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遺伝子組換え食品

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Genetically Modified Foods

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1. はじめに

遺伝子組換え食品は，遺伝子組換え技術を応用した(Genetically modified (GM)) 食品のことである．遺伝子組換え食品の開発は食糧の増産などを目的として，世界の多くの研究者が取り組んでいる．米国において平成6年5月，日持ちのよいトマトがFDAの承認を得て以来，いろいろな組換え食品の開発が進んできた．この間，種々の問題点も指摘されている．しかし，これまで開発され市販されている遺伝子組換え植物由来の食品については，わが国ではその安全性評価システム，すなわち安全性審査の義務化により，遺伝子レベル，発現タンパク質レベルまた形質変換した食品レベルで十分な審議がなされ，現在の科学的水準でその安全性は確保されていると考えて差し支えないと思われる．そのことは，これまで指摘された事項，例えば大きな成分変化やアレルギー性などがいずれも科学的検証により確認できないことからも支持されている．遺伝子組換え食品の安全性評価に関しては，国際的なハーモナイゼーションに向けた動きがコーデックス(Codex)委員会(FAO（国際連合食糧農業機関)/WHO（世界保健機関）合同食品規格会議）でなされており，ますますグローバルな安全性確認が進んでいくものと思われる．本稿では，遺伝子組換え食品の概要，安全性評価，表示制度について述べ，今後の展望についても概説してみたい．

2. 遺伝子組換え食品とは

遺伝子を操作する技術は植物の分野では古くから，紫外線照射，放射線照射や化学薬品の使用によるDNAの改変に基づく品種改良に利用されていた．そして，改良された品種を長い間の世代育種や交配育種により選抜し，良い性質を持つ新規な植物が開発され，食用にも供されてきた．しかしより短期間に品種改良が可能な手法として，新たに組換えDNA技術が登場した．農作物の分野で用いられている組換えDNA技術とは，食品としてすでに用いられている植物などの性質や機能をうまく利用するために，他の生物から有用な性質を付与する遺伝子を取り出し，その植物などに組み込む技術である．植物への遺伝子導入の方法は，アグロバクテリウム法，パーティクル・ガン法などが主に用いられている．

3. 組換え農作物の商品化の現状

わが国では，平成8年に，7種類の遺伝子組換え食品の安全性確認がなされて以来，多くの遺伝子組換え食品の安全性確認が行われてきた．平成20年2月までに，わが国において，安全性審査の手続きを経た遺伝子組換え食品は88種類に及ぶが（表1），これらはすべて国外において生産されたものであり，国内において生産されたものはない．

表1の中には実際に栽培されていないもの（ジャガイモ，一部のトウモロコシ）もあるが，この増加は国際的なGM作物の作付けの広がりと一致している．国際アグリバイオ事業団(ISAAA)の調査によると[1]，2007年度のGM作物の全世界における作付け面積は約1億1,430万ヘクタール，これらの栽培を行っている農家は23か国（先進国11，開発途上国12）で1,200万人と推測されている．この耕地面積の伸び率はここ12年間毎年2桁台を示しており，2007年度は，全世界の耕地面積の約7%に達していることになる．作付けが一番進んでいる米国では約5,770万ヘクタールでGM作物の栽培が行われている．

4. 組換え食品の安全性評価

多くの国では遺伝子組換え食品の使用に関して，決められたもののみを販売できる事前認可性を採用していることから，わが国でもこの事前認可制をとり，安全性審査の済んだポジティブリストの遺伝子組換え食品のみが販売できる制度を採用している．

図1に，わが国における遺伝子組換え作物の認可作成にかかわる行政の役割を示した．遺伝子組換え作物に関しては，まず，実験室から隔離ほ場，一般ほ場へと段階的に試験を進めていくが，遺伝子組換え作物が繁殖して，自然生態系に影響を与えてしまうことのないように，国がカルタヘナ法に基づき，生物多様性への影響が生じる恐れがないことを確認したものを承認する仕組み（平成16年2月施行）となっている．施設（実験室）内での使用は，第2種使用で，使用する者（開発者，輸入者）は，主務大臣の確認を受けた拡散防止措置をとる義務があり，次いで，環

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表 1. 安全性審査済みの遺伝子組換え食品および食品添加物（平成 20 年 2 月現在）

種類			性質
食品	ダイズ	5	除草剤（グリホサート）耐性，高オレイン酸形質
[88]	トウモロコシ	36	害虫（アワノメイガなど）抵抗性，除草剤（グルホシネート）耐性，除草剤（グリホサート）耐性，高リシン形質
	（うちスタック品種 18）		
	ジャガイモ	8	害虫（コロラドハムシ）抵抗性，ウイルス（ジャガイモ葉巻ウイルス）抵抗性
	テンサイ	3	除草剤（グルホシネート）耐性，除草剤（グリホサート）耐性
	ナタネ	15	除草剤（グルホシネート）耐性，雄性不稔性，稔性回復性，除草剤（オキシニル）耐性，除草剤（グリホサート）耐性
	ワタ	18	除草剤（グリホサート）耐性，除草剤（ブロモキシニル）耐性，害虫（オオタバコガ）抵抗性
	（うちスタック品種 7）		
	アルファルファ	3	除草剤（グリホサート）耐性
食品添加物	キモシン	2	天然添加物の代替
[14]	α-アミラーゼ	6	生産性向上
	プルナラーゼ	2	生産性向上
	リパーゼ	2	生産性向上
	リボフラビン	1	生産性向上
	グルコアミラーゼ	1	生産性向上

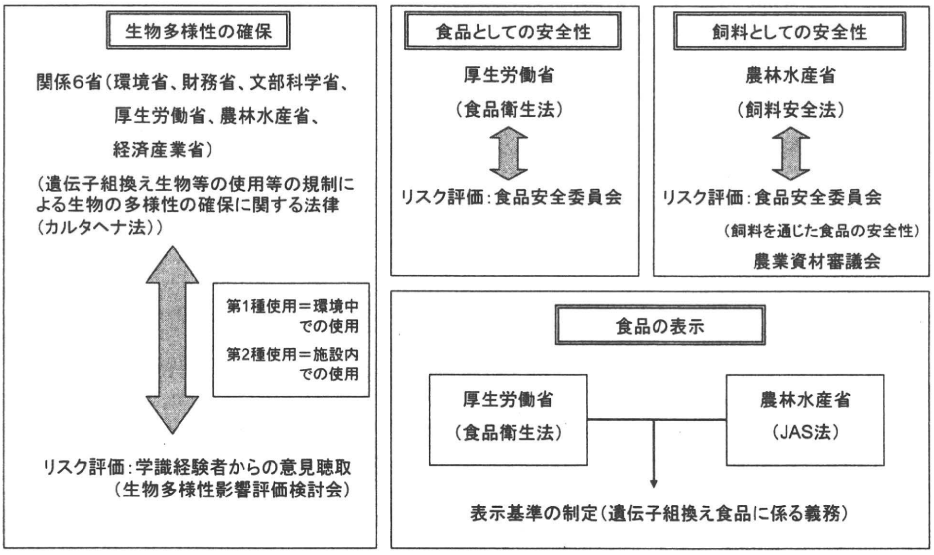

図 1. 組換え作物の開発と商品化における行政の役割

境中（ほ場）での使用は，第 1 種使用で，使用する者が，事前に使用規程を定め，生物多様性影響評価書を添付し，主務大臣の承認を受ける義務がある．遺伝子組換え生物の野生生物との競合における優位性，有害物質産生性，交雑性による生物多様性影響が生じる恐れがないことが確認され，従来の食用品種と遺伝子導入作物とがかけ離れた性質を示さないように，優秀な品種の選択が実施されている．

次いで，食品としての安全性は，厚生労働省の食品衛生法に基づいて，リスク評価機関である食品安全委員会で科学的評価が行われ，飼料としての安全性は，農林水産省の飼料安全法に基づいて，リスク評価機関として農業資材審議会または飼料を通じた食品の安全性の場合は食品安全委員会で，科学的評価が行われ，問題のないもののみが栽培，流通されるしくみとなっている．以下，食品としての安全性評価について詳しく述べていきたい．

わが国では，安全性未確認のものが国内で流通しないよう安全性審査を行う制度を法的に整備しておく必要から，食品衛生法の規定に基づく食品，食品添加物の規格基準の改正が行われ，平成 13 年 4 月より，「組換え DNA 技術応用食品及び添加物の安全性基準」(http://www.mhlw.go.jp/topics/idenshi/anzen/tuuchi2.html) が設定され，遺伝子組換え食品などの安全性審査が法的に義務づけられることとなった．一方，国際的にも，コーデックス委員会においても，平成 15 年 7 月に遺伝子組換え食品の安全性評価のガイドライン (ftp://ftp.fao.org/codex/alinorm03/al03_34e.pdf) などが作成されるに至った．平成 15 年 7 月，食品安全委員会の新設とともに，遺伝子組換え食品および食品添加物の安全性評価が，厚生労働省の意見の求めに応じて，食品安全委員会においてなされることになった（図 2）．食品安全委員会において，遺伝子組換え食品（種子植物）の安全性を評価するための基準が平成 16 年 1 月，遺伝子組換え微生物を利用して製造された添加物の安全性評価基準が平成 16 年 3 月に作成された (http://www.fsc.go.jp/senmon/idensi/index.html)．基準作成

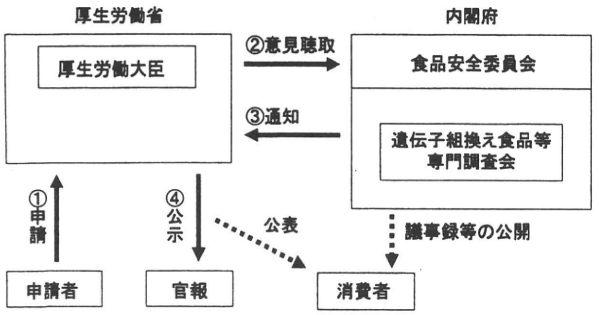

図 2.　遺伝子組換え作物の食品としての安全性評価（行政の枠組み）

に至る詳しい経緯については，他の総説を参考にされたい[2]．

1)　遺伝子組換え食品の安全性審査基準の概要

一般食品に含まれる特定の化学物質，すなわち食品添加物，残留農薬，残留動物用医薬品，環境汚染物質，自然毒（カビ毒）などについての安全性は，世界的にリスクアナリシスのアプローチで評価されている．これら化学物質は単一の化合物や単一化合物由来成分であり，評価の対象がはっきりしているためこの手法が有効である．しかし，この方法は，遺伝子組換え食品のように，丸ごとの食品に適用するものではなく，安全性評価の用語を使用するほうが適切である．食品そのものの安全性を検討する場合は，意図的，非意図的な影響の両方を考慮して，今まで安全に食品として使用されてきた既存の対応物と関連づけて組換えDNA植物を含む新しい植物品種由来の食品の安全性を評価するという原則に基づいて行われる．この考え方は，OECD（経済協力開発機構）の報告[3]に基づいて実質的同等性という言葉で用いられてきたが，平成15年のコーデックス委員会のガイドラインでは，「既存の対応物 (conventional counterpart, comparator) との比較に基づいてある食品の全体またはその食品成分を評価するものである」と再定義されている．　食品安全委員会での安全性評価においても，まず，比較対象としての既存の宿主など（組換えする前の植物や食品）があり，かつ，この宿主などと組換え体の相違点が明確であることが求められている（表2）．

