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′-TAGTGCGCTTGGTCTT GATG-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′-TGTTCACCCACCAA TAGG-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

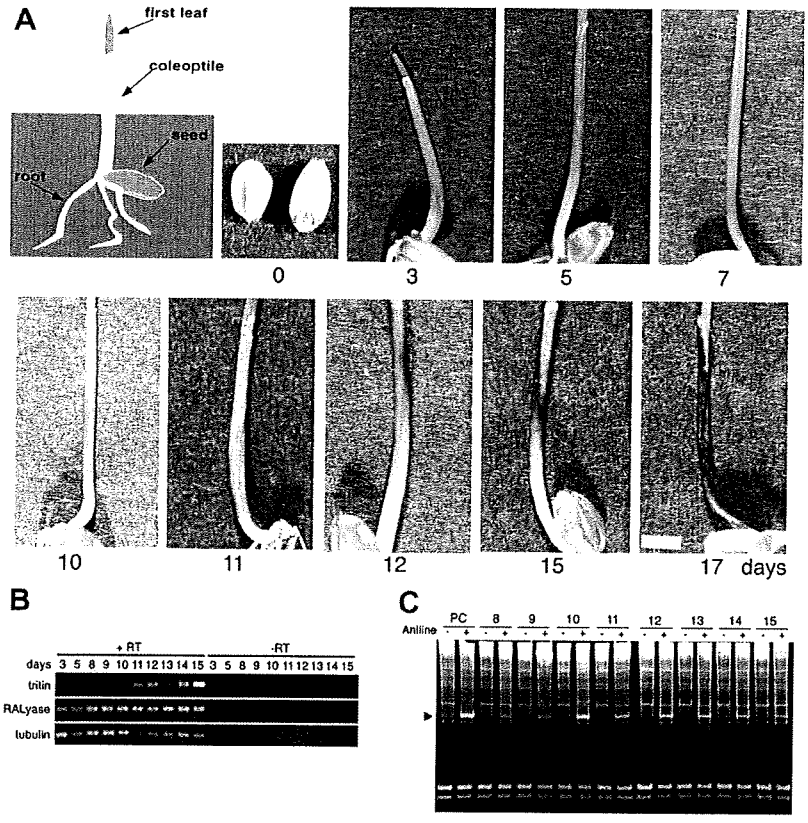

Fig. 1. Induction of RIP mRNA and activity during the senescence of wheat coleoptiles. (A) Sequential photographs showing formation of coleoptiles from sowing to systemic withering. Number under each photograph indicates the number of days from the time of sowing. Bar represents 5 mm. (B) Analysis of 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 *Gypsophiia 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

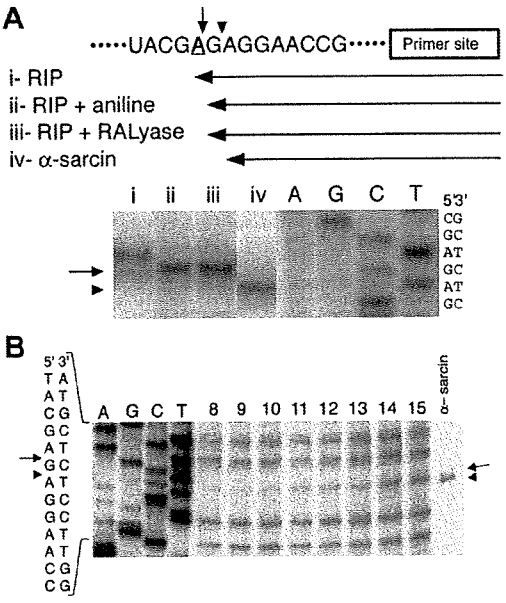

Fig. 2. Detection of RIP and RALyase mediated cleavage of SRD during the senescence of wheat coleoptiles. (A) rRNAs were isolated from the wheat ribosomes treated with RIP (lane i), RIP/aniline (lane ii), RIP–RALyase (lane iii), and α-sarcin (lane iv). The extension products shown are 97, 96, 96 and 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.

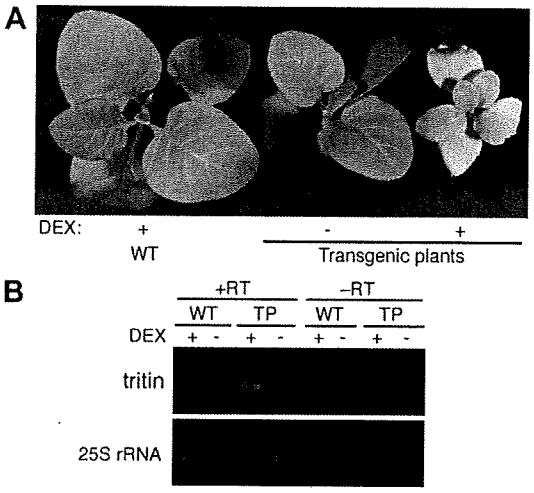

Fig. 3. Development of a senescence-like phenotype by inducible RIP in transgenic tobacco plant. (A) Photographs of dexamethasone (DEX)-treated wild-type (WT) and transgenic tobacco plants (+) and untreated control transgenic plant (−). (B) Detection of tritin mRNA expression by RT-PCR after DEX treatment. 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, *At*MPK6. 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 *At*MPK6 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

