable trait with great potential for improving seed quality in Brassica crops. Unfortunately, no natural or induced yellow seed germplasms have been found in Brassica napus, an important oil crop, which likely reflects its genome complexity and the difficulty of the simultaneous random mutagenesis of multiple gene copies with functional redundancy. Here, we demonstrate the first application of CRISPR/Cas9 for creating yellow-seeded mutants in rapeseed. The targeted mutations of the BnTT8 gene were stably transmitted to successive generations, and a range of homozygous mutants with loss-of-function alleles of the target genes were obtained for phenotyping. The yellow-seeded phenotype could be recovered only in targeted mutants of both BnTT8 functional copies, indicating that the redundant roles of BnA09.TT8 and BnC09.TT8b are vital for seed colour. The BnTT8 double mutants produced seeds with elevated seed oil and protein content and altered fatty acid (FA) composition without any serious defects in the yield-related traits, making it a valuable resource for rapeseed breeding programmes. Chemical staining and histological analysis showed that the targeted mutations of BnTT8 completely blocked the proanthocyanidin (PA)-specific deposition in the seed coat. Further, transcriptomic profiling revealed that the targeted mutations of BnTT8 resulted in the broad suppression of phenylpropanoid/flavonoid biosynthesis genes, which indicated a much more complex molecular mechanism underlying seed colour formation in rapeseed than in Arabidopsis and other Brassica species. In addition, gene expression analysis revealed the possible mechanism through which BnTT8 altered the oil content and	brassica
629	plant	Camelina sativa	CRISPR;Cas9;	cruciferin c homologs	BMC plant biology	CRISPR/Cas9 editing of three CRUCIFERIN C homoeologues alters the seed protein profile in Camelina sativa.	2019	19(1):292	[Lyzenga WJ et al.]	Agriculture and Agri-Food Canada, Saskatoon, SK, Canada.	31272394	10.1186/s12870-019-1873-0	BACKGROUND: The oilseed Camelina sativa is grown for a range of applications, including for biofuel, biolubricants, and as a source of omega-3 fatty acids for the aquaculture feed industry. The seed meal co-product is used as a source of protein for animal feed; however, the low value of the meal hinders profitability and more widespread application of camelina. The nutritional quality of the seed meal is largely determined by the abundance of specific seed storage proteins and their amino acid composition. Manipulation of seed storage proteins has been shown to be an effective means for either adjustment of nutritional content of seeds or for enhancing accumulation of high-value recombinant proteins in seeds. RESULTS: CRISPR/Cas9 gene editing technology was used to generate deletions in the first exon of the three homoeologous genes encoding the seed storage protein CRUCIFERIN C (CsCRUC), creating an identical premature stop-codon in each and resulting in a CsCRUC knockout line. The mutant alleles were detected by applying a droplet digital PCR drop-off assay. The quantitative nature of this technique is particularly valuable when applied to polyploid species because it can accurately determine the number of mutated alleles in a gene family. Loss of CRUC protein did not alter total seed protein content; however, the abundance of other cruciferin isoforms and other seed storage proteins was altered. Consequently, seed amino acid content was significantly changed with an increase in the proportion of alanine, cysteine and proline, and decrease of isoleucine, tyrosine and valine. CsCRUC knockout seeds did not have changed total oil content, but the fatty acid profile was significantly altered with increased relative abundance of all saturated fatty acids. CONCLUSIONS: This study demonstrates the plasticity of the camelina seed proteome and establishes a CRUC-devoid line, providing a framework for modifying camelina seed protein composition. The results also illustrate	Camelina sat
630	plant	carrot	agroinfiltration; CRISPR;Cas9;	DcPDS and DcMYB113-like	Molecular biotechnology	CRISPR/Cas9-Mediated Multiply Targeted Mutagenesis in Orange and Purple Carrot Plants.	2019	61(3):191-199	[Xu ZS et al.]	Nanjing Agricultural University, Nanjing, China.	30644027	10.1007/s12033-018-00150-6	The clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) system has been successfully used for precise genome editing in many plant species, including in carrot cells, very recently. However, no stable gene-editing carrot plants were obtained with CRISPR/Cas9 system to date. In the present study, four sgRNA expression cassettes, individually driven by four different promoters and assembled in a single CRISPR/Cas9 vector, were transformed into carrots using Agrobacterium-mediated genetic transformation. Four sites of DcPDS and DcMYB113-like genes were chosen as targets. Knockout of DcPDS in orange carrot 'Kurodagosun' resulted in the generation of albino carrot plantlets, with about 35.3% editing efficiency. DcMYB113-like was also successfully edited in purple carrot 'Deep purple', resulting in purple depigmented carrot plants, with about 36.4% rate of mutation. Sequencing analyses showed that insertion, deletion, and substitution occurred in the target sites, generating heterozygous, biallelic, and chimeric mutations. The highest efficiency of mutagenesis was observed in the sites targeted by AtU6-29-driven sgRNAs in both DcPDS- and DcMYB113-like-knockout T0 plants, which always induced double-strand breaks in the target sites. Our results proved that CRISPR/Cas9 system could	carrot
631	plant	carrot	CRISPR;Cas9;	DcMYB7	Plant physiology	Changing Carrot Color: Insertions in DcMYB7 Alter the Regulation of Anthocyanin Biosynthesis and Modification.	2019	181(1):195-207	[Xu ZS et al.]	Nanjing Agricultural University, Nanjing, China.	31213511	10.1104/pp.19.00523	The original domesticated carrots (Daucus carota) are thought to have been purple, accumulating large quantities of anthocyanins in their roots. A quantitative trait locus associated with anthocyanin pigmentation in purple carrot roots has been identified on chromosome 3 and includes two candidate genes, DcMYB6 and DcMYB7 Here, we characterized the functions of DcMYB6 and DcMYB7 in carrots. Overexpression of DcMYB7, but not DcMYB6, in the orange carrot 'Kurodagosun' led to anthocyanin accumulation in roots. Knockout of DcMYB7 in the solid purple (purple periderm, phloem, and xylem) carrot 'Deep Purple' using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 system resulted in carrots with yellow roots. DcMYB7 could activate the expression of its DcbHLH3 partner, a homolog of the anthocyanin-related apple (Malus x domestica) bHLH3, and structural genes in the anthocyanin biosynthetic pathway. We determined that the promoter sequence of DcMYB7 in nonpurple carrots was interrupted either by DcMYB8, a nonfunctional tandem duplication of DcMYB7, or by two transposons, leading to the transcriptional inactivation of DcMYB7 in nonpurple carrot roots. As a result, nonpurple carrots fail to accumulate anthocyanins in their roots. Our study supports the hypothesis that another genetic factor suppresses DcMYB7 expression in the phloem and xylem of purple peridermal carrot root tissues. DcMYB7 also regulated the glycosylation and acylation of anthocyanins by directly activating DcUCGXT1 and DcSAT1 We reveal the genetic factors conditioning anthocyanin pigmentation in purple versus nonpurple carrot roots. Our results also provide insights into the mechanisms	carrot

632	plant	cassava	CRISPR;Cas9;	cap-binding protein-1 (ncbp-1; ncbp-2)	Plant biotechnology journal	Simultaneous CRISPR/Cas9-mediated editing of cassava eIF4E isoforms nCBP-1 and nCBP-2 reduces cassava brown streak disease symptom severity and incidence.	2019	17(2):421-434	[Gomez MA et al.]	University of California, Berkeley, CA, USA.	30019807	10.1111/pbi.12987	Cassava brown streak disease (CBSD) is a major constraint on cassava yields in East and Central Africa and threatens production in West Africa. CBSD is caused by two species of positive-sense RNA viruses belonging to the family Potyviridae, genus Ipomovirus: Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV). Diseases caused by the family Potyviridae require the interaction of viral genome-linked protein (VPg) and host eukaryotic translation initiation factor 4E (eIF4E) isoforms. Cassava encodes five eIF4E proteins: eIF4E, eIF(iso)4E-1, eIF(iso)4E-2, novel cap-binding protein-1 (nCBP-1), and nCBP-2. Protein-protein interaction experiments consistently found that VPg proteins associate with cassava nCBPs. CRISPR/Cas9-mediated genome editing was employed to generate ncbp-1, ncbp-2, and ncbp-1/ncbp-2 mutants in cassava cultivar 60444. Challenge with CBSV showed that ncbp-1/ncbp-2 mutants displayed delayed and attenuated CBSD aerial symptoms, as well as reduced severity and incidence of storage root necrosis. Suppressed disease symptoms were correlated with reduced virus titre in storage roots relative to wild-type controls. Our results demonstrate the ability to modify multiple genes simultaneously in cassava to achieve tolerance to CBSD. Future studies will investigate the contribution of remaining eIF4E isoforms on CBSD and translate this	cassava
633	plant	Catharanthus roseus	Agroinfiltration;	introns; luciferase and GUS genes	Frontiers in plant science	EASI Transformation: An Efficient Transient Expression Method for Analyzing Gene Function in Catharanthus roseus Seedlings.	2019	10:755	[Mortensen S et al.]	Northeastern University, Boston, MA, USA.	31263474	10.3389/fpls.2019.00755	The Catharanthus roseus plant is the exclusive source of the valuable anticancer terpenoid indole alkaloids, vinblastine (VB) and vincristine (VC). The recent availability of transcriptome and genome resources for C. roseus necessitates a fast and reliable method for studying gene function. In this study, we developed an Agrobacterium-mediated transient expression method to enable the functional study of genes rapidly in planta, conserving the compartmentalization observed in the VB and VC pathway. We focused on (1) improving the transformation method (syringe versus vacuum agroinfiltration) and cultivation conditions (seedling age, Agrobacterium density, and time point of maximum transgene expression), (2) improving transformation efficiency through the constitutive expression of the virulence genes and suppressing RNA silencing mechanisms, and (3) improving the vector design by incorporating introns, quantitative and qualitative reporter genes (luciferase and GUS genes), and accounting for transformation heterogeneity across the tissue using an internal control. Of all the parameters tested, vacuum infiltration of young seedlings (10-day-old, harvested 3 days post-infection) resulted in the strongest increase in transgene expression, at 18 – 57 fold higher than either vacuum or syringe infiltration of other seedling ages. Endowing the A. tumefaciens strain with the mutated VirGN54D or silencing suppressors within the same plasmid as the reporter gene further increased expression by 2 – 10 fold. For accurate measurement of promoter transactivation or activity, we included an internal control to normalize the differences in plant mass and transformation efficiency. Including the normalization gene (Renilla luciferase) on the same plasmid as the reporter gene (firefly luciferase) consistently yielded a high signal and a high correlation between RLUC and FLUC. As proof of principle, we applied this approach to investigate the regulation of the CroSTR1 promoter with the well-known activator ORCA3 and repressor ZCT1. Our method demonstrated the quantitative assessment of both the activation and repression of promoter activity in C. roseus. Our efficient Agrobacterium-mediated seedling infiltration (EASI) protocol allows highly efficient, reproducible, and homogenous transformation of C. roseus cotyledons and provides a timely tool for the community to rapidly assess the function of genes in	Catharanthu
634	plant	chicory (Cichorium intybus L.)	agroinfiltration; CRISPR;Cas9;	chicory phytoene desaturase gene (CiPDS)	International journal of molecular sciences	Efficient Genome Editing Using CRISPR/Cas9 Technology in Chicory.	2019	20(5)	[Bernard G et al.]	Universite de Lille, INRA, ISA, Univ. Artois, Univ. Littoral Cote d'Opale, Villeneuve d'Ascq, France.	30845784	10.3390/ijms20051155	CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated with protein CAS9) is a genome-editing tool that has been extensively used in the last five years because of its novelty, affordability, and feasibility. This technology has been developed in many plant species for gene function analysis and crop improvement but has never been used in chicory (Cichorium intybus L.). In this study, we successfully applied CRISPR/Cas9-mediated targeted mutagenesis to chicory using Agrobacterium rhizogenes-mediated transformation and protoplast transfection methods. A U6 promoter (CiU6-1p) among eight predicted U6 promoters in chicory was selected to drive sgRNA expression. A binary vector designed to induce targeted mutations in the fifth exon of the chicory phytoene desaturase gene (CiPDS) was then constructed and used to transform chicory. The mutation frequency was 4.5% with the protoplast transient expression system and 31.25% with A. rhizogenes-mediated stable transformation. Biallelic mutations were detected in all the mutant plants. The use of A. rhizogenes-mediated transformation seems preferable as the regeneration of plants is faster and the mutation frequency was shown to be higher. With both transformation methods, foreign DNA was integrated in the plant genome. Hence, selection of vector (transgene)-free segregants is required. Our results showed that genome editing with CRISPR/Cas9 system can be efficiently used with chicory,	chicory
635	plant	chinese kale	CRISPR;Cas9;	phytoene desaturase (BaPDS1; BaPDS2)	Royal Society open science	Functional differences of BaPDS1 and BaPDS2 genes in Chinese kale.	2019	6(7):190260	[Sun B et al.]	Sichuan Agricultural University, Chengdu, China.	31417731	10.1098/rsos.190260	This study presents a systematic analysis of the functional differences between two genes that encode phytoene desaturase (PDS) in Chinese kale. The promoter sequences of both BaPDS1 and BaPDS2 were amplified and cloned, and their lengths were 2005 bp and 2000 bp, respectively. The mining of cis-acting elements in the promoters showed that the two BaPDS genes are mainly associated with light and phytohormone responsiveness. Light quality, light intensity and plant hormone treatments were conducted in seedlings of Chinese kale, and the results indicated that the response of the two genes to different factors differed. Among them, BaPDSs collectively respond to the treatment with salicylic acid and abscisic acid. With regard to response differences, BaPDS1 is sensitive to red and blue light, blue light, and strong light, while BaPDS2 responds to blue light, weak light, darkness, gibberellin and methyl jasmonate. In addition, both BaPDS1 and BaPDS2 are likely targeted to the chloroplast. Furthermore, single and double mutants of BaPDSs were generated via CRISPR/Cas9 technology. Phenotypic analysis showed that the double mutant with edited PDS1 and PDS2 was a pure albino, while the single mutants with edited PDS1 or PDS2 were partly whitened. In summary, BaPDS1 and BaPDS2 genes played different and indispensable roles in Chinese kale, and their functions were partially complementary.	chinese kale

636	plant	cotton	CRISPR;	CGP1	Plant biotechnology journal	The gland localized CGP1 controls gland pigmentation and gossypol accumulation in cotton.	2019		[Gao W et al.]	Henan University, Kaifeng, China.	31883409	10.1111/pbi.13323	Pigment glands, also known as black glands or gossypol glands, are specific for Gossypium spp. These glands strictly confine large amounts of secondary metabolites to the lysigenous cavity, leading to the glands' intense colour and providing defence against pests and pathogens. This study performed a comparative transcriptome analysis of glanded versus glandless cotton cultivars. Twenty-two transcription factors showed expression patterns associated with pigment glands and were characterized. Phenotypic screening of the genes, via virus-induced gene silencing, showed an apparent disappearance of pigmented glands after the silencing of a pair of homologous MYB-encoding genes in the A and D genomes (designated as CGP1). Further study showed that CGP1a encodes an active transcription factor, which is specifically expressed in the gland structure, while CGP1d encodes a non-functional protein due to a fragment deletion, which causes premature termination. RNAi-mediated silencing and CRISPR knockout of CGP1 in glanded cotton cultivars generated a glandless-like phenotype, similar to the dominant glandless mutant Gl2 (e) . Microscopic analysis showed that CGP1 knockout did not affect gland structure or density, but affected gland pigmentation. The levels of gossypol and related terpenoids were significantly decreased in cgp1 mutants, and a number of gossypol biosynthetic genes were strongly down-regulated. CGP1 is located in the nucleus where it interacts with GoPGF, a critical transcription factor for gland development and gossypol synthesis. Our data suggest that CGP1 and GoPGF form heterodimers to control the synthesis of gossypol	cotton
637	plant	cotton	CRISPR;Cas9;		Plant biotechnology journal	Whole genome sequencing reveals rare off-target mutations and considerable inherent genetic or/and somaclonal variations in CRISPR/Cas9-edited cotton plants.	2019	17(5):858-868	[Li J et al.]	Huazhong Agricultural University, Wuhan, Hubei, China.	30291759	10.1111/pbi.13020	The CRISPR/Cas9 system has been extensively applied for crop improvement. However, our understanding of Cas9 specificity is very limited in Cas9-edited plants. To identify on- and off-target mutation in an edited crop, we described whole genome sequencing (WGS) of 14 Cas9-edited cotton plants targeted to three genes, and three negative (Ne) control and three wild-type (WT) plants. In total, 4188-6404 unique single-nucleotide polymorphisms (SNPs) and 312-745 insertions/deletions (indels) were detected in 14 Cas9-edited plants compared to WT, negative and cotton reference genome sequences. Since the majority of these variations lack a protospacer-adjacent motif (PAM), we demonstrated that the most variations following Cas9-edited are due either to somaclonal variation or/and pre-existing/inherent variation from maternal plants, but not off-target effects. Of a total of 4413 potential off-target sites (allowing </=5 mismatches within the 20-bp sgRNA and 3-bp PAM sequences), the WGS data revealed that only four are bona fide off-target indel mutations, validated by Sanger sequencing. Moreover, inherent genetic variation of WT can generate novel off-target sites and destroy PAMs, which suggested great care should be taken to design sgRNA for the minimizing of off-target effect. These findings	cotton
638	plant	cotton (Gossypium hirsutum L.)	CRISPR;Cas9;	GoPGF	Plant biotechnology journal	Genes regulating gland development in the cotton plant.	2019	17(6):1142-1153	[Janga MR et al.]	Texas A&M University, College Station, TX, USA.	30467959	10.1111/pbi.13044	In seeds and other parts of cultivated, tetraploid cotton (Gossypium hirsutum L.), multicellular groups of cells lysigenously form dark glands containing toxic terpenoids such as gossypol that defend the plant against pests and pathogens. Using RNA-seq analysis of embryos from near-isogenic glanded (Gl2 Gl2 Gl3 Gl3) versus glandless (gl2 gl2 gl3 gl3) plants, we identified 33 genes that expressed exclusively or at higher levels in embryos just prior to gland formation in glanded plants. Virus-induced gene silencing against three gene pairs led to significant reductions in the number of glands in the leaves, and significantly lower levels of gossypol and related terpenoids. These genes encode transcription factors and have been designated the 'Cotton Gland Formation' (CGF) genes. No sequence differences were found between glanded and glandless cotton for CGF1 and CGF2 gene pairs. The glandless cotton has a transposon insertion within the coding sequence of the GoPGF (synonym CGF3) gene of the A subgenome and extensive mutations in the promoter of D subgenome homeolog. Overexpression of GoPGF (synonym CGF3) led to a dramatic increase in gossypol and related terpenoids in cultured cells, whereas CRISPR/Cas9 knockout of GoPGF (synonym CGF3) genes resulted in glandless phenotype. Taken collectively, the results show that the GoPGF (synonym CGF3) gene plays a critical role in the formation of glands in the cotton plant. Seed-specific silencing of CGF genes, either individually or in combination, could eliminate glands, thus gossypol, from the cottonseed to render it safe as food or feed	cotton
639	plant	cowpea (Vigna unguiculata)	CRISPR;Cas9;	symbiosis receptor-like kinase	International journal of molecular sciences	Genome Editing in Cowpea Vigna unguiculata Using CRISPR-Cas9.	2019	20(10)	[Ji J et al.]	Huazhong Agricultural University, Wuhan, China.	31109137	10.3390/ijms20102471	Cowpea (Vigna unguiculata) is widely cultivated across the world. Due to its symbiotic nitrogen fixation capability and many agronomically important traits, such as tolerance to low rainfall and low fertilization requirements, as well as its high nutrition and health benefits, cowpea is an important legume crop, especially in many semi-arid countries. However, research in Vigna unguiculata is dramatically hampered by the lack of mutant resources and efficient tools for gene inactivation in vivo. In this study, we used clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9). We applied the CRISPR/Cas9-mediated genome editing technology to efficiently disrupt the representative symbiotic nitrogen fixation (SNF) gene in Vigna unguiculata. Our customized guide RNAs (gRNAs) targeting symbiosis receptor-like kinase (SYMRK) achieved ~67% mutagenic efficiency in hairy-root-transformed plants, and nodule formation was completely blocked in the mutants with both alleles disrupted. Various types of mutations were observed near the PAM region of the respective gRNA. These results demonstrate the applicability of the CRISPR/Cas9 system in Vigna unguiculata, and therefore should significantly stimulate functional genomics analyses of many important agronomical traits in this unique crop	cowpea

640	plant	cowpea (Vigna unguiculata)	Agroinfiltration;	hpRNAi-MVR	Virusdisease	Screening of a multi-virus resistant RNAi construct in cowpea through transient vacuum infiltration method.	2019	30(2):269-278	[Prasad Babu K et al.]	ICAR-Indian Institute of Horticultural Research, Bangalore, India.	31179366	10.1007/s13337-018-00509-y	Plant viruses are the most devastating pathogens causing substantial economic losses in many crops. Current viral disease management relies on prophylactics, roguing and insect vector control, since in most crops resistant gene pools for resistance breeding are unavailable. RNA interference, a sequence dependent gene silencing mechanism holds great potential in imparting virus resistance. In this study, the efficacy of a RNAi gene construct developed against four viruses commonly infesting tomato and chilli viz., capsicum chlorosis virus, groundnut bud necrosis virus, cucumber mosaic virus and chilli veinal mottle virus was evaluated. A 3546 bp dsRNA-forming construct comprising sense-intron-antisense fragments in binary vector pBI121 (hpRNAi-MVR) was mobilized into Agrobacterium tumefaciens. Cowpea (Vigna unguiculata) was used as an indicator plant for GBNV agroinfiltration to evaluate the efficacy of hpRNAi-MVR construct in conferring GBNV resistance. The type of agroinfiltration, bacterial concentration and incubation-temperatures were optimized. Vacuum infiltration of three pulses of 20-30 s at 66.66 kPa were effective than syringe infiltration. Of the five Agrobacterial concentrations, OD600 0.5 was more efficient. Incubation temperature of 31 +/- 1 degrees C was favorable for development of disease symptoms than 20 +/- 1 degrees C and 26 +/- 1 degrees C. ELISA revealed a 35% decline in viral load in hpRNAi-MVR infiltrated plants compared to vector control plants. Quantitative real time PCR results have shown a viral gene silencing to the extent of 930-990 folds in hpRNAi-MVR infiltrated plants compared to vector control. This approach is simple, rapid and efficient to screen the efficacy of RNAi constructs developed for the RNAi	cowpea
641	plant	cucumber	Agroinfiltration;	neutralizing epitope of PRRSV glycoprotein 5	Journal of virological methods	Development of a cucumber green mottle mosaic virus-based expression vector for the production in cucumber of neutralizing epitopes against a devastating animal virus.	2019	269:18-25	[Tran HH et al.]	London Research and Development Centre, Agriculture and Agri-Food Canada, London, Ontario, Canada.	30954462	10.1016/j.jviromet.2019.04.006	Virus-based expression systems have been widely exploited for the production of recombinant proteins in plants during the last thirty years. Advances in technology have boosted scale-up manufacturing of plant-made pharmaceuticals to high levels, via the complementation of transient expression and viral vectors. This combination allows proteins of interest to be produced in plants within a matter of days and thus, is well suited for the development of plant-made vaccines or therapeutics against emerging infectious diseases and potential bioterrorism agents. Several plant-based products are currently in varying stages of clinical development. To investigate the viability of virus-based expression systems for plant-made vaccines against porcine reproductive and respiratory syndrome virus (PRRSV), the most devastating threat to the pork industry in Canada, we cloned the full-length genome of a cucumber green mottle mosaic virus (CGMMV) isolate and developed a CGMMV-based expression vector. We further employed this vector to express the neutralizing epitope (NE) of PRRSV glycoprotein 5 (GP5) in cucumber leaves via agroinfiltration. The coding region of the GP5 NE was inserted downstream of the open reading frame for coat protein (CP) and expressed by a readthrough mechanism. The chimeric virus particles were stable and the expression levels reached as high as 35.84 mg/kg of cucumber leaf fresh weight. This study offers a promising solution to the production of a low cost, versatile and robust vaccine for oral administration against PRRSV through a chimeric virus particle display system.	cucumber
642	plant	duckweed (Lemna aequinoctialis)	agroinfiltration; CRISPR;Cas9;	LaPDS	Plant biotechnology journal	Efficient genetic transformation and CRISPR/Cas9-mediated genome editing in Lemna aequinoctialis.	2019	17(11):2143-2152	[Liu Y et al.]	Qingdao Agricultural University, Qingdao, China.	30972865	10.1111/pbi.13128	The fast growth, ease of metabolic labelling and potential for feedstock and biofuels production make duckweeds not only an attractive model system for understanding plant biology, but also a potential future crop. However, current duckweed research is constrained by the lack of efficient genetic manipulation tools. Here, we report a case study on genome editing in a duckweed species, Lemna aequinoctialis, using a fast and efficient transformation and CRISPR/Cas9 tool. By optimizing currently available transformation protocols, we reduced the duration time of Agrobacterium-mediated transformation to 5-6 weeks with a success rate of over 94%. Based on the optimized transformation protocol, we generated 15 (14.3% success rate) biallelic LaPDS mutants that showed albino phenotype using a CRISPR/Cas9 system. Investigations on CRISPR/Cas9-mediated mutation spectrum among mutated L. aequinoctialis showed that most of mutations were short insertions and deletions. This study presents the first example of CRISPR/Cas9-mediated genome editing in duckweeds, which will open new research avenues in using duckweeds for both basic and applied research.	duckweed
643	plant	gentian flowers	CRISPR;Cas9;	anthocyanin 5-O-glycosyltransferase (Gt5GT); anthocyanin 3'-O-glycosyltransferase (Gt3'GT); anthocyanin 5/3'-aromatic acyltransferase (Gt5/3'AT)	Scientific reports	Effects of knocking out three anthocyanin modification genes on the blue pigmentation of gentian flowers.	2019	9(1):15831	[Tasaki K et al.]	Iwate Biotechnology Research Center, Kitakami, Iwate, Japan.	31676875	10.1038/s41598-019-51808-3	Genome editing by the CRISPR/Cas9 system has recently been used to produce gene knockout lines in many plant species. We applied this system to analyze Japanese gentian plants that produce blue flowers because of the accumulation of a polyacylated anthocyanin, gentiodelphin. Mutant lines in which anthocyanin modification genes were knocked out were examined to assess the contribution of each gene to the blue pigmentation of flowers. The targeted genes encoded anthocyanin 5-O-glycosyltransferase (Gt5GT), anthocyanin 3'-O-glycosyltransferase (Gt3'GT), and anthocyanin 5/3'-aromatic acyltransferase (Gt5/3'AT). The Gt5GT knockout lines accumulated delphinidin 3G, whereas the Gt3'GT knockout lines accumulated delphinidin 3G-5CafG as the major flower pigment. Knocking out Gt5/3'AT resulted in the accumulation of delphinidin 3G-5G-3'G and delphinidin 3G-5G as the primary and secondary pigments, respectively. These results indicated the existence of two pathways mediating the modification of delphinidin 3G-5G in flowers, with one involving a glycosylation by 3'GT and the other involving an acylation by 5/3'AT. The Gt5GT, Gt3'GT, and Gt5/3'AT transformants produced pale red violet, dull pink, and pale mauve flowers, respectively, unlike the vivid blue flowers of wild-type plants. Thus, the glycosylation and subsequent acylation of the 3'-hydroxy group of the B-ring in	gentian flowe
644	plant	grape	CRISPR;Cas9;	phytoene desaturase	Frontiers in plant science	Efficiency Optimization of CRISPR/Cas9-Mediated Targeted Mutagenesis in Grape.	2019	10:612	[Ren F et al.]	Institute of Botany, Chinese Academy of Sciences, Beijing, China.	31156675	10.3389/fpls.2019.00612	Clustered regularly interspersed short palindromic repeats (CRISPR)/Cas system is an efficient targeted genome editing method. Although CRISPR/Cas9-mediated mutagenesis has been applied successfully in grape, few studies have examined the technique's efficiency. To optimize CRISPR/Cas9 editing efficiency in Vitis vinifera, we surveyed three key parameters: GC content of single guide RNA (sgRNA), variety of transformant cells used, and SpCas9 expression levels in transgenic cell mass. Four sgRNAs with differing GC content were designed to target exon sites of the V. vinifera phytoene desaturase gene. Suspension cells of 'Chardonnay' and '41B' varieties were used as the transgenic cell mass. Both T7EI and PCR/RE assays showed that CRISPR/Cas9 editing efficiency increases proportionally with sgRNA GC content with 65% GC content yielding highest editing efficiency in both varieties. Additionally, gene editing was more efficient in '41B' than in 'Chardonnay.' CRISPR/Cas9 systems with different editing efficiency showed different SpCas9 expression level, but compared with GC content of sgRNA, SpCas9 expression level has less influence on editing efficiency. Taken together, these results help optimize of CRISPR/Cas9 performance in	grape

645	plant	Duncan grapefruit	CRISPR;Cpf1;Agroinfiltration;	CsPDS; CsLOB1	Plant biotechnology journal	CRISPR-LbCas12a-mediated modification of citrus.	2019	17(10):1928-1937	[Jia H et al.]	University of Florida, Lake Alfred, FL, USA.	30908830	10.1111/pbi.13109	Recently, CRISPR-Cas12a (Cpf1) from Prevotella and Francisella was engineered to modify plant genomes. In this report, we employed CRISPR-LbCas12a (LbCpf1), which is derived from Lachnospiraceae bacterium ND2006, to edit a citrus genome for the first time. First, LbCas12a was used to modify the CsPDS gene successfully in Duncan grapefruit via Xcc-facilitated agroinfiltration. Next, LbCas12a driven by either the 35S or Yao promoter was used to edit the PthA4 effector binding elements in the promoter (EBEP thA4 -CsLOBP) of CsLOB1. A single crRNA was selected to target a conserved region of both Type I and Type II CsLOBPs, since the protospacer adjacent motif of LbCas12a (TTTV) allows crRNA to act on the conserved region of these two types of CsLOBP. CsLOB1 is the canker susceptibility gene, and it is induced by the corresponding pathogenicity factor PthA4 in Xanthomonas citri by binding to EBEP thA4 -CsLOBP. A total of seven 35S-LbCas12a-transformed Duncan plants were generated, and they were designated as #D35 s1 to #D35 s7, and ten Yao-LbCas12a-transformed Duncan plants were created and designated as #Dyao 1 to #Dyao 10. LbCas12a-directed EBEP thA4 -CsLOBP modifications were observed in three 35S-LbCas12a-transformed Duncan plants (#D35 s1, #D35 s4 and #D35 s7). However, no LbCas12a-mediated indels were observed in the Yao-LbCas12a-transformed plants. Notably, transgenic line #D35 s4, which contains the highest mutation rate, alleviates XccDeltapthA4:dCsLOB1.4 infection. Finally, no potential off-targets were observed. Therefore, CRISPR-LbCas12a can readily be used as a powerful tool for citrus genome	grapefruit
646	plant	Hibiscus hamabo Sieb. et Zucc.	Agroinfiltration;	cloroplastos alterados 1	PeerJ	Efficient virus-induced gene silencing in Hibiscus hamabo Sieb. et Zucc. using tobacco rattle virus.	2019	7:e7505	[Wang Z et al.]	Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, Jiangsu, China.	31423365	10.7717/peerj.7505	Background: Hibiscus hamabo Sieb. et Zucc. is a semi-mangrove plant used for the ecological restoration of saline-alkali land, coastal afforestation and urban landscaping. The genetic transformation H. hamabo is currently inefficient and laborious, restricting gene functional studies on this species. In plants, virus-induced gene silencing provides a pathway to rapidly and effectively create targeted gene knockouts for gene functional studies. Methods: In this study, we tested the efficiency of a tobacco rattle virus vector in silencing the cloroplastos alterados 1 (CLA1) gene through agroinfiltration. Results: The leaves of H. hamabo showed white streaks typical of CLA1 gene silencing three weeks after agroinfiltration. In agroinfiltrated H. hamabo plants, the CLA1 expression levels in leaves with white streaks were all significantly lower than those in leaves from mock-infected and control plants. Conclusions: The system presented here can efficiently silence genes in H. hamabo and may be a powerful tool for large-scale reverse-genetic analyses of gene functions in H. hamabo.	Hibiscus han
647	plant	hybrid aspen	CRISPR;Cas9;	all NAC SECONDARY WALL THICKENING PROMOTING FACTOR3/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 1 (NST3/SND1) genes	Tree physiology	Populus NST/SND orthologs are key regulators of secondary cell wall formation in wood fibers, phloem fibers and xylem ray parenchyma cells.	2019	39(4):514-525	[Takata N et al.]	Forestry and Forest Products Research Institute, Hitachi, Ibaraki, Japan.	30806711	10.1093/treephys/tpz004	Wood fibers form thick secondary cell wall (SCW) in xylem tissues to give mechanical support to trees. NAC SECONDARY WALL THICKENING PROMOTING FACTOR3/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 1 (NST3/SND1) and NST1 were identified as master regulators of SCW formation in xylem fiber cells in the model plant Arabidopsis thaliana. In Populus species, four NST/SND orthologs have been conserved and coordinately control SCW formation in wood fibers and phloem fibers. However, it remains to be elucidated whether SCW formation in other xylem cells, such as ray parenchyma cells and vessel elements, is regulated by NST/SND orthologs in poplar. We knocked out all NST/SND genes in hybrid aspen using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) system and investigated the detailed histological appearance of stem tissues in the knockout mutants. Observation by light microscopy and transmission electron microscopy showed that SCW was severely suppressed in wood fibers, phloem fibers and xylem ray parenchyma cells in the knockout mutants. Although almost all wood fibers lacked SCW, some fiber cells formed thick cell walls. The irregularly cell wall-forming fibers retained primary wall and SCW, and were mainly located in the vicinity of vessel elements. Field emission-scanning electron microscope observation showed that there were no apparent differences in the structural features of pits such as the shape and size between irregularly SCW-forming wood fibers in the knockout mutants and normal wood fibers in wild-type. Cell wall components such as cellulose, hemicellulose and lignin were deposited in the cell wall of irregularly SCW-forming wood fibers in quadruple mutants. Our results indicate that four NST/SND orthologs are master switches for SCW formation in wood fibers, xylem ray parenchyma cells and phloem fibers in poplar, while SCW is still formed in limited wood fibers, which are located at the region adjacent to	hybrid aspen
648	plant	Kalanchoe fedtschenkoi	CRISPR;Cas9;	blue light receptor phototropin 2	Journal of experimental botany	CRISPR/Cas9-mediated targeted mutagenesis for functional genomics research of crassulacean acid metabolism plants.	2019	70(22):6621-6629	[Liu D et al.]	Oak Ridge National Laboratory, Oak Ridge, TN, USA.	31562521	10.1093/jxb/erz415	Crassulacean acid metabolism (CAM) is an important photosynthetic pathway in diverse lineages of plants featuring high water-use efficiency and drought tolerance. A big challenge facing the CAM research community is to understand the function of the annotated genes in CAM plant genomes. Recently, a new genome editing technology using CRISPR/Cas9 has become a more precise and powerful tool than traditional approaches for functional genomics research in C3 and C4 plants. In this study, we explore the potential of CRISPR/Cas9 to characterize the function of CAM-related genes in the model CAM species Kalanchoe fedtschenkoi. We demonstrate that CRISPR/Cas9 is effective in creating biallelic indel mutagenesis to reveal previously unknown roles of blue light receptor phototropin 2 (KfePHOT2) in the CAM pathway. Knocking out KfePHOT2 reduced stomatal conductance and CO2 fixation in late afternoon and increased stomatal conductance and CO2 fixation during the night, indicating that blue light signaling plays an important role in the CAM pathway. Lastly, we provide a genome-wide guide RNA database targeting 45 183 protein-coding	Kalanchoe fe

649	plant	kiwifruit (Actinidia chinensis)	CRISPR;Cas9;	CENTRORADIALIS (CEN)-like gene (AcCEN4; AcCEN)	Plant biotechnology journal	Mutagenesis of kiwifruit CENTRORADIALIS-like genes transforms a climbing woody perennial with long juvenility and axillary flowering into a compact plant with rapid terminal flowering.	2019	17(5):869-880	[Varkonyi-Gasic E et al.]	New Zealand Institute for Plant & Food Research Limited (Plant & Food Research), Auckland, New Zealand.	30302894	10.1111/pbi.13021	Annualization of woody perennials has the potential to revolutionize the breeding and production of fruit crops and rapidly improve horticultural species. Kiwifruit (Actinidia chinensis) is a recently domesticated fruit crop with a short history of breeding and tremendous potential for improvement. Previously, multiple kiwifruit CENTRORADIALIS (CEN)-like genes have been identified as potential repressors of flowering. In this study, CRISPR/Cas9- mediated manipulation enabled functional analysis of kiwifruit CEN-like genes AcCEN4 and AcCEN. Mutation of these genes transformed a climbing woody perennial, which develops axillary inflorescences after many years of juvenility, into a compact plant with rapid terminal flower and fruit development. The number of affected genes and alleles and severity of detected mutations correlated with the precocity and change in plant stature, suggesting that a bi-allelic mutation of either AcCEN4 or AcCEN may be sufficient for early flowering, whereas mutations affecting both genes further contributed to precocity and enhanced the compact growth habit. CRISPR/Cas9-mediated mutagenesis of AcCEN4 and AcCEN may be a valuable means to engineer Actinidia amenable for accelerated breeding, indoor farming and cultivation	kiwifruit (Act
650	plant	Lilium pumilum DC. Fisch.; Lilium longiflorum 'White Heaven'	CRISPR;Cas9;	LpPDS	International journal of molecular sciences	Establishment of Efficient Genetic Transformation Systems and Application of CRISPR/Cas9 Genome Editing Technology in Lilium pumilum DC. Fisch. and Lilium longiflorum White Heaven.	2019	20(12)	[Yan R et al.]	Shenyang Agricultural University, Shenyang, China.	31207994	10.3390/ijms20122920	Lilium spp. is a bulb flower with worldwide distribution and unique underground organs. The lack of an efficient genetic transformation system for Lilium has been an international obstacle. Because existing model plants lack bulbs, bulb-related gene function verification studies cannot be carried out in model plants. Here, two stable and efficient genetic transformation systems based on somatic embryogenesis and adventitious bud regeneration were established in two Lilium species. Transgenic plants and T-DNA insertion lines were confirmed by beta-glucuronidase (GUS) assay, polymerase chain reaction (PCR) and Southern blot. After condition optimization, transformation efficiencies were increased to 29.17% and 4% in Lilium pumilum DC. Fisch. and the Lilium longiflorum 'White Heaven', respectively. To further verify the validity of these transformation systems and apply the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein 9) technology in Lilium, the LpPDS gene in the two Lilium species was knocked out. Completely albino, pale yellow and albino-green chimeric mutants were observed. Sequence analysis in the transgenic lines revealed various mutation patterns, including base insertion, deletion and substitution. These results verified the feasibility and high efficiency of both transformation systems and the successful application of the CRISPR/Cas9 system to gene editing in Lilium for the first time. Overall, this study lays	Lilium pumilu
651	plant	liverwort	CRISPR;Cas9;	Mppyl1	Plant physiology	Archetypal Roles of an Abscisic Acid Receptor in Drought and Sugar Responses in Liverworts.	2019	179(1):317-328	[Jahan A et al.]	Saitama University, Saitama, Japan.	30442644	10.1104/pp.18.00761	Abscisic acid (ABA) controls seed dormancy and stomatal closure through binding to the intracellular receptor Pyrabactin resistance1 (Pyr1)/Pyr1-like/regulatory components of ABA receptors (PYR/PYL/RCAR) in angiosperms. Genes encoding PYR/PYL/RCAR are thought to have arisen in the ancestor of embryophytes, but the roles of the genes in nonvascular plants have not been determined. In the liverwort Marchantia polymorpha, ABA reduces growth and enhances desiccation tolerance through increasing accumulation of intracellular sugars and various transcripts such as those of Late Embryogenesis Abundant (LEA)-like genes. In this study, we analyzed a gene designated MpPYL1, which is closely related to PYR/PYL/RCAR of angiosperms, in transgenic liverworts. Transgenic lines overexpressing MpPYL1-GFP showed ABA-hypersensitive growth with enhanced desiccation tolerance, whereas Mppyl1 generated by CRISPR-Cas9-mediated genome editing showed ABA-insensitive growth with reduced desiccation tolerance. Transcriptome analysis indicated that MpPYL1 is a major regulator of abiotic stress-associated genes, including all 35 ABA-induced LEA-like genes. Furthermore, these transgenic plants showed altered responses to extracellular Suc, suggesting that ABA and PYR/PYL/RCAR function in sugar responses. The results presented here reveal an important role of PYR/PYL/RCAR in the ABA response, which was likely acquired in the common ancestor of land plants. The results also indicate the archetypal role of ABA and its receptor in sugar response	liverwort
652	plant	liverwort (Marchantia polymorpha)	CRISPR;Cas9;	mir529c	Current biology	An Early Arising Role of the MicroRNA156/529-SPL Module in Reproductive Development Revealed by the Liverwort Marchantia polymorpha.	2019	29(19):3307-3314.e5	[Tsuzuki M et al.]	University of Tokyo, Tokyo, Japan.	31543452	10.1016/j.cub.2019.07.084	In angiosperms, the phase transition from vegetative to reproductive growth involves the de-repression of the squamosa promoter-binding-protein-like (SPL) class of transcription factors, which is negatively regulated by the specific microRNAs (miRNAs/miRs) miR156/529 [1]. Non-vascular land plants also undergo growth-phase transition to the reproductive state, but knowledge regarding the controlling mechanisms is limited. Here, we investigate the reproductive transition in the liverwort Marchantia polymorpha, focusing on the roles of miR529c [2-4] and MpSPL2. First, we established mir529c-null mutants using CRISPR/Cas9. Even in the absence of far-red light-supplemented long-day condition, which is usually needed to induce reproductive development [5, 6], the mutant thalli developed sexual reproductive organs (gametangia) and produced gametes. Transgenic plants expressing a miR529-resistant MpSPL2 transgene also showed a similar phenotype of reproductive transition in the absence of inductive far-red light signals. In these mutants and transgenic plants, the MpSPL2 mRNA abundance was elevated. Mpspl2(ko) mutant plants showed successful gamete development and fertilization, which suggests that MpSPL2 is involved in, but not essential for, sexual reproduction in M. polymorpha. Furthermore, analysis of Mpspl2(ko) mutant and its complemented lines suggests that MpSPL2 may have a role in promotion of reproductive transition. These findings support the notion that the transition to reproductive development in liverworts is controlled by a system similar to that in angiosperms, and the miR156/529-SPL module has common significance in the control of the vegetative-to-reproductive transition during development in many land	liverwort (Ma

653	plant	Lotus japonicus	CRISPR;Cas9;	3 types of leghemoglobins	New phytologist	CRISPR/Cas9 knockout of leghemoglobin genes in Lotus japonicus uncovers their synergistic roles in symbiotic nitrogen fixation.	2019	224(2):818-832	[Wang L et al.]	Huazhong Agricultural University, Wuhan, China.	31355948	10.1111/nph.16077	Legume nodules contain high concentrations of leghemoglobins (Lbs) encoded by several genes. The reason for this multiplicity is unknown. CRISPR/Cas9 technology was used to generate stable mutants of the three Lbs of Lotus japonicus. The phenotypes were characterized at the physiological, biochemical and molecular levels. Nodules of the triple mutants were examined by electron microscopy and subjected to RNA-sequencing (RNA-seq) analysis. Complementation studies revealed that Lbs function synergistically to maintain optimal N2 fixation. The nodules of the triple mutants overproduced superoxide radicals and hydrogen peroxide, which was probably linked to activation of NADPH oxidases and changes in superoxide dismutase isoforms expression. The mutant nodules showed major ultrastructural alterations, including vacuolization, accumulation of poly-beta-hydroxybutyrate and disruption of mitochondria. RNA-seq of c. 20 000 genes revealed significant changes in expression of carbon and nitrogen metabolism genes, transcription factors, and proteinases. Lb-deficient nodules had c. 30-50-fold less heme but similar transcript levels of heme biosynthetic genes, suggesting a post-translational regulatory mechanism of heme synthesis. We conclude that Lbs act additively in nodules and that the lack of Lbs results in early nodule senescence. Our observations also provide insight into the reprogramming of the gene expression network associated with Lb deficiency, probably	Lotus japonic
654	plant	Lotus japonicus, soybean Glycine max, common bean Phaseolus vulgaris	Agroinfiltration;	GFP	Plant biotechnology	Agroinfiltration-based efficient transient protein expression in leguminous plants.	2019	36(2):119-123	[Suzaki T et al.]	University of Tsukuba, Tsukuba, Ibaraki, Japan.	31768113	10.5511/plantbiotechnology.19.0220b	Transient protein expression is an effective tool to rapidly unravel novel gene functions, such as transcriptional activity of promoters and sub-cellular localization of proteins. However, transient expression is not applicable to some species and varieties because of insufficient expression levels. We recently developed one of the strongest agroinfiltration-based transient protein expression systems for plant cells, termed 'Tsukuba system.' About 4 mg/g fresh weight of protein expression in Nicotiana benthamiana was obtained using this system. The vector pBYR2HS, which contains a geminiviral replication system and a double terminator, can be used in various plant species and varieties, including lettuces, eggplants, tomatoes, hot peppers, and orchids. In this study, we assessed the applicability of the Tsukuba system to several species of legumes, including Lotus japonicus, soybean Glycine max, and common bean Phaseolus vulgaris. The GFP protein was transiently expressed in the seedpods of all examined legume species, however, protein expression in leaves was observed only in P. vulgaris. Taken together, our system is an effective tool to examine gene function	Lotus japonic
655	plant	maize	CRISPR;Cas9;	in situ specific repeats	Cytogenetic and genome research	CRISPR/Cas9-Based RGEN-ISL Allows the Simultaneous and Specific Visualization of Proteins, DNA Repeats, and Sites of DNA Replication.	2019	159(1):48-53	[Nemeckova A et al.]	Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Seeland, Germany	31610539	10.1159/000502600	Visualizing the spatiotemporal organization of the genome will improve our understanding of how chromatin structure and function are intertwined. Here, we describe a further development of the CRISPR/Cas9-based RNA-guided endonuclease-in situ labeling (RGEN-ISL) method. RGEN-ISL allowed the differentiation between vertebrate-type (TTAGGG)n and Arabidopsis-type (TTTAGGG)n telomere repeats. Using maize as an example, we established a combination of RGEN-ISL, immunostaining, and EdU labeling to visualize in situ specific repeats, histone marks, and DNA replication sites, respectively. The effects of the non-denaturing RGEN-ISL and standard denaturing FISH on the chromatin structure were compared using super-resolution microscopy. 3D structured illumination microscopy revealed that denaturation and acetic acid fixation impaired and flattened the chromatin. The broad range of adaptability of RGEN-ISL to different combinations of	maize
656	plant	maize	CRISPR;	dek42	Journal of integrative plant biology	Dek42 encodes an RNA-binding protein that affects alternative pre-mRNA splicing and maize kernel development.	2019	61(6):728-748	[Zuo Y et al.]	China Agricultural University, Beijing, China.	30839161	10.1111/jipb.12798	RNA-binding proteins (RBPs) play an important role in post-transcriptional gene regulation. However, the functions of RBPs in plants remain poorly understood. Maize kernel mutant dek42 has small defective kernels and lethal seedlings. Dek42 was cloned by Mutator tag isolation and further confirmed by an independent mutant allele and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 materials. Dek42 encodes an RRM_RBM48 type RNA-binding protein that localizes to the nucleus. Dek42 is constitutively expressed in various maize tissues. The dek42 mutation caused a significant reduction in the accumulation of DEK42 protein in mutant kernels. RNA-seq analysis showed that the dek42 mutation significantly disturbed the expression of thousands of genes during maize kernel development. Sequence analysis also showed that the dek42 mutation significantly changed alternative splicing in expressed genes, which were especially enriched for the U12-type intron-retained type. Yeast two-hybrid screening identified SF3a1 as a DEK42-interacting protein. DEK42 also interacts with the spliceosome component U1-70K. These results suggested that DEK42 participates in the regulation of pre-messenger RNA splicing through its interaction with other spliceosome components. This study showed the function of a newly identified RBP and provided insights into	maize
657	plant	maize	CRISPR;Cas9;		Molecular plant	Development of a Haploid-Inducer Mediated Genome Editing System for Accelerating Maize Breeding.	2019	12(4):597-602	[Wang B et al.]	Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China.	30902686	10.1016/j.molp.2019.03.006	Crop breeding aims to generate pure inbred lines with multiple desired traits. Doubled haploid (DH) and genome editing using CRISPR/Cas9 are two powerful game-changing technologies in crop breeding. However, both of them still fall short for rapid generation of pure elite lines with integrated favorable traits. Here, we report the development of a Haploid-Inducer Mediated Genome Editing (IMGE) approach, which utilizes a maize haploid inducer line carrying a CRISPR/Cas9 cassette targeting for a desired agronomic trait to pollinate an elite maize inbred line and to generate genome-edited haploids in the elite maize background. Homozygous pure DH lines with the desired trait improvement could be generated within two generations, thus bypassing the lengthy procedure of repeated crossing and backcrossing used in conventional breeding for integrating a desirable trait into elite commercial backgrounds.	maize

658	plant	maize	CRISPR;Cas9;	ZmDMP	Nature plants	Mutation of ZmDMP enhances haploid induction in maize.	2019	5(6):575–580	[Zhong Y et al.]	China Agricultural University, Beijing, China.	31182848	10.1038/s41477–019–0443–7	Doubled haploid (DH) breeding based on in vivo haploid induction has led to a new approach for maize breeding(1). All modern haploid inducers used in DH breeding are derived from the haploid inducer line Stock6. Two key quantitative trait loci, qhir1 and qhir8, lead to high–frequency haploid induction(2). Mutation of the gene MTL/ZmPLA1/NLD in qhir1 could generate a ~2% haploid induction rate (HIR)(3–5); nevertheless, this mutation is insufficient for modern haploid inducers whose average HIR is ~10%(6). Therefore, cloning of the gene underlying qhir8 is important for illuminating the genetic basis of haploid induction. Here, we present the discovery that mutation of a non–Stock6–originating gene in qhir8, namely, ZmDMP, enhances and triggers haploid induction. ZmDMP was identified by map–based cloning and further verified by CRISPR–Cas9–mediated knockout experiments. A single–nucleotide change in ZmDMP leads to a 2–3–fold increase in the HIR. ZmDMP knockout triggered haploid induction with a HIR of 0.1–0.3% and exhibited a greater ability to increase the HIR by 5–6–fold in the presence of mtl/zmpla1/nld. ZmDMP was highly expressed during the late stage of pollen development and localized to the plasma membrane. These findings provide important approaches for studying the molecular mechanism of haploid	maize
659	plant	maize	agroinfiltration; CRISPR;Cas9;Cpf1;	glossy2	Plant biotechnology journal	Activities and specificities of CRISPR/Cas9 and Cas12a nucleases for targeted mutagenesis in maize.	2019	17(2):362–372	[Lee K et al.]	Iowa State University, Ames, IA, USA.	29972722	10.1111/pbi.12982	CRISPR/Cas9 and Cas12a (Cpf1) nucleases are two of the most powerful genome editing tools in plants. In this work, we compared their activities by targeting maize glossy2 gene coding region that has overlapping sequences recognized by both nucleases. We introduced constructs carrying SpCas9–guide RNA (gRNA) and LbCas12a–CRISPR RNA (crRNA) into maize inbred B104 embryos using Agrobacterium–mediated transformation. On–target mutation analysis showed that 90%–100% of the Cas9–edited T0 plants carried indel mutations and 63%–77% of them were homozygous or biallelic mutants. In contrast, 0%–60% of Cas12a–edited T0 plants had on–target mutations. We then conducted CIRCLE–seq analysis to identify genome–wide potential off–target sites for Cas9. A total of 18 and 67 potential off–targets were identified for the two gRNAs, respectively, with an average of five mismatches compared to the target sites. Sequencing analysis of a selected subset of the off–target sites revealed no detectable level of mutations in the T1 plants, which constitutively express Cas9 nuclease and gRNAs. In conclusion, our results suggest that the CRISPR/Cas9 system used in this study is highly efficient and specific for genome editing in maize, while CRISPR/Cas12a needs further optimization for improved	maize
660	plant	maize	CRISPR;Cas9;	20 genes	Plant cell reports	Single and multiple gene knockouts by CRISPR–Cas9 in maize.	2019	38(4):487–501	[Doll NM et al.]	Univ Lyon, ENS de Lyon, UCB Lyon 1, CNRS, INRA, Lyon, France.	30684023	10.1007/s00299–019–02378–1	KEY MESSAGE: The analysis of 93 mutant alleles in 18 genes demonstrated that CRISPR–Cas9 is a robust tool for targeted mutagenesis in maize, permitting efficient generation of single and multiple knockouts. CRISPR–Cas9 technology is a simple and efficient tool for targeted mutagenesis of the genome. It has been implemented in many plant species, including crops such as maize. Here we report single– and multiple–gene mutagenesis via stably transformed maize plants. Two different CRISPR–Cas9 vectors were used allowing the expression of multiple guide RNAs and different strategies to knockout either independent or paralogous genes. A total of 12 plasmids, representing 28 different single guide RNAs (sgRNAs), were generated to target 20 genes. For 18 of these genes, at least one mutant allele was obtained, while two genes were recalcitrant to sequence editing. 19% (16/83) of mutant plants showed biallelic mutations. Small insertions or deletions of less than ten nucleotides were most frequently observed, regardless of whether the gene was targeted by one or more sgRNAs. Deletions of defined regions located between the target sites of two guide RNAs were also reported although the exact deletion size was variable. Double and triple mutants were created in a single step, which is especially valuable for functional analysis of genes with strong genetic linkage. Off–target effects were theoretically limited due to rigorous sgRNA design and random experimental checks at three potential off–target sites did not reveal any editing. Sanger chromatograms allowed to unambiguously class the primary transformants; the majority (85%) were fully edited plants transmitting systematically all detected mutations to the next generation, generally following Mendelian segregation.	maize
661	plant	maize	CRISPR;Cas9;	Zmpif3; Zmpif4; Zmpif5	Plant physiology	Characterization of Maize Phytochrome–Interacting Factors in Light Signaling and Photomorphogenesis.	2019	181(2):789–803	[Wu G et al.]	Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China.	31350363	10.1104/pp.19.00239	Increasing planting density has been an effective means of increasing maize (Zea mays ssp. mays) yield per unit of land area over the past few decades. However, high–density planting will cause a reduction in the ratio of red to far–red incident light, which could trigger the shade avoidance syndrome and reduce yield. The molecular mechanisms regulating the shade avoidance syndrome are well established in Arabidopsis (Arabidopsis thaliana) but poorly understood in maize. Here, we conducted an initial functional characterization of the maize Phytochrome–Interacting Factor (PIF) gene family in regulating light signaling and photomorphogenesis. The maize genome contains seven distinct PIF genes, which could be grouped into three subfamilies: ZmPIF3s, ZmPIF4s, and ZmPIF5s Similar to the Arabidopsis PIFs, all ZmPIF proteins are exclusively localized to the nucleus and most of them can form nuclear bodies upon light irradiation. We show that all of the ZmPIF proteins could interact with ZmphyB. Heterologous expression of each ZmPIF member could partially or fully rescue the phenotype of the Arabidopsis pifq mutant, and some of these proteins conferred enhanced shade avoidance syndrome in Arabidopsis. Interestingly, all ZmPIF proteins expressed in Arabidopsis are much more stable than their Arabidopsis counterparts upon exposure to red light. Moreover, the Zmpif3, Zmpif4, and Zmpif5 knockout mutants generated via CRISPR/Cas9 technology all showed severely suppressed mesocotyl elongation in dark–grown seedlings and were less responsive to simulated shade treatment. Taken together, our results reveal both conserved and distinct molecular properties of ZmPIFs in regulating light signaling and photomorphogenesis in maize.	maize