安全性評価には，利用目的および利用方法，宿主，ベクター，挿入DNA，遺伝子産物，発現ベクターの構築，組換え体に関して詳細な情報が必要とされる．種子植物の安全性評価の主な項目は，表2に示すとおりであるが，具体的には，宿主（もとの植物）に関する食経験の有無，可食部位，有害成分などの情報が必要とされ，宿主の安全な食経験があって，宿主自体の詳細な安全性評価を行う必要がないことが確認される．次いで，導入遺伝子に関する情報，すなわち，供与体（遺伝子の由来する生物）の情報，プロモーターやターミネーターの性質，導入遺伝子の配列，発現ベクターの構築法，導入方法，遺伝子産物の機能や性質などから，導入遺伝子とその産物がヒトの健康に有害でないことが確認される．さらに，遺伝子導入により作り出された遺伝子組換え生物において，意図したとおりの導入がなされており，非意図的な悪影響がないことを確認する必要がある．わが国においては，法律に基づく安全性の確認が実施された平成13年より，挿入された遺伝子の近傍配列の情報が求められることとなった．この情報は，挿入された遺伝子が何コピーどの場所に挿入されたかを示すもので，非意図的な変化を予想するうえで非常に重要なポイントとなっている．すなわち，近傍配列情報とそれから推定されるオープンリーディングフレームの存在などから，遺伝子の挿入により，宿主の遺伝子が破壊されていないこと，および新たな転写・翻訳が起こり，新たなタンパク質が発現されることがないかどうかを推定することができる．この挿入遺伝子の近傍配列情報は，安全性が確認され認可された系統の系統特異的検知・モニタリングや後代交配種における導入遺伝子の安定性・変化などを知るうえでも大切な情報である．導入遺伝子産物の安全性に関しては，挿入遺伝子産物がヒトに対し有害性がないことを，既知の毒性物質と構造相同性がないこと，遺伝子産物が，人工消化液や加熱処理により速やかに分解されるかなどで確認を行う．遺伝子産物の安全性で問題となるアレルギー性評価については，他の総説にも詳しく記しているが[4),5)]，以下の (1)〜(4) の項目に従ってアレルギー性評価の総合的判断 (weight of evidence) を行っている．すなわち，挿入遺伝子の供与体のアレルギー誘発性（グルテン過敏性腸炎誘発性を含む．以下同じ．）に関する知見が明らかにされていること．(2) 遺伝子産物（タンパク質）についてそ

表 2.　遺伝子組換え食品（種子植物）の安全性の評価

（安全性評価の主な項目）
- 比較対象となる既存の作物（食品）があり，宿主と遺伝子組換え作物の相違点が明確であるか．
- 組換え作物の食品としての利用方法
- 宿主の食経験や有害物質生産能
- 導入される遺伝子およびその産物（タンパク質）の安全性
  導入遺伝子の性質が明らかであるか．遺伝子産物に毒性がないか．遺伝子導入方法が明らかであるか．抗生物質耐性遺伝子の場合，耐性菌を増やさないか．
- 組換え作物の安全性
  導入後の遺伝子に変化がないか．安定であるか．導入コピー数，挿入位置および周辺配列が明らかであるか．発現部位とその発現量：新たな発現可能なオープンリーディングフレームができていないか．（できている場合，その産物の毒性およびアレルゲン性は．）宿主の代謝系に大きな変化をもたらさないか．アレルギー誘発性がないか．栄養成分，有害成分，栄養阻害物質（アルカロイド，フィチン酸，トリプシンインヒビター，エルカ酸など）がもとの作物と比べて大きく変化していないか．

表 3. 遺伝子組換え食品に導入されている主なタンパク質

導入タンパク質	供与体生物	遺伝子組換え作物	商品名の例
Cry1Ab（ちょう目害虫抵抗性）	*B.t.k.* HD-1*	トウモロコシ	Mon810, Bt11, Event 176 など
Cry1Ac（ちょう目害虫抵抗性）	*B.t.k.* HD-73	トウモロコシ，ワタ	DBT418（トウモロコシ），インガード（ワタ）など
Cry1F（ちょう目害虫抵抗性）	*B.t.aizawai*	トウモロコシ，ワタ	TC1507（トウモロコシ），281（ワタ）
Cry2A（ちょう目害虫抵抗性）	*B.t.k.* NRD-12	ワタ	15985
Cry3A（甲虫目害虫抵抗性）	*B.t. tenebrionis*	トウモロコシ，ジャガイモ	MIR604（トウモロコシ），ニューリーフジャガイモなど
Cry3Bb（甲虫目害虫抵抗性）	*B.t. kumamotoensis*	トウモロコシ	Mon863, Mon88017
Cry34Ab/Cry35Ab（甲虫目害虫抵抗性）	*B.t.* PS149B1	トウモロコシ	DAS59122-7
CP4-EPSPS（グリホサート耐性）	*Agrobacterium* sp. strain CP4	ダイズ，テンサイ，トウモロコシ，ナタネ，ワタ，アルファルファ	ラウンドアップレディー（ダイズ，テンサイ，ワタ，トウモロコシ，ナタネ，アルファルファ）
PAT (pat)（グルホシネート代謝）	*Streptomyces viridochromogenes*	ダイズ，テンサイ，トウモロコシ，ナタネ，ワタ	T-25（トウモロコシ），HCN92（ナタネ）T120-7（テンサイ）など
PAT (bar)（グルホシネート代謝）	*Streptomyces hygroscopicus*	トウモロコシ，ナタネ，ワタ	LLcotton25（ワタ）など
Nitrilase（ブロモキシニル代謝）	*Klebsiella pneumoniae* subsp. *ozaenae*	ナタネ，ワタ	BXN 10211（ワタ）など
DHDPS**（リシン合成酵素）	*Corynebacterium glutamicum*	トウモロコシ	LY038

*: *Bacillus thuringiensis kurstaki* HD-1, **: Dihydrodipicolinic acid synthase

のアレルギー誘発性に関する知見が明らかにされていること．(3) 遺伝子産物（タンパク質）の物理化学的処理に対する感受性に関する事項；人工胃液，人工腸液による処理および加熱処理に対する感受性をタンパク質電気泳動法及びウェスタンブロット法により調べる．(4) 遺伝子産物（タンパク質）と既知のアレルゲンとの構造相同性に関する事項；遺伝子産物（タンパク質）について，既知のアレルゲンなどと一次構造を比較し，既知のアレルゲンなどと構造相同性を有しないこと，であり，(1) から (4) までの事項などにより，ヒトの健康を損なう恐れがないと判断できないときは，(5) の遺伝子産物（タンパク質）の IgE 結合能を検討することになっている．

なお，これまでに安全性審査のなされた遺伝子組換え食品は，急性毒性に関する試験を実施しているものもあるが，亜急性毒性試験，慢性毒性試験，生殖影響試験，変異原性試験などに関する試験は実施する必要がないと個別に判断されている．その理由は，提出された資料により既知のアレルギー物質，有害物質などヒトの健康に影響を及ぼすような新たな物質が産生されていないことを確認しているためである．また，遺伝子組換えにより付加される物質が，ヒト体内や既存の食品中に元来存在するもの（内在性物質），速やかに分解，代謝され内在性物質に変化するものである場合などには，急性毒性試験などの結果からもとの物質の安全性について評価することができると推察される．

2) 遺伝子組換え作物の作出に用いられている主な遺伝子

わが国でこれまでに安全性審査の手続きを経た遺伝子組換え食品は 88 種類，食品添加物は 14 種類であるが，遺伝子組換え作物の作出に用いられた遺伝子は，これまでのところ比較的種類が限られている（表 3）．

除草剤耐性作物に用いられている除草剤としては今のところグルホシネート，グリホサートおよびブロモキシニルの 3 農薬のみである．主な遺伝子としては植物体内でグルホシネートをアセチル化して失活させるアセチルトランスフェラーゼをコードする遺伝子 *pat* (*bar*)，シキミ酸経路に属する芳香族アミノ酸生合成に関与し，グリホサートの標的酵素である EPSPS（5-エノールピルビルシキミ酸 3-リン酸 (EPSP) 合成酵素）機能を持つが，グリホサートとは結合しないためグリホサートの阻害を受けない CP4-EPSPS（アグロバクテリウム由来）をコードする *cp4epsps* 遺伝子，ブロモキシニルを分解するニトリラーゼをコードする *bxn* 遺伝子である（表 3）．

害虫抵抗性農作物に用いられている遺伝子は，微生物農薬として用いられている *Bacillus thuringiensis*（バチラス菌）の産生するトキシン遺伝子 (*cry1Ab*, *cry1Ac*, *cry1F*, *cry2A*, *cry3A*, *cry3Bb*, *cry34Ab/Cry35Ab*) が用いられている．Cry1 および Cry2 タンパク質は，鱗翅目（ちょう目）の昆虫（アワノメイガ，オオタバコガなどの昆虫）に選択毒性があり，Cry3 タンパク質は，甲虫目の害虫（コロラドハムシ，コーンルートワームなどの昆虫）に選択毒性がある．最近の動向としては，トウモロコシおよびワタにおいて，異なる系統由来の遺伝子組換え植物同士を交配で掛け合わせた品種（スタック）の開発が進んでいることで，平成 20 年 2 月現在で，トウモロコシのスタック品種は 18 種，ワタのスタック品種は，7 種に及んでいる（表 1）．

