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RIP and RALyase cleave the sarcin/ricin domain, a critical domain for ribosome function, during senescence of wheat coleoptiles

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ABSTRACT

Type-I ribosome-inactivating protein (RIP), which is found in many plants, catalyzes depurination of a specific adenine in the sarcin/ricin domain (SRD) of the large rRNA causing loss of ribosomal activity. Previously, we found a RNA apurinic site-specific lyase (RALyase) that catalytically cleaved the phosphodiester bond at the RIP-dependent depurination site by β-elimination reaction. Here we show that both the RIP activity and RIP–RALyase-mediated cleavage of SRD in the cytoplasmic ribosome were induced at the late stage of senescence of wheat coleoptiles. Following this process, tissue death was observed. Furthermore, transgenic tobacco plants expressing glucocorticoid-induced RIP developed senescence-like phenotype. Our results suggest that ribosome inactivation due to the cleavage of SRD by the inducible RIP and constitutively expressed RALyase may be a unique plant system that mediates programmed cell death at the late senescent stage.

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Plant senescence is a key developmental event because transport of nutrients, such as carbon and nitrogen, take place from the senescing leaves to the seeds for storage [1], and is thought to accompany programmed cell death [2]. Accordingly, many nutrient metabolism-related genes have been found to be expressed during the senescence stage [3]. However, the mechanism of the programmed cell death in senescence still remains elusive.

RIPs are classified into two broad groups: type-1 is a single-chain polypeptide and type-2 is a two-chain polypeptide, such as found in *Escherichia coli* O-157 vero toxin and ricin [4,5]. Recently, Stripe [6] proposed a third type (type-3) of RIP, which contained a polypeptide with unknown activity. The molecular basis of the RIP-mediated inactivation of ribosome involves hydrolysis of the *N*-glycosidic bond between the base and the ribose at position A4324 in the 28S rRNA of rat (reviewed in [7]). The hydrolysis site is embedded in a purine-rich single-stranded segment of 14 nucleotides and is nearly universal. This conserved sequence is called sarcin/ricin domain (SRD). Although over-expression of the type-2 RIP is known to kill animal cells [7], it is not known whether the type-1 RIP, found in many plant species [8], kills the host cells.

We have previously reported that by removing the translational factors, including RIP, we were able to prepare a highly efficient and robust cell-free protein synthesis system from the wheat embryos [9,10], and demonstrated that this system was active for more than 14 days with the regular supply of substrates. Thus, it seems that the endogenous translational machinery is very robust.

RNA apurinic site-specific lyase (RALyase), found in the wheat germ extract [11] and rice plant [12], cleaves the phosphodiester bond at the RIP-dependent depurination site by a catalytic β-elimination reaction [11]. Thus, both RIP and RALyase cleave the SRD in the large rRNA. We have previously reported that the RIP–RALyase treatment inhibited the protein synthesis activity of the ribosome more strongly than the RIP treatment alone, which depurinated the rRNA but did not cleave the ribosome [13]. In mammalian cells, the RIP-induced damage to the 28S rRNA was shown to activate a novel kinase pathway known as ribotoxic stress response [14]. This ribotoxic stress response, which acts through the RNA-*N*-glycosidase, activates the cascades of the SAPK/JNK and p38-MAPK signaling pathways, and subsequently induces apoptosis [15]. These experimental evidences led us to hypothesize that in plants too RIP and/or RALyase could terminate the ribosomal activity when programmed cell death occurs in processes like plant senescence. In order to test our hypothesis, we investigated senescence in wheat coleoptiles and also in inducible RIP-expressing transgenic plants.

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Materials and methods

General. Quantifications of soluble proteins and chlorophyll from 10 coleoptiles, harvested on each successive day after sowing, were done according to a previously published method [16]. Total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction [17]. Agarose and polyacrylamide gel electrophoresis, and primer extensions were performed using standard methods [18]. EtBr-stained gels for RT-PCR and depurination assay were visualized using FAS-II system (Toyobo, Japan). The autoradiograph of the gel was obtained using a BAS-2000 phosphoimager (Fuji Photo Film, Japan).

Plant materials. Wheat (*Triticum aestivum*) seeds were sown overnight at 4 °C and then inoculated on vermiculites. Coleoptiles were grown with 0.1% Hyponex (v/v, Hyponex, Ohio) and water in a growth cabinet at 26 °C and a constant RH (60%) under 16-h photoperiod (220 $\mu E/m^2/s$; FL40SD; NEC, Japan). Hyponex and water treatments were carried out on day 0, and on days 3, 6, 9, and 12 after sowing.

RT-PCR. cDNA, synthesized from 1 μg of total RNA by RevatraAce reverse transcriptase (Toyobo, Japan), was used as a template for a 50-μl PCR reaction mixture according to the supplier's instructions (ExTaq DNA polymerase, Takara Bio, Japan). Amplification of tritin, RALyase and tubulin DNA fragments were carried out using the following sets of oligonucleotide primers: 5′-GCCAGCTCTGCCGACTATGT-3′ and 5′-GAGAGCGACGTTGGTCAGCT-3′ for tritin (GenBank Accession No. D13795); 5′-CAAGTAGGCCACACTGAATG-3′ and 5′-GACATGTCTTGCCTCTCCAC-3′ for RALyase (AB032123); and 5′-TGGTGCTCTGAATGTTGATG-3′ and 5′-TAGTGCGCTTGGTCTTGATC-3′ for α-tubulin (U76558). PCR amplification was performed using an initial denaturation step at 98 °C for 1 min, followed by an appropriate number of cycles (30 cycles for tritin, 29 cycles for RALyase and 21 cycles for tubulin) of the following steps: denaturation at 98 °C for 10 s, annealing at 60 °C for 1 min and extension at 72 °C for 30 s.