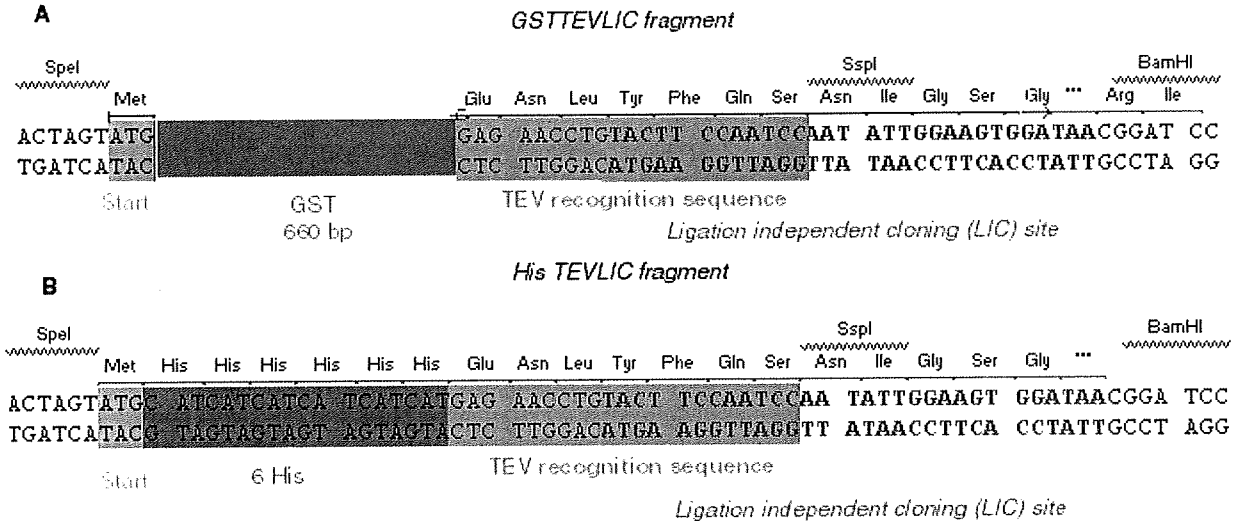

Figure 1
Nucleotide sequence of inserted cassettes.

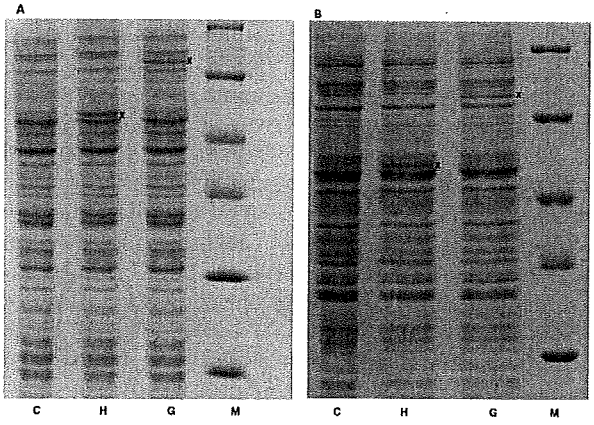

Figure 2
SDS-PAGE analysis of in vitro translated proteins. A. CEFC translation reactions with pEU3-NII backbone vector constructs: Molecular weight marker (M), wheat germ extract control (C), 1 μl out of 50 μl in vitro translation reaction mixture with pEU3-NII-HLIC (H) or pEU3-NII-GLIC (G) vector. **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.

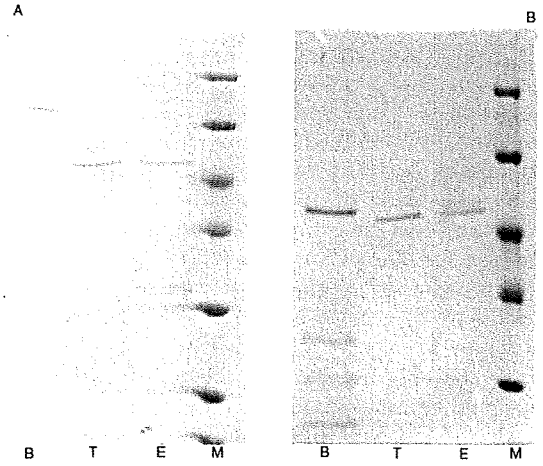

Figure 3
Affinity purification and cleavage of translated proteins. A. Purification and cleavage of GSTAtMPK6: Molecular weight marker (M), Glutathione-Sepharose coupled kinase (B), AtMPK6 cleaved from the beads (T), AtMPK6 cleaved following elution (E). **B.** Purification and cleavage of HisAtMPK6: 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 *At*MPK6 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 *At*MPK6 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-*At*MPK6 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-*At*MPK6 was tested, while the in vitro translated protein kinase displayed a clearly visible activity (Figure 4).

The high kinase activity of translated *At*MPK6 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.