遺伝子組換え食品に導入されているタンパク質としては，ジャガイモで，ウイルス遺伝子の発現を抑制するためのウイルスのコートタンパク質 (PVPcp) の遺伝子も用いられている．一方，遺伝子導入された細胞の選択のために，選択マーカー遺伝子として，抗生物質耐性遺伝子 (*npt II*) や上記の除草剤耐性遺伝子（*pat*, *bar* など）も用いられている．現在までに用いられている遺伝子の種類はそれほど多くなく，同じ遺伝子がいろいろの農作物の形質転換に

表 4.　遺伝子組換え食品安全性評価の国際的動向

- OECD（経済協力開発機構）
  1993 年；実質的同等性の概念を報告書で提案―安全性評価の原理の確立，
  1995 年；臨時会合の開催，
  1998 年～常設の TF（新食品・飼料安全性タスクフォース）を設置
  （各作物種のコンセンサスドキュメント作成（2007 年までに 16 作物種））
- FAO/WHO（国連食糧農業機関/世界保健機構）
  1999～2009 年；Codex（FAO/WHO 合同食品規格委員会）バイオテクノロジー応用食品特別部会 (TFFBT) の設置―国際的な基準や指針あるいは勧告の策定
  第 I 期 (1999～2003)
  バイオテクノロジー応用食品のリスクアセスメントに関する原則
  組換え DNA 植物由来食品の安全性評価の実施に関するガイドライン
  組換え DNA 微生物を用いて製造された食品の安全性評価の実施に関するガイドライン（2003 年に制定）
  第 II 期 (2005～2009)
  組換え DNA 動物由来食品の安全性評価の実施に関するガイドライン
  栄養または健康に資する組換え DNA 植物由来食品の安全性評価
  微量に混入した組換え DNA 植物の安全性評価（2009 年に制定予定）

用いられているのが現状である．

3)　遺伝子組換え植物の掛け合わせについての安全性評価の考え方

本考え方は，遺伝子組換え食品（種子植物）の安全性評価基準と同時に食品安全委員会において作成されたものである（平成 16 年 1 月決定）．従来も，厚労省においては，遺伝子組換え食物と従来品種との掛け合わせに関しては，新たに獲得した形質に変化がないこと，亜種間での交配でないこと，摂取量・食用部位・加工法などの変更がない限り，安全性上の問題は生じないと考えられるため，安全性審査済みとみなされてきた．平成 16 年 1 月作成の安全性評価の考え方においては，さらに，遺伝子組換え植物と遺伝子組換え植物間の掛け合わせ（スタック品種）についても，亜種レベル以上の交配でなく，さらに，摂取量・食用部位・加工法などの変更がない場合，挿入遺伝子が宿主代謝系へ影響を及ぼさないと考えられる（害虫抵抗性，除草剤耐性，ウイルス抵抗性などの形質が付与される）場合に限っては，それらの交配種の安全性の確認が必要とされないこととなった．また，それ以外の場合については，当面は安全性確認をすることとされた．スタック品種は，前述の項目でも述べたが，年々増加傾向にある．

4)　遺伝子組換え飼料および飼料添加物の安全性評価の考え方

本考え方は，平成 16 年 5 月に決定されたものである．遺伝子組換え技術を利用して製造された飼料を摂取した家畜に由来する畜産物のヒトへの健康影響評価の考え方を示したもので，組換え飼料に含まれる有害成分の畜産物への移行などを評価するが，食品としての安全性評価が済んでいるものに関しては，基本的に新たな安全性上の問題は生じないものと考えられており，家畜に用いることを主な目的とする組換え体でも，食品としての安全性評価をまず受けることが望まれている．

3.　国際的ガイドラインについて

表 4 に，遺伝子組換え食品安全性評価の国際的動向について，概要を示した．OECD は，1993 年に遺伝子組換え食品の評価の基本となる実質的同等性の概念を示して以来，1998 年からは，常設の新食品・飼料安全性タスクフォースを設置し，各作物種のコンセンサスドキュメントを作成しており[6]，2007 年までに 16 作物種について，品種間のばらつきを考慮した主要栄養素，抗栄養素などの含量が示されており，インターネット上で公開されている．

コーデックス委員会のほうでは，遺伝子組換え食品のグローバル化すなわち世界的な流通拡大と安全性への関心が高まったことから，1999 年のコーデックス総会で，「バイオテクノロジー応用食品特別部会 (TFFBT)」を設置し，特に遺伝子組換え食品について国際的な基準や指針あるいは勧告を策定することを目的とされた．第一期として，1999 年から 2002 年の間に，日本を議長国とした特別部会が 4 回開催され，「バイオテクノロジー応用食品のリスクアセスメントに関する原則」「組換え DNA 植物由来食品の安全性評価の実施に関するガイドライン及びアレルギー誘発性評価に関する付属文書」「組換え DNA 微生物を用いて製造された食品の安全性評価の実施に関するガイドライン」が議論され，2003 年の総会で採択された．また，2005 年から 2009 年にかけて，日本を議長国とした第二期の特別部会が設置されており，2007 年までに 3 回の会議が開かれ，「組換え DNA 動物由来食品の安全性評価の実施に関するガイドライン」「栄養又は健康に資する組換え DNA 植物由来食品の安全性評価」「微量に混入した組換え DNA 植物の安全性評価」の原案が，ステップ 5/8 に進み，タスクフォースとしては採択され[7]，今後の総会での検討がなされる予定となっている．

4.　組換え食品の表示

遺伝子組換え食品の表示については，改正 JAS 法，食品衛生法施行規則及び乳及び乳製品の成分規格等に関する改正省令により，平成 13 年 4 月以降表示が義務化されている．現在，ダイズ，トウモロコシ，ジャガイモ，ナタネ，ワタ，テンサイ，アルファルファの 7 種農作物と，

表 5. 遺伝子組換え作物などの研究・開発状況

〈遺伝子組換え農作物〉
- 耐病性・耐虫性，多収性-第一世代 GM 作物
  コメ，コムギ（除草剤耐性），
  カボチャ，キュウリ（ウィルス耐性）
- 食味，加工特性：トマト（日持ち向上），イチゴ（甘み増強）など
- 機能性成分（栄養成分）-第二世代 GM 作物
  ダイズ（高オレイン酸，低アレルゲン），イネ（カロテノイド）
  ナタネ（高ラウリル酸）など
- 環境ストレス耐性（耐乾性，耐塩性）
  トウモロコシ，イネ（耐乾性）
- 医薬品の製造：医薬品原料やワクチンを産生する植物

〈その他〉
- 遺伝子組換え微生物，遺伝子組換え魚

ダイズ，トウモロコシ，ジャガイモ，テンサイ，アルファルファを原材料とする加工食品のうち 32 品目が表示の対象となっている[8]．ただし，加工食品中に組換え遺伝子およびタンパク質が除去・分解されているものならびに主な原材料となっていないもの（原材料中，重量が上位 3 品目で，かつ，食品に占める重量が 5%以上のものに限り義務表示とする）は，義務表示としないことになっている．なお，義務表示に伴い，安全性が確認された GM 作物の分析法は，農林水産省および厚生労働省が，標準分析法として公表している[9),10)]．また，国際的動向としては，組換え食品の表示のあり方については，コーデックス委員会食品表示部会 (CCFL) で，分析法については，コーデックス分析・サンプリング部会 (CCMAS) で議論が続けられている．

5. これからの開発状況

これまで，耐病性，耐虫性，除草剤耐性など生産者にメリットの高いいわゆる第一世代の遺伝子組換え作物がほとんどであった．現在も，同様の手法が，コメ，コムギなどに応用され，研究が進んでいる．これら，第一世代の組換え作物の後世代の安定性の確認等の地道な継続的な研究が必要であると思われる．一方，消費者にメリットのある栄養成分を変えた第二世代の作物（高オレイン酸ダイズなど）の開発も急ピッチで進められている．その他，第三世代に位置づけられる環境ストレス耐性を付与したもの，ワクチン等医薬品を作る植物の開発も進んでいる（表 5）．遺伝子組換え食品の評価は，あくまで，比較対照との評価を基本とするものであり，comparator の選び方が重要となる．現在，コーデックス委員会のバイオテクノロジー応用食品特別部会 (TFFBT) では，栄養改変のガイドライン（2009 年最終版作成予定）を作成中で，comparator の選び方，比較する栄養素，有害物質などの範囲などの議論が進められている．比較対照にすべきデータベースの整備が必須で，今後，タンパク質，代謝物を含む低分子物質のデータベースの整備が進んでくるものと思われる．常に最新の知見に基づく安全性審査が実施できるような研究体制や情報収集体制が必要と思われる．また，消費者への新規食品に対する科学的に分かりやすい安全性に関するコミュニケーションも必要であり，さらに，わが国で遺伝子組換え食品の開発を行っていくには，EU で議論されているさまざまな農業形態の共存を確保するシステムも参考になると思われる[11)]．

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A Screening Method for the Detection of the 35S Promoter and the Nopaline Synthase Terminator in Genetically Modified Organisms in a Real-Time Multiplex Polymerase Chain Reaction Using High-Resolution Melting-Curve Analysis

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To screen for unauthorized genetically modified organisms (GMO) in the various crops, we developed a multiplex real-time polymerase chain reaction high-resolution melting-curve analysis method for the simultaneous qualitative detection of 35S promoter sequence of cauliflower mosaic virus (35SP) and the nopaline synthase terminator (NOST) in several crops. We selected suitable primer sets for the simultaneous detection of 35SP and NOST and designed the primer set for the detection of spiked ColE1 plasmid to evaluate the validity of the polymerase chain reaction (PCR) analyses. In addition, we optimized the multiplex PCR conditions using the designed primer sets and EvaGreen® as an intercalating dye. The contamination of unauthorized GMO with single copy similar to NK603 maize can be detected as low as 0.1% in a maize sample. Furthermore, we showed that the present method would be applicable in identifying GMO in various crops and foods like authorized GM soybean, authorized GM potato, the biscuit which is contaminated with GM soybeans and the rice which is contaminated with unauthorized GM rice. We consider this method to be a simple and reliable assay for screening for unauthorized GMO in crops and the processing food products.

Key words maize; nopaline synthase terminator; genetically modified organism; real-time multiplex polymerase chain reaction; high-resolution melting-curve analysis; 35S promoter

In recent years, many types of genetically modified organisms (GMO), including microorganisms, animals and plants, have been put into practical use, and the number of commercially available genetically modified (GM) crops is increasing rapidly.[1] In Japan, over 70 lines of GM crops have been approved for open field cultivation or as food, feed and ornamental plants and classified as living modified organisms (LMO) under the Cartagena Protocol domestic law that came into effect in Japan on January 31, 2008.