Depurination assay. Total protein was prepared from 10 wheat coleoptiles. Coleoptiles were pulverized under liquid nitrogen using a motor and pestle. The frozen tissue powder was extracted with 2 volumes (v/w) of ice-cold buffer (25 mM Tris-HCl, pH 7.6, 50 mM KCl, 25 mM $MgCl_2$, 4 mM DTT, and 50 μM E-64) followed by centrifugations at 30,000*g* for 15 min at 4 °C, and the supernatant was re-centrifuged again at 100,000*g* for 30 min at 4 °C. The supernatant fraction (400 μl) was applied to a DE52 column (200 μl bed volume) and the flow-through was recovered. For the depurination assay, 3 μl of the column flow-through fraction and wheat germ or rat liver ribosome (1 OD unit) were used in a total reaction volume of 25 μl. Other conditions for the assay were same as previously described [4].

Primer extension assay. Primer extension was performed using 1 μg of total RNA and the WSRD-A primer (5′-TAGAGGCGTTCAGTCATAATCC-3′, designed from the wheat 25 S rRNA sequence) in a 45-μl reaction mixture per assay, according to a previously reported method [19]. The SRD DNA fragment was amplified by RT-PCR from the primer extended product using the primers 5′-TGTTCACCCACCAATAGG-3′ and WSRD-A, and the amplified fragment was sequenced using the cycle Sequence kit (Takara Bio, Japan). The products were separated on a 6% polyacrylamide/7 M urea gel.

Dexamethasone (DEX)-inducible transgenic tobacco plant. To generate transgenic tobacco plants over-expressing the tritin gene, a dexamethasone-inducible expression system (pTA7001) was used [20]. Fourteen independent transgenic lines were developed using this tritin expression plasmid construct. T1 plants with single-copy insertions were identified on the basis of 3:1 segregation of hygromycin resistance in T2 seeds. Homozygous progeny from the second generation of these lines were used for all experiments. For induction of tritin expression, three-weeks-old tobacco plants were treated with dexamethasone (DEX), which was sprayed on the plants as described previously [20].

Results and discussion

Wheat coleoptiles as synchronized senescent tissue

Fig. 1 shows the typical morphological and physiological changes occurring during the senescence of wheat coleoptiles. The first day that wheat seeds were sown was labeled as the day 0. As shown in Fig. 1, the coleoptiles elongated until the day 5 and partial discoloration was noticed from the day 12. Finally, the coleoptiles withered on the day 17 (Fig. 1A). Soluble proteins and chlorophyll, widely used as senescence indicators [1] in

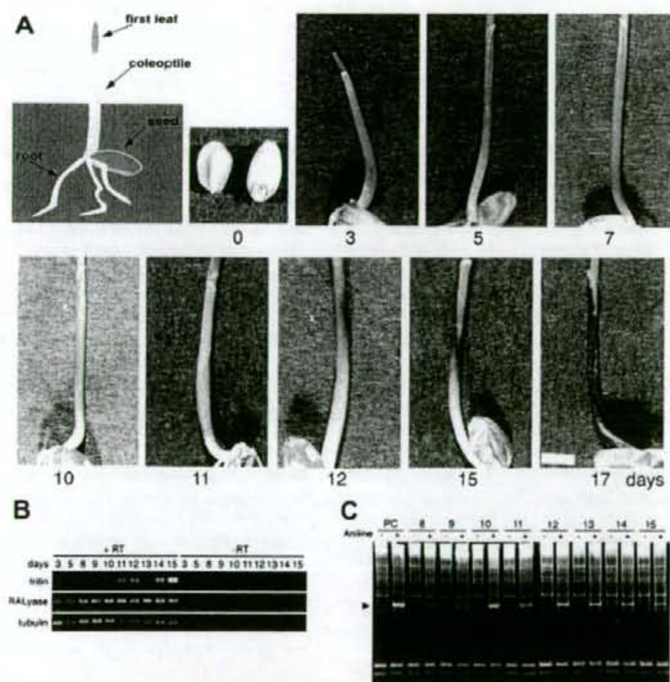

Fig. 1. Induction of RIP mRNA and activity during the senescence of wheat coleoptiles. (A) Sequential photographs showing formation of coleoptiles from sowing to systemic withering. Number under each photograph indicates the number of days from the time of sowing. Bar represents 5 mm. (B) Analysis of relative mRNA contents of tritin, RALyase and tubulin by RT-PCR. Each cDNA was synthesized with (+RT) or without (−RT) reverse transcriptase using the coleoptile total RNA as the template. Sizes of amplified products of tritin, RALyase and tubulin are 336, 239, and 268 bp, respectively. (C) Detection of RIP activity by depurination assay. As positive control (PC), rat liver ribosomes were treated with gypsophilin, RIP isolated from *Gypsophila elegans*. RNA from the reaction mixture was extracted and separated on a 4% polyacrylamide gel with (+) or without (−) aniline treatment. Arrowhead indicates α-fragment. Each number shown indicates the number of days after sowing.

physiological analysis, increased until days 5 and 7, respectively, and then both of them dramatically decreased as the discoloration progressed (data not shown). At the physiological level, senescence related changes appeared in most wheat coleoptiles as early as the day 7, but at the morphological level the senescence related changes were observed from the day 12, and subsequently the phenomenon ended on the day 17 with the death phenotype, suggesting that the changes occurred synchronously in this tissue. In general, biochemical analysis of the senescent leaves in other plant species is not easy to achieve due to non-synchronization, except in rice coleoptiles, which is reported to be synchronized and similar to those of the mature leaves according to the morphology, physiology and anatomy analyses [16]. However, as described above, it is possible to obtain synchronized senescent tissues of wheat coleoptiles for such analysis. Therefore, we used wheat coleoptiles as a model system of senescence.