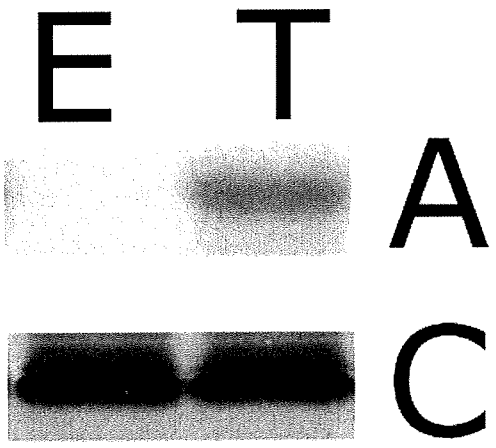

Figure 4
In vitro kinase activity of translated versus overexpressed AtMPK6. MBP phosphorylation activity of *E. coli* overexpressed (E) and translated (T) *At*MPK6 was detected by autoradiography (A). Coomassie Blue staining shows equal loading of MBP (C). *At*MPK6 was either produced in *E. coli* or 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'-ACTCTTCCTTTTTCAATGTTATGAAGCA-3' and 5'-TGAAAAAGGAAGAGTATGAGTATTCA-3';

5'-CTTCCTTTTTCAATGTTATTGAAGCATTTATCAGG-3' and 5'-CCTGATAAATGCTTCAATAACATTGAAAAAGGAAG-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 *At*MPK6 and append the sequences required for LIC:

5'-TACTTCCAATCCAATGC*AATGGACGGTGGTTCAGGT*-3' and

5'-TTATCCACTTCCAATG*TGTTTGAACGATCTGCAGTCA*-3' (gene specific sequences are indicated in italic). A vector construct comprising C-terminal HA-tagged *At*MPK6 [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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FEBS Letters 582 (2008) 221–228

Arabidopsis HY5 protein functions as a DNA-binding tag for purification and functional immobilization of proteins on agarose/DNA microplate

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Abstract **Protein microarray is considered to be one of the key analytical tools for high-throughput protein function analysis. Here, we report that the Arabidopsis HY5 functions as a novel DNA-binding tag (DBtag) for proteins. We also demonstrate that the DBtagged proteins could be immobilized and purified on a newly designed agarose/DNA microplate. Furthermore, we show three applications using the microarray: (1) detection of autophosphorylation activity of DBtagged human protein kinases and inhibition of their activity by staurosporine, (2) specific cleavage of DBtagged proteins by a virus protease and caspase 3, and (3) detection of a protein–protein interaction between the DBtagged UBE2N and UBE2v1. Thus, this method may facilitate rapid functional analysis of a wide range of proteins.**

Keywords: Arabidopsis HY5; DNA-binding tag; Cell-free protein synthesis; Protein microarray: Substrate screening; Transcription factor

1. Introduction

The successful sequencing of entire genomes of various organisms has led to the identification of a large numbers of novel genes [1]. The genomic information thus obtained so far and two other technologies in particular, DNA microarray and mass spectrometry, have largely helped advancing the analyses of transcriptome and proteome. In contrast, biochemical analysis of proteins has been hampered mainly because the traditional protein analysis methods are low-throughput in nature. Therefore, there is an urgent need to develop a high-throughput analytical tool, one analogous to the DNA microarray, for proteins. In the post-genomic era, the protein microarray is particularly promising in elucidating the biochemical properties of a large number of gene products [2]. Since the proteins found in a genome will have a wide variety of biochemical properties, it would be very useful to have several methodologies for making protein arrays.

Currently available protein microarray technology has allowed large-scale screening of biomarker proteins recognized by serum antibodies [3]. However, this method is yet to become a commonly used biochemical tool for the analysis of proteins [4]. Certainly there are rooms for further improvement before this technology could become a routinely used laboratory tool. For example, one of the problems is the difficulty in immobilizing a variety of proteins in their functionally active forms. Many proteins needed to be appropriately oriented for proper functioning [2]. However, it is not easy to control the orientation of the protein during its mobilization on the surface of the microplate. Another problem is that the high-throughput functional analysis requires freshly produced and purified proteins; however, unlike DNA, many purified proteins are not stable and thus, cannot be stored in active condition for long time. The development of functional protein microarrays for practical use, therefore, requires relatively easy methods for the functional immobilization and purification of freshly prepared proteins on the microplate. We recently developed a high-throughput method for protein synthesis using the wheat germ cell-free protein synthesis and an automatic protein synthesizer [5–7], and demonstrated that the automatic synthesizer is very useful for the production of freshly prepared proteins.

Affinity-tag purification is a conventional technique [8] that is widely used to purify recombinant proteins from the crude mixtures of lysed cells or cell-free translation systems. In this method, a target protein is expressed as a fusion protein with an affinity tag and then purified on an affinity column. A variety of affinity tags have been used, which include whole proteins such as glutathione *S*-transferase (GST) [9], maltose-binding protein (MBP) [10] and Staphylococcus protein A [11], and also include peptide tags of specific affinity such as myc-tag [12], FLAG-tag [13], polyhistidine tag [14], calmodulin-binding peptide [15], Strep-tag II [16] and SBP-tag [17]. However, these affinity tags have not been commonly used for purification of proteins on the microplate.

To create a new type of protein microarray, we developed a novel tag using a DNA-binding protein and a newly designed microplate consisting of agarose and commercial-available genomic DNAs. The new tag, named here as DBtag, is the Arabidopsis transcription factor HY5 having a basic

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leucine-zipper domain [18]. We found that the HY5 protein had high binding affinity to commercially available salmon sperm and calf DNAs. Here, we used this DNA-binding ability of HY5 to immobilize and purify the fusion protein on the microplate. Using this new protein microarray, we demonstrated (1) the autophosphorylation activity of the fusion human protein kinases, (2) specific cleavage of the fusion proteins by a virus protease and caspase 3, and (3) a protein–protein interaction between UBE2N and UBE2v1.

2. Materials and methods

2.1. General

The following procedures have been either described in detail or cited [5,7,19–21]: the isolation of wheat germs and preparation of the extract, generation of DNA template by polymerase chain reaction (PCR) using split-primers, synthesis of mRNA and protein in parallel, and estimation of the amount of protein synthesized by densitometric scanning of the Coomassie brilliant blue (CBB)-stained band and by autoradiography.