662	plant	maize	CRISPR;Cas9;	ACCELERATED CELL DEATH6 (ZmACD6)	Plant signaling & behavior	Identification and characterization of maize ACD6-like gene reveal ZmACD6 as the maize orthologue conferring resistance to Ustilago maydis.	2019	14(10):e1651604	[Zhang Z et al.]	Agricultural University of Hebei, Baoding, China.	31397626	10.1080/15592324.2019.1651604	Enhancing broad-spectrum resistance is a major goal of crop breeding. However, broad-spectrum resistance has not been thoroughly investigated, and its underlying molecular mechanisms remain elusive. In the model plant Arabidopsis (Arabidopsis thaliana), ACCELERATED CELL DEATH6 (ACD6) is a key component of broad-spectrum resistance that acts in a positive feedback loop with salicylic acid (SA) to regulate multiple pattern recognition receptors. However, the role of ACD6 in disease resistance in crop plants is unclear. Here, we show that the transcript of ANK23, one of the 15 ACD6-like genes in maize (Zea mays), is induced by SA and by infection with the pathogenic fungus Ustilago maydis. Heterologous expression of ANK23 restored disease resistance in the Arabidopsis mutant acd6-2. We show that ANK23 is a maize ortholog of ACD6 and therefore rename ANK23 as ZmACD6. Furthermore, using CRISPR/Cas9, we generated ZmACD6 knockout maize plants, which are more susceptible to U. maydis than wild-type plants. We also identified a maize line (SC-9) with relatively high ZmACD6 expression levels from a diverse natural maize population. SC-9 has increased disease resistance to U. maydis and defense activation, suggesting a practical approach to cultivate elite varieties with enhanced disease resistance.	maize
663	plant	Zea mays	CRISPR;Cas9;Cpf1;		Communications biology	The repurposing of type I-E CRISPR-Cascade for gene activation in plants.	2019	2(1):383	[Young JK et al.]	Corteva Agriscience, Johnston, IA, USA.	31925225	10.1038/s42003-019-0637-6	CRISPR-Cas systems are robust and facile tools for manipulating the genome, epigenome and transcriptome of eukaryotic organisms. Most groups use class 2 effectors, such as Cas9 and Cas12a, however, other CRISPR-Cas systems may provide unique opportunities for genome engineering. Indeed, the multi-subunit composition of class 1 systems offers to expand the number of domains and functionalities that may be recruited to a genomic target. Here we report DNA targeting in Zea mays using a class 1 type I-E CRISPR-Cas system from S. thermophilus. First, we engineer its Cascade complex to modulate gene expression by tethering a plant transcriptional activation domain to 3 different subunits. Next, using an immunofluorescent assay, we confirm Cascade cellular complex formation and observe enhanced gene activation when multiple subunits tagged with the transcriptional activator are combined. Finally, we examine Cascade mediated gene activation at	maize
664	plant	maize (Zea mays)	CRISPR;	closed stomata1	Plant cell	A Subsidiary Cell-Localized Glucose Transporter Promotes Stomatal Conductance and Photosynthesis.	2019	31(6):1328-1343	[Wang H et al.]	Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China.	30996077	10.1105/tpc.18.00736	It has long been recognized that stomatal movement modulates CO2 availability and as a consequence the photosynthetic rate of plants, and that this process is feedback-regulated by photoassimilates. However, the genetic components and mechanisms underlying this regulatory loop remain poorly understood, especially in monocot crop species. Here, we report the cloning and functional characterization of a maize (Zea mays) mutant named closed stomata1 (cst1). Map-based cloning of cst1 followed by confirmation with the clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein 9 system identified the causal mutation in a Clade I Sugars Will Eventually be Exported Transporters (SWEET) family gene, which leads to the E81K mutation in the CST1 protein. CST1 encodes a functional glucose transporter expressed in subsidiary cells, and the E81K mutation strongly impairs the oligomerization and glucose transporter activity of CST1. Mutation of CST1 results in reduced stomatal opening, carbon starvation, and early senescence in leaves, suggesting that CST1 functions as a positive regulator of stomatal opening. Moreover, CST1 expression is induced by carbon starvation and suppressed by photoassimilate accumulation. Our study thus defines CST1 as a missing link in the feedback-regulation	maize (Zea n
665	plant	maize (Zea mays)	CRISPR;Cas9;	morphogenic regulator	Plant physiology	A Novel Ternary Vector System United with Morphogenic Genes Enhances CRISPR/Cas Delivery in Maize.	2019	181(4):1441-1448	[Zhang Q et al.]	China Agricultural University, Beijing, China.	31558579	10.1104/pp.19.00767	The lack of efficient delivery methods is a major barrier to clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas)-mediated genome editing in many plant species. Combinations of morphogenic regulator (MR) genes and ternary vector systems are promising solutions to this problem. In this study, we first demonstrated that MR vectors greatly enhance maize (Zea mays) transformation. We then tested a CRISPR/Cas9 MR vector in maize and found that the MR and CRISPR/Cas9 modules have no negative influence on each other. Finally, we developed a novel ternary vector system to integrate the MR and CRISPR/Cas modules. Our ternary vector system is composed of new pGreen-like binary vectors, here named pGreen3, and a pVS1-based virulence helper plasmid, which also functions as a replication helper for the pGreen3 vectors in Agrobacterium tumefaciens The pGreen3 vectors were derived from the plasmid pRK2 and display advantages over pGreen2 vectors regarding both compatibility and stability. We demonstrated that the union of our ternary vector system with MR gene modules has additive effects in enhancing maize transformation and that this enhancement is especially evident in the transformation of recalcitrant maize inbred lines. Collectively, our ternary vector system-based tools provide a user-friendly solution to the low efficiency of CRISPR/Cas delivery in maize and represent a basic platform for developing efficient	maize (Zea n
666	plant	maize; Setaria viridis; Nicotiana benthamiana	Cas9;	N. benthamiana Phytoene desaturase gene, S. viridis Carbonic anhydrase 2 gene, and maize HKT1 gene	Plant direct	Protein expression and gene editing in monocots using foxtail mosaic virus vectors.	2019	3(11):e00181	[Mei Y et al.]	Iowa State University, Ames, IA, USA.	31768497	10.1002/pld3.181	Plant viruses can be engineered to carry sequences that direct silencing of target host genes, expression of heterologous proteins, or editing of host genes. A set of foxtail mosaic virus (FoMV) vectors was developed that can be used for transient gene expression and single guide RNA delivery for Cas9-mediated gene editing in maize, Setaria viridis, and Nicotiana benthamiana. This was accomplished by duplicating the FoMV capsid protein subgenomic promoter, abolishing the unnecessary open reading frame 5A, and inserting a cloning site containing unique restriction endonuclease cleavage sites immediately after the duplicated promoter. The modified FoMV vectors transiently expressed green fluorescent protein (GFP) and bialaphos resistance (BAR) protein in leaves of systemically infected maize seedlings. GFP was detected in epidermal and mesophyll cells by epifluorescence microscopy, and expression was confirmed by Western blot analyses. Plants infected with FoMV carrying the bar gene were temporarily protected from a glufosinate herbicide, and expression was confirmed using a rapid antibody-based BAR strip test. Expression of these proteins was stabilized by nucleotide substitutions in the sequence of the duplicated promoter region. Single guide RNAs expressed from the duplicated promoter mediated edits in the N. benthamiana Phytoene desaturase gene, the S. viridis Carbonic anhydrase 2 gene, and the maize HKT1 gene encoding a potassium transporter. The efficiency of editing was enhanced in the presence of synergistic viruses and a viral silencing suppressor. This work expands the utility of FoMV for virus-induced gene silencing	maize; Setar

667	plant	apple (Malus x domestica)	agroinfiltration; CRISPR;Cas9;	MdDIPM4	Plant biotechnology journal	Reduced fire blight susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system.	2019		[Pompili V et al.]	Fondazione Edmund Mach, San Michele all'Adige, Italy.	31495052	10.1111/pbi.13253	The bacterium Erwinia amylovora, the causal agent of fire blight disease in apple, triggers its infection through the DspA/E effector which interacts with the apple susceptibility protein MdDIPM4. In this work, MdDIPM4 knockout has been produced in two Malus x domestica susceptible cultivars using the CRISPR/Cas9 system delivered via Agrobacterium tumefaciens. Fifty-seven transgenic lines were screened to identify CRISPR/Cas9-induced mutations. An editing efficiency of 75% was obtained. Seven edited lines with a loss-of-function mutation were inoculated with the pathogen. Highly significant reduction in susceptibility was observed compared to control plants. Sequencing of five potential off-target sites revealed no mutation event. Moreover, our construct contained a heat-shock inducible FLP/FRT recombination system designed specifically to remove the T-DNA harbouring the expression cassettes for CRISPR/Cas9, the marker gene and the FLP itself. Six plant lines with reduced susceptibility to the pathogen were heat-treated and screened by real-time PCR to quantify the exogenous DNA elimination. The T-DNA removal was further validated by sequencing in one plant line. To our knowledge, this work demonstrates for the first time the development and application of a CRISPR/Cas9-FLP/FRT gene editing system for the production of edited apple plants carrying a minimal trace of exogenous	Malus x dom
668	plant	Marchantia polymorpha	CRISPR;Cas9;	MpATG8	Frontiers in plant science	Marchantia polymorpha, a New Model Plant for Autophagy Studies.	2019	10:935	[Norizuki T et al.]	University of Tokyo, Tokyo, Japan.	31379911	10.3389/fpls.2019.00935	Autophagy is a catabolic process for bulk and selective degradation of cytoplasmic components in the vacuole/lysosome. In Saccharomyces cerevisiae, ATG genes were identified as essential genes for autophagy, and most ATG genes are highly conserved among eukaryotes, including plants. Although reverse genetic analyses have revealed that autophagy is involved in responses to abiotic and biotic stresses in land plants, our knowledge of its molecular mechanism remains limited. This limitation is partly because of the multiplication of some ATG genes, including ATG8, in widely used model plants such as Arabidopsis thaliana, which adds complexity to functional studies. Furthermore, due to limited information on the composition and functions of the ATG genes in basal land plants and charophytes, it remains unclear whether multiplication of ATG genes is associated with neofunctionalization of these genes. To gain insight into the diversification of ATG genes during plant evolution, we compared the composition of ATG genes in plants with a special focus on a liverwort and two charophytes, which have not previously been analyzed. Our results showed that the liverwort Marchantia polymorpha and the charophytes Klebsormidium nitens and Chara braunii harbor fundamental sets of ATG genes with low redundancy compared with those of A. thaliana and the moss Physcomitrella patens, suggesting that multiplication of ATG genes occurred during land plant evolution. We also attempted to establish an experimental system for analyzing autophagy in M. polymorpha. We generated transgenic plants expressing fluorescently tagged MpATG8 to observe its dynamics in M. polymorpha and produced autophagy-defective mutants by genome editing using the CRISPR/Cas9 system. These tools allowed us to demonstrate that MpATG8 is transported into the vacuole in an MpATG2-, MpATG5-, and MpATG7-dependent manner, suggesting that fluorescently tagged MpATG8 can be used as an autophagosome marker in M. polymorpha. M. polymorpha can provide a powerful system	Marchantia p
669	plant	Marchantia polymorpha	CRISPR;Cas9;	MpFHY1 (ortholog of FAR-RED ELONGATED HYPOCOTYL1); MpPIF (PHYTOCHROME INTERACTING FACTOR)	Plant & cell physiology	Reproductive Induction is a Far-Red High Irradiance Response that is Mediated by Phytochrome and PHYTOCHROME INTERACTING FACTOR in Marchantia polymorpha.	2019	60(5):1136-1145	[Inoue K et al.]	Kyoto University, Kyoto, Japan.	30816950	10.1093/pcp/pcz029	Land plants have evolved a series of photoreceptors to precisely perceive environmental information. Among these, phytochromes are the sole photoreceptors for red light (R) and far-red light (FR), and play pivotal roles in modulating various developmental processes. Most extant land plants possess multiple phytochromes that probably evolved from a single phytochrome in the common ancestor of land plants. However, the ancestral phytochrome signaling mechanism remains unknown due to a paucity of knowledge regarding phytochrome functions in basal land plants. It has recently been reported that Mpphy, a single phytochrome in the liverwort Marchantia polymorpha, regulates typical photoreversible responses collectively classified as low fluence response (LFR). Here, we show that Mpphy also regulates the gametangiophore formation analogous to the mode of action of the far-red high irradiance response (FR-HIR) in angiosperms. Our phenotypic analyses using mutant plants obtained by CRISPR/Cas9-based genome editing revealed that MpFHY1, an ortholog of FAR-RED ELONGATED HYPOCOTYL1, as well as Mpphy is critical for the FR-HIR signaling in M. polymorpha. In addition, knockout of MpPIF, a single PHYTOCHROME INTERACTING FACTOR gene in M. polymorpha, completely abolished the FR-HIR-dependent gametangiophore formation, while overexpression of MpPIF accelerated the response. FR-HIR-dependent transcriptional regulation was also disrupted in the Mppif mutant. Our findings suggest that plants had already acquired the FR-HIR signaling mediated by phytochrome and PIF at a very early stage during the course of land plant evolution, and that a single phytochrome in the common ancestor of land plants could mediate	Marchantia p
670	plant	Medicago truncatula	CRISPR;Cas9;	nodule-specific Polycystin-1, Lipoxygenase, Alpha Toxin (PLAT) domain proteins (NPD)	New phytologist	Nodule-specific PLAT domain proteins are expanded in the Medicago lineage and required for nodulation.	2019	222(3):1538-1550	[Trujillo DI et al.]	University of Minnesota, Saint Paul, MN, USA.	30664233	10.1111/nph.15697	Symbiotic nitrogen fixation in legumes is mediated by an interplay of signaling processes between plant hosts and rhizobial symbionts. In legumes, several secreted protein families have undergone expansions and play key roles in nodulation. Thus, identifying lineage-specific expansions (LSEs) of nodulation-associated genes can be a strategy to discover candidate gene families. Using bioinformatic tools, we identified 13 LSEs of nodulation-related secreted protein families, each unique to either Glycine, Arachis or Medicago lineages. In the Medicago lineage, nodule-specific Polycystin-1, Lipoxygenase, Alpha Toxin (PLAT) domain proteins (NPDs) expanded to five members. We examined NPD function using CRISPR/Cas9 multiplex genome editing to create Medicago truncatula NPD knockout lines, targeting one to five NPD genes. Mutant lines with differing combinations of NPD gene inactivations had progressively smaller nodules, earlier onset of nodule senescence, or ineffective nodules compared to the wild-type control. Double- and triple-knockout lines showed dissimilar nodulation phenotypes but coincided in upregulation of a DHHC-type zinc finger and an aspartyl protease gene, possible candidates for the observed disturbance of proper nodule function. By postulating that gene family expansions can be used to detect candidate genes, we identified a family of nodule-specific PLAT domain proteins and confirmed	Medicago tru

671	plant	Medicago truncatula	Cas9;		Nucleic acids research	muLAS technology for DNA isolation coupled to Cas9-assisted targeting for sequencing and assembly of a 30 kb region in plant genome.	2019	47(15):8050-8060	[Milon N et al.]	CNRS, Toulouse, France.	31505675	10.1093/nar/gkz632	Cas9-assisted targeting of DNA fragments in complex genomes is viewed as an essential strategy to obtain high-quality and continuous sequence data. However, the purity of target loci selected by pulsed-field gel electrophoresis (PFGE) has so far been insufficient to assemble the sequence in one contig. Here, we describe the muLAS technology to capture and purify high molecular weight DNA. First, the technology is optimized to perform high sensitivity DNA profiling with a limit of detection of 20 fg/mul for 50 kb fragments and an analytical time of 50 min. Then, muLAS is operated to isolate a 31.5 kb locus cleaved by Cas9 in the genome of the plant Medicago truncatula. Target purification is validated on a Bacterial Artificial Chromosome plasmid, and subsequently carried out in whole genome with muLAS, PFGE or by combining these techniques. PacBio sequencing shows an enrichment factor of the target sequence of 84 with PFGE alone versus 892 by association of PFGE with muLAS. These performances allow us to sequence and assemble one contig of 29 441 bp with	Medicago tru
672	plant	Medicago truncatula	TALENs	basic helix-loop-helix transcription factor2	Plant physiology	Transcription Factor bHLH2 Represses CYSTEINE PROTEASE77 to Negatively Regulate Nodule Senescence.	2019	181(4):1683-1703	[Deng J et al.]	China Agricultural University, Beijing, China.	31591150	10.1104/pp.19.00574	Legume-rhizobia symbiosis is a time-limited process due to the onset of senescence, which results in the degradation of host plant cells and symbiosomes. A number of transcription factors, proteases, and functional genes have been associated with nodule senescence; however, whether other proteases or transcription factors are involved in nodule senescence remains poorly understood. In this study, we identified an early nodule senescence mutant in Medicago truncatula, denoted basic helix-loop-helix transcription factor2 (bhlh2), that exhibits decreased nitrogenase activity, acceleration of plant programmed cell death (PCD), and accumulation of reactive oxygen species (ROS). The results suggest that MtbHLH2 plays a negative role in nodule senescence. Nodules of wild-type and bhlh2-TALEN mutant plants at 28 d postinoculation were used for transcriptome sequencing. The transcriptome data analysis identified a papain-like Cys protease gene, denoted MtCP77, that could serve as a potential target of MtbHLH2. Electrophoretic mobility shift assays and chromatin immunoprecipitation analysis demonstrated that MtbHLH2 directly binds to the promoter of MtCP77 to inhibit its expression. MtCP77 positively regulates nodule senescence by accelerating plant PCD and ROS accumulation. In addition, the expression of MtbHLH2 in the nodules gradually decreased from the meristematic zone to the nitrogen fixation zone, whereas the expression of MtCP77 showed enhancement. These results indicate that MtbHLH2 and MtCP77 have opposite functions in the regulation of nodule senescence. These results reveal significant roles for MtbHLH2 and MtCP77 in plant PCD, ROS accumulation, and nodule senescence, and improve our	Medicago tru
673	plant	Medicago truncatula	agroinfiltration; CRISPR;Cas9;	Gibberellin inactivating C20-GA2-oxidase (MtGA2ox10)	Scientific reports	MtGA2ox10 encoding C20-GA2-oxidase regulates rhizobial infection and nodule development in Medicago truncatula.	2019	9(1):5952	[Kim GB et al.]	Myongji University, Yongin, Korea.	30976084	10.1038/s41598-019-42407-3	Gibberellin (GA) plays a controversial role in the legume-rhizobium symbiosis. Recent studies have shown that the GA level in legumes must be precisely controlled for successful rhizobial infection and nodule organogenesis. However, regulation of the GA level via catabolism in legume roots has not been reported to date. Here, we investigate a novel GA inactivating C20-GA2-oxidase gene MtGA2ox10 in Medicago truncatula. RNA sequencing analysis and quantitative polymerase chain reaction revealed that MtGA2ox10 was induced as early as 6 h post-inoculation (hpi) of rhizobia and reached peak transcript abundance at 12 hpi. Promoter::beta-glucuronidase fusion showed that the promoter activity was localized in the root infection/differentiation zone during the early stage of rhizobial infection and in the vascular bundle of the mature nodule. The CRISPR/Cas9-mediated deletion mutation of MtGA2ox10 suppressed infection thread formation, which resulted in reduced development and retarded growth of nodules on the Agrobacterium rhizogenes-transformed roots. Over-expression of MtGA2ox10 in the stable transgenic plants caused dwarfism, which was rescued by GA3 application, and increased infection thread formation but inhibition of nodule development. We conclude that MtGA2ox10 plays an important role in the rhizobial infection and the development of root nodules through fine catabolic tuning of	Medicago tru
674	plant	melon	agroinfiltration; CRISPR;Cas9;	phytoene desaturase	Scientific reports	Efficient knockout of phytoene desaturase gene using CRISPR/Cas9 in melon.	2019	9(1):17077	[Hooghvorst I et al.]	Universitat de Barcelona, Barcelona, Spain.	31745156	10.1038/s41598-019-53710-4	CRISPR/Cas9 system has been widely applied in many plant species to induce mutations in the genome for studying gene function and improving crops. However, to our knowledge, there is no report of CRISPR/Cas9-mediated genome editing in melon (Cucumis melo). In our study, phytoene desaturase gene of melon (CmPDS) was selected as target for the CRISPR/Cas9 system with two designed gRNAs, targeting exons 1 and 2. A construct (pHSE-CmPDS) carrying both gRNAs and the Cas9 protein was delivered by PEG-mediated transformation in protoplasts. Mutations were detected in protoplasts for both gRNAs. Subsequently, Agrobacterium-mediated transformation of cotyledonary explants was carried out, and fully albino and chimeric albino plants were successfully regenerated. A regeneration efficiency of 71% of transformed plants was achieved from cotyledonary explants, a 39% of genetic transformed plants were successful gene edited, and finally, a 42-45% of mutation rate was detected by Sanger analysis. In melon protoplasts and plants most mutations were substitutions (91%), followed by insertions (7%) and deletions (2%). We set up a CRISPR/Cas9-mediated genome editing protocol which is efficient and feasible in melon, generating multi-allelic mutations in both genomic target sites of the CmPDS gene showing an albino phenotype easily detectable after only few weeks after Agrobacterium-mediated	melon
675	plant	mini-citrus (Fortunella hindsii)	CRISPR;Cas9;		Plant biotechnology journal	Genome sequencing and CRISPR/Cas9 gene editing of an early flowering Mini-Citrus (Fortunella hindsii).	2019	17(11):2199-2210	[Zhu C et al.]	Huazhong Agricultural University, Wuhan, China.	31004551	10.1111/pbi.13132	Hongkong kumquat (Fortunella hindsii) is a wild citrus species characterized by dwarf plant height and early flowering. Here, we identified the monoembryonic F. hindsii (designated as 'Mini-Citrus') for the first time and constructed its selfing lines. This germplasm constitutes an ideal model for the genetic and functional genomics studies of citrus, which have been severely hindered by the long juvenility and inherent apomixes of citrus. F. hindsii showed a very short juvenile period (~8 months) and stable monoembryonic phenotype under cultivation. We report the first de novo assembled 373.6 Mb genome sequences (Contig-N50 2.2 Mb and Scaffold-N50 5.2 Mb) for F. hindsii. In total, 32 257 protein-coding genes were annotated, 96.9% of which had homologues in other eight Citrinae species. The phylogenomic analysis revealed a close relationship of F. hindsii with cultivated citrus varieties, especially with mandarin. Furthermore, the CRISPR/Cas9 system was demonstrated to be an efficient strategy to generate target mutagenesis on F. hindsii. The modifications of target genes in the CRISPR-modified F. hindsii were predominantly 1-bp insertions or small deletions. This genetic transformation system based on F. hindsii could shorten the whole process from explant to T1 mutant to about 15 months. Overall, due to its short juvenility, monoembryony, close genetic background to cultivated citrus and applicability of CRISPR, F. hindsii shows unprecedented potentials to be used as a model species for	mini-citrus (

676	plant	Parasponia andersonii	CRISPR;Cas9;	NODULE INCEPTION (NIN); NUCLEAR FACTOR Y (NF-YA)	New phytologist	Mutant analysis in the nonlegume Parasponia andersonii identifies NIN and NF-YA1 transcription factors as a core genetic network in nitrogen-fixing nodule symbioses.	2019		[Bu F et al.]	Wageningen University, Wageningen, the Netherlands.	31863481	10.1111/nph.16386	Nitrogen-fixing nodulation occurs in 10 taxonomic lineages, with either rhizobia or Frankia bacteria. To establish such an endosymbiosis, two processes are essential: nodule organogenesis and intracellular bacterial infection. In the legume-rhizobium endosymbiosis, both processes are guarded by the transcription factor NODULE INCEPTION (NIN) and its downstream target genes of the NUCLEAR FACTOR Y (NF-Y) complex. It is hypothesized that nodulation has a single evolutionary origin c. 110 Ma, followed by many independent losses. Despite a significant body of knowledge of the legume-rhizobium symbiosis, it remains elusive which signalling modules are shared between nodulating species in different taxonomic clades. We used Parasponia andersonii to investigate the role of NIN and NF-YA genes in rhizobium nodulation in a nonlegume system. Consistent with legumes, P. andersonii PanNIN and PanNF-YA1 are coexpressed in nodules. By analyzing single, double and higher-order CRISPR-Cas9 knockout mutants, we show that nodule organogenesis and early symbiotic expression of PanNF-YA1 are PanNIN-dependent and that PanNF-YA1 is specifically required for intracellular rhizobium infection. This demonstrates that NIN and NF-YA1 have conserved symbiotic functions. As Parasponia and legumes diverged soon after the birth of the nodulation trait, we argue that NIN and NF-YA1 represent core	Parasponia a
677	plant	Parasponia andersonii (fast-growing tropical tree)	agroinfiltration; CRISPR;Cas9;		Journal of visualized experiments	Transforming, Genome Editing and Phenotyping the Nitrogen-fixing Tropical Cannabaceae Tree Parasponia andersonii.	2019	(150)	[Wardhani TAK et al.]	Wageningen University & Research, the Netherlands.	31475981	10.3791/59971	Parasponia andersonii is a fast-growing tropical tree that belongs to the Cannabis family (Cannabaceae). Together with 4 additional species, it forms the only known non-legume lineage able to establish a nitrogen-fixing nodule symbiosis with rhizobium. Comparative studies between legumes and P. andersonii could provide valuable insight into the genetic networks underlying root nodule formation. To facilitate comparative studies, we recently sequenced the P. andersonii genome and established Agrobacterium tumefaciens-mediated stable transformation and CRISPR/Cas9-based genome editing. Here, we provide a detailed description of the transformation and genome editing procedures developed for P. andersonii. In addition, we describe procedures for the seed germination and characterization of symbiotic phenotypes. Using this protocol, stable transgenic mutant lines can be generated in a period of 2-3 months. Vegetative in vitro propagation of T0 transgenic lines allows phenotyping experiments to be initiated at 4 months after A. tumefaciens co-cultivation. Therefore, this protocol takes only marginally longer than the transient Agrobacterium rhizogenes-based root transformation method available for P. andersonii, though offers several clear advantages. Together, the procedures described here permit P. andersonii to be used as a research model for studies aimed at understanding symbiotic associations as well as potentially other aspects of the biology of this tropical tree.	Parasponia a
678	plant	peanut	CRISPR;Cas9;	fatty acid desaturase (FAD2)	BMC biotechnology	Mutagenesis of FAD2 genes in peanut with CRISPR/Cas9 based gene editing.	2019	19(1):24	[Yuan M et al.]	Tuskegee University, Tuskegee, AL, USA.	31035982	10.1186/s12896-019-0516-8	BACKGROUND: Increasing the content of oleic acid in peanut seeds is one of the major goals in peanut breeding due to consumer and industry benefits, such as anti-oxidation and long shelf-life. Homeologous ahFAD2A and ahFAD2B genes encode fatty acid desaturases, which are the key enzymes for converting oleic acid to linoleic acid that oxidizes readily. To date, all high oleic acid peanut varieties result from natural mutations occurred in both genes. A method to induce mutations in the genes of other elite cultivars could speed introgression of this valuable trait. The gene-editing approach utilizing CRISPR/Cas9 technology was employed to induce de novo mutations in the ahFAD2 genes using peanut protoplasts and hairy root cultures as models. RESULTS: The hot spot of natural mutation in these genes was selected as the target region. Appropriate sgRNAs were designed and cloned into a CRISPR/Cas9 expression plasmid. As a result of CRISPR/Cas9 activity, three mutations were identified – G448A in ahFAD2A, and 441_442insA and G451T in ahFAD2B. The G448A and 441_442insA mutations are the same as those seen in existing high oleate varieties and the G451T is new mutation. Because natural mutations appear more often in the ahFAD2A gene than in the ahFAD2B gene in subspecies A. hypogaea var. hypogaea, the mutations induced in ahFAD2B by gene editing may be useful in developing high oleate lines with many genetic backgrounds after validation of oleic acid content in the transformed lines. The appearance of the G448A mutation in ahFAD2A is a further benefit for high oleic acid oil content. CONCLUSIONS: Overall, these results showed that mutations were, for the first time, induced by CRISPR-based gene editing approach in peanut. This research demonstrated the potential application of gene	peanut
679	plant	pear (Pyrus bretschneideri)	agroinfiltration; CRISPR;Cas9;	S-acyltransferase (PbPAT14)	International journal of molecular sciences	Knockout of the S-acyltransferase Gene, PbPAT14, Confers the Dwarf Yellowing Phenotype in First Generation Pear by ABA Accumulation.	2019	20(24)	[Pang H et al.]	Hebei Agricultural University, Baoding, China.	31888281	10.3390/ijms20246347	The development of dwarf fruit trees with smaller and compact characteristics leads to significantly increased fruit production, which is a major objective of pear (Pyrus bretschneideri) breeding. We identified the S-acylation activity of PbPAT14, an S-acyltransferase gene related to plant development, using a yeast (Saccharomyces cerevisiae) complementation assay, and also PbPAT14 could rescue the growth defect of the Arabidopsis mutant atpat14. We further studied the function of PbPAT14 by designing three guide RNAs for PbPAT14 to use in the CRISPR/Cas9 system. We obtained 22 positive transgenic pear lines via Agrobacterium-mediated transformation using cotyledons from seeds of Pyrus betulifolia ('Duli'). Six of these lines exhibited the dwarf yellowing phenotype and were homozygous mutations according to sequencing analysis. Ultrastructure analysis suggested that this dwarfism was manifested by shorter, thinner stems due to a reduction in cell number. A higher level of endogenous abscisic acid (ABA) and a higher transcript level of the ABA pathway genes in the mutant lines revealed that the PbPAT14 function was related to the ABA pathway. Overall, our experimental results increase the understanding of how PATs function in plants and help elucidate the mechanism of plant dwarfism.	pear (Pyrus

680	plant	pennycress (Thlapsi arvense L.)	agroinfiltration; CRISPR;Cas9;	FATTY ACID ELONGATION1 (FAE1)	Plant biotechnology journal	Molecular tools enabling pennycress (Thlaspi arvense) as a model plant and oilseed cash cover crop.	2019	17(4):776-788	[McGinn M et al.]	Illinois State University, Normal, IL, USA.	30230695	10.1111/pbi.13014	Thlapsi arvense L. (pennycress) is being developed as a profitable oilseed cover crop for the winter fallow period throughout the temperate regions of the world, controlling soil erosion and nutrients run-off on otherwise barren farmland. We demonstrate that pennycress can serve as a user-friendly model system akin to Arabidopsis that is well-suited for both laboratory and field experimentation. We sequenced the diploid genome of the spring-type Spring 32-10 inbred line (1C DNA content of 539 Mb; 2n = 14), identifying variation that may explain phenotypic differences with winter-type pennycress, as well as predominantly a one-to-one correspondence with Arabidopsis genes, which makes translational research straightforward. We developed an Agrobacterium-mediated floral dip transformation method (0.5% transformation efficiency) and introduced CRISPR-Cas9 constructs to produce indel mutations in the putative FATTY ACID ELONGATION1 (FAE1) gene, thereby abolishing erucic acid production and creating an edible seed oil comparable to that of canola. We also stably transformed pennycress with the Euonymus alatus diacylglycerol acetyltransferase (EaDAcT) gene, producing low-viscosity acetyl-triacylglycerol-containing seed oil suitable as a diesel-engine drop-in fuel. Adoption of pennycress as a model system will accelerate oilseed-crop translational research and facilitate pennycress' rapid domestication to meet the growing sustainable food and fuel demands.	pennycress (
681	plant	Physcomitrella patens	CRISPR;	BLADE-ON-PETIOLE	Journal of plant research	BLADE-ON-PETIOLE genes are not involved in the transition from protonema to gametophore in the moss Physcomitrella patens.	2019	132(5):617-627	[Hata Y et al.]	Tohoku University, Sendai, Miyagi, Japan.	31432295	10.1007/s10265-019-01132-8	The timing of the transition between developmental phases is a critical determinant of plant form. In the moss Physcomitrella patens, the transition from protonema to gametophore is a particularly important step as it results in a change from two-dimensional to three-dimensional growth of the plant body. It is well known that this transition is promoted by cytokinin (CK), however, the underlying mechanisms are poorly understood. Previously, it was reported that P. patens orthologs of BLADE-ON-PETIOLE (BOP) genes (PpBOPs) work downstream of CK to promote the transition to gametophore. To further understand the role of PpBOPs in the control of this transition, we performed functional analyses of PpBOP genes. We simultaneously disrupted the function of all three PpBOP genes in P. patens using CRISPR technology, however, no abnormal phenotypes were observed in the triple mutant during either the gametophytic or the sporophytic growth stages. CK treatment did not alter the phase change in the triple mutant. We conclude that PpBOP genes are unnecessary in the control of P. patens development under normal conditions. We propose that BOP genes are not involved in the control of developmental processes in bryophytes and other basal land plants, but may function in physiological processes such as in the defense	Physcomitre
682	plant	Physcomitrella patens	CRISPR;Cas9;	4 loci	Plant direct	Efficient and modular CRISPR-Cas9 vector system for Physcomitrella patens.	2019	3(9):e00168	[Mallett DR et al.]	Dartmouth College, Hanover, NH, USA.	31523744	10.1002/pld3.168	CRISPR-Cas9 has been shown to be a valuable tool in recent years, allowing researchers to precisely edit the genome using an RNA-guided nuclease to initiate double-strand breaks. Until recently, classical RAD51-mediated homologous recombination has been a powerful tool for gene targeting in the moss Physcomitrella patens. However, CRISPR-Cas9-mediated genome editing in P. patens was shown to be more efficient than traditional homologous recombination (Plant Biotechnology Journal, 15, 2017, 122). CRISPR-Cas9 provides the opportunity to efficiently edit the genome at multiple loci as well as integrate sequences at precise locations in the genome using a simple transient transformation. To fully take advantage of CRISPR-Cas9 genome editing in P. patens, here we describe the generation and use of a flexible and modular CRISPR-Cas9 vector system. Without the need for gene synthesis, this vector system enables editing of up to 12 loci simultaneously. Using this system, we generated multiple lines that had null alleles at four distant loci. We also found that targeting multiple sites within a single locus can produce larger deletions, but the success of this depends on individual protospacers. To take advantage of homology-directed repair, we developed modular vectors to rapidly generate DNA donor plasmids to efficiently introduce DNA sequences encoding for fluorescent proteins at the 5' and 3' ends of gene coding regions. With regard to homology-directed repair experiments, we found that if the protospacer sequence remains on the DNA donor plasmid, then Cas9 cleaves the plasmid target as well as the genomic target. This can reduce the efficiency of introducing sequences into the genome. Furthermore, to ensure the generation of a null allele near the Cas9 cleavage site, we generated a homology	Physcomitre
683	plant	Physcomitrella patens	CRISPR;Cpf1;		Plant journal	A CRISPR/LbCas12a-based method for highly efficient multiplex gene editing in Physcomitrella patens.	2019	100(4):863-872	[Pu X et al.]	Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China.	31350780	10.1111/tpj.14478	Due to their high efficiency, specificity, and flexibility, programmable nucleases, such as those of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas12a (Cpf1) system, have greatly expanded the applicability of editing the genomes of various organisms. Genes from different gene families or genes with redundant functions in the same gene family can be examined by assembling multiple CRISPR RNAs (crRNAs) in a single vector. However, the activity and efficiency of CRISPR/Cas12a in the non-vascular plant Physcomitrella patens are largely unknown. Here, we demonstrate that LbCas12a together with its mature crRNA can target multiple loci simultaneously in P. patens with high efficiency via co-delivery of LbCas12a and a crRNA expression cassette in vivo. The mutation frequencies induced by CRISPR/LbCas12a at a single locus ranged from 26.5 to 100%, with diverse deletions being the most common type of mutation. Our method expands the repertoire of genome editing tools available for P. patens and facilitates the creation of	physcomitrel

684	plant	pomegranate (Punica granatum L.)	CRISPR;Cas9;	UDP-dependent glycosyltransferase (PgUGT84A23; PgUGT84A24)	Horticulture research	Effective genome editing and identification of a regiospecific gallic acid 4-O-glycosyltransferase in pomegranate (Punica granatum L.).	2019	6:123	[Chang L et al.]	Shanghai Chenshan Botanical Garden, Shanghai, China.	31728198	10.1038/s41438-019-0206-7	Pomegranate (Punica granatum L.) trees are woody perennials that bear colorful and nutritious fruits rich in phenolic metabolites, e.g., hydrolyzable tannins (HTs) and flavonoids. We here report genome editing and gene discovery in pomegranate hairy roots using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9), coupled with transcriptome and biochemical analyses. Single guide RNAs (sgRNAs) were designed to target two UDP-dependent glycosyltransferases (UGTs), PgUGT84A23 and PgUGT84A24, which possess overlapping activities in beta-glucogallin (a galloylglucose ester; biosynthetic precursor of HTs) biosynthesis. A unique accumulation of gallic acid 3-O- and 4-O-glucosides (galloylglucose ethers) was observed in the PgUGT84A23 and PgUGT84A24 dual CRISPR/Cas9-edited lines (i.e., ugt84a23 ugt84a24) but not the control (empty vector) or PgUGT84A23/PgUGT84A24 single edited lines (ugt84a23 or ugt84a24). Transcriptome and real-time qPCR analyses identified 11 UGTs with increased expression in the ugt84a23 ugt84a24 hairy roots compared to the controls. Of the 11 candidate UGTs, only PgUGT72BD1 used gallic acid as substrate and produced a regiospecific product gallic acid 4-O-glucoside. This work demonstrates that the CRISPR/Cas9 method can facilitate functional genomics studies in pomegranate and shows promise for capitalizing on the metabolic potential of	pomegranate
685	plant	poplar (hybrid poplar Populus Alba x Populus glandulosa Uyeki)	agroinfiltration	beta-glucuronidase (GUS)	International journal of molecular sciences	Efficient Agrobacterium-Mediated Transformation of the Commercial Hybrid Poplar Populus Alba x Populus glandulosa Uyeki.	2019	20(10)	[Song C et al.]	Beijing Forestry University, Beijing, China.	31137806	10.3390/ijms20102594	Transgenic technology is a powerful tool for gene functional characterization, and poplar is a model system for genetic transformation of perennial woody plants. However, the poplar genetic transformation system is limited to a number of model genotypes. Herein, we developed a transformation system based on efficient Agrobacterium-mediated transformation for the hybrid poplar Populus Alba x Populus glandulosa Uyeki, which is a fast-growing poplar species that is suitably grown in the northern part of China. Importantly, we optimized many independent factors and showed that the transformation efficiency was improved significantly using juvenile leaf explants. Explants were infected by an Agrobacterium suspension with the OD600 = 0.6 for 15 min and then co-cultured in dark conditions for 3 days. Using the improved transformation system, we obtained the transgenic poplar with overexpression of beta-glucuronidase (GUS) via direct organogenesis without callus induction. Furthermore, we analyzed the GUS gene in the transgenic poplars using PCR, qRT-PCR, and GUS staining. These analyses revealed that the GUS gene was efficiently transformed, and it exhibited various expression levels. Taken together, these results represent a simple, fast, and efficient transformation system of hybrid poplar plants. Our findings may facilitate future studies of gene functions in perennial woody plants and tree breeding	Populus
686	plant	Populus	CRISPR;Cas9;	PdGNC	Journal of experimental botany	A GATA transcription factor PdGNC plays an important role in photosynthesis and growth in Populus.	2019		[An Y et al.]	Beijing Forestry University, Beijing, China.	31872214	10.1093/jxb/erz564	GATA transcription factors are involved in regulation of diverse growth processes and environmental responses in Arabidopsis and rice. In this study, we conducted a comprehensive bioinformatic survey of the GATA family in the woody perennial Populus. Thirty-nine Populus GATA genes were classified into four subfamilies based on gene structure and phylogenetic relationships. Predicted cis-elements suggested potential roles of Populus GATA genes in light, phytohormone, development, and stress responses. A Populus GATA gene, PdGATA19/PdGNC (GATA nitrate-inducible carbon-metabolism-involved), was identified from a rapid-growing Populus clone. PdGNC expression was significantly up-regulated in leaves under both high (50 mM NO3-) and low (0.2 mM NO3-) nitrate concentrations. The CRISPR/Cas9-mediated mutant crispr-GNC showed severely retarded growth and enhanced secondary xylem differentiation. PdGNC-overexpressing transformants exhibited faster growth, higher biomass accumulation, and increases in chlorophyll content, photosynthetic rate, and plant height by 25%-30%, 20%-28%, and ~25%, respectively, compared with the wild type. Transcriptomic analysis showed that PdGNC was involved in photosynthetic electron transfer and carbon assimilation in the leaf, cell division and carbohydrate utilization in the stem, and nitrogen uptake in the root. These data indicated that PdGNC plays a crucial role in plant growth and is potentially useful in tree molecular breeding.	Populus
687	plant	Populus	CRISPR;Cas9;	UDP-glucose-dependent glycosyltransferase71L1	Plant journal	Discovery of salicyl benzoate UDP-glycosyltransferase, a central enzyme in poplar salicinoid phenolic glycoside biosynthesis.	2019		[Fellenberg C et al.]	University of Victoria, Victoria, British Columbia, Canada.	31736216	10.1111/tpj.14615	The salicinoids are anti-herbivore phenolic glycosides unique to the Salicaceae (Populus and Salix). They consist of a salicyl alcohol glucoside core, which is usually further acylated with benzoic, cinnamic or phenolic acids. While salicinoid structures are well known, their biosynthesis remains enigmatic. Recently, two enzymes from poplar, salicyl alcohol benzoyl transferase and benzyl alcohol benzoyl transferase, were shown to catalyze the production of salicyl benzoate, a predicted potential intermediate in salicinoid biosynthesis. Here, we used transcriptomics and co-expression analysis with these two genes to identify two UDP-glucose-dependent glycosyltransferases (UGT71L1 and UGT78M1) as candidate enzymes in this pathway. Both recombinant enzymes accepted only salicyl benzoate, salicylaldehyde and 2-hydroxycinnamic acid as glucose acceptors. Knocking out the UGT71L1 gene by CRISPR/Cas9 in poplar hairy root cultures led to the complete loss of salicortin, tremulacin and tremuloidin, and a partial reduction of salicin content. This demonstrated that UGT71L1 is required for synthesis of the major salicinoids, and suggested that an additional route can lead to salicin. CRISPR/Cas9 knockouts for UGT78M1 were not successful, and its in vivo role thus remains to be determined. Although it has a similar substrate preference and predicted structure as UGT71L1, it appears not to contribute to the synthesis of salicortin, tremulacin and tremuloidin, at least in roots. The demonstration of UGT71L1 as an enzyme of salicinoid biosynthesis will open up new avenues for the elucidation	Populus

688	plant	Populus trichocarpa	CRISPR;	AREB1; ADA2b; GCN5	Plant cell	The AREB1 Transcription Factor Influences Histone Acetylation to Regulate Drought Responses and Tolerance in Populus trichocarpa.	2019	31(3):663-686	[Li S et al.]	Northeast Forestry University, Harbin, China.	30538157	10.1105/tpc.18.00437	Plants develop tolerance to drought by activating genes with altered levels of epigenetic modifications. Specific transcription factors are involved in this activation, but the molecular connections within the regulatory system are unclear. Here, we analyzed genome-wide acetylated lysine residue 9 of histone H3 (H3K9ac) enrichment and examined its association with transcriptomes in Populus trichocarpa under drought stress. We revealed that abscisic acid-Responsive Element (ABRE) motifs in promoters of the drought-responsive genes PtrNAC006, PtrNAC007, and PtrNAC120 are involved in H3K9ac enhancement and activation of these genes. Overexpressing these PtrNAC genes in P trichocarpa resulted in strong drought-tolerance phenotypes. We showed that the ABRE binding protein PtrAREB1-2 binds to ABRE motifs associated with these PtrNAC genes and recruits the histone acetyltransferase unit ADA2b-GCN5, forming AREB1-ADA2b-GCN5 ternary protein complexes. Moreover, this recruitment enables GCN5-mediated histone acetylation to enhance H3K9ac and enrich RNA polymerase II specifically at these PtrNAC genes for the development of drought tolerance. CRISPR editing or RNA interference-mediated downregulation of any of the ternary members results in highly drought-sensitive P trichocarpa Thus, the combinatorial function of the ternary proteins establishes a coordinated histone acetylation and transcription factor-mediated gene activation for drought response	Populus
689	plant	Populus x canescens; Populus tremula	agroinfiltration; CRISPR;Cas9;	twelve genes, including SOC1, FUL, and their paralogous genes, four NFP-like genes and TOZ19	International journal of molecular sciences	Evaluating the Efficiency of gRNAs in CRISPR/Cas9 Mediated Genome Editing in Poplars.	2019	20(15)	[Bruegmann T et al.]	Thuenen Institute of Forest Genetics, Grosshansdorf, Germany.	31344908	10.3390/ijms20153623	CRISPR/Cas9 has become one of the most promising techniques for genome editing in plants and works very well in poplars with an Agrobacterium-mediated transformation system. We selected twelve genes, including SOC1, FUL, and their paralogous genes, four NFP-like genes and TOZ19 for three different research topics. The gRNAs were designed for editing, and, together with a constitutively expressed Cas9 nuclease, transferred either into the poplar hybrid Populus x canescens or into P. tremula. The regenerated lines showed different types of editing and revealed several homozygous editing events which are of special interest in perennial species because of limited back-cross ability. Through a time series, we could show that despite the constitutive expression of the Cas9 nuclease, no secondary editing of the target region occurred. Thus, constitutive Cas9 expression does not seem to pose any risk to additional editing events. Based on various criteria, we obtained evidence for a relationship between the structure of gRNA and the efficiency of gene editing. In particular, the GC content, purine residues in the gRNA end, and the free accessibility of the seed region seemed to be highly important for genome editing in poplars. Based on our findings on nine different poplar genes, efficient gRNAs can be designed for future efficient editing	Populus
690	plant	potato	CRISPR;Cas9;	coilin	Doklady. Biochemistry and biophysics	Functional Analysis of Coilin in Virus Resistance and Stress Tolerance of Potato Solanum tuberosum using CRISPR-Cas9 Editing.	2019	484(1):88-91	[Makhotenko AV et al.]	Doka Gene Technologies Ltd, Moscow oblast, Russia.	31012023	10.1134/S1607672919010241	The role of the nuclear protein coilin in the mechanisms of resistance of potato Solanum tuberosum cultivar Chicago to biotic and abiotic stresses was studied using the CRISPR-Cas9 technology. For the coilin gene editing, a complex consisting of the Cas9 endonuclease and a short guide RNA was immobilized on gold or chitosan microparticles and delivered into apical meristem cells by bioballistics or vacuum infiltration methods, respectively. Editing at least one allele of the coilin gene considerably increased the resistance of the edited lines to infection with the potato virus Y and their tolerance to salt and osmotic stress.	potato
691	plant	potato	CRISPR;Cas9;	S-RNase (S-locus RNase)	Frontiers in plant science	Overcoming Self-Incompatibility in Diploid Potato Using CRISPR-Cas9.	2019	10:376	[Enciso-Rodriguez F et al.]	Michigan State University, East Lansing, MI, USA.	31001300	10.3389/fpls.2019.00376	Potato breeding can be redirected to a diploid inbred/F1 hybrid variety breeding strategy if self-compatibility can be introduced into diploid germplasm. However, the majority of diploid potato clones (Solanum spp.) possess gametophytic self-incompatibility that is primarily controlled by a single multiallelic locus called the S-locus which is composed of tightly linked genes, S-RNase (S-locus RNase) and multiple SLFs (S-locus F-box proteins), which are expressed in the style and pollen, respectively. Using S-RNase genes known to function in the Solanaceae gametophytic SI mechanism, we identified S-RNase alleles with flower-specific expression in two diploid self-incompatible potato lines using genome resequencing data. Consistent with the location of the S-locus in potato, we genetically mapped the S-RNase gene using a segregating population to a region of low recombination within the pericentromere of chromosome 1. To generate self-compatible diploid potato lines, a dual single-guide RNA (sgRNA) strategy was used to target conserved exonic regions of the S-RNase gene and generate targeted knockouts (KOs) using a Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (Cas9) approach. Self-compatibility was achieved in nine S-RNase KO T0 lines which contained bi-allelic and homozygous deletions/insertions in both genotypes, transmitting self compatibility to T1 progeny. This study demonstrates an efficient approach to achieve stable,	potato
692	plant	potato	CRISPR;Cas9;Cpf1;TALENs;	Acetolactate synthase1 (ALS1); 5-Enolpyruvylshikimate-3-phosphate synthase1 (EPSPS1); their paralogs	Frontiers in plant science	Evaluation of Methods to Assess in vivo Activity of Engineered Genome-Editing Nucleases in Protoplasts.	2019	10:110	[Nadakuduti SS et al.]	Michigan State University, East Lansing, MI, USA.	30800139	10.3389/fpls.2019.00110	Genome-editing is being implemented in increasing number of plant species using engineered sequence specific nucleases (SSNs) such as Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated systems (CRISPR/Cas9), Transcription activator like effector nucleases (TALENs), and more recently CRISPR/Cas12a. As the tissue culture and regeneration procedures to generate gene-edited events are time consuming, large-scale screening methodologies that rapidly facilitate validation of genome-editing reagents are critical. Plant protoplast cells provide a rapid platform to validate genome-editing reagents. Protoplast transfection with plasmids expressing genome-editing reagents represents an efficient and cost-effective method to screen for in vivo activity of genome-editing constructs and resulting targeted mutagenesis. In this study, we compared three existing methods for detection of editing activity, the T7 endonuclease I assay (T7EI), PCR/restriction enzyme (PCR/RE) digestion, and amplicon-sequencing, with an alternative method which involves tagging a double-stranded oligodeoxynucleotide (dsODN) into the SSN-induced double stranded break and detection of on-target activity of gene-editing reagents by PCR and agarose gel electrophoresis. To validate these methods, multiple reagents including TALENs, CRISPR/Cas9 and Cas9 variants, eCas9(1.1) (enhanced specificity) and Cas9-HF1 (high-fidelity1) were engineered for targeted mutagenesis of Acetolactate synthase1 (ALS1), 5-Enolpyruvylshikimate- 3-phosphate synthase1 (EPSPS1) and their paralogs in potato. While all methods detected editing activity, the PCR detection of dsODN integration provided the most straightforward and easiest method to assess on-target activity of the SSN as well as a method for initial qualitative evaluation of the functionality of genome-editing constructs. Quantitative data on mutagenesis frequencies obtained by amplicon-sequencing of ALS1 revealed that the mutagenesis frequency of CRISPR/Cas9 reagents is better than TALENs. Context-based choice of method for evaluation of gene-editing reagents in protoplast	potato