Induction of RIP gene during the senescence of wheat coleoptiles

Wheat RIP gene, tritin, was previously isolated from the wheat germ [21]. RALyase cDNA was cloned from the coleoptiles [11]. Initially, to investigate whether the RIP and RALyase are expressed during the coleoptile senescence, we used a RT-PCR assay for determining their respective mRNA expression levels, and a depurination assay for the detection of their enzymatic activities. Total RNA from each sample was used for the cDNA synthesis using the random oligoprimers, and the newly synthesized cDNAs were used as templates for the PCR analysis. RT-PCR results showed that the tritin mRNA was slightly detectable on day 10 and its expression dramatically increased from the day 11; in contrast, no change was observed in the RALyase mRNA level (Fig. 1B). Furthermore, the depurination assay demonstrated that the RIP activity was induced from the day 10 (Fig. 1C). These results show that in the senescent coleoptiles, expression of the tritin gene and activity of the tritin gene product are induced from the day 10 at the transcription level. Two days later (on day 12) partial withering of the coleoptile was observed at the morphological level (Fig. 1A). In contrast, RALyase mRNA appeared to be constitutively expressed during the senescence. In this regard, we could not find any information on the expression of RIP and RALyase genes during the senescence stage in the Gene Expression Omnibus (GEO) database.

Detection of SRD cleavage

Next, we evaluated whether the senescence-induced RIP inactivates the endogenous ribosome by depurination of the specific adenine in the SRD, and also whether the RALyase over-kills the RIP-treated ribosomes. For this purpose, we carried out the primer extension assay using the total RNA isolated from each stage of the coleoptile development. We used the avian myeloblastosis virus reverse transcriptase (AMV-RT), which is known to insert an extra deoxyribonucleotide at a non-coding abasic site (such as an apurinic site, like the "A-rule" of the *Taq* DNA polymerase) and then terminates the extension [22]. The primer extension products obtained as a result of various treatments are shown in the bottom panel of Fig. 2A and the results are schematically shown on the top panel of Fig. 2A. RIP treatment caused depurination of the A residue at the SRD site of the rRNA (underlined A residue in the nucleotide sequence shown in the top panel, Fig. 2A), and the extension product from the RIP-treated ribosome (lane i, Fig. 2A) was longer than that from the RIP–RALyase treated ribosome (lane ii). Sequence of the 5′ terminus end of the α-fragment produced by RIP–RALyase treatment (lane iii) was pGAGGAA···3′OH, which was same as that of the α-fragment produced by the RIP–aniline treatment (lane ii). Sequences of the reverse strands of the RIP-treated

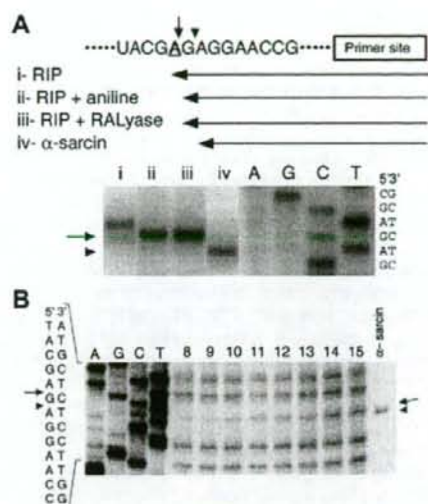

Fig. 2. Detection of RIP and RALyase mediated cleavage of SRD during the senescence of wheat coleoptiles. (A) rRNAs were isolated from the wheat ribosomes treated with RIP (lane i), RIP/aniline (lane ii), RIP–RALyase (lane iii), and α-sarcin (lane iv). The extension products shown are 97, 96, 96 and 95 nt long, respectively (schematically shown using long arrows of different lengths on the top portion of the figure). The underlined A in the partial nucleotide sequence of the rRNA shown on the top is de-adenylated by RIP. RIP–aniline and RIP–RALyase treatment cleaved at the same SRD site (small arrow). α-Sarcin treatment produced a product that was one-nucleotide shorter than the RIP/RALyase cleaved product (arrowhead). (B) Primer extension analysis of the cleavage of the SRD site in the cytoplasmic ribosome. Lanes A, G, C, and T show the nucleotide sequence of the SRD site of the wheat 25S rRNA. Lanes 8–15 indicate days from the time of sowing.

and RIP–RALyase treated fragments were 5′···CCTCT3′OH and 5′···CCTC3′OH, respectively, suggesting that the AMV-RT inserted an extra residue at the abasic site of the depurinated rRNA. Thus, when the RIP-treated ribosome was treated with aniline or the RIP–RALyase cleaved at the depurination site of the 25S rRNA (5′···GA↓GAGGA···, depurinated A is underlined; ↓ indicates the cleavage site), the extension product from the RIP–RALyase treated ribosome is one nucleotide shorter than that of the RIP treated ribosome. Since α-sarcin, which was used in this study as a control, cleaves the 3′-phosphodiester bond of the G residue one nucleotide downstream from a RIP–RALyase-recognized A residue (5′···GAG↓AGGA···, ↓ indicates the cleavage site) [23], the cleavage product is one nucleotide shorter than the RIP + RALyase treated ribosome (lane iii in Fig. 2A). Thus, the use of AMV-RT for reverse transcription enabled us to determine whether the endogenous ribosomes are catalytically depurinated by RIP or cleaved by RIP–RALyase.

RIP and RALyase mediated cleavage of the ribosome SRD during senescence of wheat coleoptiles