2.2. Template genes

The Arabidopsis HY5 gene (GenBank Accession No. AB005436) was previously cloned [5] and was inserted into a pEU-based vector (pEU-DBtag). The open reading frames of the glycogen synthase 1 (GS, muscle, NM_002103) and the following 11 human protein kinases were amplified by PCR from their respective commercially available cDNA templates (BioChain Institute, Inc., CA, USA and Takara Bio Inc., Otsu, Japan): MST3 (Accession no. NM_001032296), MST2 (NM_006281), SRPK2 (NM_182692), CK2a1 (NM_001895), PKR (NM_002759), MARK3 (NM_002376), PAK2 (NM_002577), PAK3 (NM_002578), CaMK2d (NM_001221), DAPK3 (NM_001348), MARK3 (NM_002376), CK1g3 (NM_004384), and TTK (NM_003318)]. The amplified clones were inserted into the vector pT7blue (Novagen, Merck Biosciences, Inc., Darmstadt, Germany), and their sequences were confirmed by DNA sequencing. The plasmids pEU-GFP [5] and pDsRed2 (Takara Bio Inc.) were used as the sources of GFP and RFP, respectively.

2.3. Construction of DNA template

The DBtag fragment (accession no. AB369281) for "split-primer" PCR-based fusion was amplified from the pEU-DBtag using the following primers (see Fig. 1B): deSP6E02 (5′-GGTGACACTATAGAACTCACCTATCTCTCTACACA) and DBtag-A (5′-TGGTGGTGGGTGGAAGCCCTGGAAGTACAGGTTCTC). The amplified product was then treated with exonuclease I (1 U/10 μl reac-

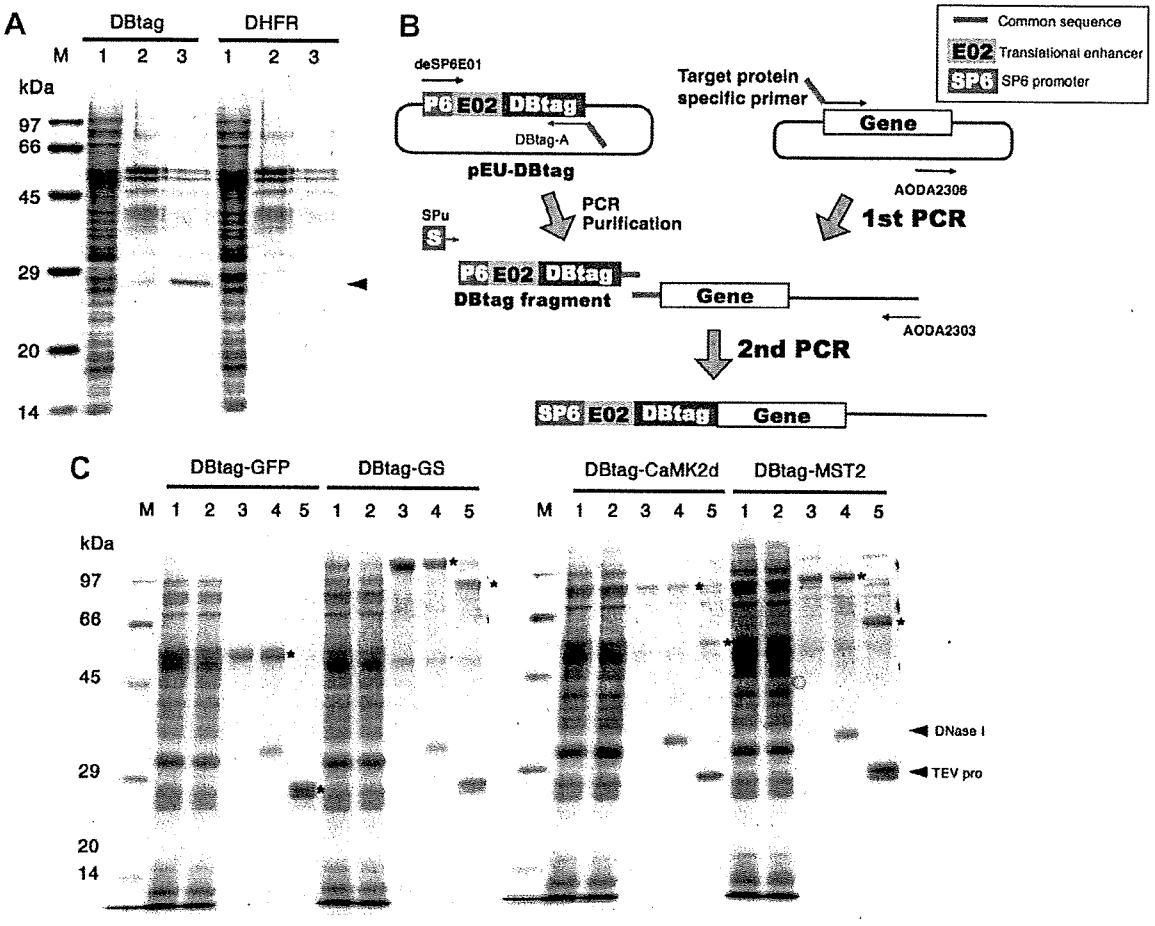

Fig. 1. Purification of DBtag and DBtag-fusion proteins, and scheme for construction of DBtag-fusion template by the PCR. (A) Purified DBtag (HY5) was found in the eluted fraction (lane 3 in DBtag, indicated by arrowhead). DHFR protein (negative control) was, however, not recovered (lane 3 in DHFR). (B) Scheme for DBtag-fusion method by the "split-primer" PCR. The DBtag fragment includes partial SP6 promoter. DNA amplification by SPu primer makes full-length SP6 promoter. (C) Purification of the DBtag-fusion proteins was observed in the eluted fraction (compare lane 3 with the crude mixture in lane 1). Lane 1: total fraction, lane 2: wash fraction (200 mM NaCl), lane 3: eluted fraction at high salt concentration (500 mM NaCl), lane 4: DNase I treatment, lane 5: TEV protease treatment to cleave between the DBtag and the target protein. Asterisks indicate purified proteins.