693	plant	potato	agroinfiltration; TALENs	SSR2	Plant biotechnology	Efficient genome engineering using Platinum TALEN in potato.	2019	36(3):167–173	[Yasumoto S et al.]	Osaka University, Suita, Osaka, Japan.	31768118	10.5511/plantbiotechnology.19.0805a	Potato (Solanum tuberosum) is one of the most important crops in the world. However, it is generally difficult to breed a new variety of potato crops because they are highly heterozygous tetraploid. Steroidal glycoalkaloids (SGAs) such as alpha-solanine and alpha-chaconine found in potato are antinutritional specialized metabolites. Because of their toxicity following intake, controlling the SGA levels in potato varieties is critical in breeding programs. Recently, genome-editing technologies using artificial site-specific nucleases such as TALEN and CRISPR-Cas9 have been developed and used in plant sciences. In the present study, we developed a highly active Platinum TALEN expression vector construction system, and applied to reduce the SGA contents in potato. Using Agrobacterium-mediated transformation, we obtained three independent transgenic potatoes harboring the TALEN expression cassette targeting SSR2 gene, which encodes a key enzyme for SGA biosynthesis. Sequencing analysis of the target sequence indicated that all the transformants could be SSR2-knockout mutants. Reduced SGA phenotype in the mutants was confirmed by metabolic analysis using LC-MS. In vitro grown SSR2-knockout mutants exhibited no differences in morphological phenotype or yields when compared with control plants, indicating that the genome editing of SGA biosynthetic genes such as SSR2 could be a suitable strategy for controlling the levels of toxic metabolites in potato. Our simple and powerful plant genome-editing system, developed in the present study, provides an	potato
694	plant	potato	agroinfiltration; Cas9;	starch-branching enzymes (SBE1; SBE2)	Plant biotechnology journal	Cas9-mediated mutagenesis of potato starch-branching enzymes generates a range of tuber starch phenotypes.	2019	17(12):2259–2271	[Tuncel A et al.]	John Innes Centre, Norwich, UK.	31033104	10.1111/pbi.13137	We investigated whether Cas9-mediated mutagenesis of starch-branching enzymes (SBEs) in tetraploid potatoes could generate tuber starches with a range of distinct properties. Constructs containing the Cas9 gene and sgRNAs targeting SBE1, SBE2 or both genes were introduced by Agrobacterium-mediated transformation or by PEG-mediated delivery into protoplasts. Outcomes included lines with mutations in all or only some of the homoeoalleles of SBE genes and lines in which homoeoalleles carried several different mutations. DNA delivery into protoplasts resulted in mutants with no detectable Cas9 gene, suggesting the absence of foreign DNA. Selected mutants with starch granule abnormalities had reductions in tuber SBE1 and/or SBE2 protein that were broadly in line with expectations from genotype analysis. Strong reduction in both SBE isoforms created an extreme starch phenotype, as reported previously for low-SBE potato tubers. HPLC-SEC and (1) H NMR revealed a decrease in short amylopectin chains, an increase in long chains and a large reduction in branching frequency relative to wild-type starch. Mutants with strong reductions in SBE2 protein alone had near-normal amylopectin chain-length distributions and only small reductions in branching frequency. However, starch granule initiation was enormously increased: cells contained many granules of <4 mum and granules with multiple hila. Thus, large reductions in both SBEs reduce amylopectin branching during granule growth, whereas reduction in SBE2 alone primarily affects numbers of starch granule initiations. Our results demonstrate that Cas9-mediated mutagenesis of SBE genes has the potential to generate new, potentially valuable starch properties without	potato
695	plant	potato	CRISPR;		Plant biotechnology journal	Generation of virus-resistant potato plants by RNA genome targeting.	2019	17(9):1814–1822	[Zhan X et al.]	Hubei University, Wuhan, China.	30803101	10.1111/pbi.13102	CRISPR/Cas systems provide bacteria and archaea with molecular immunity against invading phages and foreign plasmids. The class 2 type VI CRISPR/Cas effector Cas13a is an RNA-targeting CRISPR effector that provides protection against RNA phages. Here we report the repurposing of CRISPR/Cas13a to protect potato plants from a eukaryotic virus, Potato virus Y (PVY). Transgenic potato lines expressing Cas13a/sgRNA (small guide RNA) constructs showed suppressed PVY accumulation and disease symptoms. The levels of viral resistance correlated with the expression levels of the Cas13a/sgRNA construct in the plants. Our data further demonstrate that appropriately designed sgRNAs can specifically interfere with multiple PVY strains, while having no effect on unrelated viruses such as PVA or Potato virus S. Our findings provide a novel and highly efficient strategy for engineering crops with	potato
696	plant	potato	CRISPR;Cas9;	granular bound starch synthase gene	Scientific reports	High efficacy full allelic CRISPR/Cas9 gene editing in tetraploid potato.	2019	9(1):17715	[Johansen IE et al.]	University of Copenhagen, Frederiksberg, Denmark.	31776399	10.1038/s41598-019-54126-w	CRISPR/Cas9 editing efficacies in tetraploid potato were highly improved through the use of endogenous potato U6 promoters. Highly increased editing efficiencies in the Granular Bound Starch Synthase gene at the protoplast level were obtained by replacement of the Arabidopsis U6 promotor, driving expression of the CRISPR component, with endogenous potato U6 promotors. This translated at the ex-plant level into 35% full allelic gene editing. Indel Detection Amplicon Analysis was established as an efficient tool for fast assessment of gene editing in complex genomes, such as potato. Together, this warrants significant reduction of laborious cell culturing, ex-plant regeneration and screening procedures of plants with high complexity genomes.	potato
697	plant	potato (Solanum tuberosum)	CRISPR;Cas9;	GBSSI	Plant cell reports	The Solanum tuberosum GBSSI gene: a target for assessing gene and base editing in tetraploid potato.	2019	38(9):1065–1080	[Veillet F et al.]	INRA, Universite Rennes 1, Ploudaniel, France.	31101972	10.1007/s00299-019-02426-w	KEY MESSAGE: The StGBSSI gene was successfully and precisely edited in the tetraploid potato using gene and base-editing strategies, leading to plants with impaired amylose biosynthesis. Genome editing has recently become a method of choice for basic research and functional genomics, and holds great potential for molecular plant-breeding applications. The powerful CRISPR-Cas9 system that typically produces double-strand DNA breaks is mainly used to generate knockout mutants. Recently, the development of base editors has broadened the scope of genome editing, allowing precise and efficient nucleotide substitutions. In this study, we produced mutants in two cultivated elite cultivars of the tetraploid potato (Solanum tuberosum) using stable or transient expression of the CRISPR-Cas9 components to knock out the amylose-producing StGBSSI gene. We set up a rapid, highly sensitive and cost-effective screening strategy based on high-resolution melting analysis followed by direct Sanger sequencing and trace chromatogram analysis. Most mutations consisted of small indels, but unwanted insertions of plasmid DNA were also observed. We successfully created tetra-allelic mutants with impaired amylose biosynthesis, confirming the loss of function of the StGBSSI protein. The second main objective of this work was to demonstrate the proof of concept of CRISPR-Cas9 base editing in the tetraploid potato by targeting two loci encoding catalytic motifs of the StGBSSI enzyme. Using a cytidine base editor (CBE), we efficiently and precisely induced DNA substitutions in the KTGGL-encoding locus, leading to discrete variation in the amino acid sequence and generating a loss-of-function allele. The successful application of	potato (Sola

698	plant	potato (Solanum tubersoum L.)	CRISPR;Cas9;	StPPO2	Frontiers in plant science	Reduced Enzymatic Browning in Potato Tubers by Specific Editing of a Polyphenol Oxidase Gene via Ribonucleoprotein Complexes Delivery of the CRISPR/Cas9 System.	2019	10:1649	[Gonzalez MN et al.]	Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET), Buenos Aires, Argentina.	31998338	10.3389/fpls.2019.01649	Polyphenol Oxidases (PPOs) catalyze the conversion of phenolic substrates to quinones, leading to the formation of dark-colored precipitates in fruits and vegetables. This process, known as enzymatic browning, is the cause of undesirable changes in organoleptic properties and the loss of nutritional quality in plant-derived products. In potato (Solanum tubersoum L.), PPOs are encoded by a multi-gene family with different expression patterns. Here, we have studied the application of the CRISPR/Cas9 system to induce mutations in the StPPO2 gene in the tetraploid cultivar Desiree. We hypothesized that the specific editing of this target gene would result in a lower PPO activity in the tuber with the consequent reduction of the enzymatic browning. Ribonucleoprotein complexes (RNPs), formed by two sgRNAs and Cas9 nuclease, were transfected to potato protoplasts. Up to 68% of regenerated plants contained mutations in at least one allele of the target gene, while 24% of edited lines carried mutations in all four alleles. No off-target mutations were identified in other analyzed StPPO genes. Mutations induced in the four alleles of StPPO2 gene, led to lines with a reduction of up to 69% in tuber PPO activity and a reduction of 73% in enzymatic browning, compared to the control. Our results demonstrate that the CRISPR/Cas9 system can be applied to develop potato varieties with reduced enzymatic browning in tubers, by	potato (Sola
699	plant	pumpkin	CRISPR;Cas9;	respiratory burst oxidase homolog D (RBOHD)	Journal of experimental botany	Tissue-specific respiratory burst oxidase homolog-dependent H2O2 signaling to the plasma membrane H+-ATPase confers potassium uptake and salinity tolerance in Cucurbitaceae.	2019	70(20):5879-5893	[Huang Y et al.]	Huazhong Agricultural University and Key Laboratory of Horticultural Plant Biology, Ministry of Education, Wuhan, China.	31290978	10.1093/jxb/erz328	Potassium (K+) is a critical determinant of salinity tolerance, and H2O2 has been recognized as an important signaling molecule that mediates many physiological responses. However, the details of how H2O2 signaling regulates K+ uptake in the root under salt stress remain elusive. In this study, salt-sensitive cucumber and salt-tolerant pumpkin which belong to the same family, Cucurbitaceae, were used to answer the above question. We show that higher salt tolerance in pumpkin was related to its superior ability for K+ uptake and higher H2O2 accumulation in the root apex. Transcriptome analysis showed that salinity induced 5816 (3005 up- and 2811 down-) and 4679 (3965 up- and 714 down-) differentially expressed genes (DEGs) in cucumber and pumpkin, respectively. DEGs encoding NADPH oxidase (respiratory burst oxidase homolog D; RBOHD), 14-3-3 protein (GRF12), plasma membrane H+-ATPase (AHA1), and potassium transporter (HAK5) showed higher expression in pumpkin than in cucumber under salinity stress. Treatment with the NADPH oxidase inhibitor diphenylene iodonium resulted in lower RBOHD, GRF12, AHA1, and HAK5 expression, reduced plasma membrane H+-ATPase activity, and lower K+ uptake, leading to a loss of the salinity tolerance trait in pumpkin. The opposite results were obtained when the plants were pre-treated with exogenous H2O2. Knocking out of RBOHD in pumpkin by CRISPR/Cas9 [clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9] editing of coding sequences resulted in lower root apex H2O2 and K+ content and GRF12, AHA1, and HAK5 expression, ultimately resulting in a salt-sensitive phenotype. However, ectopic expression of pumpkin RBOHD in Arabidopsis led to the opposite effect. Taken together, this study shows that RBOHD-dependent H2O2 signaling in the root apex is important for pumpkin salt tolerance and suggests a novel mechanism that confers this trait, namely RBOHD-mediated transcriptional and post-translational activation of plasma membrane H+-	pumpkin
700	plant	red rice	CRISPR;Cas9;	red coloration gene (Rc)	Plant biotechnology journal	CRISPR/Cas9-mediated functional recovery of the recessive rc allele to develop red rice.	2019	17(11):2096-2105	[Zhu Y et al.]	Xiamen University, Xiamen, China.	31002444	10.1111/pbi.13125	Red rice contains high levels of proanthocyanidins and anthocyanins, which have been recognized as health-promoting nutrients. The red coloration of rice grains is controlled by two complementary genes, Rc and Rd. The RcRd genotype produces red pericarp in wild species Oryza rufipogon, whereas most cultivated rice varieties produce white grains resulted from a 14-bp frame-shift deletion in the seventh exon of the Rc gene. In the present study, we developed a CRISPR/Cas9-mediated method to functionally restore the recessive rc allele through reverting the 14-bp frame-shift deletion to in-frame mutations in which the deletions were in multiples of three bases, and successfully converted three elite white pericarp rice varieties into red ones. Rice seeds from T1 in-frame Rc lines were measured for proanthocyanidins and anthocyanidins, and high accumulation levels of proanthocyanidins and anthocyanidins were observed in red grains from the mutants. Moreover, there was no significant difference between wild-type and in-frame Rc mutants in major agronomic traits, indicating that restoration of Rc function had no negative effect on important agronomic traits in rice. Given that most white pericarp rice varieties are resulted from the 14-bp deletion in Rc, it is conceivable that our method could be applied to most white pericarp rice varieties and would greatly accelerate the breeding of new red rice varieties with elite agronomic traits. In addition, our study demonstrates an effective	rice
701	plant	rice	CRISPR;Cas9;	proline-rich protein (OsPRP1)	3 Biotech	Knockout of OsPRP1, a gene encoding proline-rich protein, confers enhanced cold sensitivity in rice (Oryza sativa L.) at the seedling stage.	2019	9(7):254	[Nawaz G et al.]	Guangxi University, Nanning, China.	31192079	10.1007/s13205-019-1787-4	Proline-rich proteins (PRPs) play multiple physiological and biochemical roles in plant growth and stress response. In this study, we reported that the knockout of OsPRP1 induced cold sensitivity in rice. Mutant plants were generated by CRISPR/Cas9 technology to investigate the role of OsPRP1 in cold stress and 26 mutant plants were obtained in T0 generation with the mutation rate of 85% including 15% bi-allelic, 53.3% homozygous, and 16.7% heterozygous and 16 T-DNA-free lines in T1 generation. The conserved amino acid sequence was changed and the expression level of OsPRP1 was reduced in mutant plants. The OsPRP1 mutant plants displayed more sensitivity to cold stress and showed low survival rate with decreased root biomass than wild-type (WT) and homozygous mutant line with large fragment deletion was more sensitive to low temperature. Mutant lines accumulated less antioxidant enzyme activity and lower levels of proline, chlorophyll, abscisic acid (ABA), and ascorbic acid (AsA) content relative to WT under low-temperature stress. The changes of antioxidant enzymes were examined in the leaves and roots with exogenous salicylic acid (SA) treatment which resulted in increased activity of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) under cold stress, while enzyme antioxidant activity was lower in untreated seedlings which showed that exogenous SA pretreatment could alleviate the low-temperature stress in rice. Furthermore, the expression of three genes encoding antioxidant enzyme activities (SOD4, POX1, and OsCAT3) was significantly down-regulated in the mutant lines as compared to WT. These results suggested that OsPRP1 enhances cold tolerance by modulating antioxidants and maintaining cross talk through signaling pathways. Therefore, OsPRP1 gene could be exploited for improving cold tolerance in rice and CRISPR/Cas9 technology is helpful to study the function of	rice

702	plant	rice	CRISPR;Cas9;	3 loci	3 Biotech	Dual-targeting by CRISPR/Cas9 leads to efficient point mutagenesis but only rare targeted deletions in the rice genome.	2019	9(4):158	[Pathak B et al.]	University of Arkansas, Fayetteville, AR, USA.	30944805	10.1007/s13205-019-1690-z	The present study investigated the efficiency of CRISPR/Cas9 in creating genomic deletions as the basis of its application in removing selection marker genes or the intergenic regions. Three loci, representing a transgene and two rice genes, were targeted at two sites each, in separate experiments, and the deletion of the defined fragments was investigated by PCR and sequencing. Genomic deletions were found at a low rate among the transformed callus lines that could be isolated, cultured, and regenerated into plants harboring the deletion. However, randomly regenerated plants showed mixed genomic effects, and generally did not harbor heritable genomic deletions. To determine whether point mutations occurred at each targeted site, a total of 114 plants consisting of primary transgenic lines and their progeny were analyzed. Ninety-three plants showed targeting, 60 of which were targeted at both sites. The presence of point mutations at both sites was correlated with the guide RNA efficiency. In summary, genomic deletions through dual-targeting by the paired-guide RNAs were generally observed in callus, while de novo point mutations at one or both sites occurred at high rates in transgenic plants and their progeny, generating a variety of insertion-deletions or single-nucleotide variations. In this study, point mutations were exceedingly favored over genomic deletions; therefore, for the recovery of plant lines harboring targeted deletions, identifying early transformed clones harboring the	rice
703	plant	rice	CRISPR;Cas9;	FLN2	Biomolecules	The Tolerance of Salinity in Rice Requires the Presence of a Functional Copy of FLN2.	2019	10(1)	[Chen G et al.]	China National Rice Research Institute, Hangzhou, China.	31877655	10.3390/biom10010017	A panel of ethane-methyl-sulfonate-mutagenized japonica rice lines was grown in the presence of salinity in order to identify genes required for the expression of salinity tolerance. A highly nontolerant selection proved to harbor a mutation in FLN2, a gene which encodes fructokinase-like protein2. Exposure of wild-type rice to salinity up-regulated FLN2, while a CRISPR/Cas9-generated FLN2 knockout line was hypersensitive to the stress. Both ribulose 1,5-bisphosphate carboxylase/oxygenase activity and the abundance of the transcript generated by a number of genes encoding components of sucrose synthesis were lower in the knockout line than in wild-type plants' leaves, while the sucrose contents of the leaf and root were, respectively, markedly increased and decreased. That sugar partitioning to the roots was impaired in FLN2 knockout plants was confirmed by the observation that several genes involved in carbon transport were down-regulated in both the leaf and in the leaf sheath. The levels of sucrose synthase, acid invertase, and neutral invertase activity were distinctly lower in the knockout plants' roots than in those of wild-type plants, particularly when the plants were exposed to salinity stress. The compromised salinity tolerance exhibited by the FLN2 knockout plants was likely a consequence of an inadequate supply of the assimilate required to support growth, a problem which was rectifiable by providing an exogenous supply of sucrose. The conclusion was that FLN2, on account of its influence over sugar metabolism, is important in the context of seedling growth	rice
704	plant	rice	CRISPR;Cas9;		BMC plant biology	Multiplex nucleotide editing by high-fidelity Cas9 variants with improved efficiency in rice.	2019	19(1):511	[Xu W et al.]	Beijing Academy of Agriculture & Forestry Sciences, Beijing, China.	31752697	10.1186/s12870-019-2131-1	BACKGROUND: Application of the CRISPR/Cas9 system or its derived base editors enables targeted genome modification, thereby providing a programmable tool to exploit gene functions and to improve crop traits. RESULTS: We report that PmCDA1 is much more efficient than rAPOBEC1 when fused to CRISPR/Cas9 nickase for the conversion of cytosine (C) to thymine (T) in rice. Three high-fidelity SpCas9 variants, eSpCas9(1.1), SpCas9-HF2 and HypaCas9, were engineered to serve with PmCDA1 (pBEs) as C-to-T base editors. These three high-fidelity editors had distinct multiplex-genome editing efficiencies. To substantially improve their base-editing efficiencies, a tandemly arrayed tRNA-modified single guide RNA (sgRNA) architecture was applied. The efficiency of eSpCas9(1.1)-pBE was enhanced up to 25.5-fold with an acceptable off-target effect. Moreover, two- to five-fold improvement was observed for knock-out mutation frequency by these high-fidelity Cas9s under the direction of the tRNA-modified sgRNA architecture. CONCLUSIONS: We have engineered a diverse toolkit for efficient and precise genome engineering in rice, thus making genome editing for plant research and crop improvement more flexible.	rice
705	plant	rice	CRISPR;Cas9;	prohibitin complex 2alpha subunit (NAL8)	BMC plant biology	NAL8 encodes a prohibitin that contributes to leaf and spikelet development by regulating mitochondria and chloroplasts stability in rice.	2019	19(1):395	[Chen K et al.]	Shanghai Institute for Biological Sciences, Chinese Academic of Sciences, Shanghai, China.	31510917	10.1186/s12870-019-2007-4	BACKGROUND: Leaf morphology and spikelet number are two important traits associated with grain yield. To understand how genes coordinating with sink and sources of cereal crops is important for grain yield improvement guidance. Although many researches focus on leaf morphology or grain number in rice, the regulating molecular mechanisms are still unclear. RESULTS: In this study, we identified a prohibitin complex 2alpha subunit, NAL8, that contributes to multiple developmental process and is required for normal leaf width and spikelet number at the reproductive stage in rice. These results were consistent with the ubiquitous expression pattern of NAL8 gene. We used genetic complementation, CRISPR/Cas9 gene editing system, RNAi gene silenced system and overexpressing system to generate transgenic plants for confirming the fuctions of NAL8. Mutation of NAL8 causes a reduction in the number of plastoglobules and shrunken thylakoids in chloroplasts, resulting in reduced cell division. In addition, the auxin levels in nal8 mutants are higher than in TQ, while the cytokinin levels are lower than in TQ. Moreover, RNA-sequencing and proteomics analysis shows that NAL8 is involved in multiple hormone signaling pathways as well as photosynthesis in chloroplasts and respiration in mitochondria. CONCLUSIONS: Our findings provide new insights into the way that NAL8 functions as a molecular chaperone in regulating plant leaf morphology and spikelet number through its effects	rice

706	plant	rice	CRISPR;Cas9;	Lysine/Histidine transporter (OsLHT1)	BMC plant biology	Disruption of an amino acid transporter LHT1 leads to growth inhibition and low yields in rice.	2019	19(1):268	[Wang X et al.]	Guangxi University, Nanning, China.	31221084	10.1186/s12870-019-1885-9	BACKGROUND: Research on plant amino acid transporters was mainly performed in Arabidopsis, while our understanding of them is generally scant in rice. OsLHT1 (Lysine/Histidine transporter) has been previously reported as a histidine transporter in yeast, but its substrate profile and function in planta are unclear. The aims of this study are to analyze the substrate selectivity of OsLHT1 and influence of its disruption on rice growth and fecundity. RESULTS: Substrate selectivity of OsLHT1 was analyzed in Xenopus oocytes using the two-electrode voltage clamp technique. The results showed that OsLHT1 could transport a broad spectrum of amino acids, including basic, neutral and acidic amino acids, and exhibited a preference for neutral and acidic amino acids. Two oslht1 mutants were generated using CRISPR/Cas9 genome-editing technology, and the loss-of-function of OsLHT1 inhibited rice root and shoot growth, thereby markedly reducing grain yields. QRT-PCR analysis indicated that OsLHT1 was expressed in various rice organs, including root, stem, flag leaf, flag leaf sheath and young panicle. Transient expression in rice protoplast suggested OsLHT1 was localized to the plasma membrane, which is consistent with its function as an amino acid transporter. CONCLUSIONS: Our results indicated that OsLHT1 is an amino acid transporter with wide substrate specificity and with preference for neutral and acidic amino acids, and disruption of OsLHT1 function markedly inhibited rice growth and	rice
707	plant	rice	CRISPR;Cas9;	thermo-sensitive genic male sterility (TMS5)	BMC plant biology	Generation of a new thermo-sensitive genic male sterile rice line by targeted mutagenesis of TMS5 gene through CRISPR/Cas9 system.	2019	19(1):109	[Barman HN et al.]	China National Rice Research Institute, Hangzhou, 310006, China.	30894127	10.1186/s12870-019-1715-0	BACKGROUND: Two-line hybrid rice with high yield potential is increasingly popular and the photo- and temperature-sensitive male sterile line is one of the basic components for two-line hybrid rice breeding. The development of male sterile lines through conventional breeding is a lengthy and laborious process, whereas developing thermo-sensitive genic male sterile (TGMS) lines for two-line hybrid breeding by editing a temperature-sensitivity gene by CRISPR/Cas9 is efficient and convenient. RESULTS: Here, thermo-sensitive genic male sterility (TGMS) was induced by employing the CRISPR/Cas9 gene editing technology to modify the gene TMS5. Two TGMS mutants, tms5-1 and tms5-2, both lacking any residual T-DNA, were generated in the indica rice cultivar Zhongjiazao17 (cv. YK17) background. When grown at a sub-optimal temperature (22 degrees C), both mutants produced viable pollen and successfully produced grain through self-fertilization, but at temperatures 24 and 26 degrees C, their pollen was sterile and no grain was set. F1 hybrids derived from the crosses between YK17S (tms5-1) and three different restorer lines outperformed both parental lines with respect to grain yield and related traits. CONCLUSION: The YK17S generated by CRISPR/Cas9 system was proved to be a new TGMS line with superior yield potential and can be widely utilized in two-line hybrid breeding of indica rice.	rice
708	plant	rice	CRISPR;Cas9;	NO CATALASE ACTIVITY 1 (nca1a; nca1b)	BMC plant biology	Two NCA1 isoforms interact with catalase in a mutually exclusive manner to redundantly regulate its activity in rice.	2019	19(1):105	[Liu J et al.]	South China Agricultural University, Guangzhou, Guangdong, China.	30885124	10.1186/s12870-019-1707-0	BACKGROUND: NCA1 (NO CATALASE ACTIVITY 1) was recently identified in Arabidopsis as a chaperone protein to regulate catalase (CAT) activity through maintaining the folding of CAT. The gene exists mainly in higher plants; some plants, such as Arabidopsis, contain only one NCA1 gene, whereas some others such as rice harbor two copies. It is not yet understood whether and how both isoforms have functioned to regulate CAT activity in those two-copy-containing plant species. RESULTS: In this study, we first noticed that the spatiotemporal expression patterns of NCA1a and NCA1b were very similar in rice plants. Subsequent BiFC and yeast three-hybrid experiments demonstrated that both NCA1a and NCA1b show mutually exclusive, rather than simultaneous, interaction with CAT. For a further functional analysis, nca1a and nca1b single mutants or double mutants of rice were generated by CRISPR/Cas9. Analysis on these mutants under both normal and salinity stress conditions found that, as compared with WT, either nca1a or nca1b single mutant showed no difference at phenotypes and CAT activities, whereas the double mutants constantly displayed very low CAT activity (about 5%) and serious lesion phenotypes. CONCLUSIONS: These results suggest that NCA1a and NCA1b show mutually	rice
709	plant	rice	CRISPR;Cas9;	type-B response regulators	Development	Type-B response regulators of rice play key roles in growth, development and cytokinin signaling.	2019	146(13)	[Worthen JM et al.]	Dartmouth College, Hanover, NH, USA.	31160418	10.1242/dev.174870	Cytokinins are plant hormones with crucial roles in growth and development. Although cytokinin signaling is well characterized in the model dicot Arabidopsis, we are only beginning to understand its role in monocots, such as rice (Oryza sativa) and other cereals of agronomic importance. Here, we used primarily a CRISPR/Cas9 gene-editing approach to characterize the roles of a key family of transcription factors, the type-B response regulators (RRs), in cytokinin signaling in rice. Results from the analysis of single rr mutants as well as higher-order rr21/22/23 mutant lines revealed functional overlap as well as subfunctionalization within members of the gene family. Mutant phenotypes associated with decreased activity of rice type-B RRs included effects on leaf and root growth, inflorescence architecture, flower development and fertilization, trichome formation and cytokinin sensitivity. Development of the stigma brush involved in pollen capture was compromised in the rr21/22/23 mutant, whereas anther development was compromised in the rr24 mutant. Novel as well as conserved roles for type-B RRs in the growth and development of a monocot compared with dicots were	rice
710	plant	rice	Cas9;	24 target sites selected randomly	Frontiers in genetics	Increasing Cytosine Base Editing Scope and Efficiency With Engineered Cas9-PmCDA1 Fusions and the Modified sgRNA in Rice.	2019	10:379	[Wu Y et al.]	Beijing Academy of Agriculture and Forestry Sciences, Beijing, China.	31134125	10.3389/fgene.2019.00379	Base editors that do not require double-stranded DNA cleavage or homology-directed repair enable higher efficiency and cleaner substitution of targeted single nucleotides in genomic DNA than conventional approaches. However, their broad applications are limited within the editing window of several base pairs from the canonical NGG protospacer adjacent motif (PAM) sequence. In this study, we fused the D10A nickase of several Streptococcus pyogenes Cas9 (SpCas9) variants with Petromyzon marinus cytidine deaminase 1 (PmCDA1) and uracil DNA glycosylase inhibitor (UGI) and developed two new effective PmCDA1-based cytosine base editors (pBEs), SpCas9 nickase (SpCas9n)-pBE and VQR nickase (VQRn)-pBE, which expanded the scope of genome targeting for cytosine-to-thymine (C-to-T) substitutions in rice. Four of six and 12 of 18 target sites selected randomly in SpCas9n-pBE and VQRn-pBE, respectively were base edited with frequencies of 4-90% in T0 plants. The effective deaminase window typically spanned positions 1-7 within the protospacer and the single target C showed the maximum C-to-T frequency at or near position 3, counting the end distal to PAM as position 1. In addition, the modified single guide RNA (sgRNA) improved the base editing efficiencies of VQRn-pBE with 1.3- to 7.6-fold increases compared with the native sgRNA, and targets that could not be mutated using the native sgRNA were edited successfully using the modified sgRNA. These newly developed base editors can be used to realize C-to-T substitutions and may become	rice

711	plant	rice	CRISPR;Cas9;	OsPIN5b (a panicle length gene); GS3 (a grain size gene); OsMYB30 (a cold tolerance gene)	Frontiers in plant science	Rational Improvement of Rice Yield and Cold Tolerance by Editing the Three Genes OsPIN5b, GS3, and OsMYB30 With the CRISPR-Cas9 System.	2019	10:1663	[Zeng Y et al.]	Wuhan University, Wuhan, China.	31993066	10.3389/fpls.2019.01663	Significant increases in rice yield and stress resistance are constant demands for breeders. However, high yield and high stress resistance are often antagonistic to each other. Here, we report several new rice mutants with high yield and excellent cold tolerance that were generated by simultaneously editing three genes, OsPIN5b (a panicle length gene), GS3 (a grain size gene) and OsMYB30 (a cold tolerance gene) with the CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-associated protein 9) system. We edited two target sites of each gene with high efficiency: 53% for OsPIN5b-site1, 42% for OsPIN5b-site2, 66% for GS3-site1, 63% for GS3-site2, 63% for OsMYB30-site1, and 58% for OsMYB30-site2. Consequently, the ospin5b mutants, the gs3 mutants, and the osmyb30 mutants exhibited increased panicle length, enlarged grain size and increased cold tolerance, respectively. Then nine transgenic lines of the ospin5b/gs3, six lines of ospin5b/osmyb30 and six lines of gs3/osmyb30 were also acquired, and their yield related traits and cold tolerance corresponded to the genes being edited. Additionally, we obtained eight ospin5b/gs3/osmyb30 triple mutants by editing all three genes simultaneously. Aside from the ospin5b/gs3/osmyb30-4 and ospin5b/gs3/osmyb30-25 mutants, the remaining six mutants had off-target events at the putative off-target site of OsMYB30-site1. The results also showed that the T2 generations of these two mutants exhibited higher yield and better cold tolerance compared with the wild type. Together, these results demonstrated that new and excellent rice varieties with improved yield and abiotic stress resistance can be generated through gene editing techniques and may be applied to rice breeding. Furthermore, our study proved that the	rice
712	plant	rice	CRISPR;Cas9;	two target sites	Frontiers in plant science	Bidirectional Promoter-Based CRISPR-Cas9 Systems for Plant Genome Editing.	2019	10:1173	[Ren Q et al.]	University of Electronic Science and Technology of China, Chengdu, China.	31616455	10.3389/fpls.2019.01173	CRISPR-Cas systems can be expressed in multiple ways, with different capabilities regarding tissue-specific expression, efficiency, and expression levels. Thus far, three expression strategies have been demonstrated in plants: mixed dual promoter systems, dual Pol II promoter systems, and single transcript unit (STU) systems. We explored a fourth strategy to express CRISPR-Cas9 in the model and crop plant, rice, where a bidirectional promoter (BiP) is used to express Cas9 and single guide RNA (sgRNA) in opposite directions. We first tested an engineered BiP system based on double-mini 35S promoter and an Arabidopsis enhancer, which resulted in 20.7% and 52.9% genome editing efficiencies at two target sites in T0 stable transgenic rice plants. We further improved the BiP system drastically by using a rice endogenous BiP, OsBiP1. The endogenous BiP expression system had higher expression strength and led to 75.9-93.3% genome editing efficiencies in rice T0 generation, when the sgRNAs were processed by either tRNA or Csy4. We provided a proof-of-concept study of applying BiP systems for expressing two-component CRISPR-Cas9 genome editing reagents in rice. Our work could promote future research and adoption of BiP systems for	rice
713	plant	rice	CRISPR;Cas9;	OsNramp5	Frontiers in plant science	Mutation at Different Sites of Metal Transporter Gene OsNramp5 Affects Cd Accumulation and Related Agronomic Traits in Rice (Oryza sativa L.).	2019	10:1081	[Wang T et al.]	Hunan Agricultural University, Changsha, China.	31572408	10.3389/fpls.2019.01081	OsNramp5 is a key gene involved in the control of the uptake of Cd, Mn, and other metal ions by rice root cells. The functional deficiency of this gene can significantly reduce the accumulation of Cd in rice grains, but the effects of its mutation on agronomic traits such as yield and quality have not been investigated comprehensively yet. In the present study, three Huanghuazhan-based OsNramp5 mutants [LCH1 (Low Cadmium Huanghuazhan 1), LCH2 (Low Cadmium Huanghuazhan 2), and LCH3 (Low Cadmium Huanghuazhan 3)] were obtained using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) technology. The mutation-type analysis showed that LCH1, LCH2, and LCH3 encoded defective OsNramp5 protein sequences containing only 76aa, 176aa, and 266aa, respectively. The determination of metal content and the statistics of related agronomic traits revealed that the functionally deficient OsNramp5 not only significantly reduced the accumulation of Cd in the grains of the mutants but also affected rice yield and quality. However, with the decrease of OsNramp5 mutation degree, its effects on chlorenchyma Mn accumulation, yield, and quality were also diminished. Additionally, we also found that the increase in the concentration of Mn in the soil restored the phenotype of the declined yield and quality due to the functional deficiency of OsNramp5. Our findings provide novel insights into and new materials for breeding rice varieties with low Cd accumulation and excellent agronomic traits under severe Cd	rice
714	plant	rice	CRISPR;Cas9;	monogalactosyldiacylglycerol synthase (OsMGD2)	Frontiers in plant science	Characterization and Mutational Analysis of a Monogalactosyldiacylglycerol Synthase Gene OsMGD2 in Rice.	2019	10:992	[Basnet R et al.]	Zhejiang University, Hangzhou, China.	31428115	10.3389/fpls.2019.00992	Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are the two predominant galactolipids present in the photosynthetic membrane in many photosynthetic organisms, including algae and higher plants. These galactolipids are the main constituents of thylakoid membrane and are essential for chloroplast biogenesis and photoautotrophic growth. In silico analysis revealed that rice (Oryza sativa L.) genome has three genes encoding MGDG synthase (OsMGD1, 2, and 3). Although subcellular localization analysis demonstrated that OsMGD2 is localized to chloroplast, its expression was observed mainly in anther and endosperm, suggesting that MGDG might have an important role in the development of flower and grain in rice. Knock-out mutants of OsMGD2 were generated employing the CRISPR/Cas9 system and their morphology, yield and grain quality related traits were studied. The leaf of osmgd2 mutants showed reduced MGDG (approximately 11.6%) and DGDG (approximately 9.5%) content with chlorophyll a content decreased by approximately 23%, consequently affecting the photosynthesis. The mutants also exhibited poor agronomic performance with plant height and panicle length decreased by approximately 12.2 and approximately 7.3%, respectively. Similarly, the number of filled grains per panicle was reduced by 43.8%, while the 1000 grain weight was increased by approximately 6.3% in the mutants. The milled rice of mutants also had altered pasting properties and decreased linoleic acid content (approximately 26.6%). Put together, the present study demonstrated that OsMGD2 is the predominantly expressed gene encoding MGDG synthase in anther and grain and plays important roles in plant growth	rice

715	plant	rice	CRISPR;Cas9;	inorganic pyrophosphatase (OsPPa6)	Frontiers in plant science	Mutagenesis Reveals That the OsPPa6 Gene Is Required for Enhancing the Alkaline Tolerance in Rice.	2019	10:759	[Wang B et al.]	Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, China.	31244876	10.3389/fpls.2019.00759	Alkaline stress (AS) is one of the abiotic stressful factors limiting plant's growth and development. Inorganic pyrophosphatase is usually involved in a variety of biological processes in plant in response to the abiotic stresses. Here, to clarify the responsive regulation of inorganic pyrophosphatase in rice under AS, the mutagenesis of the OsPPa6 gene encoding an inorganic pyrophosphatase in rice cv. Kitaake (Oryza sativa L. ssp. japonica) was performed by the CRISPR/Cas9 system. Two homozygous independent mutants with cas9-free were obtained by continuously screening. qPCR reveals that the OsPPa6 gene was significantly induced by AS, and the mutagenesis of the OsPPa6 gene apparently delayed rice's growth and development, especially under AS. Measurements demonstrate that the contents of pyrophosphate in the mutants were higher than those in the wild type under AS, however, the accumulation of inorganic phosphate, ATP, chlorophyll, sucrose, and starch in the mutants were decreased significantly, and the mutagenesis of the OsPPa6 gene remarkably lowered the net photosynthetic rate of rice mutants, thus reducing the contents of soluble sugar and proline, but remarkably increasing MDA, osmotic potentials and Na(+)/K(+) ratio in the mutants under AS. Metabonomics measurement shows that the mutants obviously down-regulated the accumulation of phosphorylcholine, choline, anthranilic acid, apigenin, coniferol and dodecanoic acid, but up-regulated the accumulation of L-valine, alpha-ketoglutarate, phenylpyruvate and L-phenylalanine under AS. This study suggests that the OsPPa6 gene is an important osmotic regulatory factor in rice, and the gene-editing of CRISPR/Cas9-guided is an effective method evaluating the responsive regulation of the stress-induced gene, and simultaneously provides a scientific support for the application of the gene encoding a soluble inorganic	rice
716	plant	rice	CRISPR;Cas9;	calcium-dependent protein kinase (OsCPK12)	Frontiers in plant science	Impaired Function of the Calcium-Dependent Protein Kinase, OsCPK12, Leads to Early Senescence in Rice (Oryza sativa L.).	2019	10:52	[Wang B et al.]	China National Rice Research Institute, Hangzhou, China.	30778363	10.3389/fpls.2019.00052	Premature leaf senescence affects plant yield and quality, and numerous researches about it have been conducted until now. In this study, we identified an early senescent mutant es4 in rice (Oryza sativa L.); early senescence appeared approximately at 60 dps and became increasingly senescent with the growth of es4 mutant. We detected that content of reactive oxygen species (ROS) and malondialdehyde (MDA), as well as activity of superoxide dismutase (SOD) were elevated, while chlorophyll content, soluble protein content, activity of catalase (CAT), activity of peroxidase (POD) and photosynthetic rate were reduced in the es4 mutant leaves. We mapped es4 in a 33.5 Kb physical distance on chromosome 4 by map-based cloning. Sequencing analysis in target interval indicated there was an eight bases deletion mutation in OsCPK12 which encoded a calcium-dependent protein kinase. Functional complementation of OsCPK12 in es4 completely restored the normal phenotype. We used CRISPR/Cas9 for targeted disruption of OsCPK12 in ZH8015 and all the mutants exhibited the premature senescence. All the results indicated that the phenotype of es4 was caused by the mutation of OsCPK12. Overexpression of OsCPK12 in ZH8015 enhanced the net photosynthetic rate (P n) and chlorophyll content. OsCPK12 was mainly expressed in green organs. The results of qRT-PCR analysis showed that the expression levels of some key genes involved in senescence, chlorophyll biosynthesis, and photosynthesis were significantly altered in the es4 mutant. Our results demonstrate that the mutant of OsCPK12 triggers the premature leaf senescence; however, the overexpression of OsCPK12 may delay its growth period and provide the potentially positive effect on	rice
717	plant	rice	Cas9;	OsGRAS19	Functional plant biology	Novel OsGRAS19 mutant, D26, positively regulates grain shape in rice (Oryza sativa).	2019		[Lin Z et al.]	Huaqiao University, Xiamen, China	31146805	10.1071/FP18266	Grain size is an important factor in rice yield. Several genes related to grain size have been reported, but most of them are determined by quantitative trail loci (QTL) traits. Gene D26 is a novel site mutation of OsGRAS19 and involved in the brassinosteroid (BR) signalling pathway. However, whether D26 is involved in the process of rice reproductive development remains unclear. Here, gene cloning and functional analysis revealed that D26 has an obvious regulatory effect on grain size. Overexpression or CRISP/Cas9 mutant of D26 also showed that grain size was positively influenced. Cellular analyses show that D26 modulates grain size by promoting cell division and regulating the cell number in the upper epidermis of the glume. The overexpression results further suggest that the level of D26 expression positively impacts grain length and leaf angles and that the expression of several known grain size genes is involved in the regulation. Based on our results, D26, as a transcription factor, effectively improves	rice
718	plant	rice	CRISPR;Cas9;	serine/arginine-rich locus	Genes	Multiplex CRISPR Mutagenesis of the Serine/Arginine-Rich (SR) Gene Family in Rice.	2019	10(8)	[Butt H et al.]	King Abdullah University of Science and Technology, Thuwal, Saudi Arabia.	31394891	10.3390/genes10080596	Plant growth responds to various environmental and developmental cues via signaling cascades that influence gene expression at the level of transcription and pre-mRNA splicing. Alternative splicing of pre-mRNA increases the coding potential of the genome from multiexon genes and regulates gene expression through multiple mechanisms. Serine/arginine-rich (SR) proteins, a conserved family of splicing factors, are the key players of alternative splicing and regulate pre-mRNA splicing under stress conditions. The rice (Oryza sativa) genome encodes 22 SR proteins categorized into six subfamilies. Three of the subfamilies are plant-specific with no mammalian orthologues, and the functions of these SR proteins are not well known. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a genome engineering tool that cleaves the target DNA at specific locations directed by a guide RNA (gRNA). Recent advances in CRISPR/Cas9-mediated plant genome engineering make it possible to generate single and multiple functional knockout mutants in diverse plant species. In this study, we targeted each rice SR locus and produced single knockouts. To overcome the functional redundancy within each subfamily of SR genes, we utilized a polycistronic tRNA-gRNA multiplex targeting system and targeted all loci of each subfamily. Sanger sequencing results indicated that most of the targeted loci had knockout mutations. This study provides useful resource materials for understanding the molecular role of SR proteins in plant	rice
719	plant	rice	CRISPR;Cas9;		Genome biology	Modulating chromatin accessibility by transactivation and targeting proximal dsgRNAs enhances Cas9 editing efficiency in vivo.	2019	20(1):145	[Liu G et al.]	Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.	31349852	10.1186/s13059-019-1762-8	The CRISPR/Cas9 system is unable to edit all targetable genomic sites with full efficiency in vivo. We show that Cas9-mediated editing is more efficient in open chromatin regions than in closed chromatin regions in rice. A construct (Cas9-TV) formed by fusing a synthetic transcription activation domain to Cas9 edits target sites more efficiently, even in closed chromatin regions. Moreover, combining Cas9-TV with a proximally binding dead sgRNA (dsgRNA) further improves editing efficiency up to several folds. The use of Cas9-TV/dsgRNA thus provides a novel strategy for obtaining efficient genome editing in vivo, especially at nuclease-refractory target sites.	rice

720	plant	rice	CRISPR;Cas9;	OsCAF1	International journal of molecular sciences	OsCAF1, a CRM Domain Containing Protein, Influences Chloroplast Development.	2019	20(18)	[Zhang Q et al.]	China National Rice Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China.	31500108	10.3390/ijms20184386	The chloroplast RNA splicing and ribosome maturation (CRM) domain proteins are involved in the splicing of chloroplast gene introns. Numerous CRM domain proteins have been reported to play key roles in chloroplast development in several plant species. However, the functions of CRM domain proteins in chloroplast development in rice remain poorly understood. In the study, we generated oscaf1 albino mutants, which eventually died at the seedling stage, through the editing of OsCAF1 with two CRM domains using CRISPR/Cas9 technology. The mesophyll cells in oscaf1 mutant had decreased chloroplast numbers and damaged chloroplast structures. OsCAF1 was located in the chloroplast, and transcripts revealed high levels in green tissues. In addition, the OsCAF1 promoted the splicing of group IIA and group IIB introns, unlike orthologous proteins of AtCAF1 and ZmCAF1, which only affected the splicing of subgroup IIB introns. We also observed that the C-terminal of OsCAF1 interacts with OsCRS2, and OsCAF1-OsCRS2 complex may participate in the splicing of group IIA and group IIB introns in rice chloroplasts. OsCAF1 regulates chloroplast development	rice
721	plant	rice	CRISPR;Cas9;	QTL qLL9	International journal of molecular sciences	Enhanced Expression of QTL qLL9/DEP1 Facilitates the Improvement of Leaf Morphology and Grain Yield in Rice.	2019	20(4)	[Fu X et al.]	China National Rice Research Institute, Hangzhou, China.	30781568	10.3390/ijms20040866	In molecular breeding of super rice, it is essential to isolate the best quantitative trait loci (QTLs) and genes of leaf shape and explore yield potential using large germplasm collections and genetic populations. In this study, a recombinant inbred line (RIL) population was used, which was derived from a cross between the following parental lines: hybrid rice Chunyou84, that is, japonica maintainer line Chunjiang16B (CJ16); and indica restorer line Chunhui 84 (C84) with remarkable leaf morphological differences. QTLs mapping of leaf shape traits was analyzed at the heading stage under different environmental conditions in Hainan (HN) and Hangzhou (HZ). A major QTL qLL9 for leaf length was detected and its function was studied using a population derived from a single residual heterozygote (RH), which was identified in the original population. qLL9 was delimitated to a 16.17 kb region flanked by molecular markers C-1640 and C-1642, which contained three open reading frames (ORFs). We found that the candidate gene for qLL9 is allelic to DEP1 using quantitative real-time polymerase chain reaction (qRT-PCR), sequence comparison, and the clustered regularly interspaced short palindromic repeat-associated Cas9 nuclease (CRISPR/Cas9) genome editing techniques. To identify the effect of qLL9 on yield, leaf shape and grain traits were measured in near isogenic lines (NILs) NIL-qLL9(CJ16) and NIL-qLL9(C84), as well as a chromosome segment substitution line (CSSL) CSSL-qLL9(KASA) with a Kasalath introgressed segment covering qLL9 in the Wuyunjing (WYJ) 7 backgrounds. Our results showed that the flag leaf lengths of NIL-qLL9(C84) and CSSL-qLL9(KASA) were significantly different from those of NIL-qLL9(CJ16) and WYJ 7, respectively. Compared with NIL-qLL9(CJ16), the spike length, grain size, and thousand-grain weight of NIL-qLL9(C84) were significantly higher, resulting in a significant increase in yield of 15.08%. Exploring and pyramiding beneficial genes resembling qLL9(C84) for super rice breeding could increase both the source (e.g., leaf length and leaf area) and the sink (e.g., yield traits). This study provides a foundation for future investigation of the molecular mechanisms underlying the source(-)sink balance and high-yield potential of	rice
722	plant	rice	CRISPR;Cas9;	phospholipase D	Journal of agricultural and food chemistry	Mutational Analysis of OsPLDalpha1 Reveals Its Involvement in Phytic Acid Biosynthesis in Rice Grains.	2019	67(41):11436-11443	[Khan MSS et al.]	Zhejiang University, Hangzhou, China.	31553599	10.1021/acs.jafc.9b05052	Phospholipids and phytic acid are important phosphorus (P)-containing compounds in rice grains. Phytic acid is considered as a major antinutrient, because the negatively charged phytic acid chelates cations, including essential micronutrients, and decreases their bioavailability to human beings and monogastric animals. To gain an insight into the interplay of these two kinds of phosphorus-containing metabolites, we used the CRISPR/Cas9 system to generate mutants of a phospholipase D gene (OsPLDalpha1) and analyzed the mutational effect on metabolites, including phytic acid in rice grains. Metabolic profiling of two ospldalpha1 mutants revealed depletion in the phosphatidic acid production and lower accumulation of cytidine diphosphate diacylglycerol and phosphatidylinositol. The mutants also showed significantly reduced phytic acid content as compared to their wild-type parent, and the expression of the key genes involved in the phytic acid biosynthesis was altered in the mutants. These results demonstrate that OsPLDalpha1 not only plays an important role in phospholipid metabolism but also is involved in phytic acid biosynthesis, most probably through the lipid-dependent pathway, and thus revealed a potential new route to regulate phytic acid biosynthesis	rice
723	plant	rice	CRISPR;Cas9;	Heme Oxygenase 1 (PE-1)	Journal of agricultural and food chemistry	PE-1, Encoding Heme Oxygenase 1, Impacts Heading Date and Chloroplast Development in Rice (Oryza sativa L.).	2019	67(26):7249-7257	[Rao Y et al.]	Zhejiang Normal University, Jinhua, Zhejiang, China.	31244201	10.1021/acs.jafc.9b01676	The duration of the rice growth phase has always been an important target trait. The identification of mutations in rice that alter these processes and result in a shorter growth phase could have potential benefits for crop production. In this study, we isolated an early aging rice mutant, pe-1, with light green leaves, using gamma-mutated indica rice cultivar and subsequent screening methods, which is known as the phytochrome synthesis factor Se5 that controls rice flowering. The pe-1 plant is accompanied by a decreased chlorophyll content, an enhanced photosynthesis, and a decreased pollen fertility. PE-1, a close homologue of HY1, is localized in the chloroplast. Expression pattern analysis indicated that PE-1 was mainly expressed in roots, stems, leaves, leaf sheaths, and young panicles. The knockout of PE-1 using the CRISPR/Cas9 system decreased the chlorophyll content and downregulated the expression of PE-1-related genes. Furthermore, the chloroplasts of pe-1 were filled with many large-sized starch grains, and the number of osmiophilic granules (a chloroplast lipid reservoir) was significantly decreased. Altogether, our findings suggest that PE-1 functions as a master regulator to mediate in chlorophyll biosynthesis and	rice

724	plant	rice	Cas9;	APETALA1 (AP1)/FRUITFULL (FUL)-like transcription factor OsMADS18	Journal of experimental botany	OsMADS18, a membrane-bound MADS-box transcription factor, modulates plant architecture and the abscisic acid response in rice.	2019	70(15):3895-3909	[Yin X et al.]	Wuhan University, Wuhan, China.	31034557	10.1093/jxb/erz198	The APETALA1 (AP1)/FRUITFULL (FUL)-like transcription factor OsMADS18 plays diverse functions in rice development, but the underlying molecular mechanisms are far from fully understood. Here, we report that down-regulation of OsMADS18 expression in RNAi lines caused a delay in seed germination and young seedling growth, whereas the overexpression of OsMADS18 produced plants with fewer tillers. In targeted OsMADS18 genome-edited mutants (osmads18-cas9), an increased number of tillers, altered panicle size, and reduced seed setting were observed. The EYFP-OsMADS18 (full-length) protein was localized to the nucleus and plasma membrane but the EYFP-OsMADS18-N (N-terminus) protein mainly localized to the nucleus. The expression of OsMADS18 could be stimulated by abscisic acid (ABA), and ABA stimulation triggered the cleavage of HA-OsMADS18 and the translocation of OsMADS18 from the plasma membrane to the nucleus. The inhibitory effect of ABA on seedling growth was less effective in the OsMADS18-overexpressing plants. The expression of a set of ABA-responsive genes was significantly reduced in the overexpressing plants. The phenotypes of transgenic plants expressing EYFP-OsMADS18-N resembled those observed in the osmads18-cas9 mutants. Analysis of the interaction of OsMADS18 with OsMADS14, OsMADS15, and OsMADS57 strongly suggests an essential role for	rice
725	plant	rice	CRISPR;Cas9;	1-aminocyclopropane-1-carboxylic acid synthase (OsACS)	Journal of experimental botany	Editing of the OsACS locus alters phosphate deficiency-induced adaptive responses in rice seedlings.	2019	70(6):1927-1940	[Lee HY et al.]	Purdue University, West Lafayette, IN, USA.	30810167	10.1093/jxb/erz074	Phosphate (Pi) deficiency severely influences the growth and reproduction of plants. To cope with Pi deficiency, plants initiate morphological and biochemical adaptive responses upon sensing low Pi in the soil, and the plant hormone ethylene plays a crucial role during this process. However, how regulation of ethylene biosynthesis influences the Pi-induced adaptive responses remains unclear. Here, we determine the roles of rice 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), the rate-limiting enzymes in ethylene biosynthesis, in response to Pi deficiency. Through analysis of tissue-specific expression of OsACS in response to Pi deficiency and OsACS mutants generated by CRISPR/Cas9 [clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9] genome editing, we found that two members of the OsACS family, i.e. OsACS1 and OsACS2, are involved but differed in their importance in controlling the remodeling of root system architecture, transcriptional regulation of Pi starvation-induced genes, and cellular phosphorus homeostasis. Interestingly, in contrast to the known inhibitory role of ethylene on root elongation, both OsACS mutants, especially OsACS1, almost fail to promote lateral root growth in response to Pi deficiency, demonstrating a stimulatory role for ethylene in lateral root development under Pi-deficient conditions. Together, this study provides new insights into the roles of ethylene in Pi deficiency response in rice seedlings and the isoform-specific function of OsACS genes in this process.	rice
726	plant	rice	CRISPR;Cas9;	TMS5; Pi21; Xa13	Journal of integrative plant biology	Developing disease-resistant thermosensitive male sterile rice by multiplex gene editing.	2019	61(12):1201-1205	[Li S et al.]	China National Rice Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China.	30623600	10.1111/jipb.12774	High-quality and disease-resistant male sterile lines have great potential for applications in hybrid rice breeding. We introduced specific mutations into the TMS5, Pi21, and Xa13 genes in Pinzhan intermediate breeding material using the CRISPR/Cas9 multiplex genome editing system. We found that the transgene-free homozygous triple tms5/pi21/xa13 mutants obtained in the T1 generation displayed characteristics of thermosensitive genic male sterility (TGMS) with enhanced resistance to rice blast and bacterial blight. Our study provides a convenient and effective way of converting breeding intermediate material into TGMS lines through multiplex gene editing, which could significantly accelerate the breeding of sterile lines.	rice
727	plant	rice	CRISPR;Cas9;	OsSPO11-1; OsREC8; OsOSD1; OsMATL	Journal of integrative plant biology	A strategy for generating rice apomixis by gene editing.	2019	61(8):911-916	[Xie E et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.	30697955	10.1111/jipb.12785	Apomixis is an asexual reproduction way of plants that can produce clonal offspring through seeds. In this study, we introduced apomixis into rice (Oryza sativa) by mutating OsSPO11-1, OsREC8, OsOSD1, and OsMATL through a CRISPR/Cas9 system. The quadruple mutant showed a transformation from meiosis to mitosis and produced clonal diploid gametes. With mutated Osmatl, which gives rise to haploid induction in plants, the quadruple mutant is expected to be able to be produced apomictic diploid offspring. We named this quadruple mutant as AOP (Apomictic	rice
728	plant	rice	CRISPR;Cas9;	LEAF INCLINATION 4	Journal of integrative plant biology	Rice miR394 suppresses leaf inclination through targeting an F-box gene, LEAF INCLINATION 4.	2019	61(4):406-416	[Qu L et al.]	Shanghai Institute of Plant Physiology and Ecology, the Chinese Academy of Sciences, Shanghai, China.	30144351	10.1111/jipb.12713	Rice leaf inclination is an important agronomic trait, closely related to plant architecture and yield. Identification of genes controlling leaf inclination would assist in crop improvement. Although various factors, including the plant hormones auxin and brassinosteroids, have been shown to regulate lamina joint development, the role of microRNAs in regulating leaf inclination remains largely unknown. Here, we functionally characterize the role of rice miR394 and its target, LEAF INCLINCATION 4 (LC4), which encodes an F-box protein, in the regulation of leaf inclination. We show that miR394 and LC4 work, antagonistically, to regulate leaf lamina joint development and rice architecture, by modulating expansion and elongation of adaxial parenchyma cells. Suppressed expression of miR394, or enhanced expression of LC4, results in enlarged leaf angles, whereas reducing LC4 expression by CRISPR/Cas9 leads to reduced leaf inclination, suggesting LC4 as candidate for use in rice architecture improvement. LC4 interacts with SKP1, a component of the SCF E3 ubiquitin ligase complex, and transcription of both miR394 and LC4 are regulated by auxin. Rice plants with altered expression of miR394 or LC4 have altered auxin responses, indicating that the miR394-LC4 module mediates auxin effects important for determining rice leaf inclination and	rice
729	plant	rice	CRISPR;Cas9;	OsBIG	Journal of plant physiology	Rice BIG gene is required for seedling viability.	2019	232:39-50	[Cheng R et al.]	Wuhan University, Wuhan, China.	30530202	10.1016/j.jplph.2018.11.006	Arabidopsis BIG (AtBIG) gene encodes an enormous protein that is required for auxin transport. Loss of AtBIG function not only profoundly changes plant architecture but also alters plant adaptability to environmental stimuli. A putative homolog of AtBIG exists in the rice genome, but no function has been ascribed to it. In this study, we focus on the characterization of the gene structure and function of OsBIG. Sequence and phylogenetic analysis shows that the homologs of OsBIG have high amino acid conservation in several domains across species. Transgenic rice plants in which the expression of OsBIG was disrupted through the CRISPR/Cas9 system-mediated genome editing were used for phenotypic analysis. The Osbig/- plants show high levels of cell death, enhanced electrolyte leakage and membrane lipid peroxidation, and reduced chlorophyll content, which likely accounted for the seedling lethality. Moreover, gene expression between Osbig/- and wild-type plants analyzed by RNA-seq indicates that a number of metabolic and hormonal pathways including ribosome, DNA replication, photosynthesis, and chlorophyll metabolism were significantly perturbed by OsBIG deficiency. In summary, OsBIG gene is integral to the normal	rice