To examine the damage in the endogenous ribosome, we performed the primer extension assay by first using a cytoplasmic rRNA specific primer. Our results using the coleoptile total RNA as template revealed ribosome damage occurring from the day 10, which is when the RIP expression was induced and subsequently, the expression of the damaged ribosome increased until the day 14 (lanes 8–15 in Fig. 2B). Sequence of the 3′ terminus end of the fragment was 5′···CCTC3′, indicating that it was generated as a result of catalytic cleavage by both RIP and RALyase (lane iii in Fig. 2A, and lanes 10–15 in Fig. 2B). Interestingly, the one base extended product generated by RIP catalysis alone was not

detected at all. This result suggests that the depurinated ribosome by RIP is immediately cleaved by the RALyase. In addition, although specific primers for the mitochondrial and chloroplast rRNAs were used, their extension products could not be detected (data not shown). Taken together, these results suggest that at least the endogenous cytoplasmic ribosome is inactivated by the senescence-inducible RIP, which is then simultaneously cleaved by the constitutively expressed RALyase. Thus, the RIP and RALyase mediated cleavage of SRD seems to completely inactivate the ribosome. Calculations made using the intensity of the α-sarcin band (lane α-sarcin) as control revealed that approximately 10% of the rRNA was damaged in the 13-day-old coleoptiles. It was previously shown that inactivation of 7% ribosomes strongly inhibited protein synthesis, and inactivation of 24% ribosomes completely inhibited protein synthesis [9]. In general, even with 10% damaged ribosomes, it is extremely difficult to continue protein synthesis for sustaining the cell's activity. Thus, our results suggest strong inhibition of protein synthesis in the senescing coleoptiles. Furthermore, morphological examinations of the coleoptiles showed partial withering on the day 12, which is the day when the ribosome damage was induced by the RIP–RALyase, and subsequently the withering progressed systematically (see Fig. 1A and B). It is widely known that the inactivation of ribosome by RIP and antibiotics, such as hygromycin and kanamycin, arrest protein synthesis, which is then followed by cell death [23]. Previously, we have shown that specific cleavage by RIP–RALyase inhibited ribosome's protein synthesis activity more strongly than the un-cleaved and depurinated ribosome, which was produced as a result of RIP treatment [13]. Results described above suggest that during the senescence process protein synthesis in the cell is strongly inhibited by the senescence-induced expression of RIP and the constitutively-expressed RALyase, leading to cell death.

Development of a senescence-like phonotype by inducible RIP in transgenic tobacco plant

Our results described above suggested that the induction of the RIP activity, but not that of the RALyase activity, could be one of the key steps in the development of senescence in wheat coleoptiles. To confirm this hypothesis, we created transgenic tobacco plants having RIP and RALyase expression under the control of a glucocorticoid-inducible promoter. Two-weeks-old seedlings were treated with DEX for the induction of the RIP and RALyase genes. After a week, seedlings in which the expression of RIP was induced by DEX treatment became discolored like senescent leaves (right panel in Fig. 3A) and the chlorophyll content was dramatically decreased (less than 10%). DEX-induced expression of RIP in the transgenic tobacco leaf was monitored by PCR analysis (Fig. 3B). In contrast, leaf discoloration was not observed in the seedlings in which the expression of RALyase was induced by DEX treatment (data not shown). Furthermore, leaf discoloration (Fig. 3A) and RIP gene expression (Fig. 3B) were also not observed in wild-type tobacco plants treated with DEX. These results suggest that the expression of RIP is a key step for the induction of the senescence-like phenotype in tobacco plants. In this context it is noteworthy that the RIP activity was reported to be induced in stressed leaves, including senescent leaves, of several plant species [24,25]. These findings support the idea that induction of the RIP activity followed by the RALyase-mediated inactivation of the endogenous ribosome may be commonly found in the senescing plants.

The translational machinery was originally thought to be very robust [9,10]. Therefore, the ribosome is considered one of the most important targets for inducing cell death or designing a cell suicide system. The fact that the ribosome is a popular target for antibiotics, emphasizes a central role for ribosome in cell metabolism. In this context it is noteworthy that RIP is known as a pathogen resistance gene, specially in blocking virus infection in plants [26]. This resistance may be thought to be one of the systems by which plants inhibit spreading of virus as a result of RIP–RALyase-mediated inactivation of ribosome.

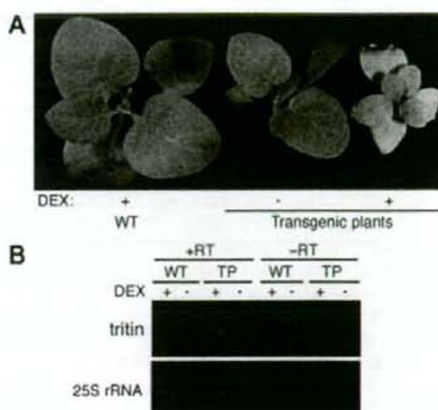

Fig. 3. Development of a senescence-like phenotype by inducible RIP in transgenic tobacco plant. (A) Photographs of dexamethasone (DEX)-treated wild-type (WT) and transgenic tobacco plants (+) and untreated control transgenic plant (−). (B) Detection of tritin mRNA expression by RT-PCR after DEX treatment. cDNAs were synthesized with (+RT) or without (−RT) reverse transcriptase using the total RNA of the transgenic (TP) and wild-type (WT) tobacco plants as templates. 25S rRNA was used as control.

Our results seem to support the idea that senescence is a developmental program, rather than a simple salvage or gratuitous program, involving ribosome inactivation by RIP and RALyase. In mammalian cells, damage to the 28S rRNA by RIPs resulted in the activation of a novel kinase pathway called ribotoxic stress response [14]. This ribotoxic stress response, mediated by the RNA-*N*-glycosidase, activates the SAPK/JNK and p38-MAPK signaling pathway cascades, and subsequently induces apoptosis [15]. Plants, like the mammalian cells, may recognize the 28S rRNA damage as the ribotoxic stress response during the senescence.

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A set of ligation-independent in vitro translation vectors for eukaryotic protein production

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Abstract

Background: The last decade has brought the renaissance of protein studies and accelerated the development of high-throughput methods in all aspects of proteomics. Presently, most protein synthesis systems exploit the capacity of living cells to translate proteins, but their application is limited by several factors. A more flexible alternative protein production method is the cell-free in vitro protein translation. Currently available in vitro translation systems are suitable for high-throughput robotic protein production, fulfilling the requirements of proteomics studies. Wheat germ extract based in vitro translation system is likely the most promising method, since numerous eukaryotic proteins can be cost-efficiently synthesized in their native folded form. Although currently available vectors for wheat embryo in vitro translation systems ensure high productivity, they do not meet the requirements of state-of-the-art proteomics. Target genes have to be inserted using restriction endonucleases and the plasmids do not encode cleavable affinity purification tags.