tion mixture, GE Healthcare, Little Chalfont, UK) and was purified with a PCR product purification kit. Next, the first round of the "split-primer" PCR was performed on each cDNA using 10 nM of each of the following primers: a target protein specific primer (5′-CCACCCACCACCACCAatgnnnnnnnnnnnnnnnnn-3′; uppercase and lowercase indicate common sequence and the 5′-coding region of the target gene, respectively) and the AODA2306 primer (5′-AGCGTCAGACCCCGTAGAAA). Then, a second round of PCR was carried out to construct the templates for the protein synthesis using a portion (5 μl) of the first PCR mix, 100 nM SPu primer (5′-GCGTAGCATTTAGGTGACACT), 100 nM AODA2303 primer (5′-GTCAGACCCCGTAGAAAAGA), and 2 nM DBtag fragment. GST and streptavidin (STA) tags were used according to methods we described previously [5,22]. Six histidine tag (His tag) was constructed by long primer (5′-GGTGACACTATAGAACTCACCTATCTCCCCAACACCTAATAACATTCAATCACTCTTTCCACTAACCACCTATCTACATCACCAAGATATCACTCGAGAATGCACCATCACCATCACCACCCACCACCACCAATG). By performing the second PCR, theses fragments or His tag were fused onto the N terminals of all the genes. The condition for the split PCR has been described in detail in a previous report [5]. The method for DBtag fusion by the PCR was illustrated in Fig. 1B.

2.4. Cell-free protein synthesis

Cell-free protein synthesis was carried out using the robotic synthesizer [7] GenDecoder® 1000 (CellFree Sciences, Yokohama, Japan) as described below. First, transcript was made from each of the DNA templates mentioned above using the SP6 RNA polymerase. The synthetic mRNAs were then precipitated with ethanol and collected by centrifugation using a Hitachi R10H rotor. Each mRNA (usually 30–35 μg) was washed and transferred into a translation mixture. The translation reaction was performed in the bilayer mode [19] with slight modifications. The translation mixture that formed the bottom layer consisted of 60 A260 units of the wheat germ extract (CellFree Sciences) and 2 μg creatine kinase (Roche Diagnostics K.K., Tokyo, Japan) in 25 μl of SUB-AMIX® (CellFree Sciences). The SUB-AMIX® contained (final concentrations) 30 mM HEPES/KOH at pH 8.0, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 4 mM DTT, 0.4 mM spermidine, 0.3 mM each of the 20 amino acids, 2.7 mM magnesium acetate, and 100 mM potassium acetate. One hundred twenty-five microliters of the SUB-AMIX® was placed on the top of the translation mixture, forming the upper layer. After incubation at 26 °C for 17 h, the synthesized proteins were confirmed by SDS–PAGE.

2.5. Purification using DNA column

To purify, 50 μl of the translational mixture was loaded onto 20 μl of DNA-conjugated column (GE Healthcare). The column was first washed three times with 100 μl of wash buffer (20 mM Tris–HCl, pH 7.8, 200 mM NaCl, 2 mM $MgCl_2$, and 1 mM DTT), and then the proteins (DBtag or DBtag-fusion protein) were eluted with an elution buffer (20 mM Tris–HCl, pH 7.8, 500 mM NaCl, 2 mM $MgCl_2$, and 1 mM DTT).

2.6. Preparation of protein microarray using agarose/DNA microplate

Agarose gel solution containing 0.2% agarose (SeaKem Gold, Takara Bio Inc., Otsu, Japan) in 20 mM Tris–HCl, pH 7.8, 2 mM $MgCl_2$, and 1 mM DTT was melted, and subsequently 1 mg/ml (final concentration) salmon sperm DNA (Sigma–Aldrich Corp, MO, USA) was added. Before the gel solidified, 600 or 400 μl of the agarose gel/DNA mixture was spread on a slide glass (Asahi Glass, Japan) or a Lab-Tek II Chamber slide (one-well, Nalge Nunc International Co.), respectively to form a thin layer (0.5–0.6 mm). The agarose/slide-coated glass was used within 1 day. Approximately 10 nl of each translational mixture per 0.2 mm^2 (~500 μm in diameter) was then directly spotted on the slide glass by using the MultiSPRinter™ spotter (Toyobo Bio Instruments, Tsuruga, Japan) according to the instruction manual. After spotting, the microplate was soaked in the wash buffer for 15 min and immediately used for assay. The microplate was washed with the wash buffer described above or detergent buffer [20 mM Tris–HCl, pH 7.8, 2 mM $MgCl_2$, 1 mM DTT and 5% Tween-20 (Sigma–Aldrich Corp.) or 5% NP-40 (Nakarai Tesque, Inc., Kyoto, Japan)]. During making of the microplate and the assay, the microplate was kept in a Tupperware box containing wet papers to prevent it from drying. Dried microplate could not use for the functional analysis of proteins.