730	plant	rice	CRISPR;Cas9;	putative sugar transporter genes (OsSWEET11; OsSWEET14; OsSWEET13)	Molecular plant	Engineering Broad-Spectrum Bacterial Blight Resistance by Simultaneously Disrupting Variable TALE-Binding Elements of Multiple Susceptibility Genes in Rice.	2019	12(11):1434-1446	[Xu Z et al.]	Shanghai Jiao Tong University, Shanghai, China.	31493565	10.1016/j.molp.2019.08.006	Xanthomonas oryzae pv. oryzae (Xoo), the causal agent of bacterial blight of rice, employs the transcription activator-like effectors (TALEs) to induce the expression of the OsSWEET family of putative sugar transporter genes, which function in conferring disease susceptibility (S) in rice plants. To engineer broad-spectrum bacterial blight resistance, we used CRISPR/Cas9-mediated gene editing to disrupt the TALE-binding elements (EBEs) of two S genes, OsSWEET11 and OsSWEET14, in rice cv. Kitaake, which harbors the recessive resistance allele of Xa25/OsSWEET13. The engineered rice line MS14K exhibited broad-spectrum resistance to most Xoo strains with a few exceptions, suggesting that the compatible strains may contain new TALEs. We identified two PthXo2-like TALEs, Tal5LN18 and Tal7PXO61, as major virulence factors in the compatible Xoo strains LN18 and PXO61, respectively, and found that Xoo encodes at least five types of PthXo2-like effectors. Given that PthXo2/PthXo2.1 target OsSWEET13 for transcriptional activation, the genomes of 3000 rice varieties were analyzed for EBE variationsin the OsSWEET13 promoter, and 10 Xa25-like haplotypes were identified. We found that Tal5LN18 and Tal7PXO61 bind slightly different EBE sequences in the OsSWEET13 promoter to activate its expression. CRISPR/Cas9 technology was then used to generate InDels in the EBE of the OsSWEET13 promoter in MS14K to creat a new germplasm with three edited OsSWEET EBEs and broad-spectrum resistance against all Xoo strains tested. Collectively, our findings illustrate how to disarm TALE-S co-evolved loci to generate	rice
731	plant	rice	Cas9;	16 possible NGN PAM (protospacer adjacent motif) combinations	Molecular plant	Improving Plant Genome Editing with High-Fidelity xCas9 and Non-canonical PAM-Targeting Cas9-NG.	2019	12(7):1027-1036	[Zhong Z et al.]	University of Electronic Science and Technology of China, Chengdu, China.	30928637	10.1016/j.molp.2019.03.011	Two recently engineered SpCas9 variants, namely xCas9 and Cas9-NG, show promising potential in improving targeting specificity and broadening the targeting range. In this study, we evaluated these Cas9 variants in the model and crop plant, rice. We first tested xCas9-3.7, the most effective xCas9 variant in mammalian cells, for targeted mutagenesis at 16 possible NGN PAM (protospacer adjacent motif) combinations in duplicates. xCas9 exhibited nearly equivalent editing efficiency to wild-type Cas9 (Cas9-WT) at most canonical NGG PAM sites tested, whereas it showed limited activity at non-canonical NGH (H = A, C, T) PAM sites. High editing efficiency of xCas9 at NGG PAMs was further demonstrated with C to T base editing by both rAPOBEC1 and PmCDA1 cytidine deaminases. With mismatched sgRNAs, we found that xCas9 had improved targeting specificity over the Cas9-WT. Furthermore, we tested two Cas9-NG variants, Cas9-NGv1 and Cas9-NG, for targeting NGN PAMs. Both Cas9-NG variants showed higher editing efficiency at most non-canonical NG PAM sites tested, and enabled much more efficient editing than xCas9 at AT-rich PAM sites such as GAT, GAA, and CAA. Nevertheless, we found that Cas9-NG variants showed significant reduced activity at the canonical NGG PAM sites. In stable transgenic rice lines, we demonstrated that Cas9-NG had much higher editing efficiency than Cas9-NGv1 and xCas9 at NG PAM sites. To expand the base-editing scope, we developed an efficient C to T base-editing system by making fusion of Cas9-NG nickase (D10A version), PmCDA1, and UGI. Taken together, our work benchmarked xCas9 as a high-fidelity nuclease for targeting canonical NGG PAMs and Cas9-NG as a preferred	rice
732	plant	rice	CRISPR;Cas9;	target sites with NG and GAT PAM sequences	Molecular plant	Genome Engineering in Rice Using Cas9 Variants that Recognize NG PAM Sequences.	2019	12(7):1003-1014	[Hua K et al.]	Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.	30928636	10.1016/j.molp.2019.03.009	CRISPR/Cas9 genome editing relies on sgRNA-target DNA base pairing and a short downstream PAM sequence to recognize target DNA. The strict protospacer adjacent motif (PAM) requirement hinders applications of the CRISPR/Cas9 system since it restricts the targetable sites in the genomes. xCas9 and SpCas9-NG are two recently engineered SpCas9 variants that can recognize more relaxed NG PAMs, implying a great potential in addressing the issue of PAM constraint. Here we use stable transgenic lines to evaluate the efficacies of xCas9 and SpCas9-NG in performing gene editing and base editing in rice. We found that xCas9 can efficiently induce mutations at target sites with NG and GAT PAM sequences in rice. However, base editors containing xCas9 failed to edit most of the tested target sites. SpCas9-NG exhibited a robust editing activity at sites with various NG PAMs without showing any preference for the third nucleotide after NG. Moreover, we showed that xCas9 and SpCas9-NG have higher specificity than SpCas9 at the CGG PAM site. We further demonstrated that different forms of cytosine or adenine base editors containing SpCas9-NG worked efficiently in rice with broadened PAM compatibility. Taken together, our work has yielded versatile genome-engineering tools that will significantly expand the target	rice
733	plant	rice	CRISPR;Cas9;	OsBZR1	Molecular plant	Cas9-NG Greatly Expands the Targeting Scope of the Genome-Editing Toolkit by Recognizing NG and Other Atypical PAMs in Rice.	2019	12(7):1015-1026	[Ren B et al.]	Sichuan University, Chengdu, China.	30928635	10.1016/j.molp.2019.03.010	CRISPR technologies enabling precise genome manipulation are valuable for gene function studies and molecular crop breeding. However, the requirement of a protospacer adjacent motif (PAM), such as NGG and TTN, for Cas protein recognition restricts the selection of targetable genomic loci in practical applications of CRISPR technologies. Recently Cas9-NG, which recognizes a minimal NG PAM, was reported to expand the targeting space of genome editing in human cells, but it remains unclear whether this Cas9 variant can be used in plants. In this study, we evaluated the nuclease activity of Cas9-NG toward various NGN PAMs by targeting endogenous genes in transgenic rice. We found that Cas9-NG edits all NGG, NGA, NGT, and NGC sites with impaired activity, while the gene-edited plants were dominated by monoallelic mutations. Cas9-NG-engineered base editors were then developed and used to generate OsBZR1 gain-of-function plants that can not be created by other available Cas9-engineered base editors. Moreover, we showed that a Cas9-NG-based transcriptional activator efficiently upregulated the expression of endogenous target genes in rice. In addition, we discovered that Cas9-NG recognizes NAC, NTG, NTT, and NCG apart from NG PAM. Together, these findings demonstrate that Cas9-NG can greatly expand the targeting scope of genome-editing tools, showing great potential for	rice

734	plant	rice	CRISPR;Cas9;	vacuolar invertase (osvin2-1; osvin2-3)	Molecules and cells	The Role of Rice Vacuolar Invertase2 in Seed Size Control.	2019	42(10):711-720	[Lee DW et al.]	Kyung Hee University, Yongin, Korea.	31607684	10.14348/molcells.2019.0109	Sink strength optimizes sucrose import, which is fundamental to support developing seed grains and increase crop yields, including those of rice (Oryza sativa). In this regard, little is known about the function of vacuolar invertase (VIN) in controlling sink strength and thereby seed size. Here, in rice we analyzed mutants of two VINs, OsVIN1 and OsVIN2, to examine their role during seed development. In a phenotypic analysis of the T-DNA insertion mutants, only the OsVIN2 mutant osvin2-1 exhibited reduced seed size and grain weight. Scanning electron microscopy analysis revealed that the small seed grains of osvin2-1 can be attributed to a reduction in spikelet size. A significant decrease in VIN activity and hexose level in the osvin2-1 spikelets interfered with spikelet growth. In addition, significant reduction in starch and increase in sucrose, which are characteristic features of reduced turnover and flux of sucrose due to impaired sink strength, were evident in the pre-storage stage of osvin2-1 developing grains. In situ hybridization analysis found that expression of OsVIN2 was predominant in the endocarp of developing grains. A genetically complemented line with a native genomic clone of OsVIN2 rescued reduced VIN activity and seed size. Two additional mutants, osvin2-2 and osvin2-3 generated by the CRISPR/Cas9 method, exhibited phenotypes similar to those of osvin2-1 in spikelet and seed size, VIN activity, and sugar metabolites. These results clearly demonstrate an important role of OsVIN2 as sink strength modulator that is critical for the maintenance of	rice
735	plant	rice	CRISPR;Cas9;	effector-binding elements in SWEET gene promoters	Nature biotechnology	Diagnostic kit for rice blight resistance.	2019	37(11):1372-1379	[Eom JS et al.]	Heinrich Heine University of Dusseldorf, Dusseldorf, Germany.	31659338	10.1038/s41587-019-0268-y	Blight-resistant rice lines are the most effective solution for bacterial blight, caused by Xanthomonas oryzae pv. oryzae (Xoo). Key resistance mechanisms involve SWEET genes as susceptibility factors. Bacterial transcription activator-like (TAL) effectors bind to effector-binding elements (EBEs) in SWEET gene promoters and induce SWEET genes. EBE variants that cannot be recognized by TAL effectors abrogate induction, causing resistance. Here we describe a diagnostic kit to enable analysis of bacterial blight in the field and identification of suitable resistant lines. Specifically, we include a SWEET promoter database, RT-PCR primers for detecting SWEET induction, engineered reporter rice lines to visualize SWEET protein accumulation and knock-out rice lines to identify virulence mechanisms in bacterial isolates. We also developed CRISPR-Cas9 genome-edited Kitaake rice to evaluate the efficacy of EBE mutations in resistance, software to predict the optimal resistance gene set for a specific geographic region, and two resistant 'mega' rice lines that will empower farmers to	rice
736	plant	rice	CRISPR;Cas9;	sucrose transporter genes (SWEET11; SWEET13; SWEET14 gene promoters)	Nature biotechnology	Broad-spectrum resistance to bacterial blight in rice using genome editing.	2019	37(11):1344-1350	[Oliva R et al.]	International Rice Research Institute, Metro Manila, Philippines.	31659337	10.1038/s41587-019-0267-z	Bacterial blight of rice is an important disease in Asia and Africa. The pathogen, Xanthomonas oryzae pv. oryzae (Xoo), secretes one or more of six known transcription-activator-like effectors (TALes) that bind specific promoter sequences and induce, at minimum, one of the three host sucrose transporter genes SWEET11, SWEET13 and SWEET14, the expression of which is required for disease susceptibility. We used CRISPR-Cas9-mediated genome editing to introduce mutations in all three SWEET gene promoters. Editing was further informed by sequence analyses of TALe genes in 63 Xoo strains, which revealed multiple TALe variants for SWEET13 alleles. Mutations were also created in SWEET14, which is also targeted by two TALes from an African Xoo lineage. A total of five promoter mutations were simultaneously introduced into the rice line Kitaake and the elite mega varieties IR64 and Ciherang-Sub1. Paddy trials showed that genome-edited SWEET promoters endow rice lines with robust,	rice
737	plant	rice	CRISPR;Cpf1;	acetolactate synthase (ALS)	Nature biotechnology	Precise gene replacement in rice by RNA transcript-templated homologous recombination.	2019	37(4):445-450	[Li S et al.]	Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China.	30886437	10.1038/s41587-019-0065-7	One of the main obstacles to gene replacement in plants is efficient delivery of a donor repair template (DRT) into the nucleus for homology-directed DNA repair (HDR) of double-stranded DNA breaks. Production of RNA templates in vivo for transcript-templated HDR (TT-HDR) could overcome this problem, but primary transcripts are often processed and transported to the cytosol, rendering them unavailable for HDR. We show that coupling CRISPR-Cpf1 (CRISPR from Prevotella and Francisella 1) to a CRISPR RNA (crRNA) array flanked with ribozymes, along with a DRT flanked with either ribozymes or crRNA targets, produces primary transcripts that self-process to release the crRNAs and DRT inside the nucleus. We replaced the rice acetolactate synthase gene (ALS) with a mutated version using a DNA-free ribonucleoprotein complex that contains the recombinant Cpf1, crRNAs, and DRT transcripts. We also produced stable lines with two desired mutations in the ALS gene using TT-HDR.	rice
738	plant	rice	CRISPR;Cas9;	REC8; PAIR1; OSD1; MATRILINEAL (MTL)	Nature biotechnology	Clonal seeds from hybrid rice by simultaneous genome engineering of meiosis and fertilization genes.	2019	37(3):283-286	[Wang C et al.]	China National Rice Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China.	30610223	10.1038/s41587-018-0003-0	Heterosis, or hybrid vigor, is exploited by breeders to produce elite high-yielding crop lines, but beneficial phenotypes are lost in subsequent generations owing to genetic segregation. Clonal propagation through seeds would enable self-propagation of F1 hybrids. Here we report a strategy to enable clonal reproduction of F1 rice hybrids through seeds. We fixed the heterozygosity of F1 hybrid rice by multiplex CRISPR-Cas9 genome editing of the REC8, PAIR1 and OSD1 meiotic genes to produce clonal diploid gametes and tetraploid seeds. Next, we demonstrated that editing the MATRILINEAL (MTL) gene (involved in fertilization) could induce formation of haploid seeds in hybrid rice. Finally, we combined fixation of heterozygosity and haploid induction by simultaneous editing of all four genes (REC8, PAIR1, OSD1 and MTL) in hybrid rice and obtained plants that could propagate clonally through seeds. Application of our method may enable self-propagation of a broad range of elite F1 hybrid crops.	rice

739	plant	rice	agroinfiltration; CRISPR;Cas9;		Nature plants	An efficient DNA- and selectable-marker-free genome-editing system using zygotes in rice.	2019	5(4):363-368	[Toda E et al.]	RIKEN Cluster for Science, Yokohama, Japan.	30911123	10.1038/s41477-019-0386-z	Technology involving the targeted mutagenesis of plants using programmable nucleases has been developing rapidly and has enormous potential in next-generation plant breeding. Notably, the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein-9 nuclease (Cas9) (CRISPR-Cas9) system has paved the way for the development of rapid and cost-effective procedures to create new mutant populations in plants(1,2). Although genome-edited plants from multiple species have been produced successfully using a method in which a Cas9-guide RNA (gRNA) expression cassette and selectable marker are integrated into the genomic DNA by Agrobacterium tumefaciens-mediated transformation or particle bombardment(3), CRISPR-Cas9 integration increases the chance of off-target modifications(4), and foreign DNA sequences cause legislative concerns about genetically modified organisms(5). Therefore, DNA-free genome editing has been developed, involving the delivery of preassembled Cas9-gRNA ribonucleoproteins (RNPs) into protoplasts derived from somatic tissues by polyethylene glycol-calcium (PEG-Ca(2+))-mediated transfection in tobacco, Arabidopsis, lettuce, rice(6), Petunia(7), grapevine, apple(8) and potato(9), or into embryo cells by biolistic bombardment in maize(10) and wheat(11). However, the isolation and culture of protoplasts is not feasible in most plant species and the frequency of obtaining genome-edited plants through biolistic bombardment is relatively low. Here, we report a genome-editing system via direct delivery of Cas9-gRNA RNPs into plant zygotes. Cas9-gRNA RNPs were transfected into rice zygotes produced by in vitro fertilization of isolated gametes(12) and the zygotes were cultured into mature plants in the absence of selection agents, resulting in the regeneration of rice plants with targeted mutations in around 14-64% of plants. This efficient plant-genome-editing system has	rice
740	plant	rice	CRISPR;Cas9;	OsOPR7	New phytologist	OsPEX5 regulates rice spikelet development through modulating jasmonic acid biosynthesis.	2019	224(2):712-724	[You X et al.]	Nanjing Agricultural University, Nanjing, China.	31264225	10.1111/nph.16037	Spikelet is the primary reproductive structure and a critical determinant of grain yield in rice. The molecular mechanisms regulating rice spikelet development still remain largely unclear. Here, we report that mutations in OsPEX5, which encodes a peroxisomal targeting sequence 1 (PTS1) receptor protein, cause abnormal spikelet morphology. We show that OsPEX5 can physically interact with OsOPR7, an enzyme involved in jasmonic acid (JA) biosynthesis and is required for its import into peroxisome. Similar to Ospex5 mutant, the knockout mutant of OsOPR7 generated via CRISPR-Cas9 technology has reduced levels of endogenous JA and also displays an abnormal spikelet phenotype. Application of exogenous JA can partially rescue the abnormal spikelet phenotype of Ospex5 and Osopr7. Furthermore, we show that OsMYC2 directly binds to the promoters of OsMADS1, OsMADS7 and OsMADS14 to activate their expression, and subsequently regulate spikelet development. Our results suggest that OsPEX5 plays a critical role in regulating spikelet development through mediating peroxisomal import of OsOPR7, therefore providing new insights into regulation of JA biosynthesis in plants and expanding our understanding of the	rice
741	plant	rice	CRISPR;Cas9;	nucleotide-binding leucine-rich repeat gene (Pizh-1; Pizh-2)	Philosophical transactions of the Royal Society of London. Series B, Biological sciences	A nucleotide-binding site-leucine-rich repeat receptor pair confers broad-spectrum disease resistance through physical association in rice.	2019	374(1767):20180308	[Xie Z et al.]	Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China.	30967012	10.1098/rstb.2018.0308	Rice blast caused by Magnaporthe oryzae is the most destructive fungal disease in crops, greatly threatening rice production and food security worldwide. The identification and utilization of broad-spectrum resistance genes are considered to be the most economic and effective method to control the disease. In the past decade, many blast resistance (R) genes have been identified, which mainly encode nucleotide-binding leucine-rich repeat (NLR) receptor family and confer limited race-specific resistance to the fungal pathogen. Resistance genes conferring broad-spectrum blast resistance are still largely lacking. In this study, we carried out a map-based cloning of the new blast R locus Pizh in variety ZH11. A bacterial artificial chromosome (BAC) clone of 165 kb spanning the Pizh locus was sequenced and identified 9 NLR genes, among which only Pizh-1 and Pizh-2 were expressed. Genetic complementation experiments indicated that Pizh-1 but not Pizh-2 alone could confer blast resistance. Intriguingly, both mutations on Pizh-1 and Pizh-2 by CRISPR-Cas9 abolished the Pizh-mediated resistance. We also observed that Pizh-1-mediated resistance was partially dependent on Pizh-2. Pizh-1 and Pizh-2 form a complex of NLRs through direct interaction. This suggests that Pizh-1 may function as the executor NLR and Pizh-2 as a 'helper' NLR that shares functional redundancy with other NLRs. Our current study provides not only a good tool for rice disease resistance breeding, but also deep insight into NLR association and function in plant immunity.	rice
742	plant	rice	CRISPR;Cas9;	pin1a, pin1b, pin1c, pin1d	Plant & cell physiology	Functional Divergence of PIN1 Paralogous Genes in Rice.	2019	60(12):2720-2732	[Li Y et al.]	Zhejiang University, Hangzhou, China.	31410483	10.1093/pcp/pcz159	Auxin is a phytohormone that plays an important role in plant growth and development by forming local concentration gradients. The regulation of auxin levels is determined by the activity of auxin efflux carrier protein PIN-formed (PIN). In Arabidopsis thaliana, PIN-formed1 (PIN1) functions in inflorescence and root development. In rice (Oryza sativa L.), there are four PIN1 homologs (OsPIN1a-1d), but their functions remain largely unexplored. Hence, in this study, we created mutant alleles of PIN1 gene-pin1a, pin1b, pin1c, pin1d, pin1a pin1b and pin1c pin1d- using CRISPR/Cas9 technology and used them to study the functions of the four OsPIN1 paralogs in rice. In wild-type rice, all four OsPIN1 genes were relatively highly expressed in the root than in other tissues. Compared with the wild type, the OsPIN1 single mutants had no dramatic phenotypes, but the pin1a pin1b double mutant had shorter shoots and primary roots, fewer crown roots, reduced root gravitropism, longer root hairs and larger panicle branch angle. Furthermore, the pin1c pin1d double mutant showed no observable phenotype at the seedling stage, but showed naked, pin-shape inflorescence at flowering. These data suggest that OsPIN1a and OsPIN1b are involved in root, shoot and inflorescence development in rice, whereas OsPIN1c and OsPIN1d mainly function in panicle formation. Our study provides basic knowledge that will facilitate the study of auxin	rice

743	plant	rice	CRISPR;Cas9	Large grain	Plant & cell physiology	LARGE GRAIN Encodes a Putative RNA-Binding Protein that Regulates Spikelet Hull Length in Rice.	2019	60(3):503-515	[Chiou WY et al.]	Okayama University, Okayama, Japan.	30690508	10.1093/pcp/pcz014	Grain size is a key determiner of grain weight, one of the yield components in rice (Oryza sativa). Therefore, to increase grain yield, it is important to elucidate the detailed mechanisms regulating grain size. The Large grain (Lgg) mutant, found in the nonautonomous DNA-based active rice transposon1 (nDart1)-tagged lines of Koshihikari, is caused by a truncated nDart1-3 and 355 bp deletion in the 5' untranslated region of LGG, which encodes a putative RNA-binding protein, through transposon display and cosegregation analysis between grain length and LGG genotype in F2 and F3. Clustered regularly interspaced short palindromic repeats/CRISPR-associated 9-mediated knockout and overexpression of LGG led to longer and shorter grains than wild type, respectively, showing that LGG regulates spikelet hull length. Expression of LGG was highest in the 0.6-mm-long young panicle and gradually decreased as the panicle elongated. LGG was also expressed in roots and leaves. These results show that LGG functions at the very early stage of panicle development. Longitudinal cell numbers of spikelet hulls of Lgg, knockout and overexpressed plants were significantly different from those of the wild type, suggesting that LGG might regulate longitudinal cell proliferation in the spikelet hull. RNA-Seq analysis of 1-mm-long young panicles from LGG knockout and overexpressing plants revealed that the expressions of many cell cycle-related genes were reduced in knockout plants relative to LGG-overexpressing plants and wild type, whereas some genes for cell proliferation were highly expressed in knockout plants. Taken together, these results suggest that	rice
744	plant	rice	CRISPR;Cas9;	Partial Resistance gene 1 (PiPR1)	Plant biotechnology journal	Genome-wide association study identifies an NLR gene that confers partial resistance to Magnaporthe oryzae in rice.	2019		[Liu MH et al.]	Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China.	31742855	10.1111/pbi.13300	Because of the frequent breakdown of major resistance (R) genes, identification of new partial R genes against rice blast disease is an important goal of rice breeding. In this study, we used a core collection of the Rice Diversity Panel II (C-RDP-II), which contains 584 rice accessions and are genotyped with 700 000 single-nucleotide polymorphism (SNP) markers. The C-RDP-II accessions were inoculated with three blast strains collected from different rice-growing regions in China. Genome-wide association study identified 27 loci associated with rice blast resistance (LABRs). Among them, 22 LABRs were not associated with any known blast R genes or QTLs. Interestingly, a nucleotide-binding site leucine-rich repeat (NLR) gene cluster exists in the LABR12 region on chromosome 4. One of the NLR genes is highly conserved in multiple partially resistant rice cultivars, and its expression is significantly up-regulated at the early stages of rice blast infection. Knockout of this gene via CRISPR-Cas9 in transgenic plants partially reduced blast resistance to four blast strains. The identification of this new non-strain specific partial R gene, tentatively named rice blast Partial Resistance gene 1 (PiPR1), provides genetic material that will be useful for understanding the partial resistance mechanism and for breeding durably resistant	rice
745	plant	rice	Cas9;		Plant biotechnology journal	Simplified adenine base editors improve adenine base editing efficiency in rice.	2019		[Hua K et al.]	Shanghai Center for Plant Stress Biology, CAS Center of Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai, China.	31469505	10.1111/pbi.13244	Adenine base editors (ABEs) have been exploited to introduce targeted adenine (A) to guanine (G) base conversions in various plant genomes, including rice, wheat and Arabidopsis. However, the ABEs reported thus far are all quite inefficient at many target sites in rice, which hampers their applications in plant genome engineering and crop breeding. Here, we show that unlike in the mammalian system, a simplified base editor ABE-P1S (Adenine Base Editor-Plant version 1 Simplified) containing the ecTadA*7.10-nSpCas9 (D10A) fusion has much higher editing efficiency in rice compared to the widely used ABE-P1 consisting of the ecTadA-ecTadA*7.10-nSpCas9 (D10A) fusion. We found that the protein expression level of ABE-P1S is higher than that of ABE-P1 in rice calli and protoplasts, which may explain the higher editing efficiency of ABE-P1S in different rice varieties. Moreover, we demonstrate that the ecTadA*7.10-nCas9 fusion can be used to improve the editing efficiency of other ABEs containing SaCas9 or the engineered SaKKH-Cas9 variant. These more efficient ABEs will help advance trait improvements in rice and other crops.	rice
746	plant	rice	CRISPR;Cas9;	OsPIL15	Plant biotechnology journal	The basic helix-loop-helix transcription factor, OsPIL15, regulates grain size via directly targeting a purine permease gene OsPUP7 in rice.	2019	17(8):1527-1537	[Ji X et al.]	Henan Agricultural University, Zhengzhou, China.	30628157	10.1111/pbi.13075	As members of the basic helix-loop-helix transcription factor families, phytochrome-interacting factors regulate an array of developmental responses ranging from seed germination to plant growth. However, little is known about their roles in modulating grain development. Here, we firstly analyzed the expression pattern of rice OsPIL genes in grains and found that OsPIL15 may play an important role in grain development. We then generated knockout (KO) OsPIL15 lines in rice using CRISPR/Cas9 technology, the silencing expression of OsPIL15 led to increased numbers of cells, which thus enhanced grain size and weight. Moreover, overexpression and suppression of OsPIL15 in the rice endosperm resulted in brown rice showing grain sizes and weights that were decreased and increased respectively. Further studies indicated that OsPIL15 binds to N1-box (CACGCG) motifs of the purine permease gene OsPUP7 promoter. Measurement of isopentenyl adenosine, a bioactive form of cytokinin (CTK), revealed increased contents in the OsPIL15-KO spikelets compared with the wild-type. Overall, our results demonstrate a possible pathway whereby OsPIL15 directly targets OsPUP7, affecting CTK transport and thereby influencing cell division and subsequent grain size. These findings provide a valuable insight into the molecular functions of OsPIL15 in rice grains, highlighting a useful genetic improvement leading to increased rice yield.	rice

747	plant	rice	CRISPR;Cas9;	ATP-citrate lyases (OsACL-A2)	Plant biotechnology journal	OsACL-A2 negatively regulates cell death and disease resistance in rice.	2019	17(7):1344-1356	[Ruan B et al.]	China National Rice Research Institute, Hangzhou, Zhejiang, China.	30582769	10.1111/pbi.13058	ATP-citrate lyases (ACL) play critical roles in tumour cell propagation, foetal development and growth, and histone acetylation in human and animals. Here, we report a novel function of ACL in cell death-mediated pathogen defence responses in rice. Using ethyl methanesulphonate (EMS) mutagenesis and map-based cloning, we identified an Oryza sativa ACL-A2 mutant allele, termed spotted leaf 30-1 (spl30-1), in which an A-to-T transversion converts an Asn at position 343 to a Tyr (N343Y), causing a recessive mutation that led to a lesion mimic phenotype. Compared to wild-type plants, spl30-1 significantly reduces ACL enzymatic activity, accumulates high reactive oxygen species and increases degradation rate of nuclear deoxyribonucleic acids. CRISPR/Cas9-mediated insertion/deletion mutation analysis and complementation assay confirmed that the phenotype of spl30-1 resulted from the defective function of OsACL-A2 protein. We further biochemically identified that the N343Y mutation caused a significant degradation of SPL30(N343Y) in a ubiquitin-26S proteasome system (UPS)-dependent manner without alteration in transcripts of OsACL-A2 in spl30-1. Transcriptome analysis identified a number of up-regulated genes associated with pathogen defence responses in recessive mutants of OsACL-A2, implying its role in innate immunity. Suppressor mutant screen suggested that OsSL, which encodes a P450 monooxygenase protein, acted as a downstream key regulator in spl30-1-mediated pathogen defence responses. Taken together, our study discovered a novel role of OsACL-A2 in negatively regulating innate immune responses	rice
748	plant	rice	CRISPR;Cas9;Cpf1;		Plant biotechnology journal	Single transcript unit CRISPR 2.0 systems for robust Cas9 and Cas12a mediated plant genome editing.	2019	17(7):1431-1445	[Tang X et al.]	University of Electronic Science and Technology of China, Chengdu, China.	30582653	10.1111/pbi.13068	CRISPR-Cas9 and Cas12a are two powerful genome editing systems. Expression of CRISPR in plants is typically achieved with a mixed dual promoter system, in which Cas protein is expressed by a Pol II promoter and a guide RNA is expressed by a species-specific Pol III promoter such as U6 or U3. To achieve coordinated expression and compact vector packaging, it is desirable to express both CRISPR components under a single Pol II promoter. Previously, we demonstrated a first-generation single transcript unit (STU)-Cas9 system, STU-Cas9-RZ, which is based on hammerhead ribozyme for processing single guide RNAs (sgRNAs). In this study, we developed two new STU-Cas9 systems and one STU-Cas12a system for applications in plants, collectively called the STU CRISPR 2.0 systems. We demonstrated these systems for genome editing in rice with both transient expression and stable transgenesis. The two STU-Cas9 2.0 systems process the sgRNAs with Csy4 ribonuclease and endogenous tRNA processing system respectively. Both STU-Cas9-Csy4 and STU-Cas9-tRNA systems showed more robust genome editing efficiencies than our first-generation STU-Cas9-RZ system and the conventional mixed dual promoter system. We further applied the STU-Cas9-tRNA system to compare two C to T base editing systems based on rAPOBEC1 and PmCDA1 cytidine deaminases. The results suggest STU-based PmCDA1 base editor system is highly efficient in rice. The STU-Cas12a system, based on Cas12a' self-processing of a CRISPR RNA (crRNA) array, was also developed and demonstrated for expression of a single crRNA and four crRNAs. Altogether, our STU CRISPR 2.0 systems further expanded the CRISPR toolbox for plant genome editing	rice
749	plant	rice	CRISPR;Cas9;	type B heterotrimeric G protein gamma subunit (zrgg2-1; zrgg2-2)	Plant biotechnology journal	Mutation of RGG2, which encodes a type B heterotrimeric G protein gamma subunit, increases grain size and yield production in rice.	2019	17(3):650-664	[Miao J et al.]	Yangzhou University, Yangzhou, China.	30160362	10.1111/pbi.13005	Heterotrimeric G proteins, which consist of Galpha , Gbeta and Ggamma subunits, function as molecular switches that regulate a wide range of developmental processes in plants. In this study, we characterised the function of rice RGG2, which encodes a type B Ggamma subunit, in regulating grain size and yield production. The expression levels of RGG2 were significantly higher than those of other rice Ggamma -encoding genes in all tissues tested, suggesting that RGG2 plays essential roles in rice growth and development. By regulating cell expansion, overexpression of RGG2 in Nipponbare (NIP) led to reduced plant height and decreased grain size. By contrast, two mutants generated by the clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system in the Zhenshan 97 (ZS97) background, zrgg2-1 and zrgg2-2, exhibited enhanced growth, including elongated internodes, increased 1000-grain weight and plant biomass and enhanced grain yield per plant (+11.8% and 16.0%, respectively). These results demonstrate that RGG2 acts as a negative regulator of plant growth and organ size in rice. By measuring the length of the second leaf sheath after gibberellin (GA3) treatment and the GA-induced alpha-amylase activity of seeds, we found that RGG2 is also involved in GA signalling. In summary, we propose that RGG2 may regulate grain and organ size via the GA pathway and that manipulation of RGG2 may provide a novel strategy for rice grain	rice
750	plant	rice	Cas9;		Plant biotechnology journal	Expanding the base editing scope in rice by using Cas9 variants.	2019	17(2):499-504	[Hua K et al.]	Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.	30051586	10.1111/pbi.12993	Base editing is a novel genome editing strategy that enables irreversible base conversion at target loci without the need for double stranded break induction or homology-directed repair. Here, we developed new adenine and cytosine base editors with engineered SpCas9 and SaCas9 variants that substantially expand the targetable sites in the rice genome. These new base editors can edit endogenous genes in the rice genome with various efficiencies. Moreover, we show that adenine and cytosine base editing can be simultaneously executed in rice. The new base editors described here will be useful in rice functional genomics research and will advance precision	rice
751	plant	rice	CRISPR;Cas9;	DEACETYLASE ON ARABINOSYL SIDECHAIN OF XYLAN1 (DARX1)	Plant cell	Arabinosyl Deacetylase Modulates the Arabinoxylan Acetylation Profile and Secondary Wall Formation.	2019	31(5):1113-1126	[Zhang L et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.	30886126	10.1105/tpc.18.00894	Acetylation, a prevalent modification of cell-wall polymers, is a tightly controlled regulatory process that orchestrates plant growth and environmental adaptation. However, due to limited characterization of the enzymes involved, it is unclear how plants establish and dynamically regulate the acetylation pattern in response to growth requirements. In this study, we identified a rice (Oryza sativa) GDSL esterase that deacetylates the side chain of the major rice hemicellulose, arabinoxylan. Acetyl esterases involved in arabinoxylan modification were screened using enzymatic assays combined with mass spectrometry analysis. One candidate, DEACETYLASE ON ARABINOSYL SIDECHAIN OF XYLAN1 (DARX1), is specific for arabinosyl residues. Disruption of DARX1 via Tos17 insertion and CRISPR/Cas9 approaches resulted in the accumulation of acetates on the xylan arabinosyl side chains. Recombinant DARX1 abolished the excess acetyl groups on arabinoxylan-derived oligosaccharides of the darx1 mutants in vitro. Moreover, DARX1 is localized to the Golgi apparatus. Two-dimensional (13)C-(13)C correlation spectroscopy and atomic force microscopy further revealed that the abnormal acetylation pattern observed in darx1 interrupts arabinoxylan conformation and cellulose microfibril orientation, resulting in compromised secondary wall patterning and reduced mechanical strength. This study provides insight into the mechanism controlling the acetylation pattern on arabinoxylan	rice

752	plant	rice	CRISPR;Cas9;	indole-3-acetic acid glucosyltransferase (OsIAAGLU)	Plant cell reports	Overexpression of OsIAAGLU reveals a role for IAA-glucose conjugation in modulating rice plant architecture.	2019	38(6):731-739	[Yu XL et al.]	South China Agricultural University, Guangzhou, China.	30903268	10.1007/s00299-019-02402-4	KEY MESSAGE: OsIAAGLU could catalyze the reaction of IAA with glucose to generate IAA-glucose. Overexpression of OsIAAGLU in rice resulted in altered rice shoot architecture and root gravitropism. The distribution and levels of indole-3-acetic acid (IAA) within plant tissues are well known to play vital roles in plant growth and development. An important mechanism of regulating free IAA levels in monocots is formation of IAA ester conjugates. In this study, a cytosol-localized protein encoded by the rice gene of indole-3-acetic acid glucosyltransferase (OsIAAGLU) was found to catalyze the reaction of free IAA with glucose to generate IAA-glucose. Expression of OsIAAGLU could be induced by IAA and NAA. The number of tillers and leaf angle was significantly increased with a concomitant decrease in plant height and panicle length in the transgenic rice lines overexpressing OsIAAGLU compared to the wild-type (WT) plants. Phenotypes of iaaglu mutants constructed using the CRISPR/Cas9 system had no obvious differences with WT plants. Furthermore, overexpression of OsIAAGLU resulted in reduced sensitivity to IAA/NAA and altered gravitropic response of the roots in the transgenic plants. Free IAA contents in the leaves, root tips, and lamina joint of OsIAAGLU-overexpressing transgenic lines were lower than those of WT plants. These results support that OsIAAGLU could play a regulatory role in IAA	rice
753	plant	rice	CRISPR;Cas9;		Plant cell reports	Development of methods for effective identification of CRISPR/Cas9-induced indels in rice.	2019	38(4):503-510	[Biswas S et al.]	Shanghai Jiao Tong University, Shanghai, China.	30783736	10.1007/s00299-019-02392-3	KEY MESSAGE: Two methods, PCR and amplicon labeling based, were developed and successfully applied to reliably detect CRISPR/Cas9 induced indels in rice. The use of CRISPR/Cas9 has emerged as a powerful nuclease-based genome editing tool in several model organisms including plants for mutagenesis by inducing precise gene editing through efficient double strand DNA breaks (DSBs) at the target site and subsequent error-prone non-homologous end joining (NHEJ) repair, leading to indel mutations. Different molecular methods including enzymatic mismatch cleavage (EMC), high-resolution melting curve analysis (HRMA) and conventional polymerase chain reaction (PCR) combined with ligation detection reaction (LDR) have been developed to quick identify CRISPR/Cas9 induced mutations. However, their intrinsic drawbacks limit their application in the identification of indel mutants in plants. Here we present two methods (one simple PCR based and the other amplicon labeling based) for effective and sensitive detection of CRISPR/Cas9 induced indels in rice. In PCR-based method, targets were amplified using two pairs of primers for each target locus and visualized on gel electrophoresis, while in amplicon labeling-based method, targets were amplified using tri-primers (with one a universal 6-FAM 5'-labelled) and detected by DNA capillary electrophoresis. Both methods can accurately define indel sizes down to +/- 1 bp, and are amenable for high throughput analysis, therefore, will significantly facilitate the identification of indel mutants generated by CRISPR/Cas9 for further	rice
754	plant	rice	CRISPR;Cas9;	Tos17 retrotransposon	Plant cell reports	Targeted deletion of rice retrotransposon Tos17 via CRISPR/Cas9.	2019	38(4):455-458	[Saika H et al.]	Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan.	30465094	10.1007/s00299-018-2357-7	KEY MESSAGE: A successful example of transposon deletion via CRISPR/Cas9-mediated genome editing suggests a novel alternative approach to plant breeding. Transposition of transposable elements (TEs) can affect adjacent genes, leading to changes in genetic traits. Expression levels and patterns, splicing and epigenetic status, and function of genes located in, or near, the inserted/excised locus can be affected. Artificial modification of loci adjacent to TEs, or TEs themselves, by genome editing could mimic the translocation of TEs that occurs in nature, suggesting that it might be possible to produce novel plants by modification of TEs via genome editing. To our knowledge, there are no reports thus far of modification of TEs by genome editing in plants. In this study, we performed targeted deletion of the Tos17 retrotransposon, which is flanked at both ends by long terminal repeat (LTR) sequences, via genome editing in rice. We succeeded in targeted mutagenesis of the LTR, and targeted deletion between LTRs, in calli transformed with CRISPR/Cas9 vectors for the Tos17 LTR. Moreover, we also successfully obtained regenerated plants derived from transformed calli and plants homozygous for lacking Tos17 in the next generation. Taken together, our results demonstrate successful deletion of the Tos17 retrotransposon from the rice genome by targeted mutagenesis using CRISPR/Cas9. We believe that this strategy could be applied to other TEs in many plant species, providing a rapid breeding technology as an alternative means to re-activate expression of agronomically important genes that have been inactivated by TE insertion, especially in plants such as fruit trees, in which it is difficult to maintain	rice
755	plant	rice	CRISPR;Cas9;	OsGS3; OsGW2; OsGn1a	Plant cell reports	Multiplex QTL editing of grain-related genes improves yield in elite rice varieties.	2019	38(4):475-485	[Zhou J et al.]	University of Electronic Science and Technology of China, Chengdu, China.	30159598	10.1007/s00299-018-2340-3	KEY MESSAGE: Significant yield increase has been achieved by simultaneous introduction of three trait-related QTLs in three rice varieties with multiplex editing by CRISPR-Cas9. Using traditional breeding approaches to develop new elite rice varieties with high yield and superior quality is challenging. It usually requires introduction of multiple trait-related quantitative trait loci (QTLs) into an elite background through multiple rounds of crossing and selection. CRISPR-Cas9-based multiplex editing of QTLs represents a new breeding strategy that is straightforward and cost effective. To test this approach, we simultaneously targeted three yield-related QTLs for editing in three elite rice varieties, namely J809, L237 and CNXJ. The chosen yield-related QTL genes are OsGS3, OsGW2 and OsGn1a, which have been identified to negatively regulate the grain size, width and weight, and number, respectively. Our approach rapidly generated all seven combinations of single, double and triple mutants for the target genes in elite backgrounds. Detailed analysis of these mutants revealed differential contributions of QTL mutations to yield performance such as grain length, width, number and 1000-grain weight. Overall, the contributions are additive, resulting in 68 and 30% yield per panicle increase in triple mutants of J809 and L237, respectively. Our data hence demonstrates a promising genome editing approach for	rice

756	plant	rice	CRISPR;Cas9;	Waxy (Wx) locus encoding granule-bound starch synthase I	Plant cell reports	CRISPR/Cas9 mutations in the rice Waxy/GBSSI gene induce allele-specific and zygosity-dependent feedback effects on endosperm starch biosynthesis.	2019	38(3):417-433	[Perez L et al.]	University of Lleida-Agrotecnio Center, Lleida, Spain.	30715580	10.1007/s00299-019-02388-z	KEY MESSAGE: Induced mutations in the waxy locus in rice endosperm did not abolish GBSS activity completely. Compensatory mechanisms in endosperm and leaves caused a major reprogramming of the starch biosynthetic machinery. The mutation of genes in the starch biosynthesis pathway has a profound effect on starch quality and quantity and is an important target for plant breeders. Mutations in endosperm starch biosynthetic genes may impact starch metabolism in vegetative tissues such as leaves in unexpected ways due to the complex feedback mechanisms regulating the pathway. Surprisingly this aspect of global starch metabolism has received little attention. We used CRISPR/Cas9 to introduce mutations affecting the Waxy (Wx) locus encoding granule-bound starch synthase I (GBSSI) in rice endosperm. Our specific objective was to develop a mechanistic understanding of how the endogenous starch biosynthetic machinery might be affected at the transcriptional level following the targeted knock out of GBSSI in the endosperm. We found that the mutations reduced but did not abolish GBSS activity in seeds due to partial compensation caused by the upregulation of GBSSII. The GBSS activity in the mutants was 61-71% of wild-type levels, similarly to two irradiation mutants, but the amylose content declined to 8-12% in heterozygous seeds and to as low as 5% in homozygous seeds, accompanied by abnormal cellular organization in the aleurone layer and amorphous starch grain structures. Expression of many other starch biosynthetic genes was modulated in seeds and leaves. This modulation of gene expression resulted in changes in AGPase and sucrose synthase	rice
757	plant	rice	CRISPR;Cas9;	2 loci	Plant direct	Heat-shock-inducible CRISPR/Cas9 system generates heritable mutations in rice.	2019	3(5):e00145	[Nandy S et al.]	University of Arkansas, Fayetteville, AR, USA.	31404128	10.1002/pld3.145	Transient expression of CRISPR/Cas9 is an effective approach for limiting its activities and improving its precision in genome editing. Here, we describe the heat-shock-inducible CRISPR/Cas9 for controlled genome editing, and demonstrate its efficiency in the model crop, rice. Using the soybean heat-shock protein gene promoter and the rice U3 promoter to express Cas9 and sgRNA, respectively, we developed the heat-shock (HS)-inducible CRISPR/Cas9 system, and tested its efficacy in targeted mutagenesis. Two loci were targeted in rice, and the presence of targeted mutations was determined before and after the HS treatment. Only a low rate of targeted mutagenesis was detected before HS (~16%), but an increased rate of mutagenesis was observed after the HS treatment among the transgenic lines (50-63%). Analysis of regenerated plants harboring HS-CRISPR/Cas9 revealed that targeted mutagenesis was suppressed in the plants but induced by HS, which was detectable by Sanger sequencing after a few weeks of HS treatments. Most importantly, the HS-induced mutations were transmitted to the progeny at a high rate, generating monoallelic and biallelic mutations that independently segregated from the Cas9 gene. Additionally, off-target mutations were either undetectable or found at a lower rate in HS-CRISPR/Cas9 lines as compared to the constitutive-overexpression CRISPR/Cas9 lines. Taken together, this work shows that HS-CRISPR/Cas9 is a controlled and reasonably efficient platform for genome editing, and therefore, a promising tool for limiting genome-wide off-target effects and	rice
758	plant	rice	CRISPR;Cas9;	OsMYB108	Plant journal	OsMYB108 loss-of-function enriches p-coumaroylated and tricin lignin units in rice cell walls.	2019	98(6):975-987	[Miyamoto T et al.]	Kyoto University, Uji, Kyoto, Japan.	30773774	10.1111/tpj.14290	Breeding approaches to enrich lignins in biomass could be beneficial to improving the biorefinery process because lignins increase biomass heating value and represent a potent source of valuable aromatic chemicals. However, despite the fact that grasses are promising lignocellulose feedstocks, limited information is yet available for molecular-breeding approaches to upregulate lignin biosynthesis in grass species. In this study, we generated lignin-enriched transgenic rice (Oryza sativa), a model grass species, via targeted mutagenesis of the transcriptional repressor OsMYB108 using CRISPR/Cas9-mediated genome editing. The OsMYB108-knockout rice mutants displayed increased expressions of lignin biosynthetic genes and enhanced lignin deposition in culm cell walls. Chemical and two-dimensional nuclear magnetic resonance (NMR) analyses revealed that the mutant cell walls were preferentially enriched in gamma-p-coumaroylated and tricin lignin units, both of which are typical and unique components in grass lignins. NMR analysis also showed that the relative abundances of major lignin linkage types were altered in the OsMYB108 mutants.	rice
759	plant	rice	CRISPR;Cas9;	coniferaldehyde 5-hydroxylase (OsCAld5H1)	Plant journal	Lignin characterization of rice CONIFERALDEHYDE 5-HYDROXYLASE loss-of-function mutants generated with the CRISPR/Cas9 system.	2019	97(3):543-554	[Takeda Y et al.]	Kyoto University, Uji, Kyoto, Japan.	30375064	10.1111/tpj.14141	The aromatic composition of lignin is an important trait that greatly affects the usability of lignocellulosic biomass. We previously identified a rice (Oryza sativa) gene encoding coniferaldehyde 5-hydroxylase (OsCAld5H1), which was effective in modulating syringyl (S)/guaiacyl (G) lignin composition ratio in rice, a model grass species. Previously characterized OsCAld5H1-knockdown rice lines, which were produced via an RNA-interference approach, showed augmented G lignin units yet contained considerable amounts of residual S lignin units. In this study, to further investigate the effect of suppression of OsCAld5H1 on rice lignin structure, we generated loss-of-function mutants of OsCAld5H1 using the CRISPR/Cas9-mediated genome editing system. Homozygous OsCAld5H1-knockout lines harboring anticipated frame-shift mutations in OsCAld5H1 were successfully obtained. A series of wet-chemical and two-dimensional NMR analyses on cell walls demonstrated that although lignins in the mutant were predictably enriched in G units all the tested mutant lines produced considerable numbers of S units. Intriguingly, lignin gamma-p-coumaroylation analysis by the derivatization followed by reductive cleavage method revealed that enrichment of G units in lignins of the mutants was limited to the non-gamma-p-coumaroylated units, whereas grass-specific gamma-p-coumaroylated lignin units were almost unaffected. Gene expression analysis indicated that no homologous genes of OsCAld5H1 were overexpressed in the mutants. These data suggested that CAld5H is mainly involved in the production of non-gamma-p-coumaroylated S lignin units, common in both eudicots and grasses, but not in the production of grass-specific gamma-p-	rice