Due to the commercialization, other GMO that have not been approved for human consumption, such as those intended for industrial processes, bioremediation, or the production of pharmaceuticals, may enter the market. Therefore, the ability to trace these organisms or to verify their absence in food will need to be assured.

GM foods have been authorized for food and/or feed by many countries based on their own criteria for safety assessment. In the EU, the authorization and use of GM foods and feed are stipulated by the provisions in regulations (EC) No. 1829/2003 and (EC) No.1830/2003.[2,3] Japan also announced a mandatory safety assessment of GM foods and processed foods containing GM ingredients. Since April 1, 2001, any GM food that has not been authorized is prohibited from import or sale in Japan. Therefore, qualitative detection methods of regulated and unauthorized GM foods are required to regulate unauthorized GM food. Previously, we reported the development of qualitative detection methods for GM maize, GM potatoes (NewLeaf Plus, NewLeaf Y), GM papayas (Line 55-1 or its derivatives) and GM rice (LL rice and Chinese Bt rice lines), including qualitative polymerase chain reaction (PCR) methods and a histochemical assay.[4—13] Especially, in China, some GM rice varieties have been developed and tested in the field and environmental trials.[14—16] Bt rice expressing the Bt toxin has been developed in China and approved for environmental release trials. We have detected at least two Bt rice lines, 'GM Shanyou 63' and 'Kemingdao,' which entered pre-production trails in 2001[16,17] and developed the detection methods for these rice.[7]

To date, no strategic method for the detection of the unauthorized GMO has been fully discussed and internationally accepted. However, to screen for unauthorized GMO in the various crops, several theoretical approaches have been proposed. One of these approaches, the "indirect subtractive approach," is based on the presumption that a positive screening test and no authorized GMO in a sample constitutes indirect evidence for the presence of unauthorized GMO.[18] In another approach, Cankar *et al.* reported the detection of unauthorized GMO based on differential quantitative PCR, which is an extension of qualitative differential PCR for detecting the 35S promoter sequence of cauliflower mosaic virus (35SP).[19] The application of this approach has been limited to a case study of GM maize events.

While, high-resolution melting-curve analysis (HRM) is a simple and cost-effective post-PCR technique that can be used.[20] The technique requires the use of standard PCR reagents and double-stranded DNA (dsDNA)-binding dyes that are used at saturating concentrations without inhibiting PCR amplification.

In the present study, we developed a screening detection method for both 35SP and the nopaline synthase terminator

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(NOST) in GM crops and the processed foods using HRM with EvaGreen® as an intercalator in a real-time multiplex PCR to predictably detect unauthorized GMO.

MATERIALS AND METHODS

Samples The rice sample (imported product from China), which was suspected to be contaminated with Bt rice based on testing at a quarantine inspection center, and the biscuit, which was contaminated with GM soybean, were obtained through the Ministry of Health, Labor and Welfare (MHLW) of Japan. The biscuit consisted mainly of wheat ingredients. Rice produced in Japan was used as a negative control and was purchased commercially in Tokyo. The certified reference materials of the representative GM maize line, NK603 (0% (w/w, non-GM), 0.1%, 0.5%, 1%, 2% and 5%) in powder and the representative GM soybean line, 40-3-2 (Roundup Ready Soybean) were purchased from the Institute for Reference Materials and Measurement (IRMM; Retieseweg, Belgium). GM potato (New Leaf) was kindly provided by the Monsanto Co. (St. Louis, MO, U.S.A.). The ColE1 plasmid was purchased from NIPPON GENE (Toyama, Japan).

Extraction and Purification of Genomic DNA The samples were ground using an electric mill. All the plant genomic DNA, except for the rice, was extracted using a silica-gel membrane-type kit (DNeasy Plant Mini; QIAGEN, Hilden, Germany). The homogenized samples (each 1 g) were incubated at 65 °C for 10 m in 10 ml of the buffer AP1 (QIAGEN) and 20 μl of RNase A (100 mg/ml; QIAGEN), and mixed 2—3 times during incubation by inverting the tube. After adding buffer AP2 (QIAGEN), the resultant mixture was incubated for 10 min on ice. The mixture was centrifuged at 4 °C for 20 min at 4000×***g***. The clear supernatant was transferred to a QIA shredder spin column (QIAGEN) and centrifuged for 4 min at 10000×***g***. The mixture was pipetted after adding 1.5 volumes of buffer AP3/ethanol, and was applied to a mini spin column. The column was centrifuged for 1 min at 10000×***g***, the flow-through was discarded, and the column was washed 3 times with buffer AW (QIAGEN). The DNA was then eluted twice from the column with 70 μl of the pre-warmed DW. The rice DNA extraction and purification were carried out using the NIPPON GENE GM quicker 2 kit (NIPPON GENE) according to the manufacturer's manual with the following modification. The ground samples (500 mg) were suspended in 2.1 ml of buffer GE1, 60 μl of proteinase K (20 mg/ml), 6 μl of α-amylase solution (attached in kit) and 30 μl of RNase A (100 mg/ml) using a vortex mixer for 30 s and then heated at 65 °C for 30 min. A 255 μl aliquot of the buffer GE2-K was added to the mixture and sufficiently mixed using a vortex mixer followed by standing on ice for 10 min. After centrifugation at 6000×***g*** for 15 min at 4 °C, the collected supernatant was transferred into a new tube, and centrifuged again at 13000×***g*** or above at 4 °C for 5 min. To 1 ml of the supernatant placed in a new tube, 375 μl of both the buffer GE3 and isopropanol were added, and then the solution was gently mixed by being shaken 10—12 times. The mixture was applied onto the spin column included in the kit and centrifuged at 13000×***g*** and 4 °C for 30 s to discard the eluate. This procedure was repeated until the entire eluate was loaded. The spin column was washed with 650 μl of the buffer GW by centrifuging at 13000×***g*** for 1 min. The column was transferred to a new tube and 50 μl of the TE buffer was added and allowed to stand for 3 min at room temperature. Finally, the tube with the spin column was centrifuged at 13000×***g*** and 4 °C for 1 min, and the eluate was then used as the DNA sample solution in the following experiments. The extracted DNA was diluted with an appropriate volume of DW to a final concentration of 10 ng/μl, and stored at −20 °C until use.

PCR Conditions The PCR reaction mixture (25 μl) in the tubes contained 50 ng genomic DNA, 2.5 μl of PCR buffer II (Applied Biosystems, CA, U.S.A.), 0.16 mM of deoxyribonucleotide triphosphate (dNTP) (Applied Biosystems), 1.5 mmol/l $MgCl_2$, 1.2 μmol/l of 5′ and 3′ primers (P35S 1—5′ and P35S 2—3′ for 35SP, NOS ter 3—5′ and NOS ter 3—3′ for NOST, ColE1 F1 and ColE1 R1 for ColE1), 2 μl of 5×10^{-2} pg/μl ColE1 plasmid (approximately 13.9×10^3 copies/reaction mixture) and 0.8 units of Ampli Taq Gold (Applied Biosystems). PCR was performed by pre-incubation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s, and terminal elongation at 72 °C for 7 min using the GeneAmp PCR System 9700 (Applied Biosystems). After PCR amplification, the amplified products were analyzed by agarose gel electrophoresis according to the previous reports.[4,7,9—12]

Multiplex Real Time PCR Using HRM The multiplex real time PCR reaction mixture (20 μl) in the tubes contained 20 ng genomic DNA, 1X Multiplex PCR Mix (QIAGEN, Hilden, Germany), 0.5X Q-solution, (QIAGEN), 1X EvaGreen® (WAKO, Tokyo, Japan), and 0.2 μmol/l of 5′ and 3′ primers (P35S 1—5′ and P35S 2—3′ for 35SP, NOS ter 3—5′ and NOS ter 3—3′ for NOST, ColE1 F1 and ColE1 R1 for ColE1). The reaction was performed by pre-incubation at 95 °C for 15 min, followed by 50 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 90 s (45 s for uniplex real time PCR), extension at 72 °C for 90 s, and terminal elongation at 72 °C for 10 min. After the latter run, the sample was held at 95 °C for 30 s and then held at 72 °C for 5 s, and melt curve data was generated by raising the temperature from 72 to 97 °C at 0.3 °C increments, while pausing for 5 s per step using the Rotor gene™ 6000 (QIAGEN). Positive identification of an isolate containing a gene target was determined by the corresponding melt temperature. The Rotor gene™ 6000 software (Ver 1.7) calculates melt peaks by plotting the negative first derivative of the primary melt curve (fluorescence *vs.* temperature).

RESULTS

Design of the PCR Systems and the Selection of EvaGreen® as an Intercalator Dye We selected primers that were appropriate for the simultaneous detection of 35SP and NOST in screening for unauthorized GMO. In addition, we further designed the primers to detect ColE1 spiked to assess the validity of the PCR run.[21] The selected primer pairs are described in Table 1. Selection of primers was performed on the basis of the results obtained from conventional PCR and electrophoresis using the NK603 maize sample. Electrophoresis analysis showed no formation of primer dimers

(Fig. 1). Next, we examined SYBR® Green I and EvaGreen® as intercalating dyes. Both fluorescence dyes provided similar amplification and melting curve data, although the shape of the melting curve using EvaGreen® was detected more clearly (data not shown). Considering the reliability and sensitivity of the analysis, we chose to use EvaGreen® in further experiments.

Table 1. Primer Sequences and Product Tm for the Multiplex Real Time PCR

Name	Oligonucleotide sequence	Product Tm (°C)
P35S 1—5′	ATT GAT GTG ATA TCT CCA CTG ACG T	80.18
P35S 2—3′	CCT CTC CAA ATG AAA TGA ACT TCC T	80.18
NOS ter 3—5′	GTC TTG CGA TGA TTA TCA TAT AAT TTC TG	77.09
NOS ter 3—3′	CGC TAT ATT TTG TTT TCT ATC GCG T	77.09
ColE1 F1	CGG TTA CTT GAA CGC TGT GA	90.59
ColE1 R1	CAC CTT ACG GGC TGT CTG AT	90.59

In uniplex PCR, the specific amplification of either 35SP, NOST or ColE1 was performed on the NK603 maize and ColE1 plasmid. All sequences were correctly identified as possessing the correct sequence for each primer set as observed by distinct melt peaks at specific temperatures. Melt peak height and shape were consistent for each sequence target. Melt temperatures relative to one another were in agreement with the expected amplicon size, with larger amplicons exhibiting higher Tms. Non-GM rice sample was considered negative due to the melt profiles.