2.7. Detection and inhibition of autophosphorylation activity of the DBtagged human protein kinases on the microplate

DBtag-fusion proteins were directly spotted on the agarose/DNA-coated glass plate as described above. The microplate was then covered with a kination solution made of 50 mM Tris–HCl, pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$ and 0.1 mM DTT, 10000 Ci/μl [γ-^{32}P] ATP, 0.05% DMSO and incubated for 30 min at 37 °C. For inhibition assay, staurosporine in a DMSO solution (at the indicated final concentration) was added to the kinase solution. After washing three times with the wash buffer, the microplate was analyzed by Typhoon 9400 imaging system (GE Healthcare). ImageQuant (ver. 5.2) in the imaging system was used for quantitative analysis of each spot.

2.8. Detection of protease activity on the microplate

Biotin protein ligase (BirA, Genbank Accession no. NP_312927) was cloned from the *Escherichia coli* strain JM109 by PCR and then inserted into a pEU vector [5]. A tobacco etch virus protease (TEVcs) recognition site, ENLYFQG, was inserted between the DBtag and the biotin ligase recognition site GLNDIFEAQKIEWHE (biotin ligation site: bls). As a negative control, we created another construct where the SARS protease recognition site (SAcs), PPQTSITSAVLQSGFRKMAFPSGKV [23], was inserted between the DBtag and bls instead of the TEVcs. The attB1-PAK2-bls-attB2 fragment was amplified by PCR with attB primers and reconstructed by Gateway system (Invitrogen Corp., CA, USA). DNA template of DBtag-PAK2-bls was constructed by the "split-primer" PCR described above. Non-biotinylated or biotinylated DBtag-TEVcs-bls, DBtag-SAcs-bls and DBtag-PAK2-bls proteins were synthesized using the cell-free system containing the synthetic BirA and without or with 500 nM biotin, respectively. After translation, the mixtures were incubated with 10 μg/ml of Alexa488-labeled streptavidin (Invitrogen Corp.) at 26 °C for 30 min and immobilized on the microplate as described above. The microplate was covered with TEV protease or caspase solution [20 mM Tris–HCl, pH 7.8, 200 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, and 2 U/μl TEV protease (Invitrogen) or 17.4 ng/μl caspase 3 (human, Sigma–Aldrich Corp.)], and incubated for 30 min at 26 °C. After washing with the washing buffer, the microplate was analyzed by Typhoon 9400 imaging system.

2.9. Detection of protein–protein interaction on the microplate

We used MGC clones of UBE2N (Accession no. NM_003348, MGC5063) and UBE2V1 (NM_022442, MGC8586). DBtag-UBE2N-bls and bls-UBE2V1 proteins were obtained according to the methods described above, and incubated for 30 min at 26 °C. Then they were mixed with Alexa488-STA (10 μg/ml) and then immobilized on the microplate as described above. The microplate was washed with 1× PBS buffer for 20 min, and was analyzed by Typhoon 9400 imaging system.

3. Results

3.1. Arabidopsis HY5 (DBtag) strongly binds to commercially available DNA column

DNA technology has progressed dramatically to meet the requirements of genetic engineering and genome projects. For example, chemically synthesized or modified DNAs have been widely used in DNA sequencing, PCR amplification and DNA microarrays. This inspired us to apply the DNA technology to comprehensive protein analysis. Several types of conserved DNA-binding domains have been found in transcription factors and nuclear hormone receptors. One of them is the bZIP domain. As described previously, the Arabidopsis HY5 transcription factor having a bZIP domain was synthesized in good quality and quantity in the wheat germ cell-free system [5]. We thus examined whether the synthesized HY5 protein could bind to a DNA-conjugated column purchased from a commercial source. Interestingly, the HY5 protein

bound to the column and was eluted at a high salt concentration (500 mM) (Fig. 1A). Furthermore, the eluted protein appeared as the major band on a SDS–PAGE because most of the endogenous proteins in the mixture were washed out with a 200 mM salt solution. This suggested the possibility of using this DNA-binding tag to purity recombinant proteins. We also found that, among 35 human nuclear hormone receptors tested, only HY5 bound to and was recovered from the DNA-conjugated column (data not shown). Since the HY5 bound to a microsatellite sequence (CA or CAA repeat) that is the highest repeat sequence in eukaryotic genome, microsatellite-binding protein may function as DNA-binding tag.

In order to investigate whether the HY5 protein was capable of purifying recombinant proteins, the HY5 DNA-binding domain (henceforth called DBtag) was fused with the green fluoresce protein (GFP), human CaMK2d, human glycogen synthase (GS), and human MST2 (see Fig. 1B), and the fusion proteins were synthesized in the wheat germ cell-free system and subjected to purification. The four fusion proteins were all purified to high quality at a high salt concentration (500 mM NaCl, lane 3 in Fig. 1C) and through DNase treatment (lane 4). On average, the purity and recovery rates of those fusion proteins were approximately 70% and 50%, respectively. In addition, the untagged forms of these proteins were recovered by TEV protease treatment (lane 5) because they contained a TEV cleavage site between the DBtag and the target protein. Taken together, these results proved that the HY5 protein was able to function as a DNA-binding tag to purify the target proteins.