760	plant	rice	CRISPR;	OsLOGL5	Plant molecular biology	A cytokinin-activation enzyme-like gene improves grain yield under various field conditions in rice.	2019		[Wang C et al.]	Sinobioway Bio-Agriculture Group Co., Ltd, Beijing, China.	31872309	10.1007/s11103-019-00952-5	KEY MESSAGE: CRISPR-edited variants at the 3'-end of OsLOGL5's coding sequence (CDS), significantly increased rice grain yield under well-watered, drought, normal nitrogen, and low nitrogen field conditions at multiple geographical locations. Cytokinins impact numerous aspects of plant growth and development. This study reports that constitutive ectopic overexpression of a rice cytokinin-activation enzyme-like gene, OsLOGL5, significantly reduced primary root growth, tiller number, and yield. Conversely, mutations at the 3'-end of OsLOGL5 CDS resulted in normal rice plant morphology but with increased grain yield under well-watered, drought, normal nitrogen, and low nitrogen field conditions at multiple geographical locations. Six gene edited variants (Edit A to F) were created and tested in the field. Edit-B and Edit-F plants increased, but Edit-D and Edit-E plants decreased, the panicle number per plant. All OsLOGL5-edited plants significantly increased seed setting rate, total grain numbers, full-filled grain numbers per panicle, and thousand seed weight under drought conditions, suggesting that OsLOGL5 is likely involved in the regulation of both seed development and grain filling processes. Our results indicate that the C-terminal end of OsLOGL5 protein plays an important role in regulating rice yield improvement under different abiotic stress conditions, and OsLOGL5 is important for rice yield	rice
761	plant	rice	CRISPR;Cas9;	OsIAA23	Plant molecular biology	Different knockout genotypes of OsIAA23 in rice using CRISPR/Cas9 generating different phenotypes.	2019	100(4-5):467-479	[Jiang M et al.]	Nanjing University, Nanjing, China.	31004275	10.1007/s11103-019-00871-5	KEY MESSAGE: We have isolated several Osiaa23 rice mutants with different knockout genotypes, resulting in different phenotypes, which suggested that different genetic backgrounds or mutation types influence gene function. The Auxin/Indole-3-Acetic Acid (Aux/IAA) gene family performs critical roles in auxin signal transduction in plants. In rice, the gene OsIAA23 (Os06t0597000) is known to affect development of roots and shoots, but previous knockouts in OsIAA23 have been sterile and difficult for research continuously. Here, we isolate new Osiaa23 mutants using the CRISPR/Cas9 system in japonica (Wuyunjing24) and indica (Kasalath) rice, with extensive genome re-sequencing to confirm the absence of off-target effects. In Kasalath, mutants with a 13-amino acid deletion showed profoundly greater dwarfing, lateral root developmental disorder, and fertility deficiency, relative to mutants with a single amino acid deletion, demonstrating that those 13 amino acids in Kasalath are essential to gene function. In Wuyunjing24, we predicted that mutants with a single base-pair frameshift insertion would experience premature termination and strong phenotypic defects, but instead these lines exhibited negligible phenotypic difference and normal fertility. Through RNA-seq, we show here that new mosaic transcripts of OsIAA23 were produced de novo, which circumvented the premature termination and thereby preserved the wild-type phenotype. This finding is a notable demonstration in plants that mutants can mask loss of function CRISPR/Cas9 editing of the target gene through de novo	rice
762	plant	rice	agroinfiltration; CRISPR;Cas9;	rice AP2 domain-containing protein gene Os01g04020	Plant molecular biology	CRISPR/Cas9-mediated targeted T-DNA integration in rice.	2019	99(4-5):317-328	[Lee K et al.]	Iowa State University, Ames, IA, USA.	30645710	10.1007/s11103-018-00819-1	KEY MESSAGE: Combining with a CRISPR/Cas9 system, Agrobacterium-mediated transformation can lead to precise targeted T-DNA integration in the rice genome. Agrobacterium-mediated T-DNA integration into the plant genomes is random, which often causes variable transgene expression and insertional mutagenesis. Because T-DNA preferentially integrates into double-strand DNA breaks, we adapted a CRISPR/Cas9 system to demonstrate that targeted T-DNA integration can be achieved in the rice genome. Using a standard Agrobacterium binary vector, we constructed a T-DNA that contains a CRISPR/Cas9 system using SpCas9 and a gRNA targeting the exon of the rice AP2 domain-containing protein gene Os01g04020. The T-DNA also carried a red fluorescent protein and a hygromycin resistance (hptII) gene. One version of the vector had hptII expression driven by an OsAct2 promoter. In an effort to detect targeted T-DNA insertion events, we built another T-DNA with a promoterless hptII gene adjacent to the T-DNA right border such that integration of T-DNA into the targeted exon sequence in-frame with the hptII gene would allow hptII expression. Our results showed that these constructs could produce targeted T-DNA insertions with frequencies ranging between 4 and 5.3% of transgenic callus events, in addition to generating a high frequency (50-80%) of targeted indel mutations. Sequencing analyses showed that four out of five sequenced T-DNA/gDNA junctions carry a single copy of full-length T-DNA at the target site. Our results indicate that Agrobacterium-mediated transformation combined with a CRISPR/Cas9 system can	rice
763	plant	rice	CRISPR;Cas9;	OsMS1	Plant molecular biology	OsMS1 functions as a transcriptional activator to regulate programmed tapetum development and pollen exine formation in rice.	2019	99(1-2):175-191	[Yang Z et al.]	China National Rice Research Institute, Hangzhou, China.	30610522	10.1007/s11103-018-0811-0	KEY MESSAGE: OsMS1 functions as a transcriptional activator and interacts with known tapetal regulatory factors through its plant homeodomain (PHD) regulating tapetal programmed cell death (PCD) and pollen exine formation in rice. The tapetum, a hallmark tissue in the stamen, undergoes degradation triggered by PCD during post-meiotic anther development. This degradation process is indispensable for anther cuticle and pollen exine formation. Previous study has shown that PTC1 plays a critical role in the regulation of tapetal PCD. However, it remained unclear how this occurs. To further investigate the role of this gene in rice, we used CRISPR/Cas9 system to generate the homozygous mutant named as osms1, which showed complete male sterility with slightly yellow and small anthers, as well as invisible pollen grains. In addition, cytological observation revealed delayed tapetal PCD, defective pollen exine formation and a lack of DNA fragmentation according to a TUNEL analysis in the anthers of osms1 mutant. OsMS1, which encodes a PHD finger protein, was located in the nucleus of rice protoplasts and functioned as a transcription factor with transcriptional activation activity. Y2H and BiFC assays demonstrated that OsMS1 can interact with OsMADS15 and TDR INTERACTING PROTEIN2 (TIP2). It has been reported that TIP2 coordinated with TDR to modulate the expression of EAT1 and further regulated tapetal PCD in rice. Results of qPCR suggested that the expression of the genes associated with tapetal PCD and pollen wall biosynthesis, such as EAT1, AP37, AP25, OsC6 and OsC4, were significantly reduced in osms1 mutant. Taken together, our results demonstrate that the interaction of OsMS1 with known tapetal regulatory factors through its PHD finger regulates tapetal PCD and pollen exine	rice

764	plant	rice	CRISPR;Cas9;	grain-filling rate1	Plant physiology	Favorable Alleles of GRAIN-FILLING RATE1 Increase the Grain-Filling Rate and Yield of Rice.	2019	181(3):1207-1222	[Liu E et al.]	Nanjing Agricultural University, Nanjing, China.	31519786	10.1104/pp.19.00413	Hybrid rice (Oryza sativa) has been cultivated commercially for 42 years in China. However, poor grain filling still limits the development of hybrid japonica rice. We report here the map-based cloning and characterization of the GRAIN-FILLING RATE1 (GFR1) gene present at a major-effect quantitative trait locus. We elucidated and confirmed the function of GFR1 via genetic complementation experiments and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing in combination with genetic and molecular biological analyses. In addition, we conducted haplotype association analysis to mine the elite alleles of GFR1 among 117 rice accessions. We observed that GFR1 was constitutively expressed and encoded a membrane-localized protein. The allele of the rice accession Ludao (GFR1 (Ludao)) improved the grain-filling rate of rice by increasing Rubisco initial activity in the Calvin cycle. Moreover, the increased expression of the cell wall invertase gene OsCIN1 in the near isogenic line NIL-GFR1 (Ludao) promoted the unloading of Suc during the rice grain-filling stage. A yeast two-hybrid assay indicated that the Rubisco small subunit interacts with GFR1, possibly in the regulation of the rice grain-filling rate. Evaluation of the grain-filling rate and grain yield of F1 plants harboring GFR1 (Ludao) and the alleles of 20 hybrids widely cultivated commercially confirmed that favorable alleles of GFR1 can be used to	rice
765	plant	rice	CRISPR;Cas9;	13 genes across four enzymatic steps in gibberellin metabolism pathway	Plant physiology	CRISPR-Based Assessment of Gene Specialization in the Gibberellin Metabolic Pathway in Rice.	2019	180(4):2091-2105	[Chen X et al.]	Nanjing University, Nanjing, China.	31160507	10.1104/pp.19.00328	Gibberellin (GA) functions as an essential natural regulator of growth and development in plants. For each step of the GA metabolic pathway, different copy numbers can be found in different species, as is the case with the 13 genes across four enzymatic steps in rice (Oryza sativa). A common view is that such gene duplication creates homologs that buffer organisms against loss-of-function (LOF) mutations. Therefore, knockouts of any single homolog might be expected to have little effect. To test this question, we generated clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) knockouts for these homologs and measured effects on growth and reproduction. Surprisingly, we report here that there is consistently one or more essential gene at each enzymatic step, for which LOF mutation induces death or sterility-suggesting that the GA pathway does not have a redundancy route and that each gene family is essential for GA metabolism. In most of these genes from the same gene family, we observed defects in plant height and infertility, suggesting that the duplicated members retain functions related to GA synthesis or degradation. We identified both subfunctionalization of the three recently diversified homologs OsKO1, OsKO2, and OsKO5 and neofunctionalization in OsKO3 and OsKO4 Thus, although the function of each step is conserved, the evolution of duplicates in that step is diversified. Interestingly, the CRISPR/Cas9 lines at the SD1 locus were typically sterile, whereas the natural sd1 mutants, related to the "Green Revolution" in rice, show normal setting rates. Collectively, our results identify candidates for control of GA production and provide insight into the evolution of four	rice
766	plant	rice	CRISPR;Cas9;	glycyl-tRNA synthetase (rice albino 1)	Plant physiology and biochemistry	Rice albino 1, encoding a glycyl-tRNA synthetase, is involved in chloroplast development and establishment of the plastidic ribosome system in rice.	2019	139:495-503	[Zheng H et al.]	Nanjing Agricultural University, Nanjing, China.	31015088	10.1016/j.plaphy.2019.04.008	The chloroplast is an important organelle that performs photosynthesis as well as biosynthesis and storage of many metabolites. Aminoacyl-tRNA synthetases (aaRSs) are key enzymes in protein synthesis. However, the relationship between chloroplast development and aaRSs still remains unclear. In this study, we isolated a rice albino 1 (ra1) mutant through methane sulfonate (EMS) mutagenesis of rice japonica cultivar Ningjing 4 (Oryza sativa L.), which displayed albinic leaves in seedling stage due to abnormal chloroplast development. Compared with wild type (WT), ra1 showed significantly decreased levels of chlorophylls (Chl) and carotenoids (Car) in 2-week-old seedlings, which also showed obvious plastidic structural defects including abnormal thylakoid membrane structures and more osmiophilic particles. These defects caused albino phenotypes in seedlings. Map-based cloning revealed that RA1 gene encodes a glycyl-tRNA synthetase (GlyRS), which was confirmed by genetic complementation and knockout by Crispr/Cas9 technology. Sequence analysis showed that a single base mutation (T to A) occurred in the sixth exon of RA1 and resulted in a change from Isoleucine (Ile) to Lysine (Lys). Real-time PCR analyses showed that RA1 expression levels were constitutive in most tissues, but most abundant in the leaves and stems. By transient expression in Nicotiana benthamiana, we found that RA1 protein was localized in the chloroplast. Expression levels of chlorophyll biosynthesis and plastid development related genes were disordered in the ra1 mutant. RNA analysis revealed biogenesis of chloroplast rRNAs was abnormal in ra1. Meanwhile, western blotting showed that synthesis of proteins associated with plastid development was significantly repressed. These results suggest that RA1 is involved in early chloroplast	rice
767	plant	rice	CRISPR;Cas9;	NF-Y transcription factor (OsNF-YC10)	Plant science	OsNF-YC10, a seed preferentially expressed gene regulates grain width by affecting cell proliferation in rice.	2019	280:219-227	[Jia S et al.]	Huazhong Agricultural University, Wuhan, China.	30824000	10.1016/j.plantsci.2018.09.021	Grain size and shape are important factors in determining the grain yield. In this study, OsNF-YC10, a member of the NF-Y transcription factor family encoding a putative histone transcription factor, was cloned and characterized. qRT-PCR and mRNA in situ hybridization analysis revealed that OsNF-YC10 was highly expressed in endosperm and spikelet hull at late developmental stages. The results showed that OsNF-YC10 was a nuclear protein showing transcription activation activity. The osnf-yc10 lines, produced using CRISPR/Cas9 technology, showed narrow, thin and light grains. Cytological experiments revealed significantly reduced cell number of spikelet hull in osnf-yc10 lines compared with that in WT. Narrow, thin, and light grains were found consistently in OsNF-YC10 RNAi transgenic lines. Moreover, the number of cells decreased in the grain-width direction than WT. These results indicated that OsNF-YC10 plays an important role in determining grain size and shape. OsNF-YC10 was further revealed to influence the expression of GW8 (a positive regulator of grain width), GW7 (a negative regulator of grain width) and cell cycle-regulated genes CYCD4, CYCA2.1, CYCB2.1, CYCB2.2, E2F2. Taken together, it is suggested that OsNF-YC10 regulates the grains size and shape by influencing the cell proliferation of	rice

768	plant	rice	CRISPR;Cas9;	OsAUX3	Plant, cell & environment	The auxin influx carrier, OsAUX3, regulates rice root development and responses to aluminium stress.	2019	42(4):1125–1138	[Wang M et al.]	Zhejiang University, Hangzhou, China.	30399648	10.1111/pce.13478	In rice, there are five members of the auxin carrier AUXIN1/LIKE AUX1 family; however, the biological functions of the other four members besides OsAUX1 remain unknown. Here, by using CRISPR/Cas9, we constructed two independent OsAUX3 knock-down lines, osaux3-1 and osaux3-2, in wild-type rice, Hwayoung (WT/HY) and Dongjin (WT/DJ). osaux3-1 and osaux3-2 have shorter primary roots (PRs), decreased lateral root (LR) density, and longer root hairs (RHs) compared with their WT. OsAUX3 expression in PRs, LRs, and RHs further supports that OsAUX3 plays a critical role in the regulation of root development. OsAUX3 locates at the plasma membrane and functions as an auxin influx carrier affecting acropetal auxin transport. OsAUX3 is up-regulated in the root apex under aluminium (Al) stress, and osaux3-2 is insensitive to Al treatments. Furthermore, 1-naphthylacetic acid accented the sensitivity of WT/DJ and osaux3-2 to respond to Al stress. Auxin concentrations, Al contents, and Al-induced reactive oxygen species-mediated damage in osaux3-2 under Al stress are lower than in WT, indicating that OsAUX3 is involved in Al-induced inhibition of root growth. This study uncovers a novel pathway alleviating Al-induced oxidative damage by inhibition of acropetal auxin transport and provides a new option for engineering Al-	rice
769	plant	rice	CRISPR;Cas9;	inositol 1,3,4-triphosphate 5/6-kinase6	Plants	Mutation of Inositol 1,3,4-trisphosphate 5/6-kinase6 Impairs Plant Growth and Phytic Acid Synthesis in Rice.	2019	8(5)	[Jiang M et al.]	Zhejiang University, Hangzhou, China.	31035443	10.3390/plants8050114	Inositol 1,3,4-trisphosphate 5/6-kinase (ITPK) is encoded by six genes in rice (OsITPK1-6). A previous study had shown that nucleotide substitutions of OsITPK6 could significantly lower the phytic acid content in rice grains. In the present study, the possibility of establishing a genome editing-based method for breeding low-phytic acid cultivars in rice was explored, in conjunction with the functional determination of OsITPK6. Four OsITPK6 mutant lines were generated by targeted mutagenesis of the gene's first exon using the CRISPR/Cas9 method, one (ositpk6_1) with a 6-bp in-frame deletion, and other three with frameshift mutations (ositpk6_2, _3, and _4). The frameshift mutations severely impaired plant growth and reproduction, while the effect of ositpk6_1 was relatively limited. The mutant lines ositpk6_1 and _2 had significantly lower levels (−10.1% and −32.1%) of phytic acid and higher levels (4.12- and 5.18-fold) of inorganic phosphorus compared with the wild-type (WT) line. The line ositpk6_1 also showed less tolerance to osmotic stresses. Our research demonstrates that mutations of OsITPK6, while effectively reducing phytic acid biosynthesis in rice grain, could	rice
770	plant	rice	CRISPR;Cas9;	OsSNB	PLoS genetics	A novel rice grain size gene OsSNB was identified by genome-wide association study in natural population.	2019	15(5):e1008191	[Ma X et al.]	Shanghai Agrobiological Gene Center, Shanghai, China.	31150378	10.1371/journal.pgen.1008191	Increasing agricultural productivity is one of the most important goals of plant science research and imperative to meet the needs of a rapidly growing population. Rice (Oryza sativa L.) is one of the most important staple crops worldwide. Grain size is both a major determinant of grain yield in rice and a target trait for domestication and artificial breeding. Here, a genome-wide association study of grain length and grain width was performed using 996,722 SNP markers in 270 rice accessions. Five and four quantitative trait loci were identified for grain length and grain width, respectively. In particular, the novel grain size gene OsSNB was identified from qGW7, and further results showed that OsSNB negatively regulated grain size. Most notably, knockout mutant plants by CRISPR/Cas9 technology showed increased grain length, width, and weight, while overexpression of OsSNB yielded the opposite. Sequencing of this gene from the promoter to the 3'-untranslated region in 168 rice accessions from a wide geographic range identified eight haplotypes. Furthermore, Hap 3 has the highest grain width discovered in japonica subspecies. Compared to other haplotypes, Hap 3 has a 225 bp insertion in the promoter. Based on the difference between Hap 3 and other haplotypes, OsSNB_Indel2 was designed as a functional marker for the improvement of rice grain width. This could be directly used to assist selection toward an improvement of grain width. These findings suggest OsSNB as useful for further improvements in	rice
771	plant	rice	CRISPR;Cas9;	CYTOKININ OXIDASE/DEHYDROGENASE 9 (OsCKX9)	Proceedings of the National Academy of Sciences of the United States of America	Strigolactone promotes cytokinin degradation through transcriptional activation of CYTOKININ OXIDASE/DEHYDROGENASE 9 in rice.	2019	116(28):14319–14324	[Duan J et al.]	Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China.	31235564	10.1073/pnas.1810980116	Strigolactones (SLs), a group of terpenoid lactones derived from carotenoids, are plant hormones that control numerous aspects of plant development. Although the framework of SL signaling that the repressor DWARF 53 (D53) could be SL-dependently degraded via the SL receptor D14 and F-box protein D3 has been established, the downstream response genes to SLs remain to be elucidated. Here we show that the cytokinin (CK) content is dramatically increased in shoot bases of the rice SL signaling mutant d53 By examining transcript levels of all the CK metabolism-related genes after treatment with SL analog GR24, we identified CYTOKININ OXIDASE/DEHYDROGENASE 9 (OsCKX9) as a primary response gene significantly up-regulated within 1 h of treatment in the wild type but not in d53 We also found that OsCKX9 functions as a cytosolic and nuclear dual-localized CK catabolic enzyme, and that the overexpression of OsCKX9 suppresses the browning of d53 calli. Both the CRISPR/Cas9-generated OsCKX9 mutants and OsCKX9-overexpressing transgenic plants showed significant increases in tiller number and decreases in plant height and panicle size, suggesting that the homeostasis of OsCKX9 plays a critical role in regulating rice shoot architecture. Moreover, we identified the CK-inducible rice type-A response regulator OsRR5 as the secondary SL-responsive gene, whose expression is significantly repressed after 4 h of GR24 treatment in the wild type but not in osckx9 These findings reveal a comprehensive plant hormone cross-talk in which SL can induce the expression of OsCKX9 to down-regulate CK content, which in turn triggers	rice
772	plant	rice	CRISPR;Cas9;	Orange gene (Osor)	Rice	A novel approach to carotenoid accumulation in rice callus by mimicking the cauliflower Orange mutation via genome editing.	2019	12(1):81	[Endo A et al.]	Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan.	31713832	10.1186/s12284-019-0345-3	BACKGROUND: beta-carotene (provitamin A) is an important target for biofortification of crops as a potential solution to the problem of vitamin A deficiency that is prevalent in developing countries. A previous report showed that dominant expression of splicing variants in the Orange (Or) gene causes beta-carotene accumulation in cauliflower curd. In this study, we focused on a putative orthologue of the cauliflower or gene in rice, Osor, and attempt to accumulate beta-carotene in rice callus by modification of the Osor gene via genome editing using CRISPR/Cas9. FINDINGS: CRISPR/Cas9 vectors for the Osor gene were constructed and transformed into rice calli. Some transformed calli showed orange color due to beta-carotene hyper-accumulation. Molecular analyses suggest that orange-colored calli are due to an abundance of in-frame aberrant Osor transcripts, whereas out-of-frame mutations were not associated with orange color. CONCLUSIONS: We demonstrate that directed gene modification of the Osor gene via CRISPR/Cas9-mediated genome editing results in beta-carotene fortification in rice calli. To date, golden rice, which accumulates beta-carotene in rice endosperm, has been developed by conventional transgenic approaches. Our results suggest an alternative approach to enhancing beta-carotene accumulation in crops.	rice

773	plant	rice	CRISPR;Cas9;	Os8N3	Rice	CRISPR/Cas9-targeted mutagenesis of Os8N3 in rice to confer resistance to Xanthomonas oryzae pv. oryzae.	2019	12(1):67	[Kim YA et al.]	Sejong University, Seoul, Korea.	31446506	10.1186/s12284-019-0325-7	BACKGROUND: Genome editing tools are important for functional genomics research and biotechnology applications. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 (Cas9) system for gene knockout has emerged as the most effective genome-editing tool. It has previously been reported that, in rice plants, knockdown of the Os8N3 gene resulted in enhanced resistance to Xanthomonas oryzae pv. oryzae (Xoo), while displaying abnormal pollen development. RESULTS: The CRISPR/Cas9 system was employed to knockout rice Os8N3, in order to confer enhanced resistance to Xoo. Analysis of the genotypes and edited Os8N3 in T0, T1, T2, and T3 transgenic rice plants showed that the mutations were transmitted to subsequent generations, and homozygous mutants displayed significantly enhanced resistance to Xoo. Stable transmission of CRISPR/Cas9-mediated Os8N3 gene editing without the transferred DNA (T-DNA) was confirmed by segregation in the T1 generation. With respect to many investigated agronomic traits including pollen development, there was no significant difference between homozygous mutants and non-transgenic control plants under greenhouse growth conditions. CONCLUSION: Data from this study indicate that the CRISPR/Cas9-mediated Os8N3 edition can be successfully employed for non-transgenic crop improvements.	rice
774	plant	rice	CRISPR;Cas9;	digalactosyldiacylglycerol	Rice	OsDGD2beta is the Sole Digalactosyldiacylglycerol Synthase Gene Highly Expressed in Anther, and its Mutation Confers Male Sterility in Rice.	2019	12(1):66	[Basnet R et al.]	Zhejiang University, Hangzhou, Zhejiang, China.	31414258	10.1186/s12284-019-0320-z	BACKGROUND: Digalactosyldiacylglycerol (DGDG) is one of the major lipids found predominantly in the photosynthetic membrane of cyanobacteria, eukaryotic algae and higher plants. DGDG, along with MGDG (Monogalactosyldiacylglycerol), forms the matrix in thylakoid membrane of chloroplast, providing the site for photochemical and electron transport reactions of oxygenic photosynthesis. RESULTS: In silico analysis reveals that rice (Oryza sativa L.) genome has 5 genes encoding DGDG synthase, which are differentially expressed in different tissues, and OsDGD2beta was identified to be the sole DGDG synthase gene expressed in anther. We then developed osdgd2beta mutants by using the CRISPR/Cas9 system and elucidate its role, especially in the development of anther and pollen. The loss of function of OsDGD2beta resulted in male sterility in rice characterized by pale yellow and shrunken anther, devoid of starch granules in pollen, and delayed degeneration of tapetal cells. The total fatty acid and DGDG content in the anther was reduced by 18.66% and 22.72% in osdgd2beta, affirming the importance of DGDG in the development of anther. The mutants had no notable differences in the vegetative phenotype, as corroborated by relative gene expression of DGDG synthase genes in leaves, chlorophyll measurements, and analysis of photosynthetic parameters, implying the specificity of OsDGD2beta in anther. CONCLUSION: Overall, we showed the importance of DGDG in pollen development and loss of function of OsDGD2beta results in male sterility. Here, we have also proposed the use of OsDGD2beta in hybrid rice breeding using the nuclear male sterility system.	rice
775	plant	rice	CRISPR;Cas9;	heterotrimeric G protein beta subunit (rgb1)	Rice	The heterotrimeric G protein beta subunit RGB1 is required for seedling formation in rice.	2019	12(1):53	[Gao Y et al.]	Yangzhou University, Yangzhou, China.	31321558	10.1186/s12284-019-0313-y	BACKGROUND: The heterotrimeric G protein beta subunit RGB1 plays an important role in plant growth and development. However, the molecular mechanisms underlying the regulation of rice growth by RGB1 remain elusive. RESULTS: Here, the rgb1 mutants rgb1-1 (+ 1 bp), rgb1-2 (- 1 bp), and rgb1-3 (- 11 bp) were isolated using the CRISPR/Cas9 system, and they were arrested at 1 day after germination and ultimately exhibited seedling lethality. The dynamic anatomical characteristics of the embryos of the rgb1 seedlings and WT during early postgermination and according to TUNEL assays showed that the suppressed growth of the rgb1 mutants was caused by cell death. In addition to the limited shoot and root development, the development of the embryo shoot-root axis was suppressed in the rgb1 mutants. RGB1 was expressed mainly in the root epidermal and vascular tissues of the embryo. Moreover, transcript profiling analysis revealed that the expression of a large number of auxin-, cytokinin-, and brassinosteroid-inducible genes was upregulated or downregulated in the rgb1 mutant compared to the wild type during seedling development. CONCLUSIONS: Overall, the rgb1 mutants provide an ideal material for exploring the molecular mechanism underlying rice seedling formation during early postgermination development by G proteins. SIGNIFICANCE STATEMENT: The heterotrimeric G protein beta subunit RGB1 acts as a crucial factor in promoting early postgermination seedling	rice
776	plant	rice	CRISPR;Cas9;	LOW SEED SETTING RATE1 (LSSR1)	Rice	LSSR1 facilitates seed setting rate by promoting fertilization in rice.	2019	12(1):31	[Xiang X et al.]	China National Rice Research Institute, Hangzhou, China.	31073866	10.1186/s12284-019-0280-3	Seed setting rate is one of the major components that determine rice (Oryza sativa L.) yield. Successful fertilization is necessary for normal seed setting. However, little is known about the molecular mechanisms governing this process. In this study, we report a novel rice gene, LOW SEED SETTING RATE1 (LSSR1), which regulates the seed setting rate by facilitating rice fertilization. LSSR1 encodes a putative GH5 cellulase, which is highly conserved in plants. LSSR1 is predominantly expressed in anthers during the microsporogenesis stage, and its encoded protein contains a signal peptide at the N-terminal, which may be a secretory protein that stores in pollen grains and functions during rice fertilization. To explore the physiological function of LSSR1 in rice, loss-of-function mutants of LSSR1 were created through the CRISPR-Cas9 system, which showed a significant decrease in rice seed setting rate. However, the morphology of the vegetative and reproductive organs appears normal in lssr1 mutant lines. In addition, lssr1 pollen grains could be normally stained by I2-KI solution. Cytological results demonstrate that the blockage of fertilization mostly accounted for the low seed setting rate in lssr1 mutant lines, which was most likely caused by abnormal pollen grain germination, failed pollen tube penetration, and retarded pollen tube elongation. Together, our results suggest that LSSR1 plays an important role in rice fertilization, which in turn is vital for maintaining rice seed setting rate.	rice

777	plant	rice	CRISPR;Cas9;	TRIANGULAR HULL1 (TH1)	Rice	Analysing the rice young panicle transcriptome reveals the gene regulatory network controlled by TRIANGULAR HULL1.	2019	12(1):6	[Wang J et al.]	Guangxi University, Nanning, China.	30725309	10.1186/s12284-019-0265-2	BACKGROUND: TRIANGULAR HULL1 (TH1), a member of the rice ALOG gene family, has been characterized as a rice lemma/palea-related gene. To understand the gene regulatory network that controlled by TH1, we analyzed the transcriptome from a TH1 knock out (KO) line, which was generated by CRISPR/Cas9. Our study may shed some light on the molecular mechanism of lemma/palea development. RESULTS: We obtained 20 T0 th1-C transgenic plants by CRISPR/Cas9. Among the 20 plants, there were eight bi-allelic mutations, five homozygous mutations, three heterozygous mutations, and four Non-KO plants. By comparing with the wild type and the heterozygous knock out (KO) line, the homozygous KO lines showed defects in lemma/palea development as well as in grain filling. Further more, we studied the gene regulatory network that controlled by TH1 by comparing the transcriptome of a homozygous TH1 KO line with its Non-KO line as a control. A total of 622 genes were identified as differentially expressed genes (DEGs), of which 297 genes were significantly up-regulated while 325 genes were down-regulated. One hundred thirty eight of the DEGs were assigned to the 59 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. Among these annotated DEGs, 15 genes were related to plant hormone signal transduction, eight genes were related to starch and sucrose metabolism. These were the two largest groups of DEGs according to the KEGG pathway analysis. CONCLUSIONS: Our results indicated that hormone related genes and starch/sucrose metabolism related genes might act as downstream targets of TH1; they might be responsible for lemma/palea	rice
778	plant	rice	CRISPR;	qPE9-1	Rice	Evaluation of differential qPE9-1/DEP1 protein domains in rice grain length and weight variation.	2019	12(1):5	[Li X et al.]	Yangzhou University, Yangzhou, China.	30706248	10.1186/s12284-019-0263-4	BACKGROUND: qPE9-1/DEP1, encoding a G protein gamma subunit, has multiple effects on plant architecture, grain size, and yield in rice. The qPE9-1 protein contains an N-terminal G gamma-like (GGL) domain, a putative transmembrane domain, and a C-terminal cysteine-rich domain. However, the roles of each domain remain unclear. RESULTS: In the present study, we focused on the genetic effects of different domains of qPE9-1 in the regulation of grain length and weight. We generated a series of transgenic plants expressing different truncated qPE9-1 proteins through constitutive expression and clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 strategies. Phenotypic analysis indicated that the complete or long-tailed qPE9-1 contributed to the elongation of grains, while the GGL domain alone and short-tailed qPE9-1 led to short grains. The long C-terminus of qPE9-1 including two or three C-terminal von Willebrand factor type C domains effectively repressed the negative effects of the GGL domain on grain length and weight. qPE9-1-overexpressing lines in a Wuxianggeng 9 (carrying a qpe9-1 allele) background showed increased grain yield per plant, but lodging occurred in some years. CONCLUSIONS: Manipulation of the C-terminal length of qPE9-1 through genetic engineering can be used to generate varieties with various grain lengths and weights according to different requirements in rice breeding. The genetic effects of qPE9-1/qpe9-1 are multidimensional, and breeders should take into account other factors including genetic backgrounds and	rice
779	plant	rice	agroinfiltration; CRISPR;		Scientific reports	High-frequency random DNA insertions upon co-delivery of CRISPR-Cas9 ribonucleoprotein and selectable marker plasmid in rice.	2019	9(1):19902	[Banakar R et al.]	Iowa State University, Ames, IA, USA.	31882637	10.1038/s41598-019-55681-y	An important advantage of delivering CRISPR reagents into cells as a ribonucleoprotein (RNP) complex is the ability to edit genes without reagents being integrated into the genome. Transient presence of RNP molecules in cells can reduce undesirable off-target effects. One method for RNP delivery into plant cells is the use of a biolistic gun. To facilitate selection of transformed cells during RNP delivery, a plasmid carrying a selectable marker gene can be co-delivered with the RNP to enrich for transformed/edited cells. In this work, we compare targeted mutagenesis in rice using three different delivery platforms: biolistic RNP/DNA co-delivery; biolistic DNA delivery; and Agrobacterium-mediated delivery. All three platforms were successful in generating desired mutations at the target sites. However, we observed a high frequency (over 14%) of random plasmid or chromosomal DNA fragment insertion at the target sites in transgenic events generated from both biolistic delivery platforms. In contrast, integration of random DNA fragments was not observed in transgenic events generated from the Agrobacterium-mediated method. These data reveal important insights that must be considered when selecting the method for genome-editing reagent delivery in plants, and emphasize the importance of employing appropriate	rice
780	plant	rice	CRISPR;Cas9;	10 heading time genes	TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik	Assessment of the effect of ten heading time genes on reproductive transition and yield components in rice using a CRISPR/Cas9 system.	2019	132(6):1887-1896	[Cui Y et al.]	Shenyang Agricultural University, Shenyang, China.	30887096	10.1007/s00122-019-03324-1	KEY MESSAGE: We demonstrated the effect of heading time genes on reproductive transition and yield components under an identical genetic background using CRISPR/Cas9 gene-editing technology, and we propose that the elite allele will provide a new breeding strategy for rice breeding in high-latitude regions. Heading date is a factor closely associated with grain yield in rice (Oryza sativa L.). In recent decades, a number of genes responsible for heading time have been identified, the variation of which contributes to the expansion of the rice cultivation area. However, it is difficult to compare the phenotypic effects of these genes due to the different genetic backgrounds. In this study, we generated 14 heading time mutants using CRISPR/Cas9 gene-editing technology and marker-assisted selection with a japonica Sasanishiki wild-type (WT) genetic background. Photoperiod sensitivity, the relationship between days to heading (DTH), and yield components of mutants were investigated. We found that the yield increases with increases in DTH, but eventually plateaus at maximum and then began to decrease, whereas the biomass continued to increase. The mutants exerted distinctly different effects on DTH and yield components. The convergent double mutants had severe yield reduction compared with single mutants, even with a DTH that was similar to that of single mutants. We also found that an elite mutant of se14 achieved a yield equal to that of the WT, but with heading occurring 10 days earlier. A sequence analysis of 72 cultivars collected from the japonica cultivated zone shows that elite se14 mutants have not been applied to rice breeding. Our study demonstrates the effect of heading time genes on reproductive transition and yield components under an identical genetic background. These results may provide new	rice

781	plant	rice (Kasalath; TeTePu)	CRISPR;Cas9;	Semi-Dwarf1	Scientific reports	Using CRISPR-Cas9 to generate semi-dwarf rice lines in elite landraces.	2019	9(1):19096	[Hu X et al.]	China National Rice Research Institute, Hangzhou, China.	31836812	10.1038/s41598-019-55757-9	Genetic erosion refers to the loss of genetic variation in a crop. In China, only a few original landraces of rice (Oryza sativa) were used in breeding and these became the primary genetic background of modern varieties. Expanding the genetic diversity among Chinese rice varieties and cultivating high-yielding and high-quality varieties with resistance to different biotic and abiotic stresses is critical. Here, we used the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9(Cas9) genome editing system to edit Semi-Dwarf1 (SD1) in the elite landraces Kasalath and TeTePu (TTP), which contain many desired agronomic traits such as tolerance to low phosphorous and broad-spectrum resistance to several diseases and insects. Mutations of SD1 confer shorter plant height for better resistance to lodging. Field trials demonstrated that the yield of the new Kasalath and TTP mutant lines was better than that of the wild type under modern cultivation and that the lines maintained the same desirable agronomic characteristics as their wild-type progenitors. Our results showed that breeding using available landraces in combination with genomic data of different landraces and gene-editing techniques is an	rice
782	plant	rice (Oryza sativa)	CRISPR;	SF3B1	Genome biology	CRISPR directed evolution of the spliceosome for resistance to splicing inhibitors.	2019	20(1):73	[Butt H et al.]	King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia.	31036069	10.1186/s13059-019-1680-9	Increasing genetic diversity via directed evolution holds great promise to accelerate trait development and crop improvement. We developed a CRISPR/Cas-based directed evolution platform in plants to evolve the rice (Oryza sativa) SF3B1 spliceosomal protein for resistance to splicing inhibitors. SF3B1 mutant variants, termed SF3B1-GEX1A-Resistant (SGR), confer variable levels of resistance to splicing inhibitors. Studies of the structural basis of the splicing inhibitor binding to SGRs corroborate the resistance phenotype. This directed evolution platform can be used to interrogate and evolve the molecular functions of key biomolecules and to engineer crop traits for improved performance and adaptation under climate change conditions.	rice
783	plant	rice (semi-dwarf)	CRISPR;Cas9;	OsGA20ox2	3 Biotech	Generation of semi-dwarf rice (Oryza sativa L.) lines by CRISPR/Cas9-directed mutagenesis of OsGA20ox2 and proteomic analysis of unveiled changes caused by mutations.	2019	9(11):387	[Han Y et al.]	Guangxi University, Nanning, China.	31656725	10.1007/s13205-019-1919-x	Plant height (PH) is one of the most important agronomic traits of rice, as it directly affects the yield potential and lodging resistance. Here, semi-dwarf mutant lines were developed through CRISPR/Cas9-based editing of OsGA20ox2 in an indica rice cultivar. Total 24 independent lines were obtained in T0 generation with the mean mutation rate of 73.5% including biallelic (29.16%), homozygous (47.91%) and heterozygous (16.66%) mutations, and 16 T-DNA-free lines (50%) were obtained in T1 generation without off-target effect in four most likely sites. Mutations resulted in a changed amino acid sequence of mutant plants and reduced gibberellins (GA) level and PH (22.2%), flag leaf length (FLL) and increased yield per plant (YPP) (6.0%), while there was no effect on other agronomic traits. Mutants restored their PH to normal by exogenous GA3 treatment. The expression of the OsGA20ox2 gene was significantly suppressed in mutant plants, while the expression level was not affected for other GA biosynthesis (OsGA2ox3 and OsGA3ox2) and signaling (D1, GIDI and SLR1) genes. The mutant lines showed decreased cell length and width, abnormal cell elongation, while increased cell numbers in the second internode sections at mature stage. Total 30 protein spots were exercised, and 24 proteins were identified, and results showed that OsGA20ox2 editing altered protein expression. Five proteins including, glyceraldehyde-3-phosphate dehydrogenase, putative ATP synthase, fructose-bisphosphate aldolase 1, S-adenosyl methionine synthetase 1 and gibberellin 20 oxidase 2, were downregulated in dwarf mutant lines which may affect the plant growth. Collectively, our results provide the insights into the role of OsGA20ox2 in PH and confirmed that CRISPR-	rice
784	plant	rice (Taichung 65)	CRISPR;Cas9;	U-box domain-containing protein 73 (OsPUB73)	BMC plant biology	Cytological and transcriptome analyses reveal OsPUB73 defect affects the gene expression associated with tapetum or pollen exine abnormality in rice.	2019	19(1):546	[Chen L et al.]	South China Agricultural University, Guangzhou, China.	31823718	10.1186/s12870-019-2175-2	BACKGROUND: As one of the main crops in the world, sterility of rice (Oryza sativa L.) significantly affects the production and leads to yield decrease. Our previous research showed that OsPUB73, which encodes U-box domain-containing protein 73, may be associated with male sterility. However, little information is available on this gene that is required for anther development. In the present study, we knocked out OsPUB73 by using the CRISPR/Cas9 system and studied the cytological and transcriptome of the gene-defect associated with pollen development and sterility in the rice variety (Taichung 65). RESULTS: The sequence analysis indicated that OsPUB73 was comprised of 3 exons and 2 introns, of which CDS encoded 586 amino acids including a U-box domain. The expression pattern of OsPUB73 showed that it was highly expressed in the anther during meiosis stage. The ospub73 displayed low pollen fertility (19.45%), which was significantly lower than wild type (WT) (85.37%). Cytological observation showed tapetum vacuolated at the meiosis stage and pollen exine was abnormal at the bi-cellular pollen stage of ospub73. RNA-seq analysis detected 2240 down and 571 up-regulated genes in anther of ospub73 compared with WT during meiosis stage. Among of 2240 down-regulated genes, seven known genes were associated with tapetal cell death or pollen exine development, including CYP703A3 (Cytochrome P450 Hydroxylase703A3), CYP704B2 (Cytochrome P450 Hydroxylase704B2), DPW (Defective Pollen Wall), PTC1 (Persistant Tapetal Cell1), UDT1 (Undeveloped Tapetum1), OsAP37 (Aspartic protease37) and OsABCG15 (ATP binding cassette G15), which were validated by quantitative real-time polymerase chain reaction (qRT-PCR). These results suggested OsPUB73 may play an important role in tapetal or pollen exine development and resulted in pollen partial sterility. CONCLUSION: Our results revealed that OsPUB73 plays an important role in rice male reproductive development, which provides valuable information about the molecular	rice
785	plant	rice; Arabidopsis	ZFN;	marker genes	BMC research notes	Utility of I-SceI and CCR5-ZFN nucleases in excising selectable marker genes from transgenic plants.	2019	12(1):272	[Pathak BP et al.]	University of Arkansas, Fayetteville, AR, USA.	31088537	10.1186/s13104-019-4304-2	OBJECTIVES: Removal of selection marker genes from transgenic plants is highly desirable for their regulatory approval and public acceptance. This study evaluated the use of two nucleases, the yeast homing endonuclease, I-SceI, and the designed zinc finger nuclease, CCR5-ZFN, in excising marker genes from plants using rice and Arabidopsis as the models. RESULTS: In an in vitro culture assay, both nucleases were effective in precisely excising the DNA fragments marked by the nuclease target sites. However, rice cultures were found to be refractory to transformation with the I-SceI and CCR5-ZFN overexpressing constructs. The inducible I-SceI expression was also problematic in rice as the progeny of the transgenic lines expressing the heat-inducible I-SceI did not inherit the functional gene. On the other hand, heat-inducible I-SceI expression in Arabidopsis was effective in creating somatic excisions in transgenic plants but ineffective in generating heritable excisions. The inducible expression of CCR5-ZFN in rice, although transmitted stably to the progeny, appeared ineffective in creating detectable excisions. Therefore, toxicity of these nucleases in plant cells poses major bottleneck in their application in plant biotechnology, which could be	rice; Arabido

786	plant	rice; Arabidopsis	CRISPR;Cas9;TALENs;	five different mature miRNA sequences; miR160*	Plant biotechnology journal	Disruption of miRNA sequences by TALENs and CRISPR/Cas9 induces varied lengths of miRNA production.	2019		[Bi H et al.]	Iowa State University, Ames, IA, USA.	31821678	10.1111/pbi.13315	MicroRNAs (miRNAs) are 20-24 nucleotides (nt) small RNAs functioning in eukaryotes. The length and sequence of miRNAs are not only related to the biogenesis of miRNAs but are also important for downstream physiological processes like ta-siRNA production. To investigate these roles, it is informative to create small mutations within mature miRNA sequences. We used both TALENs (transcription activator-like effector nucleases) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) to introduce heritable base pair mutations in mature miRNA sequences. For rice, TALEN constructs were built targeting five different mature miRNA sequences and yielding heritable mutations. Among the resulting mutants, mir390 mutant showed a severe defect in the shoot apical meristem (SAM), a shootless phenotype, which could be rescued by the wild-type MIR390. Small RNA sequencing showed the two base pair deletion in mir390 substantially interfered with miR390 biogenesis. In Arabidopsis, CRISPR/Cas9-mediated editing of the miR160* strand confirmed that the asymmetric structure of miRNA is not a necessary determinant for secondary siRNA production. CRISPR/Cas9 with double-guide RNAs successfully generated mir160a null mutants with fragment deletions, at a higher efficiency than a single-guide RNA. The difference between the phenotypic severity of miR160a mutants in Col-0 versus Ler backgrounds highlights a diverged role for miR160a in different ecotypes. Overall, we demonstrated that TALENs and CRISPR/Cas9 are both effective in modifying miRNA precursor structure, disrupting	rice; Arabido
787	plant	rice; maize; Arabidopsis	CRISPR;Cpf1;		BMC biology	Application of CRISPR-Cas12a temperature sensitivity for improved genome editing in rice, maize, and Arabidopsis.	2019	17(1):9	[Malzahn AA et al.]	University of Electronic Science and Technology of China, Chengdu, China.	30704461	10.1186/s12915-019-0629-5	BACKGROUND: CRISPR-Cas12a (formerly Cpf1) is an RNA-guided endonuclease with distinct features that have expanded genome editing capabilities. Cas12a-mediated genome editing is temperature sensitive in plants, but a lack of a comprehensive understanding on Cas12a temperature sensitivity in plant cells has hampered effective application of Cas12a nucleases in plant genome editing. RESULTS: We compared AsCas12a, FnCas12a, and LbCas12a for their editing efficiencies and non-homologous end joining (NHEJ) repair profiles at four different temperatures in rice. We found that AsCas12a is more sensitive to temperature and that it requires a temperature of over 28 degrees C for high activity. Each Cas12a nuclease exhibited distinct indel mutation profiles which were not affected by temperatures. For the first time, we successfully applied AsCas12a for generating rice mutants with high frequencies up to 93% among T0 lines. We next pursued editing in the dicot model plant Arabidopsis, for which Cas12a-based genome editing has not been previously demonstrated. While LbCas12a barely showed any editing activity at 22 degrees C, its editing activity was rescued by growing the transgenic plants at 29 degrees C. With an early high-temperature treatment regime, we successfully achieved germline editing at the two target genes, GL2 and TT4, in Arabidopsis transgenic lines. We then used high-temperature treatment to improve Cas12a-mediated genome editing in maize. By growing LbCas12a T0 maize lines at 28 degrees C, we obtained Cas12a-edited mutants at frequencies up to 100% in the T1 generation. Finally, we demonstrated DNA binding of Cas12a was not abolished at lower temperatures by using a dCas12a-SRDX-based transcriptional repression system in Arabidopsis. CONCLUSION: Our study demonstrates the use of high-temperature regimes to achieve high editing efficiencies with Cas12a systems in rice, Arabidopsis, and maize and sheds light on the mechanism of temperature	rice; maize; A
788	plant	rice; rapeseed	TALENs;	orf79; orf125	Nature plants	Curing cytoplasmic male sterility via TALEN-mediated mitochondrial genome editing.	2019	5(7):722-730	[Kazama T et al.]	Tohoku University, Sendai, Japan.	31285556	10.1038/s41477-019-0459-z	Sequence-specific nucleases are commonly used to modify the nuclear genome of plants. However, targeted modification of the mitochondrial genome of land plants has not yet been achieved. In plants, a type of male sterility called cytoplasmic male sterility (CMS) has been attributed to certain mitochondrial genes, but none of these genes has been validated by direct mitochondrial gene-targeted modification. Here, we knocked out CMS-associated genes (orf79 and orf125) of CMS varieties of rice and rapeseed, respectively, using transcription activator-like effector nucleases (TALENs) with mitochondria localization signals (mitoTALENs). We demonstrate that knocking out these genes cures male sterility, strongly suggesting that these genes are causes of CMS. Sequencing revealed that double-strand breaks induced by mitoTALENs were repaired by homologous recombination, and that during this process, the target genes and surrounding sequences were deleted. Our results show that mitoTALENs can be used to stably and heritably modify the mitochondrial genome in plants.	rice; rapesee
789	plant	Russian dandelion (Taraxacum koksaghyz)	CRISPR;Cas9;	rapid alkalinisation factor 1	PloS one	Loss of function mutation of the Rapid Alkalinization Factor (RALF1)-like peptide in the dandelion Taraxacum koksaghyz entails a high-biomass taproot phenotype.	2019	14(5):e0217454	[Wieghaus A et al.]	University of Muenster, Munster, Germany.	31125376	10.1371/journal.pone.0217454	The Russian dandelion (Taraxacum koksaghyz) is a promising source of inulin and natural rubber because large amounts of both feedstocks can be extracted from its roots. However, the domestication of T. koksaghyz requires the development of stable agronomic traits such as higher yields of inulin and natural rubber, a higher root biomass, and an agronomically preferable root morphology which is more suitable for cultivation and harvesting. Arabidopsis thaliana Rapid Alkalinisation Factor 1 (RALF1) has been shown to suppress root growth. We identified the T. koksaghyz orthologue TkRALF-like 1 and knocked out the corresponding gene (TkRALFL1) using the CRISPR/Cas9 system to determine its impact on root morphology, biomass, and inulin and natural rubber yields. The TkRALFL1 knockout lines more frequently developed a taproot phenotype which is easier to cultivate and harvest, as well as a higher root biomass and greater yields of both inulin and natural rubber. The TkRALFL1 gene could therefore be suitable as a genetic marker to support the breeding of profitable new dandelion varieties with improved agronomic traits. To our knowledge, this is the first study addressing the root system of T. koksaghyz to enhance the agronomic	Russian dan

790	plant	Sedum plumbizincicola	CRISPR;Cas9;	heavy metal ATPase 1 (SpHMA1)	Plant, cell & environment	SpHMA1 is a chloroplast cadmium exporter protecting photochemical reactions in the Cd hyperaccumulator Sedum plumbizincicola.	2019	42(4):1112-1124	[Zhao H et al.]	Institute of Botany, Chinese Academy of Sciences, Beijing, China.	30311663	10.1111/pce.13456	Sedum plumbizincicola is able to hyperaccumulate cadmium (Cd), a nonessential and highly toxic metal, in the above-ground tissues, but the mechanisms for its Cd hypertolerance are not fully understood. Here, we show that the heavy metal ATPase 1 (SpHMA1) of S. plumbizincicola plays an important role in chloroplast Cd detoxification. Compared with the HMA1 ortholog in the Cd nonhyperaccumulating ecotype of Sedum alfredii, the expression of SpHMA1 in the leaves of S. plumbizincicola was >200 times higher. Heterologous expression of SpHMA1 in Saccharomyces cerevisiae increased Cd sensitivity and Cd transport activity in the yeast cells. The SpHMA1 protein was localized to the chloroplast envelope. SpHMA1 RNA interference transgenic plants and CRISPR/Cas9-induced mutant lines showed significantly increased Cd accumulation in the chloroplasts compared with wild-type plants. Chlorophyll fluorescence imaging analysis revealed that the photosystem II of SpHMA1 knockdown and knockout lines suffered from a much higher degree of Cd toxicity than wild type. Taken together, these results suggest that SpHMA1 functions as a chloroplast Cd exporter and protects photosynthesis by preventing Cd accumulation in the chloroplast in S. plumbizincicola and hyperexpression of SpHMA1 is an important component contributing to Cd	Sedum plumbizincicola
791	plant	Silene latifolia	agroinfiltration;	GUS reporter	New biotechnology	Agrobacterium rhizogenes-mediated transformation of a dioecious plant model Silene latifolia.	2019	48:20-28	[Hudzieczek V et al.]	Institute of Biophysics, Academy of Sciences of the Czech Republic, Czech Republic.	29656128	10.1016/j.nbt.2018.04.001	Silene latifolia serves as a model species to study dioecy, the evolution of sex chromosomes, dosage compensation and sex-determination systems in plants. Currently, no protocol for genetic transformation is available for this species, mainly because S. latifolia is considered recalcitrant to in vitro regeneration and infection with Agrobacterium tumefaciens. Using cytokinins and their synthetic derivatives, we markedly improved the efficiency of regeneration. Several agrobacterial strains were tested for their ability to deliver DNA into S. latifolia tissues leading to transient and stable expression of the GUS reporter. The use of Agrobacterium rhizogenes strains resulted in the highest transformation efficiency (up to 4.7% of stable transformants) in hairy root cultures. Phenotypic and genotypic analyses of the T1 generation suggested that the majority of transformation events contain a small number of independent T-DNA insertions and the transgenes are transmitted to the progeny in a Mendelian pattern of inheritance. In short, we report an efficient and reproducible protocol for leaf disc transformation and subsequent plant regeneration in S. latifolia, based on the unique combination of infection with A. rhizogenes and plant regeneration from hairy root cultures using synthetic cytokinins. A protocol for the transient transformation of S.latifolia protoplasts was also developed and applied to demonstrate the possibility of targeted mutagenesis of the sex linked gene SlAP3 by TALENs and CRISPR/Cas9.	Silene latifol
792	plant	soybean	agroinfiltration; CRISPR;Cas9;	microsomal omega-6 desaturase (FAD2-2)	BMC biotechnology	CRISPR-Cas9 mediated targeted disruption of FAD2-2 microsomal omega-6 desaturase in soybean (Glycine max.L).	2019	19(1):9	[Al Amin N et al.]	Jilin Agricultural University, Changchun, Jilin, China.	30691438	10.1186/s12896-019-0501-2	BACKGROUND: Recent innovation in the field of genome engineering encompasses numerous levels of plant genome engineering which attract the substantial excitement of plant biologist worldwide. RNA-guided CRISPR Cas9 system has appeared a promising tool in site-directed mutagenesis due to its innovative utilization in different branches of biology. CRISPR-Cas9 nuclease system have supersedes all previously existed strategies and their associated pitfalls encountered with site-specific mutagenesis. RESULTS: Here we demonstrated an efficient sequence specific integration/mutation of FAD2-2 gene in soybean using CRISPR-Cas9 nuclease system. A single guided RNA sequence was designed with the help of a number of bioinformatics tools aimed to target distinct sites of FAD2-2 loci in soybean. The binary vector (pCas9-AtU6-sgRNA) has been successfully transformed into soybean cotyledon using Agrobacterium tumafacien. Taken together our findings complies soybean transgenic mutants subjected to targeted mutation were surprisingly detected in our target gene. Furthermore, the detection of Cas9 gene, BAR gene, and NOS terminator were carried out respectively. Southern blot analysis confirmed the stable transformation of Cas9 gene into soybean. Real time expression with qRT-PCR and Sanger sequencing analysis confirmed the efficient CRISPR-Cas9/sgRNA induced mutation within the target sequence of FAD2-2 loci. The integration of FAD2-2 target region in the form of substitution, deletions and insertions were achieved with notably high frequency and rare off-target mutagenesis. CONCLUSION: High frequent mutation efficiency was recorded as 21% out of all transgenic soybean plants subjected to targeted mutagenesis. Furthermore, Near-infrared spectroscopy (NIR) indicates the entire fatty acid profiling obtained from the mutants seeds of soybean. A considerable modulation in oleic acid content up to (65.58%) whereas the least level of linoleic acid is (16.08%) were recorded. Based on these finding CRISPR-Cas9 system can possibly sum	soybean
793	plant	soybean	agroinfiltration; CRISPR;Cas9;	late elongated hypocotyl	BMC plant biology	CRISPR/Cas9-mediated targeted mutagenesis of GmLHY genes alters plant height and internode length in soybean.	2019	19(1):562	[Cheng Q et al.]	Guangzhou University, Guangzhou, China.	31852439	10.1186/s12870-019-2145-8	BACKGROUND: Soybean (Glycine max) is an economically important oil and protein crop. Plant height is a key trait that significantly impacts the yield of soybean; however, research on the molecular mechanisms associated with soybean plant height is lacking. The CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated system 9) system is a recently developed technology for gene editing that has been utilized to edit the genomes of crop plants. RESULTS: Here, we designed four gRNAs to mutate four LATE ELONGATED HYPOCOTYL (LHY) genes in soybean. In order to test whether the gRNAs could perform properly in transgenic soybean plants, we first tested the CRISPR construct in transgenic soybean hairy roots using Agrobacterium rhizogenes strain K599. Once confirmed, we performed stable soybean transformation and obtained 19 independent transgenic soybean plants. Subsequently, we obtained one T1 transgene-free homozygous quadruple mutant of GmLHY by self-crossing. The phenotypes of the T2-generation transgene-free quadruple mutant plants were observed, and the results showed that the quadruple mutant of GmLHY displayed reduced plant height and shortened internodes. The levels of endogenous gibberellic acid (GA3) in Gmlhy1a1b2a2b was lower than in the wild type (WT), and the shortened internode phenotype could be rescued by treatment with exogenous GA3. In addition, the relative expression levels of GA metabolic pathway genes in the quadruple mutant of GmLHY were significantly decreased in comparison to the WT. These results suggest that GmLHY encodes an MYB transcription factor that affects plant height through mediating the GA pathway in soybean. We also developed genetic markers for identifying mutants for application in breeding studies. CONCLUSIONS: Our results indicate that CRISPR/Cas9-mediated targeted mutagenesis of four GmLHY genes reduces soybean plant height and shortens internodes from 20 to 35 days after emergence (DAE). These findings provide insight into the mechanisms underlying plant	soybean