Results: We designed four ligation independent cloning (LIC) vectors for wheat germ extract based in vitro protein translation. In these constructs, the RNA transcription is driven by T7 or SP6 phage polymerase and two TEV protease cleavable affinity tags can be added to aid protein purification. To evaluate our improved vectors, a plant mitogen activated protein kinase was cloned in all four constructs. Purification of this eukaryotic protein kinase demonstrated that all constructs functioned as intended: insertion of PCR fragment by LIC worked efficiently, affinity purification of translated proteins by GST-Sepharose or MagneHis particles resulted in high purity kinase, and the affinity tags could efficiently be removed under different reaction conditions. Furthermore, high in vitro kinase activity testified of proper folding of the purified protein.

Conclusion: Four newly designed in vitro translation vectors have been constructed which allow fast and parallel cloning and protein purification, thus representing useful molecular tools for high-throughput production of eukaryotic proteins.

Background

In the last decade, attention focused on functionality and structure of proteins. Accelerated proteomics studies demand high-throughput protein production methods to ensure availability of proteins of interest. Presently, overexpression in *E. coli* cells is the most preferred protein production method. Though this system has been well optimized and is suitable for the simultaneous generation of a panel of proteins, its application is often limited by the insolubility of synthesized eukaryotic proteins [1]. Although different *E. coli* strains and various protein and peptide fusion partners have been developed to increase the solubility of heterologous proteins, these methods are not universal and have to be optimized individually for efficient protein production [2].

Recently, in vitro protein translation has emerged as an alternative to cell-based protein synthesis methods. The robustness of the translation apparatus is known since the fifties, and latest technical improvements made to cell-free translation resulted in protein production methods that approach the efficiency of cell-based systems [3]. Various sources of translation machinery can be used for cell-free in vitro translation systems, but -due to its low cost and capacity for synthesizing properly folded, high molecular weight eukaryotic proteins- wheat germ derived protein extract presently seems the most promising choice [4].

Unlike prokaryotic mRNA, eukaryotic mRNA has to be extensively modified to be an effective translation template. The 5'-cap is essential to translation initiation and has to be introduced to in vitro transcribed mRNAs using RNA polymerase, which incorporates the three modified nucleotides (7-mG-5_-ppp-5_G). The efficiency of incorporation is low, and the excess of free modified nucleotides remaining in the mix dramatically decreases the productivity of translation. The 3'-end poly(A) tail of eukaryotic mRNAs also presents a technical difficulty during in vitro translation template preparation, as long polyA/T sequences of plasmids are unstable in host cells. To solve these problems, wheat germ in vitro translation vectors have been constructed with a special sequence replacing the cap. In the optimized vectors, the cap structure is substituted by either the tobacco mosaic virus translational enhancer Ω sequence with an additional GAA triplet at the 5'-end (GAA Ω) [5], or an artificial 73 nucleotides containing a leader sequence [6]. The same laboratory also examined the requirements for a poly(A) tail, and found that translation did not depend on the sequence but only on the length of 3'-UTR. An additional benefit of these plasmids is that the produced mRNAs were effective in vitro translation templates in a wider range of concentration than in vitro capped mRNAs.

Although the optimized vectors improved the productivity of in vitro translation, in order to build high-throughput protein synthesis systems, every step of the procedure must be accelerated, including the cloning of target genes and the purification of translated proteins. Ligation independent cloning (LIC) was developed to facilitate complex cloning and subcloning strategies [7], and have been applied by many laboratories since then. LIC overcomes important limitations of traditional cloning technologies, since any PCR product can be cloned into LIC compatible vectors without using restriction endonucleases and ligation. The LIC method takes advantage of the 3' exonuclease activity of T4 DNA polymerase to create complementary 12- to 15- nucleotide overhangs in the vector and PCR product. Upon transformation into *E. coli* cells, the host repair enzymes ligate at the vector-insert junction; thus, LIC produces high cloning efficiency with minimal non-recombinant background [8].

A serious bottleneck of high-throughput protein production is the fast and high level purification of target proteins. Generally, the purification step is facilitated by addition of affinity tags to the N- or C- terminus of synthesized proteins. Although the affinity tags aid the purification, it might in many cases alter the in vivo function and structure of proteins; hence, it must be removed by site specific proteases. The Tobacco Etch Virus (TEV) protease is an ideal choice because it cleaves with high specificity at a seven-amino-acid recognition sequence [9]. Furthermore, it is active under a wide range of conditions, such as low temperature and high ionic concentration, and is only mildly sensitive to many protease inhibitors which are used to prevent protein degradation by host proteases.

We have improved two commercial vectors for wheat germ in vitro protein translation to generate LIC plasmids incorporating a TEV cleavable affinity tag. The modified vectors encode a leader sequence consisting of either a GST or a His affinity purification tag, followed by a TEV protease recognition site. Experiments with the modified vectors showed that they functioned effectively in all aspects, including cloning, translation, purification and cleavage. Furthermore, we demonstrated that a protein kinase purified from a wheat germ in vitro translation reaction possessed higher in vitro kinase activity than the same kinase produced by overexpression in *E. coli*. These features make the modified vectors suitable for high-throughput production of properly folded eukaryotic proteins.

Results and Discussion

Construction and characterization of LIC vectors

mRNA templates of commercial in vitro translation systems are produced by either T7 or SP6 bacteriophage RNA polymerases, therefore we modified the pEU3N-II and

pEU01 vectors which harbor T7 and SP6 promoters, respectively. The LIC vectors with cleavable affinity tags were created by inserting an oligonucleotide cassette or PCR-generated fragment into the multicloning site of host vectors. Sequencing of the constructed pEU3-NII-HLIC, pEU3-NII-GLIC, pEU-E01-HLIC, pEU-E01-GLIC vectors showed that the DNA fragments had been correctly introduced. Inserts in HLIC vectors encode an amino acid sequence consisting of an N-terminal methionine followed by a six-histidine affinity tag and the ENLYFQS TEV recognition site. The PCR fragment introduced into GLIC constructs possesses the same components except the six histidines are replaced by Glutathione-S-Transferase protein (Figure 1). The extensively used Gateway system shows limitations for protein production since the fusion partners and amino acids encoded by the recombination site cannot be removed without inserting protease specific motif coding nucleotides downstream of the recombination site, and non-native amino acids can interfere with the structure and functionality of purified proteins [10]. To obviate this drawback, an *SspI* restriction endonuclease site involving LIC was inserted in our vector constructs. This rational design places the TEV protease site in proximity of the native protein and allows removal of the affinity tag, leaving only three extra amino acid residues in N-terminal [11].