Multiplex PCR Systems We examined the suitability of the proposed real-time PCR systems to be used in a Tm melting curve determination. After careful analysis of the respec-

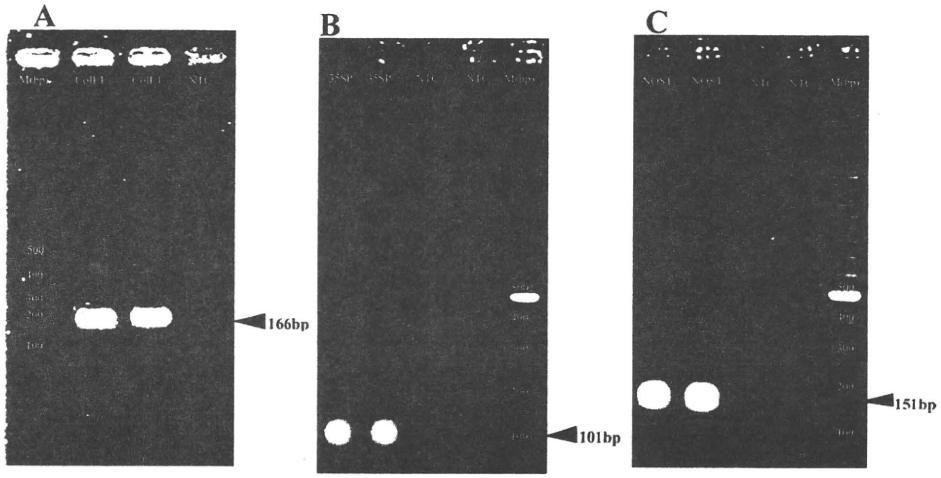

Fig. 1. Targeted Gene Amplification by PCR

PCR reactions were run using total genomic DNA from GM maize line (NK603) as the template and primers that were designed specific to ColE1 (A), 35SP (B) and NOST (C), and analyzed by 3% (w/v) agarose gel electrophoresis in TAE buffer. Arrows indicate the amplified PCR products stained by ethidium bromide. NTC, non-template control; M, 100 bp DNA marker.

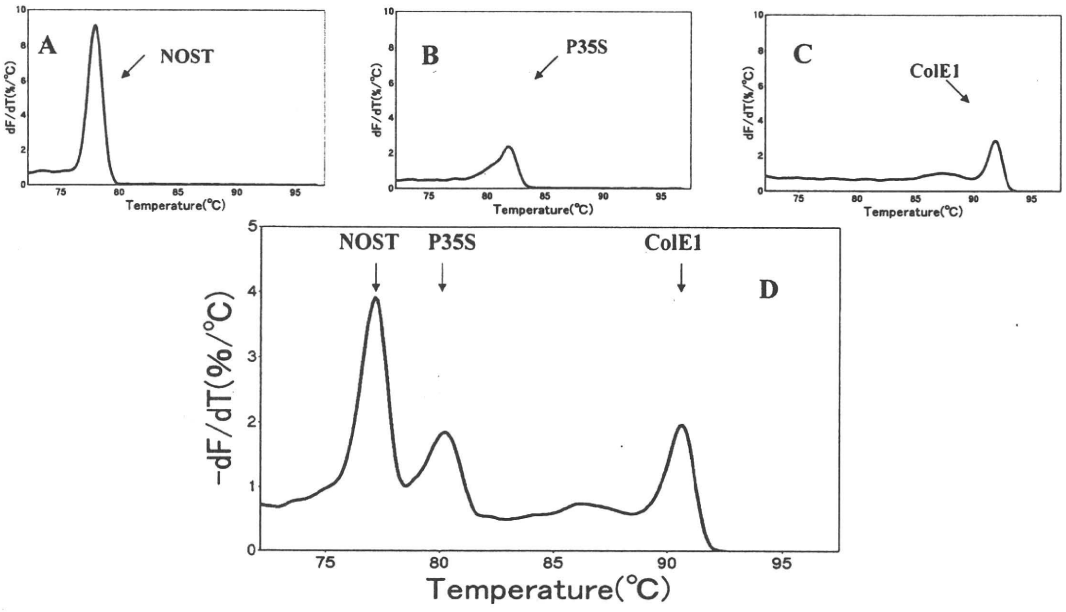

Fig. 2. Melting Curve of Each Uniplex PCR Assay for NOST (A), P35S (B), ColE1(C) and 3-Plex PCR Assay (D)

NK603 maize genomic DNA was amplified as the template by thermal conditions (initial denaturation at 95 °C for 15 m, followed by 50 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 90 s and extension at 72 °C for 90 s, and terminal elongation at 72 °C for 10 min). Melting curves were analyzed by Rotor gene™ 6000 using EvaGreen® as an intercalating dye, and the corresponding Tm was assigned to each product by arrow.

tive experimental Tm, we optimized the PCR conditions in the multiplex condition using the NK603 maize sample. In particular, we examined the time of the annealing step since that step is critical for the optimization of the multiplex condition. We found that 90 s was the optimal time for annealing in the multiplex format. Multiplex reactions using the 3 primer sets in the same reaction tube showed positive melting curves in the sample solution prepared from NK603 maize sample. For the examination of the level of spiked ColE1, we found that the optimized level of the spiked ColE1 is approximately, 2 μl of 5×10^{-2} pg/μl ColE1 plasmid (approximately 13.9×10^{3} copies) in the PCR reaction mixture (25 μl).

The Tm of the melt curve profile can identify which target sequences were amplified by PCR (Fig. 2). Peaks were clearly separated from one another and the average Tms of the different peaks were significantly different in the multiplex reaction. On the other hand, no significant shifts of the average Tms of 35SP, NOST and ColE1 were observed between the uniplex and multiplex reactions. The sample solution prepared from non-GM maize did not produce the correct amplicon when viewed on agarose gels and did not show the correct melt profiles.

To assess the sensitivity of the proposed method using the NK603 maize samples, we tested different amounts of the NK603 maize samples: 0% (non-GM), 0.1%, 0.5%, 1%, 2% and 5%. As shown in Fig. 3, all of the positive melting curves were obtained stably except for the 0% sample. These results suggest that the contamination of unauthorized GMO with single copy of the transgenic gene similar to NK603 maize as low as 0.1% can be detected in a maize sample.

Application to Various Other Samples To assess the application of the established method to other crops, the authorized GM soybean (Roundup Ready Soybean), the authorized GM potato (NewLeaf), the biscuit which is contaminated with GM soybean and the rice which is contaminated with unauthorized GM rice were analyzed using the established method. As shown in Fig. 4, the positive melting curves for 35S and NOST were detected in GM soybean,

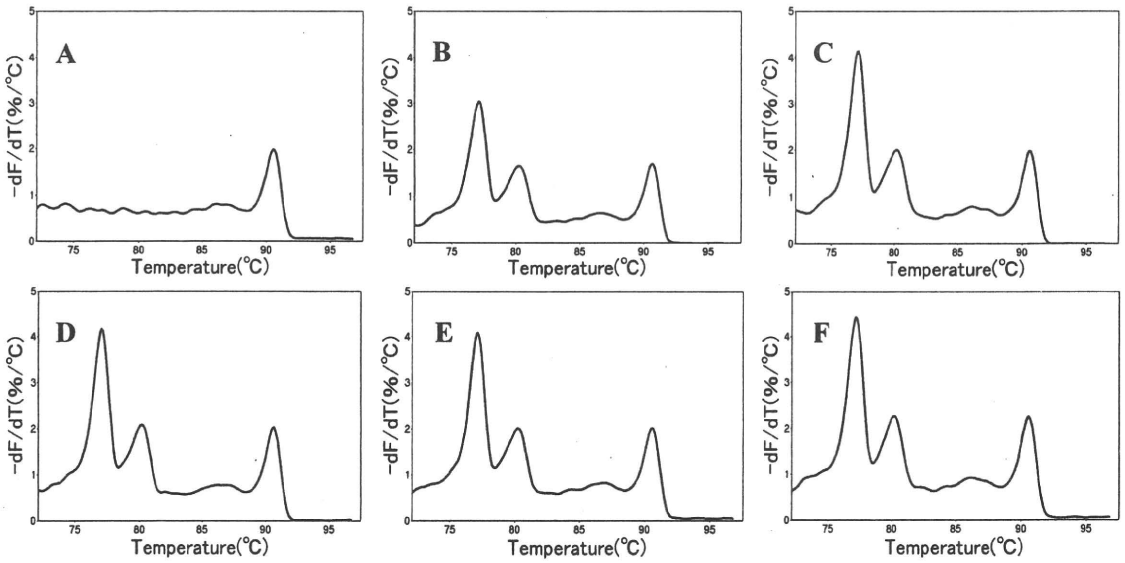

Fig. 3. Sensitivity of the 3-Plex Realtime PCR

The melting curves were tested for detection of different amounts of NK603 maize samples: A) 0% (w/w, non-GM), B) 0.1% (w/w), C) 0.5% (w/w), D) 1% (w/w), E) 2% (w/w) and F) 5% (w/w).

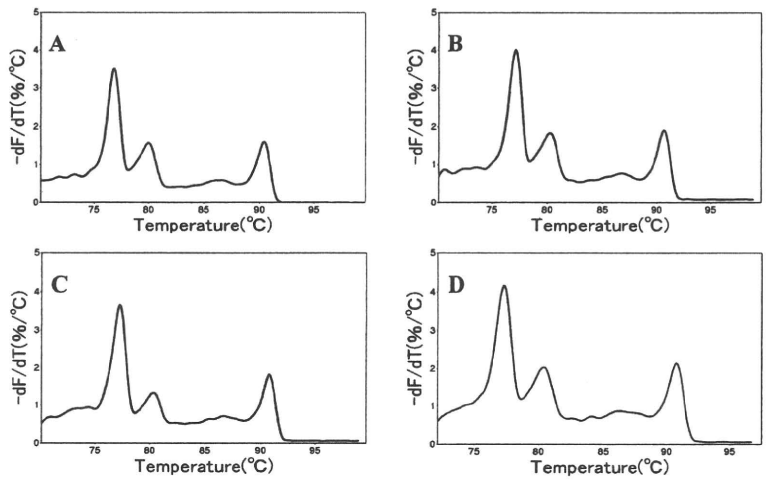

Fig. 4. Application to Other GM Products for Detection

Melting curves represent 3-plex PCR using total genomic DNA samples from: A) GM soybean (Roundup Ready Soybean), B) GM potato (NewLeaf), C) rice contaminated with unauthorized GM rice and D) biscuit contaminated with GM soybean of unknown amount.