794	plant	soybean	agroinfiltration; CRISPR;Cas9;	Fatty Acid Desaturase 2 (GmFAD2-1A; GmFAD2-1B)	BMC plant biology	Demonstration of highly efficient dual gRNA CRISPR/Cas9 editing of the homeologous GmFAD2-1A and GmFAD2-1B genes to yield a high oleic, low linoleic and alpha-linolenic acid phenotype in soybean.	2019	19(1):311	[Do PT et al.]	University of Missouri, Columbia, MO, USA.	31307375	10.1186/s12870-019-1906-8	BACKGROUND: CRISPR/Cas9 gene editing is now revolutionizing the ability to effectively modify plant genomes in the absence of efficient homologous recombination mechanisms that exist in other organisms. However, soybean is allotetraploid and is commonly viewed as difficult and inefficient to transform. In this study, we demonstrate the utility of CRISPR/Cas9 gene editing in soybean at relatively high efficiency. This was shown by specifically targeting the Fatty Acid Desaturase 2 (GmFAD2) that converts the monounsaturated oleic acid (C18:1) to the polyunsaturated linoleic acid (C18:2), therefore, regulating the content of monounsaturated fats in soybean seeds. RESULTS: We designed two gRNAs to guide Cas9 to simultaneously cleave two sites, spaced 1Kb apart, within the second exons of GmFAD2-1A and GmFAD2-1B. In order to test whether the Cas9 and gRNAs would perform properly in transgenic soybean plants, we first tested the CRISPR construct we developed by transient hairy root transformation using Agrobacterium rhizogenesis strain K599. Once confirmed, we performed stable soybean transformation and characterized ten, randomly selected T0 events. Genotyping of CRISPR/Cas9 T0 transgenic lines detected a variety of mutations including large and small DNA deletions, insertions and inversions in the GmFAD2 genes. We detected CRISPR-edited DNA in all the tested T0 plants and 77.8% of the events transmitted the GmFAD2 mutant alleles to T1 progenies. More importantly, null mutants for both GmFAD2 genes were obtained in 40% of the T0 plants we genotyped. The fatty acid profile analysis of T1 seeds derived from CRISPR-edited plants homozygous for both GmFAD2 genes showed dramatic increases in oleic acid content to over 80%, whereas linoleic acid decreased to 1.3-1.7%. In addition, transgene-free high oleic soybean homozygous genotypes were created as early as the T1 generation. CONCLUSIONS: Overall, our data showed that dual gRNA CRISPR/Cas9 system offers a rapid and	soybean
795	plant	soybean	CRISPR;Cas9;agroinfiltration	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors of the SPL9 family (GmSPL9)	BMC plant biology	CRISPR/Cas9-mediated targeted mutagenesis of GmSPL9 genes alters plant architecture in soybean.	2019	19(1):131	[Bao A et al.]	Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan, China.	30961525	10.1186/s12870-019-1746-6	BACKGROUND: The plant architecture has significant effects on grain yield of various crops, including soybean (Glycine max), but the knowledge on optimization of plant architecture in order to increase yield potential is still limited. Recently, CRISPR/Cas9 system has revolutionized genome editing, and has been widely utilized to edit the genomes of a diverse range of crop plants. RESULTS: In the present study, we employed the CRISPR/Cas9 system to mutate four genes encoding SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors of the SPL9 family in soybean. These four GmSPL9 genes are negatively regulated by GmmiR156b, a target for the improvement of soybean plant architecture and yields. The soybean Williams 82 was transformed with the binary CRISPR/Cas9 plasmid, assembled with four sgRNA expression cassettes driven by the Arabidopsis thaliana U3 or U6 promoter, targeting different sites of these four SPL9 genes via Agrobacterium tumefaciens-mediated transformation. A 1-bp deletion was detected in one target site of the GmSPL9a and one target site of the GmSPL9b, respectively, by DNA sequencing analysis of two T0-generation plants. T2-generation spl9a and spl9b homozygous single mutants exhibited no obvious phenotype changes; but the T2 double homozygous mutant spl9a/spl9b possessed shorter plastochron length. In T4 generation, higher-order mutant plants carrying various combinations of mutations showed increased node number on the main stem and branch number, consequently increased total node number per plants at different levels. In addition, the expression levels of the examined GmSPL9 genes were higher in the spl9b-1 single mutant than wild-type plants, which might suggest a feedback regulation on the expression of the investigated GmSPL9 genes in soybean. CONCLUSIONS: Our results showed that CRISPR/Cas9-mediated targeted mutagenesis of four GmSPL9 genes in different combinations altered plant architecture in soybean. The findings demonstrated that GmSPL9a, GmSPL9b, GmSPL9c and GmSPL9 function as redundant transcription factors in regulating plant architecture in	soybean
796	plant	soybean	agroinfiltration; CRISPR;Cas9;	soybean storage protein genes	BMC research notes	Mutagenesis of seed storage protein genes in Soybean using CRISPR/Cas9.	2019	12(1):176	[Li C et al.]	London Research and Development Center, Agriculture and Agri-Food Canada, London, ON, Canada.	30917862	10.1186/s13104-019-4207-2	OBJECTIVE: Soybean seeds are an important source of vegetable proteins for both food and industry worldwide. Conglycinins (7S) and glycinins (11S), which are two major families of storage proteins encoded by a small family of genes, account for about 70% of total soy seed protein. Mutant alleles of these genes are often necessary in certain breeding programs, as the relative abundance of these protein subunits affect amino acid composition and soy food properties. In this study, we set out to test the efficiency of the CRISPR/Cas9 system in editing soybean storage protein genes using Agrobacterium rhizogenes-mediated hairy root transformation system. RESULTS: We designed and tested sgRNAs to target nine different major storage protein genes and detected DNA mutations in three storage protein genes in soybean hairy roots, at a ratio ranging from 3.8 to 43.7%. Our work provides a useful resource for future soybean breeders to engineer/develop varieties with mutations in seed storage proteins.	soybean
797	plant	soybean	Agroinfiltration;	Lotus japonicus polyubiquitin gene promoter-fused MYB gene into promoter-fused beta-glucuronidase gene constructs	Plant biotechnology	Identification of novel MYB transcription factors involved in the isoflavone biosynthetic pathway by using the combination screening system with agroinfiltration and hairy root transformation.	2019	36(4):241-251	[Sarkar MAR et al.]	Kagoshima University, Kagoshima, Japan.	31983878	10.5511/plantbiotechnology.19.1025a	Soybean isoflavones are functionally important secondary metabolites that are mainly accumulated in seeds. Their biosynthetic processes are regulated coordinately at the transcriptional level; however, screening systems for key transcription factors (TFs) are limited. Here we developed a combination screening system comprising a simple agroinfiltration assay and a robust hairy root transformation assay. First, we screened for candidate MYB TFs that could activate the promoters of the chalcone synthase (CHS) gene GmCHS8 and the isoflavone synthase (IFS) genes GmIFS1 and GmIFS2 in the isoflavone biosynthetic pathway. In the agroinfiltration assay, we co-transformed a LjUbi (Lotus japonicus polyubiquitin gene) promoter-fused MYB gene with target promoter-fused GUS (beta-glucuronidase) gene constructs, and identified three genes (GmMYB102, GmMYB280, and GmMYB502) as candidate regulators of isoflavone biosynthesis. We then evaluated the functional regulatory role of identified three MYB genes in isoflavone biosynthesis using hairy roots transformation assay in soybean for the accumulation of isoflavones. Three candidate MYB genes showed an increased accumulation of total isoflavones in hairy root transgenic lines. Accumulation of total isoflavones in the three MYB-overexpressing lines was approximately 2-to 4-folds more than that in the vector control, confirming their possible role to regulate isoflavone biosynthesis. However, the significant accumulation of authentic GmCHS8, GmIFS1, and GmIFS2 transcripts could not be observed except for the GmMYB502-overexpressing line. Therefore, the analysis of isoflavone accumulation in transgenic hairy root was effective for evaluation of transactivation activity of MYB TFs for isoflavone biosynthetic genes. Our results demonstrate a simple and robust system that can potentially identify the function of orphan TFs in diverse plant metabolic	soybean

798	plant	soybean	CRISPR;Cas9;	GmF3H1; GmF3H2; GmFNSII-1	Plant biotechnology journal	Multiplex CRISPR/Cas9-mediated metabolic engineering increases soya bean isoflavone content and resistance to soya bean mosaic virus.	2019		[Zhang P et al.]	Nanjing Agricultural University, Nanjing, China.	31769589	10.1111/pbi.13302	Isoflavonoids, which include a variety of secondary metabolites, are derived from the phenylpropanoid pathway and are distributed predominantly in leguminous plants. These compounds play a critical role in plant-environment interactions and are beneficial to human health. Isoflavone synthase (IFS) is a key enzyme in isoflavonoid synthesis and shares a common substrate with flavanone-3-hydroxylase (F3H) and flavone synthase II (FNS II). In this study, CRISPR/Cas9-mediated multiplex gene-editing technology was employed to simultaneously target GmF3H1, GmF3H2 and GmFNSII-1 in soya bean hairy roots and plants. Various mutation types and frequencies were observed in hairy roots. Higher mutation efficiencies were found in the T0 transgenic plants, with a triple gene mutation efficiency of 44.44%, and these results of targeted mutagenesis were stably inherited in the progeny. Metabolomic analysis of T0 triple-mutants leaves revealed significant improvement in isoflavone content. Compared with the wild type, the T3 generation homozygous triple mutants had approximately twice the leaf isoflavone content, and the soya bean mosaic virus (SMV) coat protein content was significantly reduced by one-third after infection with strain SC7, suggesting that increased isoflavone content enhanced the leaf resistance to SMV. The isoflavone content in the seeds of T2 triple mutants was also significantly increased. This study provides not only materials for the improvement of soya bean isoflavone content and resistance to SMV but also a simple system to generate multiplex mutations in soya bean, which may be beneficial for further breeding and metabolic engineering.	soybean
799	plant	soybean	CRISPR;Cas9;	102 candidate genes and their paralogs	Plant biotechnology journal	Generation of a multiplex mutagenesis population via pooled CRISPR-Cas9 in soya bean.	2019		[Bai M et al.]	Fujian Agriculture and Forestry University, Fuzhou, China.	31452351	10.1111/pbi.13239	The output of genetic mutant screenings in soya bean [Glycine max (L.) Merr.] has been limited by its paleopolypoid genome. CRISPR-Cas9 can generate multiplex mutants in crops with complex genomes. Nevertheless, the transformation efficiency of soya bean remains low and, hence, remains the major obstacle in the application of CRISPR-Cas9 as a mutant screening tool. Here, we report a pooled CRISPR-Cas9 platform to generate soya bean multiplex mutagenesis populations. We optimized the key steps in the screening protocol, including vector construction, sgRNA assessment, pooled transformation, sgRNA identification and gene editing verification. We constructed 70 CRISPR-Cas9 vectors to target 102 candidate genes and their paralogs which were subjected to pooled transformation in 16 batches. A population consisting of 407 T0 lines was obtained containing all sgRNAs at an average mutagenesis frequency of 59.2%, including 35.6% lines carrying multiplex mutations. The mutation frequency in the T1 progeny could be increased further despite obtaining a transgenic chimera. In this population, we characterized gmric1/gmric2 double mutants with increased nodule numbers and gmrdn1-1/1-2/1-3 triple mutant lines with decreased nodulation. Our study provides an advanced strategy for the generation of a targeted multiplex mutant population to overcome the gene redundancy problem in	soybean
800	plant	soybean	ZFN;	multiple transgenes into FAD2-1a locus	Plant biotechnology journal	Zinc finger nuclease-mediated targeting of multiple transgenes to an endogenous soybean genomic locus via non-homologous end joining.	2019	17(4):750-761	[Bonawitz ND et al.]	Dow AgroSciences LLC, Indianapolis, IN, USA.	30220095	10.1111/pbi.13012	Emerging genome editing technologies hold great promise for the improvement of agricultural crops. Several related genome editing methods currently in development utilize engineered, sequence-specific endonucleases to generate DNA double strand breaks (DSBs) at user-specified genomic loci. These DSBs subsequently result in small insertions/deletions (indels), base substitutions or incorporation of exogenous donor sequences at the target site, depending on the application. Targeted mutagenesis in soybean (Glycine max) via non-homologous end joining (NHEJ)-mediated repair of such DSBs has been previously demonstrated with multiple nucleases, as has homology-directed repair (HDR)-mediated integration of a single transgene into target endogenous soybean loci using CRISPR/Cas9. Here we report targeted integration of multiple transgenes into a single soybean locus using a zinc finger nuclease (ZFN). First, we demonstrate targeted integration of biolistically delivered DNA via either HDR or NHEJ to the FATTY ACID DESATURASE 2-1a (FAD2-1a) locus of embryogenic cells in tissue culture. We then describe ZFN- and NHEJ-mediated, targeted integration of two different multigene donors to the FAD2-1a locus of immature embryos. The largest donor delivered was 16.2 kb, carried four transgenes, and was successfully transmitted to T1 progeny of mature targeted plants obtained via somatic embryogenesis. The insertions in most plants with a targeted, 7.1 kb, NHEJ-integrated donor were perfect or near-perfect, demonstrating that NHEJ is a viable alternative to HDR for gene targeting in soybean. Taken together, these results show that ZFNs can be used to generate fertile transgenic soybean plants with NHEJ-mediated targeted	soybean
801	plant	soybean	CRISPR;Cas9;	Heat shock protein 90 (GmHsp90A2)	Plant science	GmHsp90A2 is involved in soybean heat stress as a positive regulator.	2019	285:26-33	[Huang Y et al.]	Nanjing Agricultural University, Nanjing, China.	31203891	10.1016/j.plantsci.2019.04.016	Heat shock protein 90 s (Hsp90s), one of the most conserved and abundant molecular chaperones, is an essential component of the protective stress response. A previous study reported at least 12 genes in the GmHsp90s family in soybean and that GmHsp90A2 overexpression enhanced thermotolerance in Arabidopsis thaliana. Here, we investigate the roles of GmHsp90A2 in soybean by utilizing stable transgenic soybean lines overexpressing GmHsp90A2 and mutant lines generated by the CRISPR/Cas9 system. The results showed that compared with wild-type plants (WT) and empty vector control plants (VC), T3 transgenic soybean plants overexpressing GmHsp90A2 exhibited increased tolerance to heat stress through higher chlorophyll and lower malondialdehyde (MDA) contents in plants. Conversely, reduced chlorophyll and increased MDA contents in T2 homozygous GmHsp90A2-knockout mutants indicated decreased tolerance to heat stress. GmHsp90A2 was found to interact with GmHsp90A1 in yeast two-hybrid assays. Furthermore, subcellular localization analyses revealed that GmHsp90A2 was localized to the cytoplasm and cell membrane; as shown by bimolecular fluorescence complementation (BiFC) assays, GmHsp90A2 interacted with GmHsp90A1 in the nucleus and cytoplasm and cell membrane. Hence, we conclude that GmHsp90A1 is able to bind to GmHsp90A2 to form a complex and that this complex enters the nucleus. In summary, GmHsp90A2 might respond to heat stress	soybean

802	plant	soybean	CRISPR;	GmLCLa1; LCLa2; LCLb1; LCLb2	Plant, cell & environment	Light- and temperature-entrainable circadian clock in soybean development.	2019		[Wang Y et al.]	Hebei Normal University, Shijiazhuang, China.	31724182	10.1111/pce.13678	In plants, the spatiotemporal expression of circadian oscillators provides adaptive advantages in diverse species. However, the molecular basis of circadian clock in soybean is not known. In this study, we used soybean hairy roots expression system to monitor endogenous circadian rhythms and the sensitivity of circadian clock to environmental stimuli. We discovered in experiments with constant light and temperature conditions that the promoters of clock genes GmLCLb2 and GmPRR9b1 drive a self-sustained, robust oscillation of about 24-h in soybean hairy roots. Moreover, we demonstrate that circadian clock is entrainable by ambient light/dark or temperature cycles. Specifically, we show that light and cold temperature pulses can induce phase shifts of circadian rhythm, and we found that the magnitude and direction of phase responses depends on the specific time of these two zeitgeber stimuli. We obtained a quadruple mutant lacking the soybean gene GmLCLa1, LCLa2, LCLb1, and LCLb2 using CRISPR, and found that loss-of-function of these four GmLCL orthologs leads to an extreme short-period circadian rhythm and late-flowering phenotype in transgenic soybean. Our study establishes that the morning-phased GmLCLs genes act constitutively to maintain circadian rhythmicity and demonstrates that their absence	soybean
803	plant	soybean	CRISPR;Cas9;	CPR5	Scientific reports	Functional analysis and development of a CRISPR/Cas9 allelic series for a CPR5 ortholog necessary for proper growth of soybean trichomes.	2019	9(1):14757	[Campbell BW et al.]	University of Minnesota, St. Paul, MN, USA.	31611562	10.1038/s41598-019-51240-7	Developments in genomic and genome editing technologies have facilitated the mapping, cloning, and validation of genetic variants underlying trait variation. This study combined bulked-segregant analysis, array comparative genomic hybridization, and CRISPR/Cas9 methodologies to identify a CPR5 ortholog essential for proper trichome growth in soybean (Glycine max). A fast neutron mutant line exhibited short trichomes with smaller trichome nuclei compared to its parent line. A fast neutron-induced deletion was identified within an interval on chromosome 6 that co-segregated with the trichome phenotype. The deletion encompassed six gene models including an ortholog of Arabidopsis thaliana CPR5. CRISPR/Cas9 was used to mutate the CPR5 ortholog, resulting in five plants harboring a total of four different putative knockout alleles and two in-frame alleles. Phenotypic analysis of the mutants validated the candidate gene, and included intermediate phenotypes that co-segregated with the in-frame alleles. These findings demonstrate that the CPR5 ortholog is essential for proper growth and development of soybean trichomes, similar to observations in A. thaliana. Furthermore, this work demonstrates the value of using CRISPR/Cas9 to generate an allelic series and intermediate phenotypes for functional analysis of	soybean
804	plant	soybean (cultivar Jack)	agroinfiltration; CRISPR;Cas9;	E1	Frontiers in plant science	Creation of Early Flowering Germplasm of Soybean by CRISPR/Cas9 Technology.	2019	10:1446	[Han J et al.]	Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China.	31824524	10.3389/fpls.2019.01446	Soybean is an important economic crop and a typical short-day crop, sensitive to photoperiod, and has narrow geographical adaptative region, which limit the creation of transgenic materials and reduce the breeding efficiency of new varieties. In addition, the genetic transformation efficiency of soybean is lower than that of many other crops, and the available receptor genotypes are limited. In this study, Agrobacterium-mediated transformation were used to introduce the CRISPR/Cas9 expression vector into soybean cultivar Jack and generated targeted mutants of E1 gene controlling soybean flowering. We obtained two novel types of mutations, 11 bp and 40 bp deletion at E1 coding region, respectively, and frameshift mutations produced premature translation termination codons and truncated E1 proteins, causing obvious early flowering under long day condition. In addition, no off-target effects were observed by predicting and analyzing the potential off-target sites of E1 targets. Significant decreased E1 gene expression of two novel mutants showed that the truncated E1 protein disinhibited GmFT2a/5a and increasing GmFT2a/5a gene expressions resulted obvious early flowering. Homozygous trans-clean mutants without T-DNA elements were also obtained and showed early flowering under long day condition. The photo-insensitive soybean transformation receptor we created laid a foundation for breeding	soybean
805	plant	strawberry (Fragaria vesca ssp. vesca 'Hawaii 4' and F. x ananassa 'Calypso')	agroinfiltration; CRISPR;Cas9;	phytoene desaturase	Plant methods	CRISPR/Cas9-mediated mutagenesis of phytoene desaturase in diploid and octoploid strawberry.	2019	15:45	[Wilson FM et al.]	NIAB EMR, Kent, UK.	31068975	10.1186/s13007-019-0428-6	Background: Gene editing using CRISPR/Cas9 is a simple and powerful tool for elucidating genetic controls and for crop improvement and its use has been reported in a growing number of important food crops, including recently Fragaria. In order to inform application of the technology in Fragaria, we targeted the visible endogenous marker gene PDS (phytoene desaturase) in diploid Fragaria vesca ssp. vesca 'Hawaii 4' and octoploid F. x ananassa 'Calypso'. Results: Agrobacterium-mediated transformation of leaf and petiole explants was used for efficient stable integration of constructs expressing plant codon-optimised Cas9 and single guide sequences under control of the Arabidopsis U6-26 consensus promoter and terminator or Fragaria vesca U6III regulatory sequences. More than 80% ('Hawaii 4') and 50% ('Calypso') putative transgenic shoot lines (multiple shoots derived from a single callus) exhibited mutant phenotypes. Of mutant shoot lines selected for molecular analysis, approximately 75% ('Hawaii 4') and 55% ('Calypso') included albino regenerants with bi-allelic target sequence variants. Our results indicate the PDS gene is functionally diploid in 'Calypso'. Conclusion: We demonstrate that CRISPR/Cas9 may be used to generate biallelic mutants at high frequency within the genomes of diploid and octoploid strawberry. The methodology, observations and comprehensive data set presented will facilitate routine application of this technology in Fragaria to single and multiple gene	strawberry

806	plant	strawberry (Fragaria vesca)	CRISPR;Cas9;	Reduced Anthocyanins in Petioles	Plant biotechnology journal	Genetic modulation of RAP alters fruit coloration in both wild and cultivated strawberry.	2019		[Gao Q et al.]	Huazhong Agricultural University, Wuhan, China.	31845477	10.1111/pbi.13317	Fruit colour affects consumer preference and is an important trait for breeding in strawberry. Previously, we isolated the Reduced Anthocyanins in Petioles (RAP) gene encoding a glutathione S-transferase (GST) that binds anthocyanins to facilitate their transport from cytosol to vacuole in the diploid strawberry Fragaria vesca. The parent of rap was the F. vesca variety 'Yellow Wonder' that develops white fruit due to a natural mutation in the FveMYB10 gene. Here, we investigated the application potential of RAP in modulating fruit colours by overexpression of RAP in F. vesca and knockout of RAP in the cultivated strawberry Fragaria x ananassa. Unexpectedly, the RAP overexpression in Yellow Wonder background caused formation of red fruit. In addition, the red coloration occurs precociously at floral stage 10 and continues in the receptacle during early fruit development. Transcriptome analysis revealed that the anthocyanin biosynthesis genes were not up-regulated in RAP-ox; rap myb10 flowers at anthesis and largely inhibited at the turning stage in fruit, suggesting a coloration mechanism independent of FveMYB10. Moreover, we used CRISPR/Cas9 to knockout RAP in cultivated strawberry which is octoploid. Six copies of RAP were simultaneously knocked out in the T0 generation leading to the green stem and white-fruited phenotype. Several T1 progeny have segregated away the CRISPR/Cas9 transgene but maintain the green stem trait. Our results indicate that enhancing the anthocyanin transport could redirect the metabolic flux from proanthocyanidin to anthocyanin production at early developmental stages of fruit and that RAP is one	strawberry
807	plant	strawberry (Fragaria x ananassa)	Agroinfiltration;	FaPRE1	BMC plant biology	An atypical HLH transcriptional regulator plays a novel and important role in strawberry ripened receptacle.	2019	19(1):586	[Medina-Puche L et al.]	Universidad de Cordoba, Cordoba, Spain.	31881835	10.1186/s12870-019-2092-4	BACKGROUND: In soft fruits, the differential expression of many genes during development and ripening is responsible for changing their organoleptic properties. In strawberry fruit, although some genes involved in the metabolic regulation of the ripening process have been functionally characterized, some of the most studied genes correspond to transcription factors. High throughput transcriptomics analyses performed in strawberry red receptacle (Fragaria x ananassa) allowed us to identify a ripening-related gene that codes an atypical HLH (FaPRE1) with high sequence homology with the PACLOBUTRAZOL RESISTANCE (PRE) genes. PRE genes are atypical bHLH proteins characterized by the lack of a DNA-binding domain and whose function has been linked to the regulation of cell elongation processes. RESULTS: FaPRE1 sequence analysis indicates that this gene belongs to the subfamily of atypical bHLHs that also includes ILI-1 from rice, SlPRE2 from tomato and AtPRE1 from Arabidopsis, which are involved in transcriptional regulatory processes as repressors, through the blockage by heterodimerization of bHLH transcription factors. FaPRE1 presented a transcriptional model characteristic of a ripening-related gene with receptacle-specific expression, being repressed by auxins and activated by abscisic acid (ABA). However, its expression was not affected by gibberellic acid (GA3). On the other hand, the transitory silencing of FaPRE1 transcription by agroinfiltration in receptacle produced the down-regulation of a group of genes related to the ripening process while inducing the transcription of genes involved in receptacle growth and development. CONCLUSIONS: In summary, this work presents for the first time experimental data that support an important novel function for the atypical HLH FaPRE1 during the strawberry fruit ripening. We hypothesize that FaPRE1 modulates antagonistically the transcription of genes related to both receptacle growth and ripening. Thus, FaPRE1 would repress the expression of receptacle growth promoting	strawberry
808	plant	strawberry (Fragaria x ananassa)	CRISPR;Cas9;	MADS-box gene TM6	Journal of experimental botany	Functional analysis of the TM6 MADS-box gene in the octoploid strawberry by CRISPR/Cas9-directed mutagenesis.	2019	70(3):885-895	[Martin-Pizarro C et al.]	Universidad de Malaga-Consejo Superior de Investigaciones Cientificas, Malaga, Spain.	30428077	10.1093/jxb/ery400	The B-class of MADS-box transcription factors has been studied in many plant species, but remains functionally uncharacterized in Rosaceae. APETALA3 (AP3), a member of this class, controls petal and stamen identities in Arabidopsis. In this study, we identified two members of the AP3 lineage in cultivated strawberry, Fragaria x ananassa, namely FaAP3 and FaTM6. FaTM6, and not FaAP3, showed an expression pattern equivalent to that of AP3 in Arabidopsis. We used the CRISPR/Cas9 genome editing system for the first time in an octoploid species to characterize the function of TM6 in strawberry flower development. An analysis by high-throughput sequencing of the FaTM6 locus spanning the target sites showed highly efficient genome editing already present in the T0 generation. Phenotypic characterization of the mutant lines indicated that FaTM6 plays a key role in anther development in strawberry. Our results validate the use of the CRISPR/Cas9 system for gene functional analysis in F. x ananassa as an octoploid species, and offer new opportunities for engineering	strawberry
809	plant	wild strawberry (Fragaria vesca)	CRISPR;	FveYUC10	Journal of experimental botany	Reporter gene expression reveals precise auxin synthesis sites during fruit and root development in wild strawberry.	2019	70(2):563-574	[Feng J et al.]	Huazhong Agricultural University, Wuhan, China.	30371880	10.1093/jxb/ery384	The critical role of auxin in strawberry fruit set and receptacle enlargement was demonstrated previously. While fertilization is known to trigger auxin biosynthesis, the specific tissue source of fertilization-induced auxin is not well understood. Here, the auxin reporter DR5ver2::GUS was introduced into wild strawberry (Fragaria vesca) to reveal auxin distribution in the seed and fruit receptacle pre- and post-fertilization as well as in the root. In addition, the expression of TAR and YUCCA genes coding for enzymes catalysing the two-step auxin biosynthesis pathway was investigated using their respective promoters fused to the beta-glucuronidase (GUS) reporter. Two FveTARs and four FveYUCs were shown to be expressed primarily in the endosperm and embryo inside the achenes as well as in root tips and lateral root primordia. Expression of these reporters in dissected tissues provided more detailed and precise spatial (cell and tissue) and temporal (pre- and post-fertilization) information on where auxin is synthesized and accumulates than previous studies in strawberry. Moreover, we generated CRISPR-mediated knock-out mutants of FveYUC10, the most abundant YUC in seeds; the mutants had a lower free auxin level in young fruit, but displayed no obvious morphological phenotypes. However, overexpression of FveYUC10 resulted in elongated hypocotyls in Arabidopsis caused by elevated auxin level. Overall, the study revealed auxin accumulation in the chalazal seed coat, embryo, receptacle vasculature, root tip, and lateral root primordia and highlighted the endosperm as the main auxin	strawberry

810	plant	sweet potato (Ipomoea batatas)	CRISPR;Cas9;	IbGBSSI (encoding granule-bound starch synthase I); IbSBEII (encoding starch branching enzyme II)	International journal of molecular sciences	CRISPR/Cas9-Based Mutagenesis of Starch Biosynthetic Genes in Sweet Potato (Ipomoea Batatas) for the Improvement of Starch Quality.	2019	20(19)	[Wang H et al.]	Shanghai Institutes for Biological Sciences, Chinese Academy of Science, Shanghai, China.	31547486	10.3390/ijms20194702	CRISPR/Cas9-mediated genome editing is a powerful technology that has been used for the genetic modification of a number of crop species. In order to evaluate the efficacy of CRISPR/Cas9 technology in the root crop, sweet potato (Ipomoea batatas), two starch biosynthetic pathway genes, IbGBSSI (encoding granule-bound starch synthase I), and IbSBEII (encoding starch branching enzyme II), were targeted in the starch-type cultivar Xushu22 and carotenoid-rich cultivar Taizhong6. I. batatas was transformed using a binary vector, in which the Cas9 gene is driven by the Arabidopsis AtUBQ promoter and the guide RNA is controlled by the Arabidopsis AtU6 promoter. A total of 72 Xushu22 and 35 Taizhong6 transgenic lines were generated and analyzed for mutations. The mutation efficiency was 62-92% with multi-allelic mutations in both cultivars. Most of the mutations were nucleotide substitutions that lead to amino acid changes and, less frequently, stop codons. In addition, short nucleotide insertions or deletions were also found in both IbGBSSI and IbSBEII. Furthermore, a 2658 bp deletion was found in one IbSBEII transgenic line. The total starch contents were not significantly changed in IbGBSSI- and IbSBEII-knockout transgenic lines compared to the wild-type control. However, in the allopolyploid sweet potato, the IbGBSSI-knockout reduced, while the IbSBEII-knockout increased, the amylose percentage. Our results demonstrate that CRISPR/Cas9 technology is an effective tool for the improvement of starch qualities in sweet potato and breeding of polyploid root crops.	sweet potato
811	plant	Nicotiana benthamiana	Agroinfiltration;	RNA1 (RdRp, p22) and RNA2 (CP, CPm and p26) of cucurbit chlorotic yellows virus	Archives of virology	Cucurbit chlorotic yellows virus p22 is a suppressor of local RNA silencing.	2019	164(11):2747-2759	[Orfanidou CG et al.]	Aristotle University of Thessaloniki, Thessaloniki, Greece.	31502079	10.1007/s00705-019-04391-x	RNA silencing is a major antiviral mechanism in plants, which is counteracted by virus-encoded proteins with silencing suppression activity. ORFs encoding putative silencing suppressor proteins that share no structural or sequence homology have been identified in the genomes of four criniviruses. In this study, we investigated the RNA silencing suppression activity of several proteins encoded by the RNA1 (RdRp, p22) and RNA2 (CP, CPm and p26) of cucurbit chlorotic yellows virus (CCYV) using co-agroinfiltration assays on Nicotiana benthamiana plants. Our results indicate that p22 is a suppressor of local RNA silencing that does not interfere with cell-to-cell movement of the RNA silencing signal or with systemic silencing. Furthermore, comparisons of the suppression activity of CCYV p22 with that of two other well-known crinivirus suppressors (CYSDV p25 and ToCV p22) revealed that CCYV p22 is a weaker suppressor of local RNA silencing than the other two proteins. Finally, a comparative sequence analysis of the p22 genes of seven Greek CCYV isolates was performed, revealing a high level of conservation. Taken together, our research advances our knowledge about plant-virus interactions of criniviruses, an emergent group of	tobacco
812	plant	Nicotiana benthamiana	agroinfiltration	chimaeric RVFV virus-like particles	Biotechnology journal	Chimaeric Rift Valley Fever Virus-Like Particle Vaccine Candidate Production in Nicotiana benthamiana.	2019	14(4):e1800238	[Mbewana S et al.]	University of Cape Town, Cape Town, South Africa.	30488669	10.1002/biot.201800238	Rift Valley fever virus (RVFV) is an emerging mosquito-borne virus and hemorrhagic fever agent, which causes abortion storms in farmed small ruminants and potentially causes miscarriages in humans. Although live-attenuated vaccines are available for animals, they can only be used in endemic areas and there are currently no commercially available vaccines for humans. Here the authors describe the production of chimaeric RVFV virus-like particles transiently expressed in Nicotiana benthamiana by Agrobacterium tumefaciens-mediated gene transfer. The glycoprotein (Gn) gene is modified by removing its ectodomain (Gne) and fusing it to the transmembrane domain and cytosolic tail-encoding region of avian influenza H5N1 hemagglutinin. This is expressed transiently in N. benthamiana with purified protein yields calculated to be approximately 57 mg kg(-1) fresh weight. Transmission electron microscopy shows putative chimaeric RVFV Gne-HA particles of 49-60 nm which are immunogenic, eliciting Gn-specific antibody responses in vaccinated mice without the use of adjuvant. To our knowledge, this is the first demonstration of the synthesis of Gne-HA chimaeric RVFV VLPs and the first demonstration of a detectable yield of RVFV Gn in	tobacco
813	plant	Nicotiana benthamiana	agroinfiltration; CRISPR;Cas9;	NbPDS; NbRRA	BMC biotechnology	Improved CRISPR/Cas9 gene editing by fluorescence activated cell sorting of green fluorescence protein tagged protoplasts.	2019	19(1):36	[Petersen BL et al.]	University of Copenhagen, Frederiksberg, Denmark.	31208390	10.1186/s12896-019-0530-x	BACKGROUND: CRISPR/Cas9 is widely used for precise genetic editing in various organisms. CRISPR/Cas9 editing may in many plants be hampered by the presence of complex and high ploidy genomes and inefficient or poorly controlled delivery of the CRISPR/Cas9 components to gamete cells or cells with regenerative potential. Optimized strategies and methods to overcome these challenges are therefore in demand. RESULTS: In this study we investigated the feasibility of improving CRISPR/Cas9 editing efficiency by Fluorescence Activated Cell Sorting (FACS) of protoplasts. We used Agrobacterium infiltration in leaves of Nicotiana benthamiana for delivery of viral replicons for high level expression of gRNAs designed to target two loci in the genome, NbPDS and NbRRA, together with the Cas9 nuclease in fusion with the 2A self-splicing sequence and GFP (Cas9-2A-GFP). Protoplasts isolated from the infiltrated leaves were then subjected to FACS for selection of GFP enriched protoplast populations. This procedure resulted in a 3-5 fold (from 20 to 30% in unsorted to more than 80% in sorted) increase in mutation frequencies as evidenced by restriction enzyme analysis and the Indel Detection by Amplicon Analysis, which allows for high throughput profiling and quantification of the generated mutations. CONCLUSIONS: FACS of protoplasts expressing GFP tagged CRISPR/Cas9, delivered through A. tumefaciens leaf infiltration, facilitated clear CRISPR/Cas9 mediated	tobacco

814	plant	Nicotiana benthamiana	RdDM;	green fluorescent protein (GFP)	BMC plant biology	Transcriptional silencing of 35S driven-transgene is differentially determined depending on promoter methylation heterogeneity at specific cytosines in both plus- and minus-sense strands.	2019	19(1):24	[Matsunaga W et al.]	Hokkaido University, Sapporo, Japan.	30642254	10.1186/s12870-019-1628-y	BACKGROUND: De novo DNA methylation triggered by short interfering RNAs is called RNA-directed DNA methylation (RdDM). Transcriptional gene silencing (TGS) through RdDM can be induced using a viral vector. We have previously induced RdDM on the 35S promoter in the green fluorescent protein (GFP)-expressing Nicotiana benthamiana line 16c using the cucumber mosaic virus vector. The GFP fluorescence phenotype segregated into two types, "red" and "orange" in the first self-fertilized (S1) progeny plants by the difference in degree of recovery from TGS on GFP expression. In the second self-fertilized generation (S2 plants), the phenotypes again segregated. Explaining what generates the red and orange types could answer a very important question in epigenetics: How is the robustness of TGS maintained after RdDM induction? RESULTS: In bisulfite sequencing analyses, we found a significant difference in the overall promoter hypermethylation pattern between the red and orange types in S1 plants but little difference in S2 plants. Therefore, we assumed that methylation at some specific cytosine residues might be important in determining the two phenotypes. To find the factor that discriminates stable, robust TGS from the unstable TGS with incomplete inheritance, we analyzed the direct effect of methylated cytosine residues on TGS. Because it has not yet been demonstrated that DNA methylation at a few specific cytosine residues on known sequence elements can indeed determine TGS robustness, we newly developed a method by which we can directly evaluate the effect of specific methylation on promoter activity. In this assay, we found that the effects of the specific cytosine methylation on TGS differed between the plus- and minus-strands. CONCLUSIONS: We found two distinct phenotypes, the stable and unstable TGS in the progenies of virus-induced TGS plants. Our bisulfite sequencing analyses suggested that methylation at some specific cytosine residues in the 35S promoter played a role in determining whether stable or unstable TGSs are induced. Using the developed method, we inferred that DNA methylation heterogeneity	tobacco
815	plant	Nicotiana benthamiana	Agroinfiltration;	Plasmopara viticola RxLR effector, PvAvh74	Frontiers in microbiology	The Nuclear-Localized RxLR Effector PvAvh74 From Plasmopara viticola Induces Cell Death and Immunity Responses in Nicotiana benthamiana.	2019	10:1531	[Yin X et al.]	Northwest A&F University, Yangling, China.	31354650	10.3389/fmicb.2019.01531	Downy mildew is one of the most serious diseases of grapevine (Vitis spp). The causal agent of grapevine downy mildew, Plasmopara viticola, is an obligate biotrophic oomycete. Although oomycete pathogens such as P. viticola are known to secrete RxLR effectors to manipulate host immunity, there have been few studies of the associated mechanisms by which these may act. Here, we show that a candidate P. viticola RxLR effector, PvAvh74, induces cell death in Nicotiana benthamiana leaves. Using agroinfiltration, we found that nuclear localization, two putative N-glycosylation sites, and 427 amino acids of the PvAvh74 carboxyl terminus were necessary for cell-death-inducing activity. Using virus-induced gene silencing (VIGS), we found that PvAvh74-induced cell death in N. benthamiana requires EDS1, NDR1, SGT1, RAR1, and HSP90, but not BAK1. The MAPK cascade components MEK2, WIPK, and SIPK were also involved in PvAvh74-induced cell death in N. benthamiana. Transient expression of PvAvh74 could suppress Phytophthora capsici colonization of N. benthamiana, which suggests that PvAvh74 elicits plant immune responses. Suppression of P. capsici colonization also was dependent on nuclear localization of PvAvh74. Additionally, PvAvh74-triggered cell death could be suppressed by another effector, PvAvh8, from the same isolate. This work provides a framework to further investigate the interactions	tobacco
816	plant	Nicotiana benthamiana	Agroinfiltration;	SCI-57	Frontiers in pharmacology	Expression of the Biologically Active Insulin Analog SCI-57 in Nicotiana Benthamiana.	2019	10:1335	[Munoz-Talavera A et al.]	University of Guadalajara, Guadalajara, Mexico.	31798448	10.3389/fphar.2019.01335	Diabetes mellitus is a growing problem worldwide; however, only 23% of low-income countries have access to insulin, and ironically it costs higher in such countries than high-income ones. Therefore, new strategies for insulin and insulin analogs production are urgently required to improve low-cost access to therapeutic products, so as to contain the diabetes epidemic. SCI-57 is an insulin analog with a greater affinity for the insulin receptor and lower thermal degradation than native insulin. It also shows native mitogenicity and insulin-like biological activity. In this work, SCI-57 was transiently expressed in the Nicotiana benthamiana (Nb) plant, and we also evaluated some of its relevant biological effects. An expression plasmid was engineered to translate an N-terminal ubiquitin and C-terminal endoplasmic reticulum-targeting signal KDEL, in order to increase protein expression and stability. Likewise, the effect of co-expression of influenza M2 ion channel (M2) on the expression of insulin analog SCI-57 (SCI-57/M2) was evaluated. Although using M2 increases yield, it tends to alter the SCI-57 amino acid sequence, possibly promoting the formation of oligomers. Purification of SCI-57 was achieved by FPLC cation exchange and ultrafiltration of N. benthamiana leaf extract (NLE). SCI-57 exerts its anti-diabetic properties by stimulating glucose uptake in adipocytes, without affecting the lipid accumulation process. Expression of the insulin analog in agroinfiltrated plants was confirmed by SDS-PAGE, RP-HPLC, and MS. Proteome changes related to the expression of heterologous proteins on N. benthamiana were not observed; up-regulated proteins were related to the agroinfiltration process. Our results demonstrate the potential for producing a	tobacco

817	plant	Nicotiana benthamiana	Agroinfiltration;	60 putative virulence factors fo candidatus liberibacter asiaticus	International journal of molecular sciences	Identification of the Virulence Factors of Candidatus Liberibacter asiaticus via Heterologous Expression in Nicotiana benthamiana using Tobacco Mosaic Virus.	2019	20(22)	[Ying X et al.]	University of Florida, Wimauma, FL, USA.	31717281	10.3390/ijms20225575	Huanglongbing (HLB), also known as citrus greening, is the most destructive disease of citrus worldwide. HLB is associated with the non-culturable bacterium, Candidatus Liberibacter asiaticus (CaLas) in the United States. The virulence mechanism of CaLas is largely unknown, partly because of the lack of a mutant library. In this study, Tobacco mosaic virus (TMV) and Nicotiana benthamiana (N. benthamiana) were used for large-scale screening of the virulence factors of CaLas. Agroinfiltration of 60 putative virulence factors in N. benthamiana led to the identification of four candidates that caused severe symptoms in N. benthamiana, such as growth inhibition and cell death. CLIBASIA_05150 and CLIBASIA_04065C (C-terminal of CLIBASIA_04065) could cause cell death in the infiltrated leaves at five days post infiltration. Two low-molecular-weight candidates, CLIBASIA_00470 and CLIBASIA_04025, could inhibit plant growth. By converting start codon to stop codon or frameshifting, the four genes lost their harmful effects to N. benthamiana. It indicated that the four virulence factors functioned at the protein level rather than at the RNA level. The subcellular localization of the four candidates was determined by confocal laser scanning microscope. CLIBASIA_05150 located in the Golgi apparatus; CLIBASIA_04065 located in the mitochondrion; CLIBASIA_00470 and CLIBASIA_04025 distributed in cells as free GFP. The host proteins interacting with the four virulence factors were identified by yeast two-hybrid. The host proteins interacting with CLIBASIA_00470 and CLIBASIA_04025 were overlapping. Based on the phenotypes, the subcellular localization and the host proteins identified by yeast two-hybrid, CLIBASIA_00470 and CLIBASIA_04025, functioned redundantly. The hypothesis of CaLas virulence was proposed. CaLas affects citrus development and suppresses citrus disease resistance, comprehensively, in a complicated manner. Ubiquitin-mediated protein degradation might play a vital role in CaLas virulence. Deep characterization of the interactions between the identified virulence factors and their prey will shed light on HLB. Eventually, it will help in	tobacco
818	plant	Nicotiana benthamiana	Agroinfiltration;	NbSGT1	Journal of proteomics	In planta proximity-dependent biotin identification (BioID) identifies a TMV replication co-chaperone NbSGT1 in the vicinity of 126kDa replicase.	2019	204:103402	[Das PP et al.]	National University of Singapore (NUS), Singapore.	31158515	10.1016/j.jprot.2019.103402	Tobacco mosaic virus (TMV) is a positive, single-stranded RNA virus. It encodes two replicases (126kDa and 183kDa), a movement protein and a coat protein. These proteins interact with host proteins for successful infection. Some host proteins such as eEF1alpha, Tm-1, TOM1, 14-3-3 proteins directly interact with Tobamovirus replication proteins. There are host proteins in the virus replication complex which do not interact with viral replicases directly, such as pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase. We have used Proximity-dependent biotin identification (BioID) technique to screen for transient or weak protein interactions of host proteins and viral replicase in vivo. We transiently expressed BirA* tagged TMV 126kDa replicase in TMV infected Nicotiana benthamiana plants. Among 18 host proteins, we identified NbSGT1 as a potential target for further characterization. Silencing of NbSGT1 in N. benthamiana plants increased its susceptibility to TMV infection, and overexpression of NbSGT1 increased resistance to TMV infection. There were weak interactions between NbSGT1 and TMV replicases but no interaction between them was found in Y2H assay. It suggests that the interaction might be transient or indirect. Therefore, the BioID technique is a valuable method to identify weak or transient/indirect interaction(s) between pathogen proteins and host proteins in plants. BIOLOGICAL SIGNIFICANCE: TMV is a well characterized positive-strand RNA virus model for study of virus-plant host interactions. It infects >350 plant species and is one of the significant pathogens of crop loss globally. Many host proteins are involved in TMV replication complex formation. To date there are few techniques available for identifying interacting host proteins to viral proteins. There is limited knowledge on transient or non-interacting host proteins during virus infection/replication. In this study, we used agroinfiltration-mediated in planta BioID technique to identify transiently or non-interacting host proteins to viral proteins in TMV-infected N. benthamiana plants. This technique allowed us to identify potential candidate proteins in the vicinity of TMV 126kDa replicase. We have selected NbSGT1 and its overexpression suppresses TMV replication and increase plant resistance. NbSGT1 is believed to interact transiently or indirectly with TMV replicases in the presence of	tobacco
819	plant	Nicotiana benthamiana	CRISPR;Cas9;	oxidosqualane cyclase friedelin synthase	Plant & cell physiology	CYP712K4 Catalyzes the C-29 Oxidation of Friedelin in the Maytenus ilicifolia Quinone Methide Triterpenoid Biosynthesis Pathway.	2019	60(11):2510-2522	[Bicalho KU et al.]	Ghent University, Ghent, Belgium.	31350564	10.1093/pcp/pcz144	The native Brazilian plant Maytenus ilicifolia accumulates a set of quinone methide triterpenoids with important pharmacological properties, of which maytenin, pristimerin and celastrol accumulate exclusively in the root bark of this medicinal plant. The first committed step in the quinone methide triterpenoid biosynthesis is the cyclization of 2,3-oxidosqualene to friedelin, catalyzed by the oxidosqualene cyclase friedelin synthase (FRS). In this study, we produced heterologous friedelin by the expression of M. ilicifolia FRS in Nicotiana benthamiana leaves and in a Saccharomyces cerevisiae strain engineered using CRISPR/Cas9. Furthermore, friedelin-producing N. benthamiana leaves and S. cerevisiae cells were used for the characterization of CYP712K4, a cytochrome P450 from M. ilicifolia that catalyzes the oxidation of friedelin at the C-29 position, leading to maytenoic acid, an intermediate of the quinone methide triterpenoid biosynthesis pathway. Maytenoic acid produced in N. benthamiana leaves was purified and its structure was confirmed using high-resolution mass spectrometry and nuclear magnetic resonance analysis. The three-step oxidation of friedelin to maytenoic acid by CYP712K4 can be considered as the second step of the quinone methide triterpenoid biosynthesis pathway, and may form the basis for further discovery of the pathway and heterologous production of friedelanes and ultimately	tobacco

820	plant	Nicotiana benthamiana	Agroinfiltration;	porcine circovirus type 2 capsid protein	Plant biotechnology journal	Immunogenicity of plant-produced porcine circovirus-like particles in mice.	2019	17(9):1751-1759	[Gunter CJ et al.]	University of Cape Town, Cape Town, South Africa.	30791210	10.1111/pbi.13097	Porcine circovirus type 2 (PCV-2) is the main causative agent associated with a group of diseases collectively known as porcine circovirus-associated disease (PCAD). There is a significant economic strain on the global swine industry due to PCAD and the production of commercial PCV-2 vaccines is expensive. Plant expression systems are increasingly regarded as a viable technology to produce recombinant proteins for use as pharmaceutical agents and vaccines. However, successful production and purification of PCV-2 capsid protein (CP) from plants is an essential first step towards the goal of a plant-produced PCV-2 vaccine candidate. In this study, the PCV-2 CP was transiently expressed in Nicotiana benthamiana plants via agroinfiltration and PCV-2 CP was successfully purified using sucrose gradient ultracentrifugation. The CP self-assembled into virus-like particles (VLPs) resembling native virions and up to 6.5 mg of VLPs could be purified from 1 kg of leaf wet weight. Mice immunized with the plant-produced PCV-2 VLPs elicited specific antibody responses to PCV-2 CP. This is the first report describing the expression of PCV-2 CP in plants, the confirmation of its assembly into VLPs and the demonstration of their use to elicit a strong immune	tobacco
821	plant	Nicotiana benthamiana	CRISPR;Cas9;	6 genes that cause alpha-1,3-fucosyltransferase and beta-1,2-xylosyltransferase deficiency	Plant biotechnology journal	CRISPR/Cas9-mediated knockout of six glycosyltransferase genes in Nicotiana benthamiana for the production of recombinant proteins lacking beta-1,2-xylose and core alpha-1,3-fucose.	2019	17(2):350-361	[Jansing J et al.]	RWTH Aachen University, Aachen, Germany.	29969180	10.1111/pbi.12981	Plants offer fast, flexible and easily scalable alternative platforms for the production of pharmaceutical proteins, but differences between plant and mammalian N-linked glycans, including the presence of beta-1,2-xylose and core alpha-1,3-fucose residues in plants, can affect the activity, potency and immunogenicity of plant-derived proteins. Nicotiana benthamiana is widely used for the transient expression of recombinant proteins so it is desirable to modify the endogenous N-glycosylation machinery to allow the synthesis of complex N-glycans lacking beta-1,2-xylose and core alpha-1,3-fucose. Here, we used multiplex CRISPR/Cas9 genome editing to generate N. benthamiana production lines deficient in plant-specific alpha-1,3-fucosyltransferase and beta-1,2-xylosyltransferase activity, reflecting the mutation of six different genes. We confirmed the functional gene knockouts by Sanger sequencing and mass spectrometry-based N-glycan analysis of endogenous proteins and the recombinant monoclonal antibody 2G12. Furthermore, we compared the CD64-binding affinity of 2G12 glycovariants produced in wild-type N. benthamiana, the newly generated FX-KO line, and Chinese hamster ovary (CHO) cells, confirming that the glyco-engineered antibody performed as well as its CHO-produced counterpart.	tobacco
822	plant	Nicotiana benthamiana	Agroinfiltration;	NbUGT73A24; NbUGT73A25	Plant journal	Glucosylation of the phytoalexin N-feruloyl tyramine modulates the levels of pathogen-responsive metabolites in Nicotiana benthamiana.	2019	100(1):20-37	[Sun G et al.]	Technische Universitat Munchen, Freising, Germany.	31124249	10.1111/tpj.14420	Enzyme promiscuity, a common property of many uridine diphosphate sugar-dependent glycosyltransferases (UGTs) that convert small molecules, significantly hinders the identification of natural substrates and therefore the characterization of the physiological role of enzymes. In this paper we present a simple but effective strategy to identify endogenous substrates of plant UGTs using LC-MS-guided targeted glycoside analysis of transgenic plants. We successfully identified natural substrates of two promiscuous Nicotiana benthamiana UGTs (NbUGT73A24 and NbUGT73A25), orthologues of pathogen-induced tobacco UGT (TOGT) from Nicotiana tabacum, which is involved in the hypersensitive reaction. While in N. tabacum, TOGT glucosylated scopoletin after treatment with salicylate, fungal elicitors and the tobacco mosaic virus, NbUGT73A24 and NbUGT73A25 produced glucosides of phytoalexin N-feruloyl tyramine, which may strengthen cell walls to prevent the intrusion of pathogens, and flavonols after agroinfiltration of the corresponding genes in N. benthamiana. Enzymatic glucosylation of fractions of a physiological aglycone library confirmed the biological substrates of UGTs. In addition, overexpression of both genes in N. benthamiana produced clear lesions on the leaves and led to a significantly reduced content of pathogen-induced plant metabolites such as phenylalanine and tryptophan. Our results revealed some additional biological functions of TOGT enzymes and indicated a multifunctional role of UGTs in plant resistance.	tobacco
823	plant	Nicotiana benthamiana	CRISPR;Cas9;	RNA-dependent RNA polymerase 6	Planta	CRISPR/Cas9-mediated knockout of the RDR6 gene in Nicotiana benthamiana for efficient transient expression of recombinant proteins.	2019	250(2):463-473	[Matsuo K et al.]	National Institute of Advanced Industrial Science and Technology (AIST), Sapporo, Japan.	31065786	10.1007/s00425-019-03180-9	MAIN CONCLUSION: RDR6 gene knockout Nicotiana benthamiana plant was successfully produced using CRISPR/Cas9 technology. The production of recombinant proteins in plants has many advantages, such as safety and reduced costs. However, there are several problems with this technology, especially low levels of protein production. The dysfunction of the RNA silencing mechanism in plant cells would be effective to improve recombinant protein production because the RNA silencing mechanism efficiently degrades transgene-derived mRNAs. Therefore, to overcome this problem, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology was used to develop RNA silencing-related gene knockout transgenic Nicotiana benthamiana. We successfully produced RNA-dependent RNA polymerase 6 (RDR6), one of the most important components of the RNA silencing mechanism-knockout N. benthamiana (DeltaRDR6 plants). The DeltaRDR6 plants had abnormal flowers and were sterile, as with the Arabidopsis RDR6 mutants. However, a transient gene expression assay showed that the DeltaRDR6 plants accumulated larger amounts of green fluorescent protein (GFP) and GFP mRNA than the wild-type (WT) plants. Small RNA sequencing analysis revealed that levels of small interfering RNA against the GFP gene were greatly reduced in the DeltaRDR6 plants, as compared to that of the WT plants. These findings demonstrate that the DeltaRDR6 plants can express larger amounts of recombinant proteins than WT plants and, therefore, would be useful for recombinant protein production and understanding the contributions of RDR6 to	tobacco
824	plant	Nicotiana benthamiana	CRISPR;Cas9;	RNA dependent RNA polymerase (rdr6)	Virology	The virological model plant, Nicotiana benthamiana expresses a single functional RDR6 homeolog.	2019	537:143-148	[Ludman M et al.]	National Agricultural Research and Innovation Centre, Godollo, Hungary.	31493652	10.1016/j.virol.2019.08.017	The RNA dependent RNA polymerase, RDR6 is involved in a variety of processes including the biogenesis of endogenous regulatory small RNAs, maintaining post-transcriptional gene silencing of transgenes and establishing efficient antiviral RNA silencing. In the virological model plant, Nicotiana benthamiana, functional studies of RDR6 has so far only been depended on RNAi based methodologies. These techniques however have inherent limitations, especially in the context of antiviral RNA silencing. To overcome this issue, we created rdr6 mutant N. benthamiana by the CRISPR/Cas9 genome editing system. Using the mutant, most of the proposed functions of RDR6 was confirmed. Additionally, the rdr6 N. benthamiana plant recapitulated closely the phenotype of the equivalent Arabidopsis mutant. In summary, the rdr6 N. benthamiana described here may be employed as a model system not only for the better understanding of the role of RDR6 in pathogen elicited immune responses but in	tobacco