The created vectors were tested by generating constructs encoding a plant mitogen activated protein kinase, AtMPK6. The vectors were cleaved in the middle of the LIC site by *SspI* digestion, and the linearized plamids were incubated with T4 DNA polymerase in presence of dGTP. Due to the 3'-5' exonuclease activity of T4 DNA polymerase, this protocol generated 15-base long single-stranded overhangs. To insert the target gene, the *AtMPK6* specific primers were designed with a 5'-end extension complementary to the LIC site, and the PCR products were treated with T4 DNA polymerase in presence of dCTP. The generated complementary overhangs allowed introduction of the target gene into the vectors treated with T4 DNA polymerase using a simple annealing step, without use of any other enzyme. The reaction mixture was directly transformed into competent cells, and colony PCR analysis showed than more than 90% of tested colonies carried the target gene, proving the LIC procedure to be very effective.

In vitro *translation of a mitogen activated protein kinase*

In order to test the protein synthesizing capacity of constructed vectors, two commercial wheat germ in vitro protein translation kits were used. These companies use different approaches to extend the lifetime and thus the productivity of in vitro translation reactions. The continuous supply of feeding solution is provided by diffusion through either a dialysis membrane [12] or simply the phase of different density solution [13], and they require

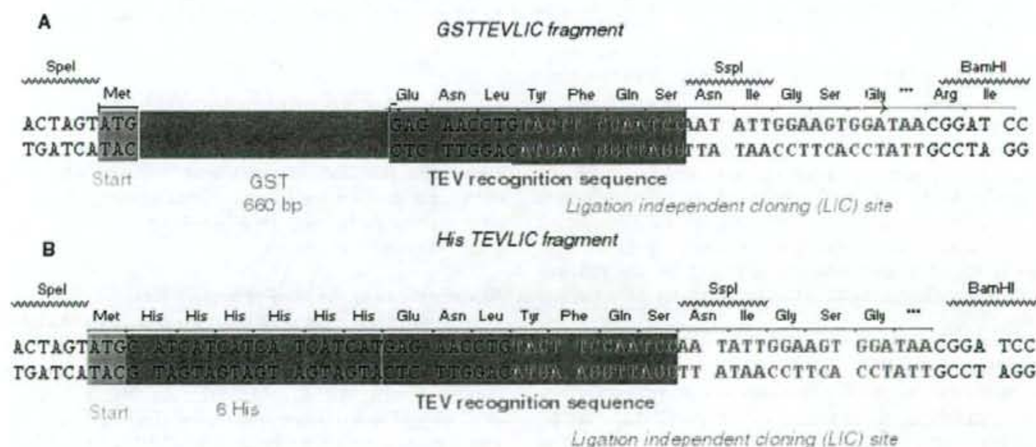

Figure 1
Nucleotide sequence of inserted cassettes.

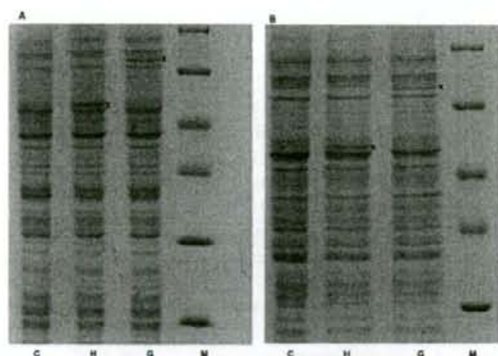

Figure 2

SDS-PAGE analysis of in vitro translated proteins. A. CEFC translation reactions with pEU3-NII backbone vector constructs: Molecular weight marker (M), wheat germ extract control (C), 1 μl out of 50 μl in vitro translation reaction mixture with pEU3-NII-HLIC (H) or pEU3-NII-GLIC (G) vector. **B.** Bilayer translation reactions with pEU-E01 backbone vector constructs: Molecular weight marker (M), wheat germ extract (C), 5 μl out of 225 μl in vitro translation reaction mixture with pEU-E01-HLIC (H) or pEU-E01-GLIC (G) vector. Proteins present in the translation mixtures were separated on 12% SDS-PAGE gel and detected by Coomassie Blue staining. Vector encoded-kinases are indicated by asterisks.

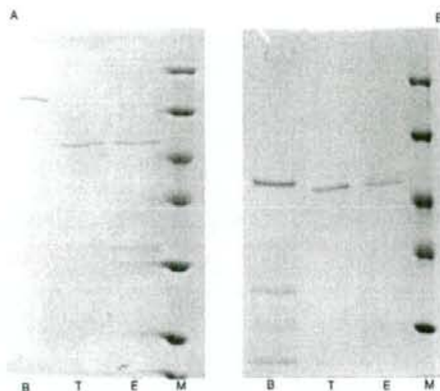

Figure 3

Affinity purification and cleavage of translated proteins. A. Purification and cleavage of GSTAtMPK6: Molecular weight marker (M), Glutathione-Sepharose coupled kinase (B), AtMPK6 cleaved from the beads (T), AtMPK6 cleaved following elution (E). **B.** Purification and cleavage of HisAtMPK6: Molecular weight marker (M), MagneHis particle coupled kinase (B), AtMPK6 cleaved from the beads (T), AtMPK6 cleaved following elution (E). Proteins were translated by CEFC and bilayer method, respectively. A quarter of the total amount of purified protein was separated on 12% SDS-PAGE gel and detected by Coomassie Blue staining.