GM potato, the biscuit which is contaminated with GM soybean and the rice which is contaminated with unauthorized GM rice. The melt profiles did not produce peaks, except for ColE1 as a reference, in non-GM soybean, non-GM rice and non-GM potato as negative samples (data not shown). These results suggest that the method established here may be applicable in identifying GMO in various crops and foods.

DISCUSSION

A simultaneous qualitative detection method for 35SP and NOST using HRM was developed for the screening detection of unauthorized GMO in several crops. We selected suitable primer sets for the simultaneous detection of 35SP and NOST and designed the primer set for the detection of spiked ColE1 plasmid to evaluate the validity of the PCR analyses. In addition, we optimized in the multiplex PCR conditions using the designed primer sets and EvaGreen® as an intercalating dye. We considered that using EvaGreen® would be more accurate because EvaGreen® is a generally less inhibitory toward PCR and less likely to cause nonspecific amplification compared with SYBRGreen I®.

Simple and accurate screening methods for detection are necessary to search the unauthorized GM crops in various foods. Many studies have employed PCR to target multiple genes for the detection of GM crops. However, conventional PCR requires post-PCR processing to visualize the amplification products, and usually including agarose gel electrophoresis and nucleic acid staining. The handling of PCR products is not only time-consuming, but also has the potential of cross-contamination of the amplified products. Real-time PCR has solved many of these problems, allowing faster cycling times and closed-tube detection during the amplification process. Previous studies have demonstrated the detection of GM crops using specific probes.[7,13] However, the use of fluorescence labeled probes adds significant cost to an assay comprising multiple targets. The closed-tube screening method using HRM has advantages over current techniques because it requires no post-PCR handling, which minimize the risk of PCR contamination, and no separation step, both of which improve analysis time.

The intercalating dyes, such as SYBR® Green I and EvaGreen®, have been used successfully in real-time PCR HRM.[22—24] They bind all amplified dsDNA and do not confer sequence specificity to a desired target as oligonucleotide probes. However, the amplicons can be distinguished by melt curve analysis, whereby the melting temperature of PCR products is determined by the reduction in relative fluorescence as all dsDNA is denatured to the single-stranded form. The shape and peak location of the melt curve are functions of the GC/AT ratio, length, and sequence of the fragment.[23] Therefore, optimized reaction conditions and sufficient differences in amplicon length and GC/AT ratio are necessary to discriminate clearly between amplification products in a melt analysis. A real-time PCR HRM using EvaGreen® was developed to simultaneously detect 35SP, NOST and ColE1. Each product differed sufficiently in size and sequence to be distinguished by melt curve analysis and agarose gel electrophoresis.

In addition, we applied the method established in this study to several GM crops. To assess unauthorized GM crop contamination, GM maize, GM soybean, GM rice and the biscuits contaminated with GM soybeans were used as selected targets. We showed that the present method is applicable for GM maize, GM soybean, GM rice and even the detection of GM soybean in the biscuit, which consisted mainly of wheat. The present method has the potential to screen for novel types of unauthorized GM crops, as well as authorized GM crops, since 35SP and NOST are major sequences in GM crops and widely inserted into various GM crops.

Recently, we detected unauthorized GM crops that have become major concerns such as CBH 351 maize, Bt10 maize, LLRice601, and Chinese Bt rice. The present method may not meet the absolute detection limit of unauthorized GM crops, because the contaminated crops may contain unknown recombinant-DNA (r-DNA) sequence or r-DNA sequences with modified nucleotide sequences. However, after confirming the contamination of unauthorized GM crops using the present method, it may be necessary to analyze the detailed construct sequence close to 35SP and NOST using inversed PCR and anchored PCR techniques.

In conclusion, we developed a multiplex real-time PCR HRM method for the simultaneous qualitative detection of 35SP and NOST in several crops. We consider this method to be a simple and reliable assay for screening for unauthorized GMO in crops and the processing food products.

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Note

Optical Detection of Specific Genes for Genetically Modified Soybean and Maize Using Multiplex PCR Coupled with Primer Extension on a Plastic Plate

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A novel DNA microarray method to detect one line of genetically modified (GM) soybean and five lines of GM maize was developed using multiplex PCR coupled with primer extension on a plastic plate. Multiplex PCR products were applied on an extension primer-immobilized plate and the spots corresponding to the DNA sequences were visualized. This method is a rapid and simple way to detect GM soybean and GM maize optically.

Key words: arrayed primer extension; genetically modified organism; multiple primer extension; oligonucleotide microarray

The numbers and uses of genetically modified organisms (GMOs) have increased in recent years, but controversy continues to surround their increased distribution and use due to concerns about food safety, environmental risks, and ethical issues. Many countries and regions have issued GMO labeling regulations, for example thresholds of 5% in Japan.[1)] The primary means of GMO detection to determine whether such labeling requirements have been met is the polymerase chain reaction (PCR) technique, but this method has an obvious limitation in that it can detect only one gene at a time. Even for multiplex PCR, due to the limit of the separation of the amplified products for ordinary electrophoresis, it can detect only five to six genes simultaneously.

Oligonucleotide microarrays show great advantages in the detection of many target genes simultaneously. In fact, microarrays have been applied to simultaneous detection of GMOs.[2–7)] These microarrays are based on the hybridization of labeled target oligonucleotides to probes on the chip, but the hybridization method is generally time-consuming, especially the hybridization step itself. In addition, many of these microarrays require laser scanning. On the other hand, primer extension methods on a microarray, such as the arrayed primer extension method and the multiple primer extension method, are used mainly for mutation and single nucleotide polymorphism analysis.[8,9)] The extension method is relatively quick and can be adapted to optical detection.[10)] This method was recently used for rapid identification of bacteria.[11)]

In this study, we adapted this rapid, simple method to detect target DNA sequences optically and simultaneously, as shown in Fig. 1. We developed a method of identifying different GMO events, one line of genetically modified (GM) soybean (Roundup Ready soybean, RRS) and five lines of GM maize (Event176, Mon810, Bt11, GA21, and T25). The method is based on specific integration junction sequences between the host plant genome DNAs, and it uses multiplex (8-plex) PCR together with primer extension on a plastic plate.

Conventional soybean and maize seeds were purchased from a local market in Hyogo, Japan, and were ground to a powder (AM-3, Nihon Seiki Seisakusho, Tokyo). The powdered certified reference materials of RRS, Event176, Mon810, Bt11, and GA21 were obtained from the Institute for Reference Materials and Measurements (Geel, Belgium) and commercialized by Fluka (Buchs, Switzerland). DNA was extracted from powder samples using a silica membrane-type kit (DNeasy Plant Mini Kit, Qiagen, Hilden, Germany) according to a previously reported procedure.[12)] The concentration of DNA was calculated from the absorbance at 260 nm, as measured with a UV spectrophotometer. On the other hand, T25 DNA was purchased from Generon (Modena, Italy). Samples (1% and 5%) of T25 were prepared by mixing T25 DNA with non-GM maize DNA. A GM maize mixture containing 1% each of Event176, Mon810, Bt11, GA21, and T25 was also prepared by mixing GM maize DNAs with non-GM maize DNA.

The PCR primer sets for taxon specific sequences in soybean (the lectin gene, *Le1*) and maize (the starch synthase IIb gene, *SSIIb*), and construct specific GM sequences, *RRS*, *Event176*, *Mon810*, *GA21*, and *T25*, had been used to quantify GMOs using real-time PCR (Table 1).[12)] The set for *Bt11* has also been reported.[5)] The primers were synthesized by Texas Genomics Japan (Tokyo). A 25-μl reaction mixture contained 12.5 μl, QuantiTect Multiplex PCR Master Mix (Qiagen),

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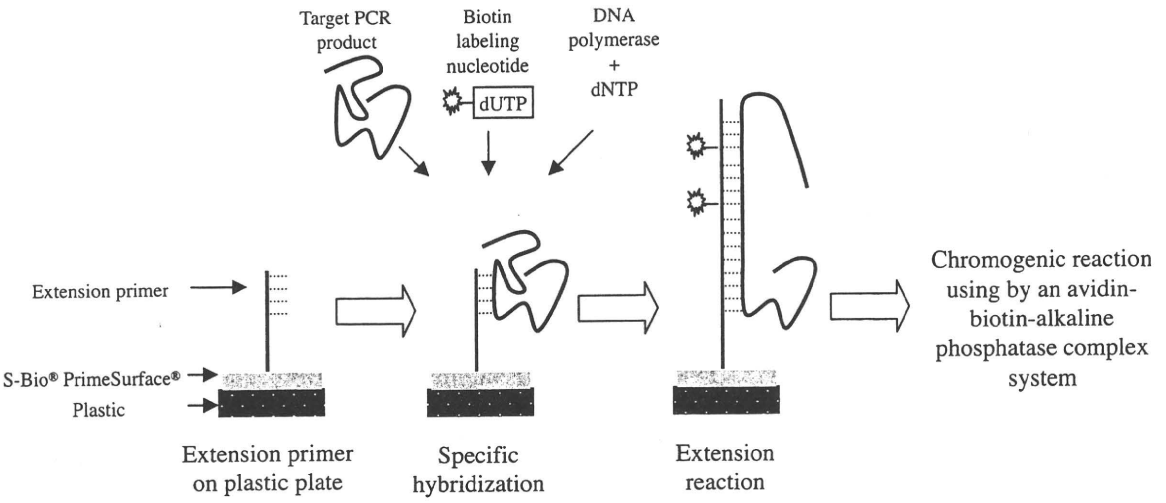

Fig. 1. Process for Primer Extension on a Plastic Plate.
An extension primer is immobilized on the S-Bio® PrimeSurface®-treated plastic plate. Target PCR product amplified by multiplex PCR hybridizes to the primer. Extension and incorporation of biotin-dUTP are carried out by DNA polymerase. The extension reaction is optically detected by an avidin-biotin-alkaline phosphatase complex system.