825	plant	Nicotiana benthamiana	Agroinfiltration;	green fluorescent protein (GFP)	Virology	Identification and subcellular location of an RNA silencing suppressor encoded by mulberry crinkle leaf virus.	2019	526:45-51	[Lu QY et al.]	Jiangsu University of Science and Technology, Zhenjiang, Jiangsu, China.	30342301	10.1016/j.virol.2018.10.007	Mulberry crinkle leaf virus (MCLV) is a novel geminivirus recently identified from the woody plant mulberry (Morus alba L.). Little is known about the functions of the proteins encoded by the MCLV genome. Here, all the MCLV-encoded proteins were examined for the ability to suppress gene silencing by an agroinfiltration assay in combination with northern blot analysis of green fluorescent protein (GFP) mRNA and western blot analysis. Of the six proteins, only one protein, V3, which has been predicted to play a role in viral movement, was found to suppress the gene silencing induced by a sense GFP gene in Nicotiana benthamiana 16c. The minimal amino acid sequence of V3 that maintains suppressor activity was also determined by constructing truncated mutants lacking different lengths of the amino acid sequences at the N- or C-terminus of the V3 protein. The results showed that the 94 N-terminal amino acid residues of V3 are sufficient to maintain V3 suppressor activity. In addition, the subcellular location of the V3 protein was investigated by confocal laser scanning microscopy after the expression of a V3-RFP fused protein in leaf epidermal cells of N. benthamiana. The results indicated that the V3 protein localized not only to the cytoplasm but also to the nucleus of N. benthamiana, implying that V3 can shuttle between the nucleus and the cytoplasm. Deletion mutant analysis indicated that a putative nuclear localization signal (NLS) between aa 118-134 might be responsible for the nuclear distribution of the V3 protein. Given the importance of RNA silencing in plant-virus interactions, the identification of a silencing suppressor of MCLV should be	tobacco
826	plant	Nicotiana benthamiana; Arabidopsis thaliana	CRISPR;Cas9;TALENs;	telomeric hisotnes and chromatin	Plant journal	Two combinatorial patterns of telomere histone marks in plants with canonical and non-canonical telomere repeats.	2019		[Adamusova K et al.]	Masaryk University, Brno, Czech Republic.	31834959	10.1111/tpj.14653	Telomeres, nucleoprotein structures at the ends of linear eukaryotic chromosomes, are crucial for the maintenance of genome integrity. In most plants, telomeres consist of conserved tandem repeat units comprising the TTTAGGG motif. Recently, non-canonical telomeres were described in several plants and plant taxons, including the carnivorous plant Genlisea hispidula (TTCAGG/TTTCAGG), the genus Cestrum (Solanaceae; TTTTTTAGGG), and plants from the Asparagales order with either a vertebrate-type telomere repeat TTAGGG or Allium genus-specific CTCGGTTATGGG repeat. We analyzed epigenetic modifications of telomeric histones in plants with canonical and non-canonical telomeres, and further in telomeric chromatin captured from leaves of Nicotiana benthamiana transiently transformed by telomere CRISPR-dCas9-eGFP, and of Arabidopsis thaliana stably transformed with TALE_telo C-3xGFP. Two combinatorial patterns of telomeric histone modifications were identified: (i) an Arabidopsis-like pattern (A. thaliana, G. hispidula, Genlisea nigrocaulis, Allium cepa, Narcissus pseudonarcissus, Petunia hybrida, Solanum tuberosum, Solanum lycopersicum) with telomeric histones decorated predominantly by H3K9me2; (ii) a tobacco-like pattern (Nicotiana tabacum, N. benthamiana, C. elegans) with a strong H3K27me3 signal. Our data suggest that epigenetic modifications of plant telomere-associated histones are related neither to the sequence of the telomere motif nor to the lengths of the telomeres. Nor the phylogenetic position of the species plays the role; representatives of the Solanaceae family are included in both groups. As both patterns of histone marks are compatible with fully functional telomeres in respective plants, we conclude that the described specific differences in histone marks are not	tobacco
827	plant	Nicotiana benthamiana; Arabidopsis thaliana	Agroinfiltration;	GAL4-VP16	Transgenic research	Development of a Gateway-compatible two-component expression vector system for plants.	2019	28(5-6):561-572	[Li N et al.]	Key Laboratory of Cultivation and Protection for Non-Wood Forest Trees (Central South University of Forestry and Technology), Ministry of Education, Changsha, Hunan, China.	31435821	10.1007/s11248-019-00167-w	Genetic transformation of plants offers the possibility of functional characterization of individual genes and the improvement of plant traits. Development of novel transformation vectors is essential to improve plant genetic transformation technologies for various applications. Here, we present the development of a Gateway-compatible two-component expression vector system for Agrobacterium-mediated plant transformation. The expression system contains two independent plasmid vector sets, the activator vector and the reporter vector, based on the concept of the GAL4/UAS trans-activation system. The activator vector expresses a modified GAL4 protein (GAL4-VP16) under the control of specific promoter. The GAL4-VP16 protein targets the UAS in the reporter vector and subsequently activates reporter gene expression. Both the activator and reporter vectors contain the Gateway recombination cassette, which can be rapidly and efficiently replaced by any specific promoter and reporter gene of interest, to facilitate gene cloning procedures. The efficiency of the activator-reporter expression system has been assessed using agroinfiltration mediated transient expression assay in Nicotiana benthamiana and stable transgenic expression in Arabidopsis thaliana. The reporter genes were highly expressed with precise tissue-specific and subcellular localization. This Gateway-compatible two-component expression vector system will be a useful tool for	tobacco
828	plant	Nicotiana benthamiana; Nicotiana excelsiana	Agroinfiltration;	recombinant human plasminogen activator (rhPA)	Nan fang yi ke da xue xue bao = Journal of Southern Medical University	[Transient expression of bioactive recombinant human plasminogen activator in tobacco leaf].	2019	39(5):515-522	[Ma J et al.]	Key Laboratory of Ministry of Education for Protection and Utilization of Special Biological Resources in the Western China, Yinchuan, China.	31140413	10.12122/j.issn.1673-4254.2019.05.03	OBJECTIVE: To assess the potential of transient expression of recombinant human plasminogen activator (rhPA) in plants as a cost-effective approach for recombinant rhPA production. METHODS: Tobacco mosaic virus-based expression vector pTMV rhPA-NSK and plant binary expression vector pJ Zera-rhPA were constructed by in vitro sequence synthesis and subcloning. The two vectors were inoculated on either Nicotiana benthamiana or N. excelsiana leaves via agroinfiltration. The expression of recombinant rhPA in Nicotiana leaves was examined using Western blotting and ELISA, and the in vitro fibrinolysis activity of plant-produced rhPA was assessed by fibrin agarose plate assay (FAPA). RESULTS: Five to nine days after infiltration with an Agrobacterium inoculum containing pTMV rhPA-NSK, necrosis appeared in the infiltrated area on the leaves of both Nicotiana plants, but intact recombinant rhPA was still present in the necrotic leaf tissues. The accumulation level of recombinant rhPA in infiltrated N. benthamiana leaves was significantly higher than that in N. excelsiana leaves (P < 0.05). The yield of recombinant rhPA was up to 0.6% of the total soluble protein (or about 60.0 mug per gram) in the fresh leaf biomass at 7 days post-inoculation. The plant-derived rhPA was bioactive to convert inactive plasminogen to active plasmin. No necrosis occurred in pJ Zera-rhPA-infiltrated leaves. The Zera-rhPA protein was partially cleaved between the site of Zera tag and rhPA sequence in both Nicotiana leaves. We speculated that the formation of Zera tags-induced particles in the plant cells was a dynamic process of progressive aggregation in which some of the soluble polypeptides were encapsulated in these particles. CONCLUSIONS: Enzymatically active recombinant rhPA can be rapidly expressed in tobacco plants using the plant viral amplicon-based system, which offers a promising alternative for	tobacco

829	plant	Nicotiana benthamiana; Nicotiana occidental	Agroinfiltration;	YFP-ASPV-CPs; PVX-ASPV-CPs	Virology journal	Functional analysis of apple stem pitting virus coat protein variants.	2019	16(1):20	[Ma X et al.]	Huazhong Agricultural University, Wuhan, Hubei, China.	30736799	10.1186/s12985-019-1126-8	BACKGROUND: Although the canonical function of viral coat protein (CP) is to encapsidate the viral genome, they have come to be recognized as multifunctional proteins, involved in almost every stage of the viral infection cycle. However, CP functions of Apple stem pitting virus (ASPV) has not been comprehensively documented. This study aimed to characterize the functions of ASPV CP and any functional diversification caused by sequence diversity of six ASPV CP variants and studied their biological, serological, pathogenic and viral suppressor of RNA silencing (VSR) functions. METHODS: Six ASPV CP variants that have previously been shown to belong to different subgroups were selected here to study their diversity functions. Agrobacterium mediated infiltration (Agroinfiltration) was used to express YFP-ASPV-CPs in Nicotiana. benthamiana and infect Nicotiana. occidental with PVX-ASPV-CPs in. Confocal microscopy was used to detect YFP-ASPV-CPs florescence. CPs expressed in Escherichia coli BL21 (DE3) were induced by IPTG. RESULTS: In this study, we showed that recombinant CPs expressed in Escherichia coli BL21 (DE3) had different levels of serological reactivity to three anti-ASPV antibodies used to detect ASPV. Furthermore, fusion CPs with YFP (YFP-CPs) expressed in N. benthamiana cells differed in their ability to form aggregates. We also showed that ASPV isolates that harbour these CPs induced different biological symptoms on its herbaceous host N. occidentalis. At the same time, we found that all six CPs when expressed in PVX vector showed similar VSR activity and produced similar symptoms in N. occidentalis, despite their differences in amino acids. CONCLUSIONS: Different ASPV isolates induced different symptoms in N. occidentalis, however, ASPV CP variants expressed in PVX vector showed the same symptoms in N. occidentalis plants. Also, we showed	tobacco
830	plant	Nicotiana benthamiana; Solanum lycopersicum; Arabidopsis thaliana	CRISPR;Cas9;Cpf1;	8 Nicotiana benthamiana loci	Plant biotechnology journal	Assessment of Cas12a-mediated gene editing efficiency in plants.	2019	17(10):1971-1984	[Bernabe-Orts JM et al.]	Universidad Politecnica de Valencia, Valencia, Spain.	30950179	10.1111/pbi.13113	The CRISPR/Cas12a editing system opens new possibilities for plant genome engineering. To obtain a comparative assessment of RNA-guided endonuclease (RGEN) types in plants, we adapted the CRISPR/Cas12a system to the GoldenBraid (GB) modular cloning platform and compared the efficiency of Acidaminococcus (As) and Lachnospiraceae (Lb) Cas12a variants with the previously described GB-assembled Streptococcus pyogenes Cas9 (SpCas9) constructs in eight Nicotiana benthamiana loci using transient expression. All three nucleases showed drastic target-dependent differences in efficiency, with LbCas12 producing higher mutagenesis rates in five of the eight loci assayed, as estimated with the T7E1 endonuclease assay. Attempts to engineer crRNA direct repeat (DR) had little effect improving on-target efficiency for AsCas12a and resulted deleterious in the case of LbCas12a. To complete the assessment of Cas12a activity, we carried out genome editing experiments in three different model plants, namely N. benthamiana, Solanum lycopersicum and Arabidopsis thaliana. For the latter, we also resequenced Cas12a-free segregating T2 lines to assess possible off-target effects. Our results showed that the mutagenesis footprint of Cas12a is enriched in deletions of −10 to −2 nucleotides and included in some instances complex rearrangements in the surroundings of the target sites. We found no evidence of off-target mutations neither in related sequences nor somewhere else in the genome. Collectively, this study shows that LbCas12a is a viable alternative to	tobacco
831	plant	Nicotiana benthamiana; sugar beet	Cas9;	NbPDS	Plant biotechnology journal	Development of Beet necrotic yellow vein virus-based vectors for multiple-gene expression and guide RNA delivery in plant genome editing.	2019	17(7):1302-1315	[Jiang N et al.]	China Agricultural University, Beijing, China.	30565826	10.1111/pbi.13055	Many plant viruses with monopartite or bipartite genomes have been developed as efficient expression vectors of foreign recombinant proteins. Nonetheless, due to lack of multiple insertion sites in these plant viruses, it is still a big challenge to simultaneously express multiple foreign proteins in single cells. The genome of Beet necrotic yellow vein virus (BNYVV) offers an attractive system for expression of multiple foreign proteins owning to a multipartite genome composed of five positive-stranded RNAs. Here, we have established a BNYVV full-length infectious cDNA clone under the control of the Cauliflower mosaic virus 35S promoter. We further developed a set of BNYVV-based vectors that permit efficient expression of four recombinant proteins, including some large proteins with lengths up to 880 amino acids in the model plant Nicotiana benthamiana and native host sugar beet plants. These vectors can be used to investigate the subcellular co-localization of multiple proteins in leaf, root and stem tissues of systemically infected plants. Moreover, the BNYVV-based vectors were used to deliver NbPDS guide RNAs for genome editing in transgenic plants expressing Cas9, which induced a photobleached phenotype in systemically infected leaves. Collectively, the BNYVV-based vectors will facilitate genomic research and	tobacco
832	plant	Nicotiana tabacum	CRISPR;Cas9;	NtFT5	Frontiers in plant science	The Major Floral Promoter NtFT5 in Tobacco (Nicotiana tabacum) Is a Promising Target for Crop Improvement.	2019	10:1666	[Schmidt FJ et al.]	University of Munster, Munster, Germany.	31998348	10.3389/fpls.2019.01666	The FLOWERING LOCUS T (FT)-like gene family encodes key regulators of flower induction that affect the timing of reproduction in many angiosperm species. Agricultural research has therefore focused on such genes to improve the success of breeding programs and enhance agronomic traits. We recently identified a novel FT-like gene (NtFT5) that encodes a day-neutral floral activator in the model tobacco crop Nicotiana tabacum. However, further characterization is necessary to determine its value as a target for breeding programs. We therefore investigated the function of NtFT5 by expression analysis and mutagenesis. Expression analysis revealed that NtFT5 is transcribed in phloem companion cells, as is typical for FT-like genes. However, high levels of NtFT5 mRNA accumulated not only in the leaves but also in the stem. Loss-of-function mutants (generated using CRISPR/Cas9) were unable to switch to reproductive growth under long-day conditions, indicating that NtFT5 is an indispensable major floral activator during long-days. Backcrossing was achieved by grafting the mutant scions onto wild-type rootstock, allowing the restoration of flowering and pollination by a wild-type donor. The resulting heterozygous Ntft5(−)/NtFT5(+) plants flowered with a mean delay of only ~2 days, demonstrating that one functional allele is sufficient for near-normal reproductive timing. However, this minor extension of the vegetative growth phase also conferred beneficial agronomic traits, including a >10% increase in vegetative leaf biomass on the main shoot and the production of more seeds. The agronomic benefits of the heterozygous plants persisted under various abiotic stress conditions, confirming that NtFT5 is a promising target for	tobacco

833	plant	Nicotiana tabacum	ZFN;	neomycin phosphotransferase II; Discosoma sp. red fluorescent protein	Plant direct	Targeted insertion of large DNA sequences by homology-directed repair or non-homologous end joining in engineered tobacco BY-2 cells using designed zinc finger nucleases.	2019	3(7):e00153	[Schiermeyer A et al.]	Fraunhofer Institute for Molecular Biology and Applied Ecology, Aachen Germany.	31360827	10.1002/pld3.153	Targeted integration of recombinant DNA fragments into plant genomes by DNA double-strand break (DSB) repair mechanisms has become a powerful tool for precision engineering of crops. However, many targeting platforms require the screening of many transgenic events to identify a low number of targeted events among many more random insertion events. We developed an engineered transgene integration platform (ETIP) that uses incomplete marker genes at the insertion site to enable rapid phenotypic screening and recovery of targeted events upon functional reconstitution of the marker genes. The two marker genes, encoding neomycin phosphotransferase II (nptII) and Discosoma sp. red fluorescent protein (DsRed) enable event selection on kanamycin-containing selective medium and subsequent screening for red fluorescent clones. The ETIP design allows targeted integration of donor DNA molecules either by homology-directed repair (HDR) or non-homologous end joining (NHEJ)-mediated mechanisms. Targeted donor DNA integration is facilitated by zinc finger nucleases (ZFN). The ETIP cassette was introduced into Nicotiana tabacum BY-2 suspension cells to generate target cell lines containing a single copy locus of the transgene construct. The utility of the ETIP platform has been demonstrated by targeting DNA constructs containing up to 25-kb payload. The success rate for clean targeted DNA integration was up to 21% for HDR and up to 41% for NHEJ based on the total number of calli analyzed by next-generation sequencing (NGS). The rapid generation of targeted events with large DNA constructs expands the utility of the nuclease-	tobacco
834	plant	Nicotiana tabacum	CRISPR;Cas9;Cpf1;	ethylene receptor 1	Plant molecular biology	Application of Cas12a and nCas9-activation-induced cytidine deaminase for genome editing and as a non-sexual strategy to generate homozygous/multiplex edited plants in the allotetraploid genome of tobacco.	2019	101(4-5):355-371	[Hsu CT et al.]	Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan.	31401729	10.1007/s11103-019-00907-w	KEY MESSAGE: Protoplasts can be used for genome editing using several different CRISPR systems, either separately or simultaneously, and that the resulting mutations can be recovered in regenerated non-chimaeric plants. Protoplast transfection and regeneration systems are useful platforms for CRISPR/Cas mutagenesis and genome editing. In this study, we demonstrate the use of Cpf1 (Cas12a) and nCas9-activation-induced cytidine deaminase (nCas9-Target-AID) systems to mutagenize Nicotiana tabacum protoplasts and to regenerate plants harboring the resulting mutations. We analyzed 20 progeny plants of Cas12a-mediated phytoene desaturase (PDS) mutagenized regenerants, as well as regenerants from wild-type protoplasts, and confirmed that their genotypes were inherited in a Mendelian manner. We used a Cas9 nickase (nCas9)-cytidine deaminase to conduct C to T editing of the Ethylene receptor 1 (ETR1) gene in tobacco protoplasts and obtained edited regenerates. It is difficult to obtain homozygous edits of polyploid genomes when the editing efficiency is low. A second round of mutagenesis of partially edited regenerants (a two-step transfection protocol) allowed us to derive ETR1 fully edited regenerants without the need for sexual reproduction. We applied three different Cas systems (SaCas9, Cas12a, and nCas9-Traget AID) using either a one-step or a two-step transfection platform to obtain triply mutated and/or edited tobacco regenerants. Our results indicate that	tobacco
835	plant	Nicotiana tabacum L.	Agroinfiltration;	hepatitis B surface antigen (HBsAg), mouse granulocyte macrophage colony stimulating factor (mGM-CSF) (mono or bicistronic cassettes)	Pharmaceutical biology	Construction of bicistronic cassette for co-expressing hepatitis B surface antigen and mouse granulocyte-macrophage colony stimulating factor as adjuvant in tobacco plant.	2019	57(1):669-675	[Mohammadzadeh S et al.]	Kermanshah University of Medical Sciences, Kermanshah, Iran.	31549887	10.1080/13880209.2019.1662458	Context: The co-delivery of adjuvant and antigen has shown to be more effective for targeting the immune response than antigen alone. Therefore, designing an efficient bicistronic system is more assuring for production of both elements in the same tobacco cells as a plant model system. Objective: Comparing the efficient transient co-expression of hepatitis B surface antigen (HBsAg) and mouse granulocyte macrophage colony stimulating factor (mGM-CSF) in tobacco leaves by designing either mono or bicistronic cassettes. Materials and methods: Four expression cassettes containing tobacco etch virus (TEV) leader sequence were constructed with and without above genes in different orders. The cassettes were transferred into tobacco, Nicotiana tabacum L. (Solanaceae), leaves by agroinfiltration technique. The expression levels were compared using ELISA and western blotting and bioactivity of cytokine was assessed by in vitro proliferation of mouse GM-CSF-responsive progenitor cells. Results: Agroinfiltrated leaves contained recombinant HBsAg protein at 20-50 ng/mg and mGM-CSF at 0.2-4 ng/mg in both nonglycosylated and glycosylated forms. The highest expression obtained in HBsAg and mGM-CSF monocistronic co-agroinfiltrated leaves. The expression of mGM-CSF was 1.1 and 0.2 ng/mg in two different orders of bicistronic cassettes. The growth frequency of GM progenitors was approximately 1/187 cells for standard rGM-CSF and 3.2 times less activity for the plant produced. Discussion and conclusions: The recombinant mGM-CSF was produced less in bicistronic cassette than other forms; however, co-presenting of both vaccine candidate and adjuvant is confirmed and could be promising for amelioration of plant	tobacco
836	plant	Nicotiana tabacum L. 'SR-1'	CRISPR;Cas9;agroinfiltration	acetolactate synthase (SuRB); MYB transcription factor (An2)	Plant cell reports	CRISPR/Cas9-mediated homologous recombination in tobacco.	2019	38(4):463-473	[Hirohata A et al.]	Osaka Prefecture University, Sakai, Osaka, Japan.	30006757	10.1007/s00299-018-2320-7	KEY MESSAGE: Co-transformation of multiple T-DNA in a binary vector enabled CRISPR/Cas9-mediated HR in tobacco. HR occurred in a limited region around the gRNA target site. In this study, CRISPR/Cas9-mediated homologous recombination (HR) in tobacco (Nicotiana tabacum L. 'SR-1') was achieved using binary vectors comprising two (T1-T2) or three (T1-T2-T3) independent T-DNA regions. For HR donor with the tobacco acetolactate synthase gene, SuRB, T-DNA1 contained DeltaSuRB(W568L), which lacked the N-terminus region of SuRB and was created by three nucleotide substitutions (ATG to GCT; W568L), leading to herbicide chlorsulfuron (Cs) resistance, flanked by the hygromycin (Hm)-resistant gene. T-DNA2 consisted of the hSpCas9 gene and two gRNA inserts targeting SuRB and An2. For the 2nd HR donor with the tobacco An2 gene encoding a MYB transcription factor involved in anthocyanin biosynthesis, T-DNA3 had a 35S promoter-driven An2 gene lacking the 3rd exon resulting in anthocyanin accumulation after successful HR. After selecting for Hm and Cs resistance from among the 7462 Agrobacterium-inoculated explants, 77 independent lines were obtained. Among them, the ATG to GCT substitution of endogenous SuRB was detected in eight T1-T2-derived lines and two T1-T2-T3-derived lines. Of these mutations, four T1-T2-derived lines were bi-allelic. All the HR events occurred across the endogenous SuRB and 5' homology arm of the randomly integrated T-DNA1. HR of the SuRB paralog, SuRA, was also found in one of the T1-T2-derived lines. Sequence analysis of its SuRA-targeted region indicated that the HR occurred in a limited (< 153 bp) region around the gRNA target site. Even though some T1-T2-T3-derived lines introduced three different T-DNAs and modified the An2	tobacco

837	plant	Nicotiana tabacum Xanthi	Agroinfiltration;	anthocyanin VlmybA1-2	Plant signaling & behavior	Use of a visible reporter marker- myb-related gene in crop plants to minimize herbicide usage against weeds.	2019	14(4):e1581558	[Aly R et al.]	Agricultural Research Organization, Newe Ya'ar Research Center , Ramat Yishay , Israel.	30806150	10.1080/15592324.2019.1581558	Weeds, a main threat to agricultural productivity worldwide, are mostly controlled by herbicides. To minimize herbicide usage by targeting only weedy areas, we developed a new methodology for robust weed detection that relies on manipulating the crop plant's leaf hue, without affecting crop fitness. We generated transgenic tobacco (Nicotiana tabacum Xanthi) lines overexpressing the anthocyanin pigment as a traceable marker that differentiates transgenes from the surrounding weeds at an early stage. Transformation with the anthocyanin VlmybA1-2 gene produced purple-colored leaves. Subsequent gene silencing with vector pTRV2:VlmybA1-2 significantly reduced anthocyanin pigments in tobacco leaves 40 days after agroinfiltration, with a concomitant reduction in VlmybA1-2 transcript levels. Purple hue faded gradually, and there were no fitness costs in terms of plant height or leaf number in the silenced vs. non-silenced tobacco transgenes. These results could lead to a new sustainable weed-control method that will alleviate weed-related ecological, agricultural and economic	tobacco
838	plant	Nicotiana tabacum; grape	CRISPR;Cas9;	enhanced green fluorescence protein (EGFP); phytoene desaturase gene (PDS)	Plant cell reports	Recovery of the non-functional EGFP-assisted identification of mutants generated by CRISPR/Cas9.	2019	38(12):1541-1549	[Ren C et al.]	Institute of Botany, Chinese Academy of Science, Beijing, China.	31446470	10.1007/s00299-019-02465-3	KEY MESSAGE: The recovery of non-functional-enhanced green fluorescence protein can be used as indicator to facilitate the identification of mutants generated by CRISPR/Cas9. The CRISPR/Cas9 system is a powerful tool for genome editing and it has been employed to knock out genes of interest in multiple plant species. Identification of desired mutants from regenerated plants is necessary prior to functional study. Current screening methods work based on the purification of genomic DNA and it would be laborious and time consuming using these methods to screen mutants from a large population of seedlings. Here, we developed the non-functional enhanced green fluorescence protein (nEGFP) reporter gene by inserting a single guide RNA (sgRNA) and the protospacer adjacent motif in the 5' coding region of EGFP, and the activity of nEGFP could be recovered after successful targeted editing. Using the nEGFP as the reporter gene in Nicotiana tabacum, we found that over 94% of the plants exhibiting EGFP fluorescence were confirmed to be desired mutants. The use of this nEGFP reporter construct had limited negative effect on editing efficiency, and the expression of Cas9 and sgRNA was not affected. Moreover, this method was also applied in grape by targeting the phytoene desaturase gene (PDS), and the grape cells with EGFP signal were revealed to contain targeted mutations in VvPDS. Our results show that the nEGFP gene can be used as reporter to help screen mutants according to the recovered EGFP fluorescence during the application of CRISPR/Cas9 in plants.	tobacco
839	plant	tobacco (Xanthi Brad)	Agroinfiltration;	victoviral Vin gene (encoding Victoriocin)	Journal of biotechnology	Synthetic Salicylic acid inducible recombinant promoter for translational research.	2019	297:9-18	[Deb D et al.]	Institute of Life Sciences, Government of India, Chandrasekharpur, Bhubaneswar, Odisha, India.	30880184	10.1016/j.jbiotec.2019.03.004	In the present study, we have developed an inter-molecularly shuffled caulimoviral promoter for protein over-expression by placing the Upstream Activation Sequence (UAS) of Figwort Mosaic Virus (FMV; -249 to -54) at the 5'-end of the Cassava Vein Mosaic Virus (CsVMV) promoter fragment 8 (CsVMV8; -215 to +166) to design a hybrid promoter; FUASCsV8CP. The FUASCsV8CP promoter exhibited approximately 2.1 and 2.0 times higher GUS-activities than that obtained from the CaMV35S promoter, in tobacco (Xanthi Brad) protoplasts and in Agroinfiltration assays respectively. Hereto, when FUASCsV8CP was assayed using transgenic tobacco plants (T2- generation), it showed 2.0 times stronger activity than CaMV35S promoter and almost equivalent activity to that of CaMV35S(2) promoter. The promoter displayed Salicylic acid (SA) inducibility and hence can also be used for ensuring effective gene expression in plants under constitutive as well as specific inducible conditions. Furthermore, FUASCsV8CP was used to drive the expression of victoviral Vin gene (encoding Victoriocin) transiently in tobacco. The recombinant Victoriocin could be successfully detected by western blotting three days post infiltration. Also, the in vitro Agar-based killing zone assays employing plant-derived Victoriocin-His (obtained from transient expression of Vin) revealed enhanced antifungal activity of Victoriocin against hemi-biotrophic	tobacco
840	plant	tomato	CRISPR;Cas9;	6 lncRNAs including lncRNA2155	Annals of botany	Genome-wide identification of long non-coding RNA targets of the tomato MADS box transcription factor RIN and function analysis.	2019	123(3):469-482	[Yu T et al.]	China Agricultural University, Beijing, China.	30376036	10.1093/aob/mcy178	BACKGROUND AND AIMS: In recent years, increasing numbers of long non-coding RNAs (lncRNAs) have been identified in humans, animals and plants, and several of them have been shown to play important roles in diverse biological processes. However, little work has been performed on the regulation mechanism of lncRNA biogenesis and expression, especially in plants. Compared with studies of tomato MADS-box transcription factor RIPENING INHIBITOR (RIN) target coding genes, there are few reports on its relationship to non-coding RNAs. The aim of the present study was to identify and explore the specific role of RIN target lncRNAs in tomato fruit development and ripening. METHODS: lncRNA targets of RIN were identified by chromatin immunoprecipitation sequencing (ChIP-seq) combined with RNA deep sequencing analysis. Six selected lncRNA targets were validated by quantitative real-time PCR, ChIP and electrophoretic mobility shift assays, and we further confirmed differential expression between wild-type and ripening-deficient mutant fruit, and RIN direct binding in the promoter regions. By means of virus-induced gene silencing (VIGS) assays and a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome editing strategy, the ripening-related function of a specific target lncRNA (lncRNA2155) was studied. KEY RESULTS: We identified 187 lncRNAs as direct RIN targets, which exhibited RIN binding sites in their promoters and showed different expression between the wild-type and rin mutant. Six target lncRNAs were shown to bind with RIN directly in their promoters in vivo and in vitro. Moreover, using CRISPR/Cas9 technology to knock out the locus of the target lncRNA2155 indicated that it delayed fruit ripening in tomato. CONCLUSIONS: Collectively, these findings provide new insight into RIN in the transcriptional regulation of lncRNAs and suggest that lncRNAs will contribute to a better understanding of the	tomato

841	plant	tomato	agroinfiltration	A region of cell surface protein PAc (PAcA) coding gene of mutans streptococci with cholera toxin B subunit coding gene (CTB) fusion	Biotechnology and applied biochemistry	Construction of a fusion anti-caries DNA vaccine in transgenic tomato plants for PAcA gene and cholera toxin B subunit.	2019	66(6):924-929	[Bai G et al.]	Zunyi Medical University, Zunyi, China.	31434162	10.1002/bab.1806	Chronic bacterial infections in the oral cavity influence the development of dental caries. Mutans streptococci are the major pathogenic cause of dental caries. The World Health Organization (WHO) ranks dental caries, cancer, and cardiovascular diseases as the three major global diseases that need urgent preventative and curative measures. However, substantial evidence suggests that traditional prevention and treatment strategies are inefficient in reducing the prevalence of dental caries. For protection against caries, it is important to develop effective vaccines that induce anticolonizing immunity against Streptococcus mutans infections. In the present investigation, we constructed a fusion anti-caries DNA vaccine (PAcA-ctxB) through fusing A region of cell surface protein PAc (PAcA) coding gene of mutans streptococci with cholera toxin B subunit coding gene (CTB). Afterward, the plasmids were integrated into tomato genomes through agrobacterium-mediated plant transformation technology. The presence of transgenes in the tomato genome was confirmed by PCR, beta-glucuronidase gene (GUS), and western blot. The expression of genes was confirmed at transcription and protein level. Altogether, the results presented herein showed that transgenic tomatoes may provide a useful system for the production of human caries	tomato
842	plant	tomato	CRISPR;Cas9;	Mitogen-activated protein kinase (SlMAPK3)	BMC plant biology	Knockout of SlMAPK3 enhances tolerance to heat stress involving ROS homeostasis in tomato plants.	2019	19(1):354	[Yu W et al.]	China Agricultural University, Beijing, China.	31412779	10.1186/s12870-019-1939-z	BACKGROUND: High temperature is a major environmental stress that limits plant growth and agriculture productivity. Mitogen-activated protein kinases (MAPKs) are highly conserved serine and threonine protein kinases that participate in response to diverse environmental stresses in plants. A total of 16 putative SlMAPK genes are identified in tomato, and SlMAPK3 is one of the most extensively studied SlMAPKs. However, the role of SlMAPK3 in response to heat stress is not clearly understood in tomato plants. In this study, we performed functional analysis of SlMAPK3 for its possible role in response to heat stress. RESULTS: qRT-PCR analyses revealed that SlMAPK3 relative expression was depressed by heat stress. Here, wild-type (WT) tomato plants and CRISPR/Cas9-mediated slmapk3 mutant lines (L8 and L13) were used to investigate the function of SlMAPK3 in response to heat stress. Compared with WT plants, slmapk3 mutants exhibited less severe wilting and less membrane damage, showed lower reactive oxygen species (ROS) contents, and presented higher both activities and transcript levels of antioxidant enzymes, as well as elevated expressions of genes encoding heat stress transcription factors (HSFs) and heat shock proteins (HSPs). CONCLUSIONS: CRISPR/Cas9-mediated slmapk3 mutants exhibited more tolerance to heat stress than WT plants, suggesting that SlMAPK3 was a negative regulator of thermotolerance. Moreover, antioxidant enzymes and HSPs/HSFs genes expression were involved in SlMAPK3-mediated heat stress response in tomato plants.	tomato
843	plant	tomato	CRISPR;Cas9;	nonexpressor of pathogenesis-related gene 1 (SlNPR1)	BMC plant biology	CRISPR/Cas9-Mediated SlNPR1 mutagenesis reduces tomato plant drought tolerance.	2019	19(1):38	[Li R et al.]	China Agricultural University, Beijing, China.	30669982	10.1186/s12870-018-1627-4	BACKGROUND: NPR1, nonexpressor of pathogenesis-related gene 1, is a master regulator involved in plant defense response to pathogens, and its regulatory mechanism in the defense pathway has been relatively clear. However, information about the function of NPR1 in plant response to abiotic stress is still limited. Tomato is the fourth most economically crop worldwide and also one of the best-characterized model plants employed in genetic studies. Because of the lack of a stable tomato NPR1 (SlNPR1) mutant, little is known about the function of SlNPR1 in tomato response to biotic and abiotic stresses. RESULTS: Here we isolated SlNPR1 from tomato 'Ailsa Craig' and generated slnpr1 mutants using the CRISPR/Cas9 system. Analysis of the cis-acting elements indicated that SlNPR1 might be involved in tomato plant response to drought stress. Expression pattern analysis showed that SlNPR1 was expressed in all plant tissues, and it was strongly induced by drought stress. Thus, we investigated the function of SlNPR1 in tomato-plant drought tolerance. Results showed that slnpr1 mutants exhibited reduced drought tolerance with increased stomatal aperture, higher electrolytic leakage, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels, and lower activity levels of antioxidant enzymes, compared to wild type (WT) plants. The reduced drought tolerance of slnpr1 mutants was further reflected by the down-regulated expression of drought related key genes, including SlGST, SlDHN, and SlDREB. CONCLUSIONS: Collectively, the data suggest that SlNPR1 is involved in regulating tomato plant drought response. These results aid in	tomato
844	plant	tomato	CRISPR;Cas9;	transglutaminases	Horticulture research	TGase positively regulates photosynthesis via activation of Calvin cycle enzymes in tomato.	2019	6:92	[Zhong M et al.]	Nanjing Agricultural University, Nanjing, China.	31645950	10.1038/s41438-019-0173-z	Transglutaminases (TGases), which are widespread cross-linking enzymes in plants, play key roles in photosynthesis and abiotic/biotic stress responses; however, evidence concerning the genetics underlying how TGase improves the capability of photosynthesis and the mechanism of TGase-mediated photosynthesis are not clear in this crop species. In this study, we clarified the function of TGase in the regulation of photosynthesis in tomato by comparing wild-type (WT) plants, tgase mutants generated by the CRISPR/Cas9 system and TGase-overexpressing (TGaseOE) plants. Our results showed that increasing the transcript level of TGase resulted in an enhanced net photosynthetic rate (Pn), whereas the tgase mutants presented significantly inhibited Pns and CO_2 assimilation compared with the WT. Although the total RuBisCO activity was not affected by TGase, the initial and activation status of RuBisCO and the activity of RuBisCO activase (RCA) and fructose-1,6-bisphosphatase (FBPase) in TGaseOE plants were significantly higher than that in WT plants. Except for RuBisCO small subunit (RbcS), the transcription levels of Benson-Calvin cycle-related genes were positively related to the endogenous TGase activity. Furthermore, TGaseOE plants had higher protein levels of RuBisCO large subunit (RbcL) and RCA than did WT plants and showed a reduced redox status by enhancing the activity of dehydroascorbate reductase (DHAR) and glutathione reductase (GR), which was compromised in TGase-deficient plants. Overall, TGase positively regulated photosynthesis by maintaining the activation states of the Benson-Calvin cycle and	tomato

845	plant	tomato	CRISPR;Cas9;	SBP-CNR and NAC-NOR transcription factors	Horticulture research	Diversity and redundancy of the ripening regulatory networks revealed by the fruitENCODE and the new CRISPR/Cas9 CNR and NOR mutants.	2019	6:39	[Gao Y et al.]	China Agricultural University, Beijing, China.	30774962	10.1038/s41438-019-0122-x	Tomato is considered as the genetic model for climacteric fruits, in which three major players control the fruit ripening process: ethylene, ripening transcription factors, and DNA methylation. The fruitENCODE project has now shown that there are multiple transcriptional circuits regulating fruit ripening in different species, and H3K27me3, instead of DNA methylation, plays a conserved role in restricting these ripening pathways. In addition, the function of the core tomato ripening transcription factors is now being questioned. We have employed CRISPR/Cas9 genome editing to mutate the SBP-CNR and NAC-NOR transcription factors, both of which are considered as master regulators in the current tomato ripening model. These plants only displayed delayed or partial non-ripening phenotypes, distinct from the original mutant plants, which categorically failed to ripen, suggesting that they might be gain-of-function mutants. Besides increased DNA methylation genome-wide, the original mutants also have hyper-H3K27me3 in ripening gene loci such as ACS2, RIN, and TDR4. It is most likely that multiple genetic and epigenetic factors have contributed to their strong non-ripening phenotypes. Hence, we propose that the field should move beyond these linear and two-dimensional models and embrace the fact that important biological processes such as ripening are often regulated by highly redundant network with inputs from	tomato
846	plant	tomato	Agroinfiltration;	PaM6PR; PaCWI; PaSUS1	Molecular biotechnology	Host-Induced Silencing of Some Important Genes Involved in Osmoregulation of Parasitic Plant Phelipanche aegyptiaca.	2019	61(12):929-937	[Farrokhi Z et al.]	University of Tehran, Karaj, Iran.	31564035	10.1007/s12033-019-00215-0	Broomrape is an obligate root-parasitic weed that acts as a competitive sink for host photoassimilates. Disruption of essential processes for growth of broomrape using host plant-mediated systemic signals can help to implement more specific and effective management plans of this parasite. Accordingly, we tested the possibility of transient silencing three involved genes (PaM6PR, PaCWI, and PaSUS1) in osmoregulation process of broomrape using syringe agroinfiltration of dsRNA constructs in tomato. The highest decrease in mRNA levels, enzyme activity, and amount of total reducing sugars was observed in Phelipanche aegyptiaca when grown on agroinfiltrated tomato plants by PaM6PR dsRNA construct than control. In addition, PaSUS1 dsRNA construct showed high reduction in mRNA abundance (32-fold fewer than control). The lowest decrease in mRNA levels was observed after infiltration of PaCWI dsRNA construct (eightfold fewer than control). While the highest reduction in PaM6PR and PaSUS1 expression levels was detected in the parasite at 3 days post-infiltration (dpi), the maximum reduction in both of the total reducing sugars amount and M6PR and SUS1 activities was observed at 8 dpi. On the contrary, CWI activity, PaCWI expression level, and amount of total reducing sugars in broomrape shoots simultaneously decreased at the day 3 after the dsRNA construct infiltration against PaCWI. On the whole, our results indicated that the three studied genes especially PaM6PR may constitute appropriate targets for the development of transgenic	tomato
847	plant	tomato	CRISPR;Cas9;	ripening inhibitor	New phytologist	Roles of RIN and ethylene in tomato fruit ripening and ripening-associated traits.	2019		[Li S et al.]	Zhejiang University, Zijingang Campus, Hangzhou, China.	31814125	10.1111/nph.16362	RIPENING INHIBITOR (RIN)-deficient fruits generated by CRISPR/Cas9 initiated partial ripening at a similar time to wild-type (WT) fruits but only 10% WT concentrations of carotenoids and ethylene (ET) were synthesized. RIN-deficient fruit never ripened completely, even when supplied with exogenous ET. The low amount of endogenous ET that they did produce was sufficient to enable ripening initiation and this could be suppressed by the ET perception inhibitor 1-MCP. The reduced ET production by RIN-deficient tomatoes was due to an inability to induce autocatalytic system-2 ET synthesis, a characteristic feature of climacteric ripening. Production of volatiles and transcripts of key volatile biosynthetic genes also were greatly reduced in the absence of RIN. By contrast, the initial extent and rates of softening in the absence of RIN were similar to WT fruits, although detailed analysis showed that the expression of some cell wall-modifying enzymes was delayed and others increased in the absence of RIN. These results support a model where RIN and ET, via ERFs, are required for full expression of ripening genes. Ethylene initiates ripening of mature green fruit, upregulates RIN expression and other changes, including system-2 ET production. RIN, ET and other factors are required for completion of the full fruit-ripening programme.	tomato
848	plant	tomato	CRISPR;Cas9;	PHOSPHATE 1 (SlPHO1;1)	Physiologia plantarum	A CRISPR/Cas9 deletion into the phosphate transporter SlPHO1;1 reveals its role in phosphate nutrition of tomato seedlings.	2019	167(4):556-563	[Zhao P et al.]	Nanchang Normal University, Nanchang, China.	30537089	10.1111/ppl.12897	In vascular (Arabidopsis thaliana) and non-vascular (Physcomitrella patens) plants, PHOSPHATE 1 (PHO1) homologs play important roles in the acquisition and transfer of phosphate. The tomato genome contains six genes (SlPHO1;1-SlPHO1;6) homologous to AtPHO1. The six proteins have typical characteristics of the plant PHO1 family, such as the three Syg1/Pho81/XPRI (SPX) subdomains in the N-terminal portion and one ERD1/XPR1/SYG1 (EXS) domain in the C-terminal portion. Phylogenetic analysis revealed that the SlPHO1 family is subdivided into three clusters. A pairwise comparison indicated that SlPHO1;1 showed the highest level of sequence identity/similarity (67.39/76.21%) to AtPHO1. SlPHO1;1 deletion mutants induced by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 displayed typical phenotypes of Pi starvation, such as decreased shoot fresh weight and increased root fresh weight, therefore having a greater root-to-shoot ratio. Mutants also accumulated more anthocyanin and had more soluble Pi content in the root and less in the shoot. These results indicate that SlPHO1;1 plays an important role in Pi	tomato
849	plant	tomato	CRISPR;Cas9;	bZIP transcription factor (SlHY5)	Plant & cell physiology	Identification of Candidate HY5-Dependent and -Independent Regulators of Anthocyanin Biosynthesis in Tomato.	2019	60(3):643-656	[Qiu Z et al.]	South China Agricultural University, Guangzhou, China.	30597099	10.1093/pcp/pcy236	High quantities of anthocyanins in plants confer potential protective benefits against biotic and abiotic stressors. Studies have shown that the bZIP transcription factor HY5 plays a key role in controlling anthocyanin accumulation in response to light. However, in hy5 mutants, residual anthocyanins have been detected, indicating that other regulators exist to regulate anthocyanin biosynthesis in an HY5-independent manner. Here, we employed the CRISPR/Cas9 (clustered regularly interspersed short palindromic repeats/CRISPR-associated protein 9) system specifically to induce targeted mutagenesis of SlHY5 in the purple tomato cultivar 'Indigo Rose'. The T2 generation of tomato plants homozygous for the null allele of the SlHY5 frameshift mutated by a 1 bp insertion contained a lower anthocyanin content. Transcriptional analysis showed that most of the anthocyanin biosynthesis structural genes and several regulatory genes were down-regulated in the hy5 mutant lines. With transcriptome analyses of the various tissues from hy5 mutant lines, eight candidate transcription factors were identified that may regulate anthocyanin biosynthesis in an HY5-independent manner. These findings deepen our understanding of how light controls anthocyanin accumulation and facilitate the identification of the regulators of	tomato

850	plant	tomato	CRISPR;Cas9;	SlS5alphaR1; SlS5alphaR2	Plant biotechnology	Characterization of steroid 5alpha-reductase involved in alpha-tomatine biosynthesis in tomatoes.	2019	36(4):253-263	[Akiyama R et al.]	Kobe University, Kobe, Hyogo, Japan.	31983879	10.5511/plantbiotechnology.19.1030a	alpha-tomatine and dehydrotomatine are steroidal glycoalkaloids (SGAs) that accumulate in the mature green fruits, leaves, and flowers of tomatoes (Solanum lycopersicum) and function as defensive compounds against pathogens and predators. The aglycones of alpha-tomatine and dehydrotomatine are tomatidine and dehydrotomatidine (5,6-dehydrogenated tomatidine), and tomatidine is derived from dehydrotomatidine via four reaction steps: C3 oxidation, isomerization, C5alpha reduction, and C3 reduction. Our previous studies (Lee et al. 2019) revealed that Sl3betaHSD is involved in the three reactions except for C5alpha reduction, and in the present study, we aimed to elucidate the gene responsible for the C5alpha reduction step in the conversion of dehydrotomatidine to tomatidine. We characterized the two genes, SlS5alphaR1 and SlS5alphaR2, which show high homology with DET2, a brassinosteroid 5alpha reductase of Arabidopsis thaliana. The expression pattern of SlS5alphaR2 is similar to those of SGA biosynthetic genes, while SlS5alphaR1 is ubiquitously expressed, suggesting the involvement of SlS5alphaR2 in SGA biosynthesis. Biochemical analysis of the recombinant proteins revealed that both of SlS5alphaR1 and SlS5alphaR2 catalyze the reduction of tomatid-4-en-3-one at C5alpha to yield tomatid-3-one. Then, SlS5alphaR1- or SlS5alphaR2-knockout hairy roots were constructed using CRISPR/Cas9 mediated genome editing. In the SlS5alphaR2-knockout hairy roots, the alpha-tomatine level was significantly decreased and dehydrotomatine was accumulated. On the other hand, no change in the amount of alpha-tomatine was observed in the SlS5alphaR1-knockout hairy root. These results indicate that SlS5alphaR2 is responsible for the C5alpha reduction in	tomato
851	plant	tomato	CRISPR;Cas9;	SlJAZ2	Plant biotechnology journal	Design of a bacterial speck resistant tomato by CRISPR/Cas9-mediated editing of SlJAZ2.	2019	17(3):665-673	[Ortigosa A et al.]	Centro Nacional de Biotecnologia, Consejo Superior de Investigaciones Cientificas (CNB-CSIC), Madrid, Spain.	30183125	10.1111/pbi.13006	Due to their different lifestyles, effective defence against biotrophic pathogens normally leads to increased susceptibility to necrotrophs, and vice versa. Solving this trade-off is a major challenge for obtaining broad-spectrum resistance in crops and requires uncoupling the antagonism between the jasmonate (JA) and salicylate (SA) defence pathways. Pseudomonas syringae pv. tomato (Pto) DC3000, the causal agent of tomato bacterial speck disease, produces coronatine (COR) that stimulates stomata opening and facilitates bacterial leaf colonization. In Arabidopsis, stomata response to COR requires the COR co-receptor AtJAZ2, and dominant AtJAZ2Deltajas repressors resistant to proteasomal degradation prevent stomatal opening by COR. Here, we report the generation of a tomato variety resistant to the bacterial speck disease caused by PtoDC3000 without compromising resistance to necrotrophs. We identified the functional ortholog of AtJAZ2 in tomato, found that preferentially accumulates in stomata and proved that SlJAZ2 is a major co-receptor of COR in stomatal guard cells. SlJAZ2 was edited using CRISPR/Cas9 to generate dominant JAZ2 repressors lacking the C-terminal Jas domain (SlJAZ2Deltajas). SlJAZ2Deltajas prevented stomatal reopening by COR and provided resistance to PtoDC3000. Water transpiration rate and resistance to the necrotrophic fungal pathogen Botrytis cinerea, causal agent of the tomato gray mold, remained unaltered in Sljaz2Deltajas plants. Our results solve the defence trade-off in a crop, by spatially uncoupling the SA-JA hormonal antagonism at the stomata, entry gates of specific microbes such as PtoDC3000. Moreover, our results also constitute a novel CRISPR/Cas-based strategy for crop protection that	tomato
852	plant	tomato	CRISPR;Cas9;	PROCERA	Plant biotechnology journal	Using CRISPR/Cas9 genome editing in tomato to create a gibberellin-responsive dominant dwarf DELLA allele.	2019	17(1):132-140	[Tomlinson L et al.]	Sainsbury Laboratory, Norwich, UK.	29797460	10.1111/pbi.12952	The tomato PROCERA gene encodes a DELLA protein, and loss-of-function mutations derepress growth. We used CRISPR/Cas9 and a single guide RNAs (sgRNA) to target mutations to the PROCERA DELLA domain, and recovered several loss-of-function mutations and a dominant dwarf mutation that carries a deletion of one amino acid in the DELLA domain. This is the first report of a dominant dwarf PROCERA allele. This allele retains partial responsiveness to exogenously applied gibberellin. Heterozygotes show an intermediate phenotype at the seedling stage, but adult heterozygotes are as dwarfed as homozygotes.	tomato
853	plant	tomato	CRISPR;Cas9;	MYB transcription factor SlMYB21	Plant cell	Tomato MYB21 Acts in Ovules to Mediate Jasmonate-Regulated Fertility.	2019	31(5):1043-1062	[Schubert R et al.]	Institute of Plant Biochemistry, Halle, Germany.	30894458	10.1105/tpc.18.00978	The function of the plant hormone jasmonic acid (JA) in the development of tomato (Solanum lycopersicum) flowers was analyzed with a mutant defective in JA perception (jasmonate-insensitive1-1, jai1-1). In contrast with Arabidopsis (Arabidopsis thaliana) JA-insensitive plants, which are male sterile, the tomato jai1-1 mutant is female sterile, with major defects in female development. To identify putative JA-dependent regulatory components, we performed transcriptomics on ovules from flowers at three developmental stages from wild type and jai1-1 mutants. One of the strongly downregulated genes in jai1-1 encodes the MYB transcription factor SlMYB21. Its Arabidopsis ortholog plays a crucial role in JA-regulated stamen development. SlMYB21 was shown here to exhibit transcription factor activity in yeast, to interact with SlJAZ9 in yeast and in planta, and to complement Arabidopsis myb21-5 To analyze SlMYB21 function, we generated clustered regularly interspaced short palindromic repeats(CRISPR)/CRISPR associated protein 9 (Cas9) mutants and identified a mutant by Targeting Induced Local Lesions in Genomes (TILLING). These mutants showed female sterility, corroborating a function of MYB21 in tomato ovule development. Transcriptomics analysis of wild type, jai1-1, and myb21-2 carpels revealed processes that might be controlled by SlMYB21. The data suggest positive regulation of JA biosynthesis by SlMYB21, but negative regulation of auxin and gibberellins. The results demonstrate that SlMYB21 mediates at least partially the	tomato