3.2. Newly designed agarose/DNA microplate for the immobilization and purification of DBtag-fusion proteins

Miniaturized and arrayed assay systems are indispensable tools for large-scale and high-throughput biochemical analyses. Among them, microarrays are especially powerful tools because they are economic, and also because of the speed by which they could be used to interpret gene functions [2]. In the process of developing a protein microarray, we designed a new microplate that carries a thin layer of agarose gel containing DNA (agarose/DNA microplate) to immobilize and purify the DBtagged proteins on the microplate (Fig. 2A). We hypothesized that a DBtag-fusion protein would be immobilized on the agarose/DNA microplate and the other proteins would washed out. To test our hypothesis, we fused 6× histidine, GST, streptavidin, and DBtag to the N-terminal end of GFP to produce His-GFP, GST-GFP, STA-GFP, and DBtag-GFP, respectively. Those tagged GFPs were then spotted in an array on an agaraose/DNA microplate. Consistent with our hypothesis, subsequent washing of the plate removed all the spots except those of the DBtag-GFP (Fig. 2B). Fluorescence from the red fluorescence protein (RFP), which was used as a marker endogenous protein, was found to be diffused, and was removed by washing (Fig. 2C). This result suggested that the endogenous proteins from the cell-free system would most likely wash out. Also the DBtag can immobilize the GFP fusion even by treatment of high-concentration detergent buffer (5% Tween-20 or 5% NP-40) (Fig. 2D). Quantification of the DBtag-GFP spots after wash revealed that 25 ± 8% (average ± S.D.) of spotted DBtag-GFP remained immobilized on the microplate. This indicates that approximately

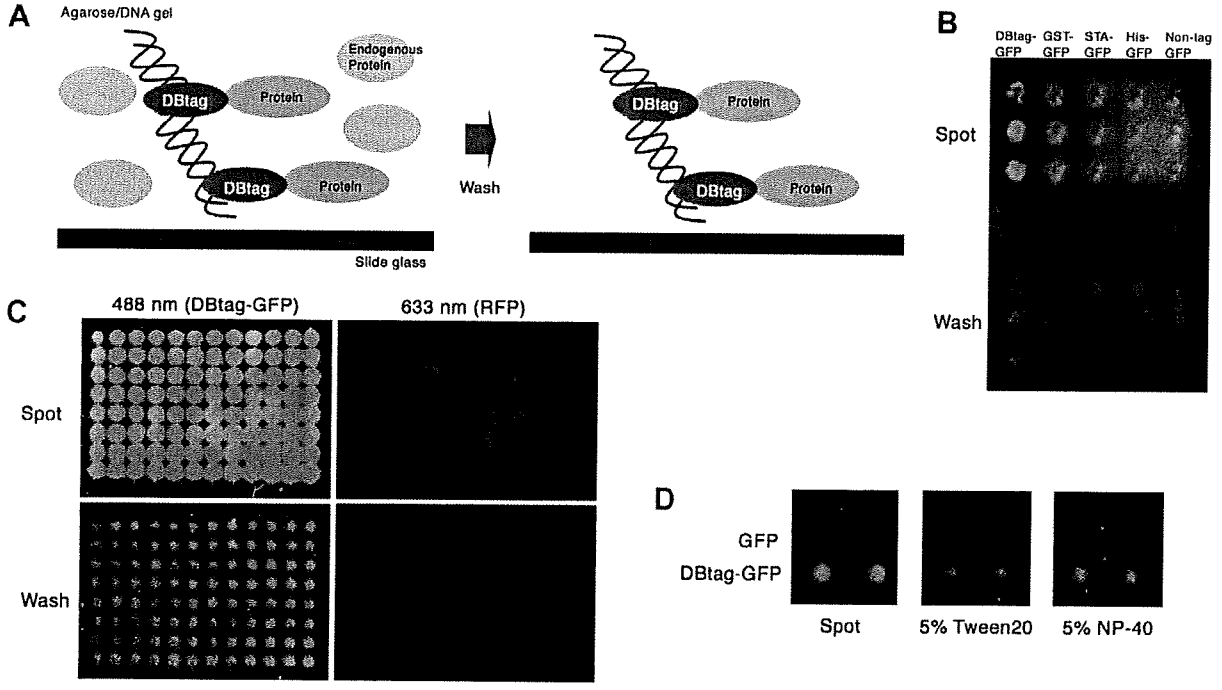

Fig. 2. Immobilization and purification of DBtag-fusion proteins on agarose/DNA microplate. (A) Schematic diagram showing immobilized DBtag-fusion proteins on the microplate before and after washing. (B) Only DBtag-GFP was immobilized on the microplate, while washing removed all other tagged GFPs and the untagged GFP. (C) DBtag-GFP and RFP (untagged) were mixed together and the mixture was spotted on the microplate. The DBtag-GFP was immobilized on the microplate, while the RFP was washed out and no RFP fluorescence was observed. (D) GFP (untagged, upper spot) and DBtag-GFP (lower spot) were spotted on the microplate. The DBtag-GFP was immobilized on the microplate by 5% of detergent treatment (5% Tween-20 and 5% NP40 panels), while the GFP was washed out and no GFP fluorescence was observed.

160 pg of DBtag-GFP was immobilized, which compares favorably to existing method [2]. The concentration of the DBtag-GFP in the spot was calculated as16 pg/nl (see Section 2.6). This result suggested that the DBtagged proteins could be immobilized and purified on the microplate.