T7 and SP6 RNA polymerase, respectively. The DNA templates were purified from *AtMPK6* comprising constructs with a commercial plasmid DNA isolation kit. The translation reactions were carried out according to protocols suggested by manufacturers. Polyacrylamide gel electrophoresis analysis of total protein samples demonstrated that all four vector encoded proteins with their expected size (Figure 2). The in vitro translated target proteins were detectable with Coomassie Blue staining, and the yields of different constructs and translation mixtures were comparable.

Purification and TEV cleavage of translated proteins

To further verify the functionality of the created vectors, the synthesized proteins were affinity purified and cut by TEV protease. The GST- and His-tagged in vitro translation products were separated by batch incubation with Glutathione Sepharose and MagneHis particles, respectively. According to PAGE analysis, highly purified proteins were obtained within one hour of incubation for both affinity purification protocols (Figure 3). The TEV protease cleavage site was tested under different conditions. In the first case, the purified proteins were eluted with appropriate buffers and digested with His-tagged TEV protease. In the second case, the kinases were cut directly by TEV on the beads, without elution (Figure 3). The results demonstrated that the TEV protease worked effectively on both constructs under a wide range of conditions, since they were cleaved completely in different buffers, either coupled to beads or in solution.

Kinase activity of the purified protein kinase

Previously, it has been demonstrated that 207 out of 439 wheat germ in vitro translated plant protein kinases displayed autophosphorylation activity [14]. In order to compare the in vitro kinase activities of proteins produced in *E.coli* and in a cell-free system, the His tagged *AtMPK6* cassette was transferred from pEU3-NII-HLIC into pET11a expression vector. The kinase was isolated by metal chelate affinity chromatography either from *E. coli* or from the in vitro translation reaction mixture. Equal amounts of His-*AtMPK6* were used to determine the in vitro kinase activities of overexpressed and translated proteins using myelin basic protein (MBP) as a substrate. According to autoradiography results, phospholabeling of MBP was

hardly detectable when bacterially overexpressed His-AtMPK6 was tested, while the in vitro translated protein kinase displayed a clearly visible activity (Figure 4).

The high kinase activity of translated AtMPK6 indicates proper folding of the kinase domain, although posttranslational modification(s) of the translated protein are also likely to be responsible for elevated kinase activity; indeed, proper phosphorylation of MAP kinases is essential to gain their full kinase activity and kinases present in wheat germ extract could perform these phosphorylations.

Conclusion

We have constructed a set of four vectors to facilitate the cloning and purification steps of wheat germ extract protein in vitro translation systems. The presented vectors eliminate the traditional cloning steps and aid the purification of translated proteins by incorporation of a LIC site and a TEV cleavable affinity tag, respectively. Purification of a plant mitogen activated protein kinase demonstrated that the vectors functioned as intended. Furthermore, proper folding of the purified protein was indicated by high in vitro kinase activity. We have successfully used our vectors for purification of proteins with different sizes from various organisms, too (data not shown). In summary, these vectors allow fast and parallel cloning and protein purification, and hence represent useful molecular tools for in vitro translation of eukaryotic proteins.

Figure 4
In vitro kinase activity of translated versus overexpressed AtMPK6. MBP phosphorylation activity of *E. coli* overexpressed (E) and translated (T) *AtMPK6* was detected by autoradiography (A). Coomassie Blue staining shows equal loading of MBP (C). *AtMPK6* was either produced in *E. coli* or translated by bilayer method, and 100 ng of purified kinase was used in kinase reactions.

Methods

Vector construction

The vectors pEU3-NII (Invitrotech) and pEU-E01 (Cell-Free Sciences) were used as backbones for plasmid construction. In order to remove the intrinsic *SspI* restriction endonuclease sites in pEU3-NII and pEU-E01, the vectors were mutated using Gene Tailor kit (Invitrogen) according to the provided manual with the 5'-ACTCTTCCTTTT-TCAATGTTATGAAGCA-3' and 5'-TGAAAAAGGAAGAG-TATGAGTATTCA-3';

5'-CTTCCTTTTTCAATGTTATTGAAGCATTTATCAGG-3' and 5'-CCTGATAAATGCTTCAATAACATTGAAAAAG-GAAG-3' primers, respectively.

The mutated vectors were further manipulated to produce four different constructs.

pEU3-NII-GLIC, pEU-E01-GLIC

The LIC site combined GST fragment was generated by PCR. pGEX-2T (GE Healthcare) was used as template with primers 5'-ACTAGTATGTCCCCTATACTAGGTT-3'and 5'-GGATCCGTTATCCACTTCCAATATTGGATTGGAAGTAC AGGTTCTCATCCGATTTTGGAGGATGGTC-3'. The PCR product was digested with *BamHI* and ligated into the *EcoRV-BamHI* digested pEU-3-NII *SspI-* vector, and transformed into DH10B competent cells. Ampicillin-resistant colonies were selected, and the purified plasmids were sequenced to confirm the PCR accuracy. The pEU-E01-GLIC plasmid was constructed by transferring GSTTEVLIC fragment from pEU3-NII-GLIC into pEU-E01*SspI-* vector. The fragment was obtained by *SpeI-BamHI* digestion and gel purification. The isolated fragment was inserted into pEU-E01*SspI-* vector treated with the same restrction endonucleases.