854	plant	tomato	CRISPR;Cas9;	zinc-finger transcription factor LOL1 (LSD ONE LIKE1; CcLOL1) tomato ortholog	Plant journal	The zinc-finger transcription factor CcLOL1 controls chloroplast development and immature pepper fruit color in Capsicum chinense and its function is conserved in tomato.	2019	99(1):41-55	[Borovsky Y et al.]	Institute of Plant Science, Agricultural Research Organization, Rishon LeZion, Israel.	30828904	10.1111/tpj.14305	Chloroplast development and chlorophyll content in the immature fruit has a major impact on the morphology and quality in pepper (Capsicum spp.) fruit. Two major quantitative trait loci (QTLs), pc1 and pc10 that affect chlorophyll content in the pepper fruit by modulation of chloroplast compartment size were previously identified in chromosomes 1 and 10, respectively. The pepper homolog of GOLDEN2-LIKE transcription factor (CaGLK2) has been found as underlying pc10, similar to its effect on tomato chloroplast development. In the present study, we identified the pepper homolog of the zinc-finger transcription factor LOL1 (LSD ONE LIKE1; CcLOL1) as the gene underlying pc1. LOL1 has been identified in Arabidopsis as a positive regulator of programmed cell death and we report here on its role in controlling fruit development in the Solanaceae in a fruit-specific manner. The light-green C. chinense parent used for QTL mapping was found to carry a null mutation in CcLOL1. Verification of the function of the gene was done by generating CRISPR/Cas9 knockout mutants of the orthologous tomato gene resulting in light-green tomato fruits, indicating functional conservation of the orthologous genes in controlling chlorophyll content in the Solanaceae. Transcriptome profiling of light and dark-green bulks differing for pc1, showed that the QTL affects multiple photosynthesis and oxidation-reduction associated genes in the immature green fruit. Allelic diversity of three known genes CcLOL1, CaGLK2, and CcAPRR2 that influence pepper immature fruit color, was found	tomato
855	plant	tomato	CRISPR;Cas9;	Brassinosteroid-insensitive 1 (SlBRI1)	Planta	Brassinosteroids facilitate xylem differentiation and wood formation in tomato.	2019	249(5):1391-1403	[Lee J et al.]	Chungbuk National University, Cheongju, Korea.	30673841	10.1007/s00425-019-03094-6	MAIN CONCLUSION: BR signaling pathways facilitate xylem differentiation and wood formation by fine tuning SlBZR1/SlBZR2-mediated gene expression networks involved in plant secondary growth. Brassinosteroid (BR) signaling and BR crosstalk with diverse signaling cues are involved in the pleiotropic regulation of plant growth and development. Recent studies reported the critical roles of BR biosynthesis and signaling in vascular bundle development and plant secondary growth; however, the molecular bases of these roles are unclear. Here, we performed comparative physiological and anatomical analyses of shoot morphological growth in a cultivated wild-type tomato (Solanum lycopersicum cv. BGA) and a BR biosynthetic mutant [Micro Tom (MT)]. We observed that the canonical BR signaling pathway was essential for xylem differentiation and sequential wood formation by facilitating plant secondary growth. The gradual retardation of xylem development phenotypes during shoot vegetative growth in the BR-deficient MT tomato mutant recovered completely in response to exogenous BR treatment or genetic complementation of the BR biosynthetic DWARF (D) gene. By contrast, overexpression of the tomato Glycogen synthase kinase 3 (SlGSK3) or CRISPR-Cas9 (CR)-mediated knockout of the tomato Brassinosteroid-insensitive 1 (SlBRI1) impaired BR signaling and resulted in severely defective xylem differentiation and secondary growth. Genetic modulation of the transcriptional activity of the tomato Brassinazole-resistant 1/2 (SlBZR1/SlBZR2) confirmed the positive roles of BR signaling pathways for xylem differentiation and secondary growth. Our data indicate that BR signaling pathways directly promote xylem	tomato
856	plant	tomato	CRISPR;Cas9;	carotenoid cleavage dioxygenase 8	Scientific reports	CRISPR/Cas9-mediated mutagenesis of CAROTENOID CLEAVAGE DIOXYGENASE 8 in tomato provides resistance against the parasitic weed Phelipanche aegyptiaca.	2019	9(1):11438	[Bari VK et al.]	Agricultural Research Organization (ARO), Volcani Center, Ramat Yishay, Israel.	31391538	10.1038/s41598-019-47893-z	Broomrapes (Phelipanche aegyptiaca and Orobanche spp.) are obligate plant parasites that cause extreme damage to crop plants. The parasite seeds have strict requirements for germination, involving preconditioning and exposure to specific chemicals strigolactones [SLs] exuded by the host roots. SLs are plant hormones derived from plant carotenoids via a pathway involving the Carotenoid Cleavage Dioxygenase 8 (CCD8). Having no effective means to control parasitic weeds in most crops, and with CRISPR/Cas9 being an effective gene-editing tool, here we demonstrate that CRISPR/Cas9-mediated mutagenesis of the CCD8 gene can be used to develop host resistance to the parasitic weed P. aegyptiaca. Cas9/single guide (sg) RNA constructs were targeted to the second exon of CCD8 in tomato (Solanum lycopersicum L.) plants. Several (CCD8)Cas9 mutated tomato lines with variable insertions or deletions in CCD8 were obtained with no identified off-targets. Genotype analysis of T1 plants showed that the introduced CCD8 mutations are inherited. Compared to control tomato plants, the (CCD8)Cas9 mutant had morphological changes that included dwarfing, excessive shoot branching and adventitious root formation. In addition, SL-deficient (CCD8)Cas9 mutants showed a significant reduction in parasite infestation compared to non-mutated tomato plants. In the (CCD8)Cas9 mutated lines, orobanchol (SL) content was significantly reduced but total carotenoids level and expression of genes related to carotenoid biosynthesis were increased, as compared to control plants. Taking into account, the impact of plant parasitic weeds on agriculture and difficulty to constitute efficient control methods, the current study offers insights into the development of a new, efficient method that could	tomato
857	plant	tomato	CRISPR;Cas9;	Transcription Factors APETALA2a (AP2a), NON-RIPENING (NOR) and FRUITFULL (FUL1/TDR4 and FUL2/MBP7)	Scientific reports	Re-evaluation of transcription factor function in tomato fruit development and ripening with CRISPR/Cas9-mutagenesis.	2019	9(1):1696	[Wang R et al.]	Wageningen University, Wageningen, The Netherlands.	30737425	10.1038/s41598-018-38170-6	Tomato (Solanum lycopersicum) is a model for climacteric fleshy fruit ripening studies. Tomato ripening is regulated by multiple transcription factors together with the plant hormone ethylene and their downstream effector genes. Transcription Factors APETALA2a (AP2a), NON-RIPENING (NOR) and FRUITFULL (FUL1/TDR4 and FUL2/MBP7) were reported as master regulators controlling tomato fruit ripening. Their proposed functions were derived from studies of the phenotype of spontaneous mutants or RNAi knock-down lines rather than, as it appears now, actual null mutants. To study TF function in tomato fruit ripening in more detail, we used CRISPR/Cas9-mediated mutagenesis to knock out the encoding genes, and phenotypes of these mutants are reported for the first time. While the earlier ripening, orange-ripe phenotype of ap2a mutants was confirmed, the nor null mutant exhibited a much milder phenotype than the spontaneous nor mutant. Additional analyses revealed that the severe phenotype in the spontaneous mutant is caused by a dominant-negative allele. Our approach also provides new insight into the independent and overlapping functions of FUL1 and FUL2. Single and combined null alleles of FUL1 and FUL2 illustrate that these two genes have partially redundant functions in fruit ripening, but also unveil an	tomato

858	plant	tomato (Solanum lycopersicum and S. pimpinellifolium)	CRISPR;Cas9;	RecQ4	Plant biotechnology journal	CRISPR/Cas inactivation of RECQ4 increases homeologous crossovers in an interspecific tomato hybrid.	2019		[de Maagd RA et al.]	Wageningen University & Research, Wageningen, The Netherlands.	31483929	10.1111/pbi.13248	Crossover formation during meiosis in plants is required for proper chromosome segregation and is essential for crop breeding as it allows an (optimal) combination of traits by mixing parental alleles on each chromosome. Crossover formation commences with the production of a large number of DNA double-strand breaks, of which only a few result in crossovers. A small number of genes, which drive the resolution of DNA crossover intermediate structures towards non-crossovers, have been identified in Arabidopisis thaliana. In order to explore the potential of modification of these genes in interspecific hybrids between crops and their wild relatives towards increased production of crossovers, we have used CRISPR/Cas9-mutagenesis in an interspecific tomato hybrid to knockout RecQ4. A biallelic recq4 mutant was obtained in the F1 hybrid of Solanum lycopersicum and S. pimpinellifolium. Compared with the wild-type F1 hybrid, the F1 recq4 mutant was shown to have a significant increase in crossovers: a 1.53-fold increase when directly observing ring bivalents in male meiocytes microscopically and a 1.8-fold extension of the genetic map when measured by analysing SNP markers in the progeny (F2) plants. This is one of the first demonstrations of increasing crossover frequency in interspecific hybrids by manipulating genes in crossover intermediate resolution pathways and the first to do so by directed mutagenesis. SIGNIFICANCE STATEMENT: Increasing crossover frequency during meiosis can speed up or simplify crop breeding that relies on meiotic crossovers to introduce favourable alleles controlling important traits from wild relatives into crops. Here we show for the first time that knocking out an inhibitor of crossovers in an interspecific hybrid between tomato and its relative wild species using CRISPR/Cas9-	tomato
859	plant	tomato (Solanum lycopersicum)	CRISPR;Cas9;	Methyltransferase 1 (SlMET1)	Journal of integrative plant biology	Critical function of DNA methyltransferase 1 in tomato development and regulation of the DNA methylome and transcriptome.	2019	61(12):1224-1242	[Yang Y et al.]	Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.	30652405	10.1111/jipb.12778	DNA methylation confers epigenetic regulation on gene expression and thereby on various biological processes. Tomato has emerged as an excellent system to study the function of DNA methylation in plant development. To date, regulation and function of DNA methylation maintenance remains unclear in tomato plants. Here, we report the critical function of tomato (Solanum lycopersicum) Methyltransferase 1 (SlMET1) in plant development and DNA methylome and transcriptome regulation. Using CRISPR-Cas9 gene editing, we generated slmet1 mutants and observed severe developmental defects with a frame-shift mutation, including small and curly leaves, defective inflorescence, and parthenocarpy. In leaf tissues, mutations in SlMET1 caused CG hypomethylation and CHH hypermethylation on a whole-genome scale, leading to a disturbed transcriptome including ectopic expression of many RIN target genes such as ACC2 in leaf tissues, which are normally expressed in fruits. Neither the CG hypomethylation nor CHH hypermethylation in the slmet1 mutants is related to tissue culture. Meanwhile, tissue culture induces non-CG hypomethylation, which occurs more frequently at gene regions than at TE regions. Our results depict SlMET1- and tissue culture-dependent tomato DNA methylomes, and that SlMET1 is required for	tomato
860	plant	tomato (Solanum lycopersicum)	CRISPR;	gibberellin-insensitive dwarf1	Plant cell	Multiple Gibberellin Receptors Contribute to Phenotypic Stability under Changing Environments.	2019	31(7):1506-1519	[Illouz-Eliaz N et al.]	Hebrew University of Jerusalem, Rehovot, Israel.	31076539	10.1105/tpc.19.00235	The pleiotropic and complex gibberellin (GA) response relies on targeted proteolysis of DELLA proteins mediated by a GA-activated GIBBERELLIN-INSENSITIVE DWARF1 (GID1) receptor. The tomato (Solanum lycopersicum) genome encodes for a single DELLA protein, PROCERA (PRO), and three receptors, SlGID1a (GID1a), GID1b1, and GID1b2, that may guide specific GA responses. In this work, clustered regularly interspaced short palindromic repeats (CRISPR) /CRISPR associated protein 9-derived gid1 mutants were generated and their effect on GA responses was studied. The gid1 triple mutant was extremely dwarf and fully insensitive to GA. Under optimal growth conditions, the three receptors function redundantly and the single gid1 mutants exhibited very mild phenotypic changes. Among the three receptors, GID1a had the strongest effects on germination and growth. Yeast two-hybrid assays suggested that GID1a has the highest affinity to PRO. Analysis of lines with a single active receptor demonstrated a unique role for GID1a in protracted response to GA that was saturated only at high doses. When the gid1 mutants were grown in the field under ambient changing environments, they showed phenotypic instability, the high redundancy was lost, and gid1a exhibited dwarfism that was strongly exacerbated by the loss of another GID1b receptor gene. These results suggest that multiple GA receptors contribute to	tomato
861	plant	tomato (Solanum lycopersicum)	CRISPR;	pectate lyase; polygalacturonase 2a; beta-galactanase	Plant physiology	Characterization of CRISPR Mutants Targeting Genes Modulating Pectin Degradation in Ripening Tomato.	2019	179(2):544-557	[Wang D et al.]	University of Nottingham, Loughborough, UK.	30459263	10.1104/pp.18.01187	Tomato (Solanum lycopersicum) is a globally important crop with an economic value in the tens of billions of dollars, and a significant supplier of essential vitamins, minerals, and phytochemicals in the human diet. Shelf life is a key quality trait related to alterations in cuticle properties and remodeling of the fruit cell walls. Studies with transgenic tomato plants undertaken over the last 20 years have indicated that a range of pectin-degrading enzymes are involved in cell wall remodeling. These studies usually involved silencing of only a single gene and it has proved difficult to compare the effects of silencing these genes across the different experimental systems. Here we report the generation of CRISPR-based mutants in the ripening-related genes encoding the pectin-degrading enzymes pectate lyase (PL), polygalacturonase 2a (PG2a), and beta-galactanase (TBG4). Comparison of the physiochemical properties of the fruits from a range of PL, PG2a, and TBG4 CRISPR lines demonstrated that only mutations in PL resulted in firmer fruits, although mutations in PG2a and TBG4 influenced fruit color and weight. Pectin localization, distribution, and solubility in the pericarp cells of the CRISPR mutant fruits were investigated using the monoclonal antibody probes LM19 to deesterified homogalacturonan, INRA-RU1 to rhamnogalacturonan I, LM5 to beta-1,4-galactan, and LM6 to arabinan epitopes, respectively. The data indicate that PL, PG2a, and TBG4 act on separate cell wall domains and the importance of cellulose microfibril-associated pectin is reflected in its	tomato

862	plant	tomato (Solanum neorickii)	Agroinfiltration;	phenylalanine ammonia-lyase and cystathionine gamma-lyase	Plant journal	A Solanum neorickii introgression population providing a powerful complement to the extensively characterized Solanum pennellii population.	2019	97(2):391-403	[Brog YM et al.]	Hebrew University of Jerusalem, Rehovot, Israel.	30230636	10.1111/tpj.14095	We present a complementary resource for trait fine-mapping in tomato to those based on the intra-specific cross between cultivated tomato and the wild tomato species Solanum pennellii, which have been extensively used for quantitative genetics in tomato over the last 20 years. The current population of backcross inbred lines (BILs) is composed of 107 lines derived after three backcrosses of progeny of the wild species Solanum neorickii (LA2133) and cultivated tomato (cultivar TA209) and is freely available to the scientific community. These S. neorickii BILs were genotyped using the 10K SolCAP single nucleotide polymorphism chip, and 3111 polymorphic markers were used to map recombination break points relative to the physical map of Solanum lycopersicum. The BILs harbor on average 4.3 introgressions per line, with a mean introgression length of 34.7 Mbp, allowing partitioning of the genome into 340 bins and thereby facilitating rapid trait mapping. We demonstrate the power of using this resource in comparison with archival data from the S. pennellii resources by carrying out metabolic quantitative trait locus analysis following gas chromatography-mass spectrometry on fruits harvested from the S. neorickii BILs. The metabolic candidate genes phenylalanine ammonia-lyase and cystathionine gamma-lyase were then tested and validated in F2 populations and via agroinfiltration-based overexpression in order to exemplify the fidelity of this method in identifying the genes that drive tomato	tomato
863	plant	tomato (Solanum pimpinellifolium (0043) and S. pimpinellifolium (0049-w1))	Agroinfiltration;	green fluorescent protein (GFP)	Plant cell reports	Efficient transient protein expression in tomato cultivars and wild species using agroinfiltration-mediated high expression system.	2019	38(1):75-84	[Hoshikawa K et al.]	University of Tsukuba, Tsukuba, Ibaraki, Japan.	30328507	10.1007/s00299-018-2350-1	KEY MESSAGE: The new transient protein expression system using the pBYR2HS vector is applicable to several tomato cultivars and wild species with high level of protein expression. Innovation and improvement of effective tools for transient protein expression in plant cells is critical for the development of plant biotechnology. We have created the new transient protein expression system using the pBYR2HS vector that led to about 4 mg/g fresh weight of protein expression in Nicotiana benthamiana. In this study, we validated the adaptability of this transient protein expression system by agroinfiltration to leaves and fruits of several tomato cultivars and wild species. Although the GFP protein was transiently expressed in the leaves and fruits of all tomato cultivars and wild species, we observed species-specific differences in protein expression. In particular, GFP protein expression was higher in the leaves and fruits of Micro-Tom, Solanum pimpinellifolium (0043) and S. pimpinellifolium (0049-w1) than in those of cultivars and wild species. Furthermore, Agrobacterium with GABA transaminase enhanced transient expression in tomato fruits of Micro-Tom. Taken together with these results, our system is applicable to several tomato cultivars and species as well as a model tomato, even though characteristics are often different among tomato cultivars or species. Thus, the system is an effective, simple, and valuable tool to achieve rapid transgene expression to examine gene function in tomato	tomato
864	plant	tomato; Arabidopsis	Cas9;		Journal of integrative plant biology	Expanding the scope of CRISPR/Cas9-mediated genome editing in plants using an xCas9 and Cas9-NG hybrid.	2019		[Niu Q et al.]	Center of Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai, China.	31702097	10.1111/jipb.12886	The widely used Streptococcus pyogenes Cas9 (SpCas9) requires NGG as a protospacer adjacent motif (PAM) for genome editing. Although SpCas9 is a powerful genome-editing tool, its use has been limited on the targetable genomic locus lacking NGG PAM. The SpCas9 variants xCas9 and Cas9-NG have been developed to recognize NG, GAA, and GAT PAMs in human cells. Here, we show that xCas9 cannot recognize NG PAMs in tomato, and Cas9-NG can recognize some of our tested NG PAMs in the tomato and Arabidopsis genomes. In addition, we engineered SpCas9 (XNG-Cas9) based on mutations from both xCas9 and Cas9-NG, and found that XNG-Cas9 can efficiently mutagenize endogenous target sites with NG, GAG, GAA, and GAT PAMs in the tomato or Arabidopsis genomes. The PAM compatibility of XNG-Cas9 is the broadest reported to date among Cas9s (SpCas9 and Cas9-NG) active in plant.	tomato; Arab
865	plant	tomato; groundcherry	CRISPR;Cas9;	SlER (targeted); SP5G; SP (new traits)	Nature biotechnology	Rapid customization of Solanaceae fruit crops for urban agriculture.	2019		[Kwon CT et al.]	Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.	31873217	10.1038/s41587-019-0361-2	Cultivation of crops in urban environments might reduce the environmental impact of food production(1-4). However, lack of available land in cities and a need for rapid crop cycling, to yield quickly and continuously, mean that so far only lettuce and related 'leafy green' vegetables are cultivated in urban farms(5). New fruit varieties with architectures and yields suitable for urban farming have proven difficult to breed(1,5). We identified a regulator of tomato stem length (SlER) and devised a trait-stacking strategy to combine mutations for condensed shoots, rapid flowering (SP5G) and precocious growth termination (SP). Application of our strategy using one-step CRISPR-Cas9 genome editing restructured vine-like tomato plants into compact, early yielding plants suitable for urban agriculture. Field data confirmed that yields were maintained, and we demonstrated cultivation in indoor farming systems. Targeting the same stem length regulator alone in groundcherry, another Solanaceae plant, also enabled engineering to a compact stature. Our approach can expand the repertoire of	tomato; grou
866	plant	tomato; potato	agroinfiltration; CRISPR;Cas9;	acetolactate synthase (ALS)	International journal of molecular sciences	Transgene-Free Genome Editing in Tomato and Potato Plants Using Agrobacterium-Mediated Delivery of a CRISPR/Cas9 Cytidine Base Editor.	2019	20(2)	[Veillet F et al.]	INRA, Universite Rennes 1, Ploudaniel, France.	30669298	10.3390/ijms20020402	Genome editing tools have rapidly been adopted by plant scientists for gene function discovery and crop improvement. The current technical challenge is to efficiently induce precise and predictable targeted point mutations valuable for crop breeding purposes. Cytidine base editors (CBEs) are CRISPR/Cas9 derived tools recently developed to direct a C-to-T base conversion. Stable genomic integration of CRISPR/Cas9 components through Agrobacterium-mediated transformation is the most widely used approach in dicotyledonous plants. However, elimination of foreign DNA may be difficult to achieve, especially in vegetatively propagated plants. In this study, we targeted the acetolactate synthase (ALS) gene in tomato and potato by a CBE using Agrobacterium-mediated transformation. We successfully and efficiently edited the targeted cytidine bases, leading to chlorsulfuron-resistant plants with precise base edition efficiency up to 71% in tomato. More importantly, we produced 12.9% and 10% edited but transgene-free plants in the first generation in tomato and potato, respectively. Such an approach is expected to decrease deleterious effects due to the random integration of transgene(s) into the host genome. Our successful approach opens up new perspectives for genome engineering by the co-edition of the ALS with other gene(s), leading to transgene-free plants harboring new traits of	tomato;potat

867	plant	wheat	CRISPR;Cas9;	5-enolpyruvylshikimate-3-phosphate synthase	BMC biotechnology	gRNA validation for wheat genome editing with the CRISPR-Cas9 system.	2019	19(1):71	[Arndell T et al.]	CSIRO, Agriculture and Food, Canberra, ACT, Australia.	31684940	10.1186/s12896-019-0565-z	BACKGROUND: The CRISPR-Cas9 system is a powerful and versatile tool for crop genome editing. However, achieving highly efficient and specific editing in polyploid species can be a challenge. The efficiency and specificity of the CRISPR-Cas9 system depends critically on the gRNA used. Here, we assessed the activities and specificities of seven gRNAs targeting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in hexaploid wheat protoplasts. EPSPS is the biological target of the widely used herbicide glyphosate. RESULTS: The seven gRNAs differed substantially in their on-target activities, with mean indel frequencies ranging from 0% to approximately 20%. There was no obvious correlation between experimentally determined and in silico predicted on-target gRNA activity. The presence of a single mismatch within the seed region of the guide sequence greatly reduced but did not abolish gRNA activity, whereas the presence of an additional mismatch, or the absence of a PAM, all but abolished gRNA activity. Large insertions (>/=20 bp) of DNA vector-derived sequence were detected at frequencies up to 8.5% of total indels. One of the gRNAs exhibited several properties that make it potentially suitable for the development of non-transgenic glyphosate resistant wheat. CONCLUSIONS: We have established a rapid and reliable method for gRNA validation in hexaploid wheat protoplasts. The method can be used to identify gRNAs that have favourable properties. Our approach is particularly suited to polyploid species, but should be applicable to any plant species amenable to protoplast	wheat
868	plant	wheat	CRISPR;Cas9;	alpha- and gamma-gliadin	BMC plant biology	Outlook for coeliac disease patients: towards bread wheat with hypoimmunogenic gluten by gene editing of alpha- and gamma-gliadin gene families.	2019	19(1):333	[Jouanin A et al.]	Wageningen University and Research, Wageningen, The Netherlands.	31370789	10.1186/s12870-019-1889-5	BACKGROUND: Wheat grains contain gluten proteins, which harbour immunogenic epitopes that trigger Coeliac disease in 1-2% of the human population. Wheat varieties or accessions containing only safe gluten have not been identified and conventional breeding alone struggles to achieve such a goal, as the epitopes occur in gluten proteins encoded by five multigene families, these genes are partly located in tandem arrays, and bread wheat is allohexaploid. Gluten immunogenicity can be reduced by modification or deletion of epitopes. Mutagenesis technologies, including CRISPR/Cas9, provide a route to obtain bread wheat containing gluten proteins with fewer immunogenic epitopes. RESULTS: In this study, we analysed the genetic diversity of over 600 alpha- and gamma-gliadin gene sequences to design six sgRNA sequences on relatively conserved domains that we identified near coeliac disease epitopes. They were combined in four CRISPR/Cas9 constructs to target the alpha- or gamma-gliadins, or both simultaneously, in the hexaploid bread wheat cultivar Fielder. We compared the results with those obtained with random mutagenesis in cultivar Paragon by gamma-irradiation. For this, Acid-PAGE was used to identify T1 grains with altered gliadin protein profiles compared to the wild-type endosperm. We first optimised the interpretation of Acid-PAGE gels using Chinese Spring deletion lines. We then analysed the changes generated in 360 Paragon gamma-irradiated lines and in 117 Fielder CRISPR/Cas9 lines. Similar gliadin profile alterations, with missing protein bands, could be observed in grains produced by both methods. CONCLUSIONS: The results demonstrate the feasibility and efficacy of using CRISPR/Cas9 to simultaneously edit multiple genes in the large alpha- and gamma-gliadin gene families in polyploid bread wheat. Additional methods, generating genomics and proteomics data, will be necessary	wheat
869	plant	wheat	agroinfiltration; CRISPR;Cas9;		International journal of molecular sciences	Highly Efficient and Heritable Targeted Mutagenesis in Wheat via the Agrobacterium tumefaciens-Mediated CRISPR/Cas9 System.	2019	20(17)	[Zhang S et al.]	Shandong Academy of Agricultural Sciences, Jinan, Shandong, China.	31480315	10.3390/ijms20174257	The CRISPR/Cas9 system has been successfully used in hexaploid wheat. Although it has been reported that the induced mutations can be passed to the next generation, gene editing and transmission patterns in later generations still need to be studied. In this study, we demonstrated that the CRISPR/Cas9 system could achieve efficient mutagenesis in five wheat genes via Agrobacterium-mediated transformation of an sgRNA targeting the D genome, an sgRNA targeting both the A and B homologues and three tri-genome guides targeting the editing of all three homologues. High mutation rates and putative homozygous or biallelic mutations were observed in the T0 plants. The targeted mutations could be stably inherited by the next generation, and the editing efficiency of each mutant line increased significantly across generations. The editing types and inheritance of targeted mutagenesis were similar, which were not related to the targeted subgenome number. The presence of Cas9/sgRNA could cause new mutations in subsequent generations, while mutated lines without Cas9/sgRNA could retain the mutation type. Additionally, off-target mutations were not found in sequences that were highly homologous to the selected sgRNA sequences. Overall, the results suggested that CRISPR/Cas9-induced gene editing via Agrobacterium-mediated transformation plays important roles in wheat genome engineering.	wheat
870	plant	wheat	agroinfiltration; CRISPR;Cas9;CPF1;	OsU6a, TaU3 and TaU6 (promoters); TaWaxy and TaMTL (target)	Journal of experimental botany	Editing TaMTL gene induces haploid plants efficiently by optimized Agrobacterium-mediated CRISPR system in wheat.	2019		[Liu H et al.]	Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China.	31760434	10.1093/jxb/erz529	CRISPR/LbCpf1 and CRISPR/xCas9 systems in wheat have not yet been reported. In this study we compared the efficiencies of three CRISPR editing systems (SpCas9, LbCpf1 and xCas9), and three different promoters (OsU6a, TaU3 and TaU6) driving sgRNA which were introduced into wheat via Agrobacterium-mediated transformation. Results indicated that TaU3 is a better choice than OsU6a or TaU6; the editing efficiency was higher using two sgRNAs than one sgRNA, and the mutants with a large fragment deletion between the two sgRNAs were produced. The LbCpf1 and xCas9 systems were successfully used in wheat. Two endogenous wheat genes, TaWaxy and TaMTL, were edited by the optimized SpCas9 system with high efficiency; the highest efficiency of 80.5% was achieved when using TaU3 and two sgRNAs to target TaWaxy. Seed-set rates of the TaMTL-edited T0 transgenic plants were much lower than that of the wild-type. A haploid induction rate of 18.9% was determined in the TaMTL-edited T1 plants using CRISPR/SpCas9 system. The mutants with reverse insertion of the deleted sequence of the TaMTL and TaWaxy between the two sgRNAs were identified in the edited T1 plants. Additionally, the wheat grains lacking embryo or endosperm	wheat
871	plant	wheat	CRISPR;Cas9;		Nature biotechnology	One-step genome editing of elite crop germplasm during haploid induction.	2019	37(3):287-292	[Kelliher T et al.]	Syngenta Crop Protection, Research Triangle Park, NC, USA.	30833776	10.1038/s41587-019-0038-x	Genome editing using CRISPR-Cas9 works efficiently in plant cells(1), but delivery of genome-editing machinery into the vast majority of crop varieties is not possible using established methods(2). We co-opted the aberrant reproductive process of haploid induction (HI)(3-6) to induce edits in nascent seeds of diverse monocot and dicot species. Our method, named HI-Edit, enables direct genomic modification of commercial crop varieties. HI-Edit was tested in field and sweet corn using a native haploid-inducer line(4) and extended to dicots using an engineered CENH3 HI system(7). We also recovered edited wheat embryos using Cas9 delivered by maize pollen. Our data indicate that a transient hybrid state precedes uniparental chromosome elimination in maize HI. Edited haploid plants lack both the haploid-inducer parental DNA and the editing machinery. Therefore, edited plants could be used in trait testing and directly integrated into commercial variety development.	wheat

872	plant	wheat	ZFN;	IPK1	Plant biotechnology journal	Genome editing in wheat microspores and haploid embryos mediated by delivery of ZFN proteins and cell-penetrating peptide complexes.	2019		[Bilichak A et al.]	Lethbridge Research and Development Center, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada.	31729822	10.1111/pbi.13296	Recent advances in genome engineering technologies based on designed endonucleases (DE) allow specific and predictable alterations in plant genomes to generate value-added traits in crops of choice. The EXZACT Precision technology, based on zinc finger nucleases (ZFN), has been successfully used in the past for introduction of precise mutations and transgenes to generate novel and desired phenotypes in several crop species. Current methods for delivering ZFNs into plant cells are based on traditional genetic transformation methods that result in stable integration of the nuclease in the genome. Here, we describe for the first time, an alternative ZFN delivery method where plant cells are transfected with ZFN protein that eliminates the need for stable nuclease genomic integration and allows generation of edited, but not transgenic cells or tissues. For this study, we designed ZFNs targeting the wheat IPK1 locus, purified active ZFN protein from bacterial cultures, complexed with cell-penetrating peptides (CPP) and directly transfected the complex into either wheat microspores or embryos. NGS analysis of ZFN-treated material showed targeted edits at the IPK1 locus in independent experiments. This is the first description of plant microspore genome editing by a ZFN when delivered as a protein	wheat
873	plant	wheat	CRISPR;Cas9;	wheat male fertility gene Ms1	Plant biotechnology journal	CRISPR/Cas9-mediated knockout of Ms1 enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production.	2019	17(10):1905-1913	[Okada A et al.]	University of Adelaide, Urrbrae, South Australia, Australia.	30839150	10.1111/pbi.13106	The development and adoption of hybrid seed technology have led to dramatic increases in agricultural productivity. However, it has been a challenge to develop a commercially viable platform for the production of hybrid wheat (Triticum aestivum) seed due to wheat's strong inbreeding habit. Recently, a novel platform for commercial hybrid seed production was described. This hybridization platform utilizes nuclear male sterility to force outcrossing and has been applied to maize and rice. With the recent molecular identification of the wheat male fertility gene Ms1, it is now possible to extend the use of this novel hybridization platform to wheat. In this report, we used the CRISPR/Cas9 system to generate heritable, targeted mutations in Ms1. The introduction of biallelic frameshift mutations into Ms1 resulted in complete male sterility in wheat cultivars Fielder and Gladius, and several of the selected male-sterile lines were potentially non-transgenic. Our study demonstrates the utility of the CRISPR/Cas9 system for the rapid generation of male sterility in commercial wheat cultivars. This represents an important step towards capturing heterosis to improve	wheat
874	plant	wheat	agroinfiltration; CRISPR;Cas9;	TaCKX2-1; TaGLW7; TaGW2; TaGW8	Plant biotechnology journal	Development of an Agrobacterium-delivered CRISPR/Cas9 system for wheat genome editing.	2019	17(8):1623-1635	[Zhang Z et al.]	University of Missouri, Columbia, MO, USA.	30706614	10.1111/pbi.13088	CRISPR/Cas9 has been widely used for genome editing in many organisms, including important crops like wheat. Despite the tractability in designing CRISPR/Cas9, efficacy in the application of this powerful genome editing tool also depends on DNA delivery methods. In wheat, the biolistics based transformation is the most used method for delivery of the CRISPR/Cas9 complex. Due to the high frequency of gene silencing associated with co-transferred plasmid backbone and low edit rate in wheat, a large T0 transgenic plant population are required for recovery of desired mutations, which poses a bottleneck for many genome editing projects. Here, we report an Agrobacterium-delivered CRISPR/Cas9 system in wheat, which includes a wheat codon optimized Cas9 driven by a maize ubiquitin gene promoter and a guide RNA cassette driven by wheat U6 promoters in a single binary vector. Using this CRISPR/Cas9 system, we have developed 68 edit mutants for four grain-regulatory genes, TaCKX2-1, TaGLW7, TaGW2, and TaGW8, in T0 , T1 , and T2 generation plants at an average edit rate of 10% without detecting off-target mutations in the most Cas9-active plants. Homozygous mutations can be recovered from a large population in a single generation. Different from most plant species, deletions over 10 bp are the dominant mutation types in wheat. Plants homozygous of 1160-bp deletion in TaCKX2-D1 significantly increased grain number per spikelet. In conclusion, our Agrobacterium-delivered CRISPR/Cas9 system provides an alternative option for wheat genome editing, which requires a small number of transformation events because CRISPR/Cas9 remains active for novel	wheat
875	plant	wheat	CRISPR;Cas9;	TONNEAU1-recruiting motif (TRM) protein gene homolog (TaGW7)	Plant journal	Gene editing of the wheat homologs of TONNEAU1-recruiting motif encoding gene affects grain shape and weight in wheat.	2019	100(2):251-264	[Wang W et al.]	Kansas State University, Manhattan, KS, USA.	31219637	10.1111/tpj.14440	Grain size and weight are important components of a suite of yield-related traits in crops. Here, we showed that the CRISPR-Cas9 gene editing of TaGW7, a homolog of rice OsGW7 encoding a TONNEAU1-recruiting motif (TRM) protein, affects grain shape and weight in allohexaploid wheat. By editing the TaGW7 homoeologs in the B and D genomes, we showed that mutations in either of the two or both genomes increased the grain width and weight but reduced the grain length. The effect sizes of mutations in the TaGW7 gene homoeologs coincided with the relative levels of their expression in the B and D genomes. The effects of gene editing on grain morphology and weight traits were dosage dependent with the double-copy mutant showing larger effect than the respective single copy mutants. The TaGW7-centered gene co-expression network indicated that this gene is involved in the pathways regulating cell division and organ growth, also confirmed by the cellular co-localization of TaGW7 with alpha- and beta-tubulin proteins, the building blocks of microtubule arrays. The analyses of exome capture data in tetraploid domesticated and wild emmer, and hexaploid wheat revealed the loss of diversity around TaGW7-associated with domestication selection, suggesting that TaGW7 is likely to play an important role in the evolution of yield component traits in wheat. Our study showed how integrating CRISPR-Cas9 system with cross-species comparison can help to uncover the function of a gene fixed in wheat for allelic variants targeted by domestication selection and select targets for	wheat

876	plant	wheat	CRISPR;Cas9;	TaABCC6; TaNFXL1; TansLTP9.4	Plant methods	An optimised CRISPR/Cas9 protocol to create targeted mutations in homoeologous genes and an efficient genotyping protocol to identify edited events in wheat.	2019	15:119	[Cui X et al.]	Ottawa Research and Development Centre, Ottawa, ON, Canada.	31673276	10.1186/s13007-019-0500-2	Background: Targeted genome editing using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system has been applied in a large number of plant species. Using a gene-specific single guide RNA (sgRNA) and the CRISPR/Cas9 system, small editing events such as deletions of few bases can be obtained. However larger deletions are required for some applications. In addition, identification and characterization of edited events can be challenging in plants with complex genomes, such as wheat. Results: In this study, we used the CRISPR/Cas9 system and developed a protocol that yielded high number of large deletions employing a pair of co-expressed sgRNA to target the same gene. The protocol was validated by targeting three genes, TaABCC6, TaNFXL1 and TansLTP9.4 in a wheat protoplast assay. Deletions of sequences located between the two sgRNA in each gene were the most frequent editing events observed for two of the three genes. A comparative assessment of editing frequencies between a codon-optimized Cas9 for expression in algae, crCas9, and a plant codon-optimized Cas9, pcoCas9, showed more consistent results with the vector expressing pcoCas9. Editing of TaNFXL1 by co-expression of sgRNA pair was investigated in transgenic wheat plants. Given the ploidy of bread wheat, a rapid, robust and inexpensive genotyping protocol was also adapted for hexaploid genomes and shown to be a useful tool to identify homoeolog-specific editing events in wheat. Conclusions: Co-expressed pairs of sgRNA targeting single genes in conjunction with the CRISPR/Cas9 system produced large deletions in wheat. In addition, a genotyping protocol to identify editing events in homoeologs of TaNFXL1	wheat
877	plant	wheat; maize	CRISPR;Cas9;	TaGASR7; ZmTMS5	Molecular plant pathology	A barley stripe mosaic virus-based guide RNA delivery system for targeted mutagenesis in wheat and maize.	2019	20(10):1463-1474	[Hu J et al.]	China Agricultural University, Beijing, China.	31273916	10.1111/mpp.12849	Plant RNA virus-based guide RNA (gRNA) delivery has substantial advantages compared to that of the conventional constitutive promoter-driven expression due to the rapid and robust amplification of gRNAs during virus replication and movement. To date, virus-induced genome editing tools have not been developed for wheat and maize. In this study, we engineered a barley stripe mosaic virus (BSMV)-based gRNA delivery system for clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9-mediated targeted mutagenesis in wheat and maize. BSMV-based delivery of single gRNAs for targeted mutagenesis was first validated in Nicotiana benthamiana. To extend this work, we transformed wheat and maize with the Cas9 nuclease gene and selected the wheat TaGASR7 and maize ZmTMS5 genes as targets to assess the feasibility and efficiency of BSMV-mediated mutagenesis. Positive targeted mutagenesis of the TaGASR7 and ZmTMS5 genes was achieved for wheat and maize with efficiencies of up to 78% and 48%. Our results provide a useful tool for fast and efficient delivery of gRNAs into economically important crops.	wheat; maize
878	plant		Agroinfiltration;		Autophagy	Actin filaments are dispensable for bulk autophagy in plants.	2019	15(12):2126-2141	[Zheng X et al.]	Tsinghua University, Beijing, China.	30907219	10.1080/15548627.2019.1596496	Actin filament, also known as microfilament, is one of two major cytoskeletal elements in plants and plays important roles in various biological processes. Like in animal cells, actin filaments have been thought to participate in autophagy in plants. However, surprisingly, in this study we found that actin filaments are dispensable for the occurrence of autophagy in plants. Disruption of actin filaments by short term treatment with actin polymerization inhibitors, cytochalasin D and latrunculin B, or transient overexpression of Profilin 3 in Nicotiana benthamiana had no effect on basal autophagy as well as the upregulation of nocturnal autophagy and salt stress-induced autophagy. Furthermore, anti-microfilament drug treatment affected neither basal nor salt stress-induced autophagy in Arabidopsis. In addition, prolonged perturbation of actin filaments by silencing Actin7 or 24-h treatment with microfilament-disrupting agents in N. benthamiana caused endoplasmic reticulum (ER) disorganization and subsequent degradation via autophagy involving ATG2, 3, 5, 6 and 7. Our findings reveal that, unlike mammalian cells, actin filaments are unnecessary for bulk autophagy in plants.Abbreviations: ATG: autophagy-related; CD: cytochalasin D; Cvt pathway: cytoplasm to vacuole targeting pathway; DMSO: dimethyl sulfoxide; ER: endoplasmic reticulum; LatB: latrunculin B; Nb: Nicotiana benthamiana; PAS: phagophore assembly site; PRF3: Profilin 3; RER: rough ER; SER: smooth ER; TEM: transmission electron microscopy; TRV: Tobacco rattle virus; VIGS: virus-induced gene silencing; wpi: weeks	
879	plant		CRISPR;Cas9;		Journal of experimental botany	Decrosslinking enabled visualization of RGEN-ISL signals for DNA sequences in plant tissues.	2019		[Nagaki K et al.]	Okayama University, Kurashiki, Japan.	31784756	10.1093/jxb/erz534	Information about the positioning of individual loci in the nucleus and the status of epigenetic modification at such loci in each cell contained in plant tissue expand our understanding of how cells in tissue coordinate gene expression. To obtain such information, a less damaging DNA visualization method in tissue that can be used with immunohistochemistry is required. Recently, a less damaging DNA visualization method using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/associated caspase 9) system, named RNA-guided endonuclease – in situ labeling (RGEN-ISL), was reported. This system made it possible to visualize the target DNA locus in the nucleus fixed on the slide glass with a set of simple operations, but this system could not apply to cells in plant tissues. In this report, we have developed a modified RGEN-ISL method with decrosslinking that made it possible to simultaneously detect target the DNA loci and immunohistochemistry signals, including histone modification, in various types of plant tissues and species.	
880	plant		CRISPR;Cas9;		New phytologist	TIR-NB-LRR immune receptor SOC3 pairs with truncated TIR-NB protein CHS1 or TN2 to monitor the homeostasis of E3 ligase SAUL1.	2019	221(4):2054-2066	[Liang W et al.]	University of British Columbia, Vancouver, BC, Canada.	30317650	10.1111/nph.15534	Intracellular nucleotide binding (NB) and leucine-rich repeat (NLR) proteins function as immune receptors to recognize effectors from pathogens. They often guard host proteins that are the direct targets of those effectors. Recent findings have revealed that a typical NLR sometimes cooperates with another atypical NLR for effector recognition. Here, by using the CRISPR/Cas9 gene editing method, knockout analysis and biochemical assays, we uncovered differential pairings of typical Toll Interleukin1 receptor (TIR) type NLR (TNL) receptor SOC3 with atypical truncated TIR-NB (TN) proteins CHS1 or TN2 to guard the homeostasis of the E3 ligase SAUL1. Overaccumulation of SAUL1 is monitored by the SOC3-TN2 pair, while SAUL1's disappearance is guarded by the SOC3-CHS1 pair. SOC3 forms a head-to-head genomic arrangement with CHS1 and TN2, indicative of transcriptional co-regulation. Such intricate cooperative interactions can probably enlarge the recognition spectrum and increase the functional flexibility of NLRs, which can partly explain the	

881	plant		Agroinfiltration;	CVYV P1b	Plant, cell & environment	Sterol isomerase HYDRA1 interacts with RNA silencing suppressor P1b and restricts potyviral infection.	2019	42(11):3015-3026	[Ochoa J et al.]	Campus Universidad Autonoma de Madrid, Madrid, Spain.	31286514	10.1111/pce.13610	Plants use RNA silencing as a strong defensive barrier against virus challenges, and viruses counteract this defence by using RNA silencing suppressors (RSSs). With the objective of identifying host factors helping either the plant or the virus in this interaction, we have performed a yeast two-hybrid screen using P1b, the RSS protein of the ipomovirus Cucumber vein yellowing virus (CVYV, family Potyviridae), as a bait. The C-8 sterol isomerase HYDRA1 (HYD1), an enzyme involved in isoprenoid biosynthesis and cell membrane biology, and required for RNA silencing, was isolated in this screen. The interaction between CVYV P1b and HYD1 was confirmed in planta by Bimolecular Fluorescence Complementation assays. We demonstrated that HYD1 negatively impacts the accumulation of CVYV P1b in an agroinfiltration assay. Moreover, expression of HYD1 inhibited the infection of the potyvirus Plum pox virus, especially when antiviral RNA silencing was boosted by high temperature or by coexpression of homologous sequences. Our results reinforce previous evidence highlighting the relevance of particular composition and structure of cellular membranes for RNA silencing and viral infection. We report a new interaction of an RSS protein from the Potyviridae family with a member of the isoprenoid biosynthetic	
882	plant		Agroinfiltration;	multifunctional viral translation transactivator/viroplasmin (TAV) protein from Cauliflower mosaic virus	Scientific reports	Cauliflower mosaic virus transactivator protein (TAV) can suppress nonsense-mediated decay by targeting VARICOSE, a scaffold protein of the decapping complex.	2019	9(1):7042	[Lukhovitskaya N et al.]	CNRS, Universite de Strasbourg, Strasbourg, France.	31065034	10.1038/s41598-019-43414-0	During pathogenesis, viruses hijack the host cellular machinery to access molecules and sub-cellular structures needed for infection. We have evidence that the multifunctional viral translation transactivator/viroplasmin (TAV) protein from Cauliflower mosaic virus (CaMV) can function as a suppressor of nonsense-mediated mRNA decay (NMD). TAV interacts specifically with a scaffold protein of the decapping complex VARICOSE (VCS) in the yeast two-hybrid system, and co-localizes with components of the decapping complex in planta. Notably, plants transgenic for TAV accumulate endogenous NMD-elicited mRNAs, while decay of AU-rich instability element (ARE)-signal containing mRNAs are not affected. Using an agroinfiltration-based transient assay we confirmed that TAV specifically stabilizes mRNA containing a premature termination codon (PTC) in a VCS-dependent manner. We have identified a TAV motif consisting of 12 of the 520 amino acids in the full-length sequence that is critical for both VCS binding and the NMD suppression effect. Our data suggest that TAV can intercept NMD by targeting the decapping machinery through the scaffold protein VARICOSE, indicating that 5'-3' mRNA decapping is a late step in NMD-related	
883	plant		CRISPR;Cas9;agroinfiltration		Transgenic research	DNA-free genome editing with preassembled CRISPR/Cas9 ribonucleoproteins in plants.	2019	28(Suppl 2):61-64	[Park J et al.]	Naturegenic Inc., West Lafayette, IN, USA.	31321685	10.1007/s11248-019-00136-3	Processes of traditional trait development in plants depend on genetic variations derived from spontaneous mutation or artificial random mutagenesis. Limited availability of desired traits in crossable relatives or failure to generate the wanted phenotypes by random mutagenesis led to develop innovative breeding methods that are truly cross-species and precise. To this end, we devised novel methods of precise genome engineering that are characterized to use pre-assembled CRISPR/Cas9 ribonucleoprotein (RNP) complex instead of using nucleic ands or Agrobacterium. We found that our methods successfully engineered plant genomes without leaving any foreign DNA footprint in the genomes. To facilitate introduction of RNP into plant nucleus, we first obtained protoplasts after removing the transfection barrier, cell wall. Whole plants were regenerated from the single cell of protoplasts that has been engineered with the RNP. Pending the improved way of protoplast regeneration technology especially in crop plants, our methods should help develop novel traits in	

研究成果の刊行に関する一覧表
（令和元年度）

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木下政人	養殖業へのゲノム編集技術活用のために	月刊養殖ビジネス	緑書房	東京都	2020	１月号, 61-64
木下政人	ゲノム編集技術を使った肉厚マダイの作出と品種改良期間の短縮	JATAFF ジャーナル	農林水産・食品産業技術振興協会	東京都	2020	8巻2号, 8-12

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所属研究機関長　職　名　所　長

氏　名　奥田　晴宏[redacted]

次の職員の令和元年度厚生労働行政推進調査事業費補助金の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　食品の安全確保推進研究事業

２．研究課題名　新たなバイオテクノロジーを用いて得られた食品の安全性確保とリスクコミュニケーションのための研究

３．研究者名　（所属部局・職名）　生化学部　部長

（氏名・フリガナ）　近藤　一成　〈コンドウ　カズナリ〉

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入（※1）		
	有	無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	☑	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□	☑	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。

（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年　４月　８日

厚生労働大臣
~~(国立医薬品食品衛生研究所長)~~　殿
~~(国立保健医療科学院長)~~

機関名　大阪府立大学[redacted]

所属研究機関長　職　名　学長

氏　名　辰巳砂　昌弘[redacted]

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　食品の安全確保推進研究事業

２．研究課題名　新たなバイオテクノロジーを用いて得られた食品の安全性確保とリスクコミュニケーションのための研究

３．研究者名　（所属部局・職名）　生命環境科学研究科・教授

（氏名・フリガナ）　小泉　望・コイズミ　ノゾム

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入（※1）		
	有　無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□　■	□		□
遺伝子治療等臨床研究に関する指針	□　■	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□　■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　■	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　）	□　■	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講　■　　未受講　□

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有　■　無　□（無の場合はその理由：　）
当研究機関におけるＣＯＩ委員会設置の有無	有　■　無　□（無の場合は委託先機関：　）
当研究に係るＣＯＩについての報告・審査の有無	有　■　無　□（無の場合はその理由：　）
当研究に係るＣＯＩについての指導・管理の有無	有　□　無　■（有の場合はその内容：　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年４月９日

厚生労働大臣
~~(国立医薬品食品衛生研究所長)~~ 殿
~~(国立保健医療科学院長)~~

機関名　京都大学　大学院　農学研究科

所属研究機関長　職　名　研究科長

氏　名　村上　章　[redacted]

次の職員の平成３１年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反
いては以下のとおりです。

１．研究事業名　食品の安全確保推進研究事業

２．研究課題名　新たなバイオテクノロジーを用いて得られた食品の安全性確保と
リスクコミュニケーションのための研究

３．研究者名　(所属部局・職名)　農学研究科・助教

(氏名・フリガナ)　木下　政人　(キノシタ　マサト)

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
人を対象とする医学系研究に関する倫理指針 (※3)	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	☑	□		□
その他、該当する倫理指針があれば記入すること (指針の名称：　)	□	☑	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

(※2) 未審査に場合は、その理由を記載すること。
(※3) 廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由：　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関：　)
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □(無の場合はその理由：　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑ (有の場合はその内容：　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

２０２０年４月９日

厚生労働大臣　殿

機関名　名古屋工業大学

所属研究機関長　職　名　学長

氏　名　木下　隆利[redacted]

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益[redacted]いては以下のとおりです。

１．研究事業名　食品の安全確保推進研究事業

２．研究課題名　新たなバイオテクノロジーを用いて得られた食品の安全性確保とリスクコミュニケーションのための研究

３．研究者名　（所属部局・職名）工学研究科・教授

（氏名・フリガナ）竹内　一郎・タケウチ　イチロウ

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入（※1）		
	有　無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□　■	□		□
遺伝子治療等臨床研究に関する指針	□　■	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□　■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　■	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□　■	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ■　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ■　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ■　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ■　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ■（有の場合はその内容：　　）

令和２年　　４月　　２６　日

厚生労働大臣
(~~国立医薬品食品衛生研究所長~~)　殿
(~~国立保健医療科学院長~~)

機関名　学校法人沖縄科学技術大学院大学学園

所属研究機関長　職　名　理事長

氏　名　ピーター・グルース　[redacted]

次の職員の平成　　年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　食品の安全確保推進研究　事業

２．研究課題名　新たなバイオテクノロジーを用いて得られた食品の安全性確保とリスクコミュニケーションのための研究

３．研究者名　(所属部局・職名)　進化神経生物学ユニット　グループリーダー

(氏名・フリガナ)　早川英介　(ハヤカワ　エイスケ)

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	■	□		□
遺伝子治療等臨床研究に関する指針	□	■	□		□
人を対象とする医学系研究に関する倫理指針 (※3)	□	■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	■	□		□
その他、該当する倫理指針があれば記入すること (指針の名称：　　)	□	■	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

(※2) 未審査に場合は、その理由を記載すること。
(※3) 廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ■　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ■　無 □(無の場合はその理由：　　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ■　無 □(無の場合は委託先機関：　　)
当研究に係るＣＯＩについての報告・審査の有無	有 ■　無 □(無の場合はその理由：　　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ■(有の場合はその内容：　　)

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年３月２７日

厚生労働大臣　殿

機関名　国立医薬品[redacted]

所属研究機関長　職　名　所　長

氏　名　奥田　晴宏

次の職員の令和元年度厚生労働行政推進調査事業費補助金の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　食品の安全確保推進研究事業

２．研究課題名　新たなバイオテクノロジーを用いて得られた食品の安全性確保とリスクコミュニケーションのための研究

３．研究者名　（所属部局・職名）　食品部　第５室長

（氏名・フリガナ）　中村　公亮　〈ナカムラ　コウスケ〉

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入（※1）		
	有	無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	☑	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□	☑	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年３月２７日

厚生労働大臣　殿

機関名　国立医薬品[redacted]

所属研究機関長　職　名　所　長

氏　名　奥田　晴宏[redacted]

次の職員の令和元年度厚生労働行政推進調査事業費補助金の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　食品の安全確保推進研究事業

２．研究課題名　新たなバイオテクノロジーを用いて得られた食品の安全性確保とリスクコミュニケーションのための研究

３．研究者名　（所属部局・職名）　生化学部　主任研究官

（氏名・フリガナ）　為広　紀正　〈タメヒロ　ノリマサ〉

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入（※1）		
	有	無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	☑	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□	☑	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。