Table 1. Forward and Reverse Primers Used in Multiplex (8-plex) PCR and Extension Primers Used in an Extension Reaction on a Plastic Plate

Target	Orientation	Sequence (5′-3′)	Reference
Le1	Forward	GCCCTCTACTCCACCCCCA	12
	Reverse	GCCCATCTGCAAGCCTTTTT	12
	Extension[a]	AGCTTCGCCGCTTCCTTCAACTTCAC	12
RRS	Forward	CCTTTAGGATTTCAGCATCAGTGG	12
	Reverse	GACTTGTCGCCGGAAATG	12
	Extension[a]	CGCAACCGCCCGCAAATCC	12
SSIIb	Forward	CTCCCAATCCTTTGACATCTGC	12
	Reverse	TCGATTTCTCTCTTGGTGACAGG	12
	Extension[a]	GCAATGCAAAACGCAACGAGTGGGGG	This study
Event176	Forward	TGTTCACCAGCAGCAACCAG	12
	Reverse	ACTCCACTTTGTGCAGAACAGATCT	12
	Extension[a]	TCGATGTGGTAGTCGGTCACGTCGG	12[b]
Mon810	Forward	GATGCCTTCTCCCTAGTGTTGA	12
	Reverse	GGATGCACTCGTTGATGTTTG	12
	Extension[a]	TTGTTGTCCATGGCCGCTTGGTATCT	12[b]
Bt11	Forward	ACATTTAATACGCGATAGAAAAC	5
	Reverse	ACACCTACAGATTTTAGACCAAG	5
	Extension[a]	TATGTTACTAGATCTGGGCCTCGTG	5
GA21	Forward	GAAGCCTCGGCAACGTCA	12
	Reverse	ATCCGGTTGGAAAGCGACTT	12
	Extension[a]	CGGCCATGCACCGGATCCTT	12[b]
T25	Forward	GCCAGTTAGGCCAGTTACCCA	12
	Reverse	TGAGCGAAACCCTATAAGAACCCT	12
	Extension[a]	TGCAGGCATGCCCGCTGAAATC	12

[a]Modification with amino linkers at the 5′ end due to immobilization of the extension primer on a plastic plate.
[b]Using the complement sequence of the reference.

0.05 μM each of primer for *SSII*, *Event176*, and *Bt11*, 0.1 μM each of primer for *Le1*, *RRS*, *Mon810*, and *T25*, 0.5 μM each of primer for *GA21*, a 100-ng DNA sample, and sterilized water to make up the final reaction volume. Eight-plex PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal cycle program was as follows: 2 min at 50 °C and 15 min at 95 °C, followed by amplification of DNA for 35 cycles of 60 s at 94 °C and 60 s at 61 °C. The PCR products were used for the primer extension reaction.

The sequences of the extension primers, which were immobilized on a plastic plate (for *Le1*, *RRS*, *Event176*, *Mon810*, *GA21*, and *T25*) were those previously used as TaqMan probes in real-time PCR (Table 1).[12] The sequence of the TaqMan probe for *SSIIb* was modified for use in the primer extension reaction. The modified sequence was checked for specificity by BLAST search. The primer extension sequence for *Bt11* has also been reported.[5] These primers were modified with amino linkers at the 5′ end, and were synthesized and purified on a reverse-phase column by Nippon EGT (Toyama, Japan). The plastic plate was treated with S-Bio® PrimeSurface® (Sumitomo Bakelite, Hyogo, Japan), providing a unique biocompatible phospholipid polymer and a highly active functional ester moiety to bind the attachment site covalently for amino-linked oligonucleotides under alkaline conditions.[13] One microliter of

10 μM 5′-amino-link primer in alkaline solution was spotted onto the plate. After incubation for 3 h, the primer-immobilized plate was treated with 0.1 M sodium hydroxide solution to block the remaining functional ester moieties, and was washed with hot water. The plate was dried in air and stored at 4 °C.

Fifty μl of reaction mixture of primer extension consisted of 5 U TERMIPol DNA polymerase (Solis Biodyne, Tartu, Estonia), 1 × reaction buffer C, 2 mM $MgCl_2$, 50 μg/ml, salmon sperm DNA, 0.05% triton-X 100, 100 μM dATP, 100 μM dGTP, 100 μM dCTP, 65 μM dTTP (each dNTP, Indianapolis, Roche, IN), 35 μM biotin-11-dUTP (Bioron, Ludwigshafen, Germany), 5 μl of PCR products, and sterilized water to make up the final reaction volume. The sample was incubated for 5 min at 95 °C, and then was applied to the extension primer-immobilized plastic plate (preheated to 72 °C). The reaction was allowed to proceed at 72 °C for 3 min under a coverslip. The plate was washed in 0.1% triton X-100 in 50 mM tris buffer (pH 7.5), and then in 50 mM tris buffer (pH 7.5). It was incubated at 37 °C for 20 min with the addition of alkaline phosphatase-conjugated biotin and avidin complex (VectaStain ABC-AP Kit, Burlingame, Vector Laboratories, CA), which was prepared by dilution of A and B reagents with 200 times the volume of 2% BSA and 0.01% salmon sperm DNA in 50 mM tris buffer (pH 7.5) and preincubated for 20 min at room temperature before use. The plate was washed in 0.1% triton X-100 in 50 mM tris buffer (pH 7.5) and then in 50 mM tris buffer (pH 7.5). The plate was incubated at 37 °C for 10 min with the addition of NBT/BCIP solution (Roche). Then it was washed in water and dried in air. The image on the plate was scanned using an MP 600 scanner (Cannon, Tokyo).

The principle of the present method, based on multiplex PCR amplification and identification of the primer extension reaction on a plastic plate, is shown in Fig. 1, but it is necessary to optimize all reaction procedures. In multiplex PCR, the primer sets were tested and the primer concentration and thermal cycle programs were optimized to minimize non-specific amplification and variation in amplification efficiency between the pairs of primers. In the primer extension reaction, the oligonucleotide lengths and orientations of the extension primers were examined, and incubation time and temperature were optimized to minimize cross hybridization and self-extension.

Under optimized conditions, *Le1* and *SSIIb* spots were visible to the naked eye in the non-GM soybean and non-GM maize samples (Fig. 2). Accordingly, detection of these species-specific genes can be used as a positive control. In the samples containing 1% and 5% of GM soybean and GM maize, except for 4.3% GA21, the corresponding spots were visible to the naked eye. In addition, in the mixture sample containing 1% each of the five lines of GM maize, the spots corresponding to *SSIIb* and five lines of GM maize were simultaneously visible. These results indicate that this method can identify GM soybean lines and GM maize lines in seed and grain samples containing 1% GM material. However, spots with a lower signal intensity than those containing 1% GMO, especially *Bt11* and *GA21*, are invisible to the naked eye. In fact, when the DNA amount used for multiplex PCR decreased from 100 ng to 50 ng, the corresponding spot was frequently invisible in the sample containing 1% GA21 (data not shown).

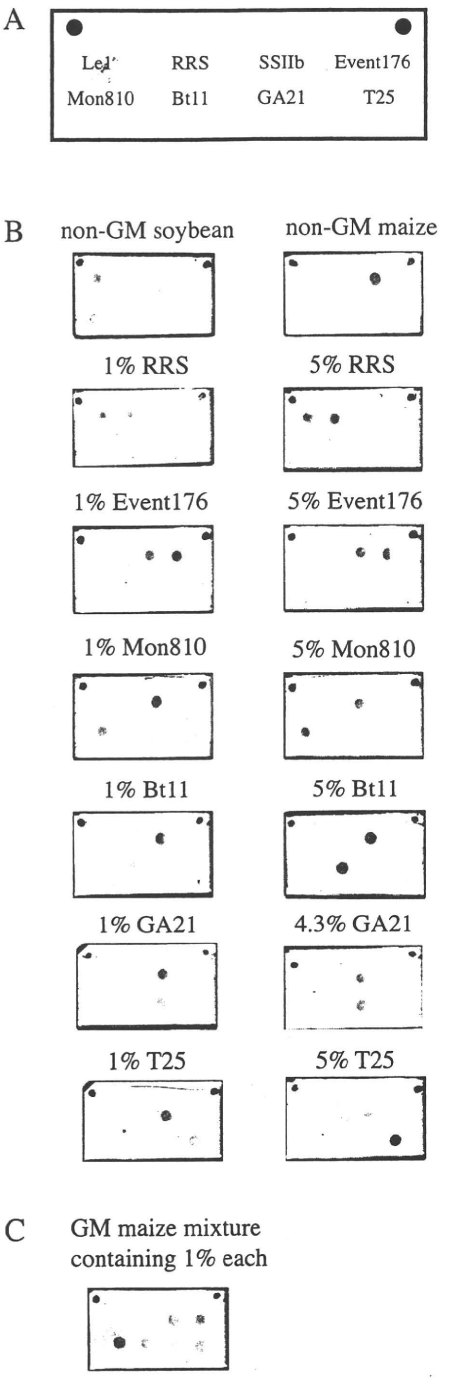

Fig. 2. Detection of GMO Genes Using Multiplex (8-plex) PCR Coupled with Primer Extension.

A, Layout of the extension primer-immobilized plastic plate. The sequences of the primer are given in Table 1. B, Image of the primer extension reaction of non-GM soybean, GM soybean (Roundup Ready soybean), non-GM maize and GM maize (Event176, Mon810, Bt11, GA21, and T25). The GMO contents of the samples are 1 and 5%, except for 4.3% GA21. In the case of T25, the percentages indicate the percentages of GM DNA relative to non-GM DNA. C, Image of the primer extension reaction of the GM maize mixture containing 1% each of Event176, Mon810, Bt11, GA21, and T25. The percentages indicate the percentages of GM DNA relative to non-GM DNA. The positive signals appear as gray spots. The dots in the upper corners denote the position of the plate.

In previous reports, the detection limits of microarrays for GMO were 0.1%.[2–4] These amplification processes were the four separated PCR,[3] PCR amplifi-

cation with a tag primer in tag-labeled samples prepared by polymerase reaction with a bipartite primer containing the tag sequence,[2] and nucleic acid sequence-based amplification (NASBA) in promoter-conjugated samples prepared by polymerase reaction with a bipartite primer containing the promoter sequence.[4] These methods are sensitive and quantitative, but are time-consuming and require more manipulation. On the other hand, the amplifying and fluorescent-labeling system using multiplex PCR detected 0.5% and 1% of GMOs.[5,6] The method had low sensitivity, but was rapid and simple. The present method also used multiplex PCR amplification and optically detected samples containing 1% GM soybean and GM maize. In addition, microarrays for GMO detection in all previous reports were based on the hybridization of labeled target oligonucleotide to a probe on the chip.[2–7] The hybridization times were from 1 h to 18 h as compared with the time of primer extension reaction in the present method of 3 min.

In conclusion, we have developed a new detection method for GMOs using multiplex PCR coupled with a primer extension method in a microarray. This rapid and simple method should be useful for optical identification of GM soybean and GM maize.