pEU3-NII-HLIC, pEU-E01-HLIC

The synthetic oligonucleotides

5'-TACTAGTATGCATCATCATCATCATCATGAGAACCTG-TACTTCCAAT

CCAATATTGGAAGTGGATAACGGATCCA-3'and

5'-TGGATCCGTTATCCACTTCCAATATTGGATTGGAAG-TACA

GGTTCTCATGATGATGATGATGATGCATACTAGTA-3'

encoding HisTEVLIC fragment were mixed, heated at 95°C for 30 sec, then incubated at 60°C for 3 minutes. The annealed, double stranded DNA was cut with *SpeI* and *BamHI*, and phenol-chloroform treatment of digested fragment was followed by ethanol precipitation. Finally, the fragment was ligated into *SpeI-BamHI* digested *SspI*-backbone vectors, and transformed into DH10B competent cells. The HisTEVLIC cassette comprising constructs were selected by colony PCR with primers specific for the backbone vectors:

pEUE01 forward: CGATTTAGGTGACACTATAGAACTC

pEU3-NII forward: CACTATAGGGTACACGGAATTCGC

pEU rev: TATAGGAAGGCCGGATAAGACG

Ligation independent cloning

The following primers were designed to amplify *AtMPK6* and append the sequences required for LIC:

5'-TACTTCCAATCCAATGCA*ATGGACGGTGGTTCAGGT*-3' and

5'-TTATCCACTTCCAATG*TGTTTGAACGATCTGCAGTCA*-3' (gene specific sequences are indicated in italic). A vector construct comprising C-terminal HA-tagged *AtMPK6* [15] was used as template. The resultant PCR product was cleaned up by PEG precipitation protocol [16].

20 μg of pEU-LIC vectors were hydrolyzed by 100 U SspI (Fermentas) for 2 hours at 37°C separated on agarose gel, and purified with QUIAquick Gel Extraction Kit (Quiagen). The cohesive ends were generated by T4 DNA polymerase (Promega) treatment. Briefly, 1 μg linearized vector or PCR product was incubated for 10 minutes at 37°C with 1 U enzyme in presence of provided buffer and 1 mM dGTP or dCTP, respectively. The reaction was stopped by heat inactivation for 20 minutes at 75°C. 60 ng vector and 30 ng PCR fragment resulting from T4 DNA polymerase treatment were mixed and adjusted to 12.5 μl final volume with 5 mM EDTA concentration. The annealing mixture was incubated at RT for 20 minutes. The annealing mix was directly transformed into competent cells.

Protein in vitro translation

2 μg of pEU-LIC*At*MPK6 constructs were used for mRNA synthesis, and the translation reactions were carried out according to the CEFC or bilayer protocol using commercial kits. In case of pEU3-NII backbone vector constructs, transcription and translation were done simultaneously at 23°C for 20 hours using RTS 100 WG CECF Kit (Roche). Constructs originating from pEU-E01 vector were transcribed by SP6 RNA polymerase, and half of the mRNA was added to the translation mixture of ENDEXT® Wheat Germ Expression S Kit (CellFree Sciences). Transcription and translation were carried out as described in manual, and protein translation took place at 23°C for 20 hours.

His affinity purification

The bilayer translation reaction mixture was incubated with 10 μl of MagneHis Protein Purification System (Promega) particles at 4°C for 30 minutes in 250 μl translation buffer completed with NaCl to 500 mM final concentration. After binding, the beads were washed five times with buffer containing 20 mM Tris, 10 mM imidazole, 500 mM NaCl, pH 7.5. The coupled His*At*MPK6 protein was removed from the beads either by elution with 20 mM Tris, 500 mM imidazole pH 7.5 buffer, or by cleavage with TEV protease treatment. TEV protease was purified essentially following a previously published protocol [17]. In order to directly digest kinase coupled to the beads, 2.5 μl beads were incubated with 1 μM TEV protease in washing buffer at 4°C, overnight. The eluted proteins were cleaved in elution buffer under the same condition.

GST affinity purification

The translation reaction mixture was incubated with 10 μl Glutathione Sepharose 4B at 4°C for 1 hour in translation buffer, and the resin was washed four times with PBS to expel unspecific proteins. GST*At*MPK6 was eluted either by 20 minutes incubation in 50 mM Tris, 20 mM Reduced Glutathione pH 8.0 at 4°C, or cleaved on beads by TEV protease. TEV protease treatments were performed as described above.

Production of AtMPK6 by bacterial overexpression

In order to compare the kinase activities of *E. coli* produced *At*MPK6 and in vitro translated *At*MPK6, the HisTEVLICMPK6 cassette was cloned into pET11a bacterial overexpression vector. Briefly, pEU-E01-HLICMPK6 was digested with *SpeI* and *BamHI*, and the resulting fragment was inserted into pET11a vector (Novagen) hydrolyzed with *NheI* and *BamHI* restriction endonucleases. The vector construct was transformed into *E. coli* BL21 (DE3) strain, and the cells were induced according to previously published protocol [18]. Briefly, the cells were grown at 37°C in 500 ml LB containing 100 μg/ml ampicillin until OD600 = 0.5, and incubated further 20 hours at 20°C with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacterial cells were collected by centrifugation at 4500 rpm for 20 minutes, resuspended in buffer containing 20 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8.0 and disrupted by sonication in the presence of 1 mM PMSF. Following centrifugation at 13000 rpm for 30 minutes the supernatant was mixed with 80 μl MagneHis particles for 30 minutes. Unspecific proteins were removed by washing the beads five times with lysis buffer,

and the bound protein was eluted with 80 µl of 20 mM Tris, 500 mM imidazole pH 7.5.

Kinase assay

100 ng in vitro translated or *E. coli* overexpressed *At*MPK6 protein was added to 20 µl kinase assay mixture (25 mM Tris, 1 mM EGTA, 1 mM DTT, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 20 µM ATP, 1 mg/ml Myelin Basic Protein, 5 µCi [γ-^{32}P]ATP, pH 7.5) [19]. The reaction mixture was incubated at room temperature for 30 minutes, then stopped by addition of 5 × Laemmli SDS buffer. The samples were separated on 15% SDS-PAGE gel and analysed by autoradiography.

Authors' contributions

VB and VG implemented the cloning, expression and translation studies, and participated in the design of inserted cassettes. TS and YE designed the original vectors and helped the translation studies with essential advices. TM conceived of studies, carried out the kinase assays and drafted the manuscript. All authors read and approved the final manuscript.

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