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Original

Development of Quantitative Duplex Real-Time PCR Method for Screening Analysis of Genetically Modified Maize

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A duplex real-time PCR method was developed for quantitative screening analysis of GM maize. The duplex real-time PCR simultaneously detected two GM-specific segments, namely the cauliflower mosaic virus (CaMV) 35S promoter (P35S) segment and an event-specific segment for GA21 maize which does not contain P35S. Calibration was performed with a plasmid calibrant specially designed for the duplex PCR. The result of an in-house evaluation suggested that the analytical precision of the developed method was almost equivalent to those of simplex real-time PCR methods, which have been adopted as ISO standard methods for the analysis of GMOs in foodstuffs and have also been employed for the analysis of GMOs in Japan. In addition, this method will reduce both the cost and time requirement of routine GMO analysis by half. The high analytical performance demonstrated in the current study would be useful for the quantitative screening analysis of GM maize. We believe the developed method will be useful for practical screening analysis of GM maize, although interlaboratory collaborative studies should be conducted to confirm this.

Key words: genetically modified (GM); maize (*Zea mays*); duplex real-time PCR

Introduction

Recombinant DNA (r-DNA) technologies have been used in modern farming and have provided many advantages in related industries. In fact, the global area of genetically modified (GM) crops exceeded 120 million hectares in 2007, and is expected to continue to rise[1]. GM crops have been authorized for use as food and/or feed in many countries based on each country's own criteria for safety assessment. However, consumers have demanded appropriate information and labeling for foods derived from GM crops. Thus, various labeling systems have been introduced for GM foods in the European Union (EU)[*1], Korea[*2], Japan[*3,*4], and other countries (reviewed by Hino[2]). In addition, many countries have found ways for the farming of conventional crops and GM crops to coexist. In these situations, the growing importance of scientific GM detection methods has been recognized.

For the detection of GM maize in foods and food materials, PCR-based detection methods, which are able to detect even small amounts of transgenes in raw materials and processed foods, have been routinely used[3)-5)]. We have also developed simplex and multiplex qualitative PCR methods[6)-11)], simplex real-time PCR methods[12),13)], and an individual kernel-based detection method including a qualitative multiplex real-time PCR[14)]. In particular, six real-time PCR methods for five events of GM maize, *i.e.*, Bt11, Bt176, GA21, MON810,

[*1] Regulation (EC) No. 1829/2003. Official J. Eur. Union L268, 1-23 (2003).
[*2] Notification No. 2000-31; Ministry of Agriculture and Forestry of Korea (2000).
[*3] Notification No. 110 (Mar. 27, 2001); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2001).
[*4] Notification No. 517 (Mar. 31, 2000); Ministry of Agriculture, Forestry and Fisheries of Japan (2000).

and T25, and a Roundup Ready soy, were fully validated by an international interlaboratory collaborative study[12),15)], and have been not only employed as Japanese official analytical methods[*3, *4], but also internationally recognized as ISO standard methods for the analysis of GMOs in foodstuffs[*5]. On the other hand, the cost and time required for GM analysis could constitute a major bottleneck in the process of providing consumers with cost-effective labeling[16)]. The conventional or non-GM food supply chains, however, must be secured by affordable detection methods. Thus, the development of cost- and time-effective detection methods is important to ensure freedom of choice for consumers.

Maize is the major agricultural crop to which GM technology has been applied. In Japan, sixteen events of GM maize and twenty varieties of their hybrid progenies have been authorized as of 2008[*6], and in fact, some of them, *e.g.*, Bt11, Bt176, GA21, MON810, MON 863, NK603, T25 and TC1506, have been used for commercial purposes in Japan. Analysis of the r-DNA constructions of these eight GM maize events elucidated that all the events except GA21 maize contained the same constitutive promoter, which originated from the cauliflower mosaic virus (CaMV) and has been designated as CaMV35S promoter (P35S). The Ministry of Health, Labour and Welfare of Japan (MHLW) announced a combinational method for the quantitation of P35S and a construct-specific quantitation for GA21 maize, and it has been officially used as a screening method for GM maize[12),*3,*4], though it requires two independent quantitations. Thus, to improve the efficiency of the GM analysis method, we have been trying to develop a screening analytical method for GM maize based on our real-time PCR methodologies. In this study, we developed a duplex real-time PCR method for simultaneous quantitation of both P35S and an event-specific sequence for GA21 maize, and evaluated the performance characteristics of the newly developed method by means of a single-laboratory validation procedure.

Materials and Methods

Maize (Zea mays) and other plant materials

Dry seeds of GM maize, i.e., one progeny each of Bt11 and Event176 developed by Syngenta Seeds AG (Basel, Switzerland), a progeny of TC1507 developed by Dow Agrosciences LLC (Indianapolis, IN, USA), one progeny each of MON810, MON863, GA21, and NK603 developed by Monsanto Company (St. Louis, MO, USA) were provided by their respective developers, and a progeny of T25 developed by Bayer CropScience AG (Monheim am Rhein, Germany) was directly imported from the USA. Dry seeds of QC9651 maize (Quality Technology International, Inc., Elgy, IL, USA) were used as a non-GM control. Dry seeds of Roundup Ready® (RR) soy, and non-GM cereal materials, *i.e.*, dry soybeans harvested in Ohio in 1998, rice (*Oryza sativa*) variety Kinuhikari, wheat (*Triticum aestivum*) variety Haruyutaka, and barley (*Hordeum vulgare*) variety Harrington (these were harvested in Japan) were used as experimental controls.

DNA extraction

Maize and other plant seed materials were ground with a P-14 seed rotor mill (Fritsch GmbH, Ibar-Oberstein, Germany) and used for DNA extraction. DNA extraction was performed with the DNeasy Plant Maxi kit (QIAGEN GmbH, Hilden, Germany) as described in our previous report[12)]. The DNA concentration of solutions was determined by measuring UV absorption at 260 nm, and the quality of DNA solutions was evaluated in terms of the absorption ratios at 260/280 nm and 260/230 nm. DNA solutions whose absorption ratios were between 1.7 and 2.0 at 260/280 nm and were >1.7 at 260/230 nm were used for the subsequent experiments.

Oligonucleotide primers and probes

The primer pairs and TaqMan® probes used in this study are listed in Table 1. The primers were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan) and purified by HPLC. TaqMan probes with a carboxytetramethylrhodamine (TAMRA) quencher and with a Blackhole Quencher (BHQ) were synthesized by Applied Biosystems, Inc. (Foster City, CA, USA) and Biosearch Technologies, Inc. (Novato, CA, USA), respectively. Each oligonucleotide was diluted to the appropriate concentration for real-time PCR assays with sterilized water and stored at $-30°C$ until use.

Qualitative PCR conditions

A 25 μL reaction solution contained 25 ng genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.5 μmol/L of each of the primers, and 0.625 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). The reactions were buffered with PCR buffer II (Applied Biosystems) and amplified in a thermal cycler, the Silver 96-Well GeneAmp PCR System 9700 (Applied Biosystems) in Max mode, according to the following step-cycle program: pre-incubation at 95°C for 10 min; 40 cycles consisting of denaturation at 95°C for 0.5 min, annealing at 60°C for 1 min and extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis buffered with 1×Tris–Acetate–EDTA (TAE) solution.

Real-time PCR condition and calculation of GM contents

In general, the experimental conditions of real-time PCRs followed those of our previous methods[12)], which have been employed as the Japanese official method and the ISO standard method for GM detection[*3–*5]. A

[*5] Foodstuffs—methods of analysis for the detection of genetically modified organisms and derived products—quantitative nucleic acid based methods. Annex of ISO 21570: 2005 (2005).

[*6] http://www.mhlw.go.jp/english/topics/food/pdf/sec01-2.pdf

Table 1. Primers and probes used in this study

Method	Target segment	Name	Sequence (5′→3′)	Length of amplicon	Specificity	Reference
MON810 construct sp.	MON810 construct sp.	M810 2-5′	GATGCCTTCTCCCTAGTGTTGA	113 bp	hsp70 cryIA(b)	(12)
		M810 2-3′	GGATGCACTCGTTGATGTTTG			
	M810-Taq	(12)	FAM-AGATACCAAGCGGCCATGGACAACAA-TAMRA		hsp70–cryIA(b)	(12)
	SSIIb	SSIIb03-5′	CCAATCCTTTGACATCTGCTCC	114 bp	maize SSIIb	(16)
		SSIIb03-3′	GATCAGCTTTGGGTCCGGA		maize SSIIb	(16)
		SSII-Taq	FAM-AGCAAAGTCAGAGCGCTGCAATGCA-TAMRA		maize SSIIb	(16)
GA21 construct sp.	GA21 construct sp.	GA21 3-5′	GAAGCCTCGGCAACGTCA	133 bp	OTP	(12)
		GA21 3-3′	ATCCGGTTGGAAAGCGACTT		m-epsps/anti-sense primer	(12)
		GA21-2-Taq	FAM-AAGGATCCGGTGCATGGCCG-TAMRA		OTP-m-epsps/sense probe	(12)
	SSIIb	SSIIb03-5′	CCAATCCTTTGACATCTGCTCC	114 bp	maize SSIIb	(16)
		SSIIb03-3′	GATCAGCTTTGGGTCCGGA		maize SSIIb	(16)
		SSII-Taq	FAM-AGCAAAGTCAGAGCGCTGCAATGCA-TAMRA		maize SSIIb	(16)
Duplex	P35S	P35S 1-5′	ATTGATGTGATATCTCCACTGACGT	101 bp	CaMV 35S promoter	(12)
		P35S 1-3′	CCTCTCCAAATGAAATGAACTTCCT		CaMV 35S promoter	(12)
		P35S-TaqFB	FAM-CCCACTATCCTTCGCAAGACCCTTCCT-BHQ1		CaMV 35S promoter	in this study
	GA21 event sp.	GA21esp 5′-1	TGGGACCTTATCGTTATGCTATTTG	111 bp	maize genome	(13)
		GA21esp 3′-1	CGATCCTCCTCGCGTTTCC		rice *ACT1* 5′-UTR[a]	(13)
		GA21es-TaqHB	HEX-CCGGACCCACCTGCTGTTGAGAAAG-BHQ1		maize genome/ rice *ACT1* 5′-UTR[a]	in this study
	SSIIb	SSIIb03-5′	CCAATCCTTTGACATCTGCTCC	114 bp	maize SSIIb	(16)
		SSIIb03-3′	GATCAGCTTTGGGTCCGGA		maize SSIIb	(16)
		SSII-TaqFB	FAM-AGCAAAGTCAGAGCGCTGCAATGCA-BHQ1		maize SSIIb	in this study

[a] Rice *ACT1* 5′-untranscripted region (UTR) sequence contains the promoter and first exon of rice *ACTIN1* gene.