3.3. Functional analysis of DBtag-human protein kinases on the agarose/DNA microplate

Next, we investigated whether we could detect the autophosphorylation activity of DBtagged protein kinases immobilized on the microplate. Out of a large number of protein kinases that are known to exhibit autophosphorylation activity [22], we selected 11 human protein kinases, which had exhibited autophosphorylation in an earlier experiment (data not shown). The selected kinases were synthesized as DBtag-fusion proteins using the wheat germ cell-free system and were then spotted in an array on an agarose/DNA microplate. After the plate was washed, a drop of [γ-^{32}P] ATP containing reaction mixture was applied on each spot, and the plate was incubated at 37 °C for 30 min. Fig. 3 shows the results of a typical autophosphorylation assay where all 11 human protein kinases on the microplate showed positive activity. The background phosphorylation activity was very low (see DBtag spots on Fig. 3). The low-level contaminants detected in the test were attributed to be due to the endogenous wheat germ proteins.

To investigate the possibility of using this autophosphorylation assay for inhibitor screening, the kinase assay was carried out in the presence of staurosporine, a widely used protein kinase inhibitor [24]. The activity of most of the protein kinases tested was inhibited by staurosporine at a high concentration (Fig. 3, middle and lower panels). MST2 was especially sensitive to the staurosporine treatment and its activity drastically decreased to less than 2% of the control value by treatment with 0.1 μM staurosporine (Fig. 3, middle panel). The activities of PAK3 and DAPK3 were inhibited by 70% and 80%, respectively, when treated with 0.1 μM staurosporine, and by 97% and 98%, respectively, when treated with 1 μM staurosporine. Eight out of the 11 human protein kinases were inhibited by staurosporine in this microarray assay. However, staurosporine did not inhibit the kinase activities of CK1g3, CK2a1, and TTK in our assay. Non-inhibition of CK1 and CK2 was reasonable because inhibition of CK1 and CK2 by staurosporine was reported as 163.5 μM and 19.5 μM of IC50, respectively [25]. To our knowledge about inhibition of TTK by staurosporine, this is the first report. Our study suggested low sensitivity of TTK to staurosporine. Also staurosporine inhibition from non-tag form proteins synthesized by the cell-free system indicated the similar data (data not shown). Together, these results suggest that our newly designed protein microarray could not only immobilize proteins in active forms but could also be used as a platform for designing assays for inhibitor screening.

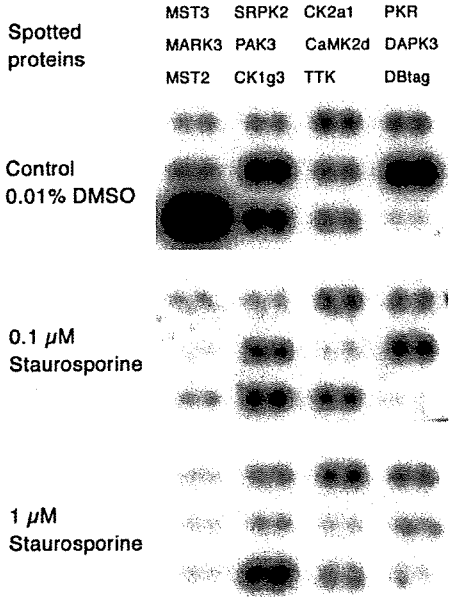

Fig. 3. Detection and specific inhibition of the autophosphorylation activity of 11 human protein kinases on agarose/DNA microplate. The DBtagged human protein kinases were analyzed in duplicates. DBtag indicates the tag protein by itself (control).

3.4. Specific cleavage of DBtag-fusion proteins on the agarose/DNA microplate by a site-specific protease

We next examined if the immobilized proteins on the microplate could be cleaved by site-specific proteases. For this assay, we used fluorescence-labeled proteins as substrates, which had been immobilized on the microplate. A TEV protease recognition site was inserted between the DBtag and biotin ligation site (bls) of these test proteins. The substrate proteins were fluorescence-labeled by co-translationally biotinylating them in the cell-free protein synthesis and then adding Alexa488-STA to bind with the biotin (schematically shown in Fig. 4A). The labeled proteins and non-biotinylated proteins (negative controls) were mixed with Alexa488-STA and then the mixture was spotted in an array on a microplate. When the plate was washed, fluorescence of the non-biotinylated proteins disappeared, which indicated that only biotinylated proteins were labeled with fluorescence (Fig. 4B, middle panel). When incubated with the TEV protease, the proteins having the TEV protease cleavage sites lost fluorescence, while the protein having other protease cleavage sites retained fluorescence (Fig. 4B, lower panel). We next used the approach for detection of caspase-3 (casp3) cleavage. The casp3, a key protease in apoptosis event, activates PAK2 protein kinase by cleavage [26]. Like above substrate proteins, the PAK2 was labeled with the DBtag and the biotin on bls in N and C-terminals respectively (DBtag-PAK2-bls) and spotted with Alexa488-STA on the microplate. The microplate was incubated with casp3 and washed. Like the assay of TEV protease above, fluorescence of DBtag-PAK2-bls lost (Fig. 4C, middle panel), while DBtag-bls (negative control) or buffer without casp3 retained fluorescence (middle and lower panels). By Western blotting, the cleavage of DBtag-PAK2-bls by casp3 was confirmed (data not shown). Thus the lost of fluorescence indicated the cleavage of the PAK2. The data showed that the DBtag-protein-bls could function as a good material for substrate screening of protease. Thus these results suggested that this microarray system could also be used for screening substrate proteins that are specifically cleaved by proteases.

3.5. Detection of a protein–protein interaction on the agarose/DNA microplate

Next, we investigated whether we could detect a protein–protein interaction of DBtagged proteins immobilized on the

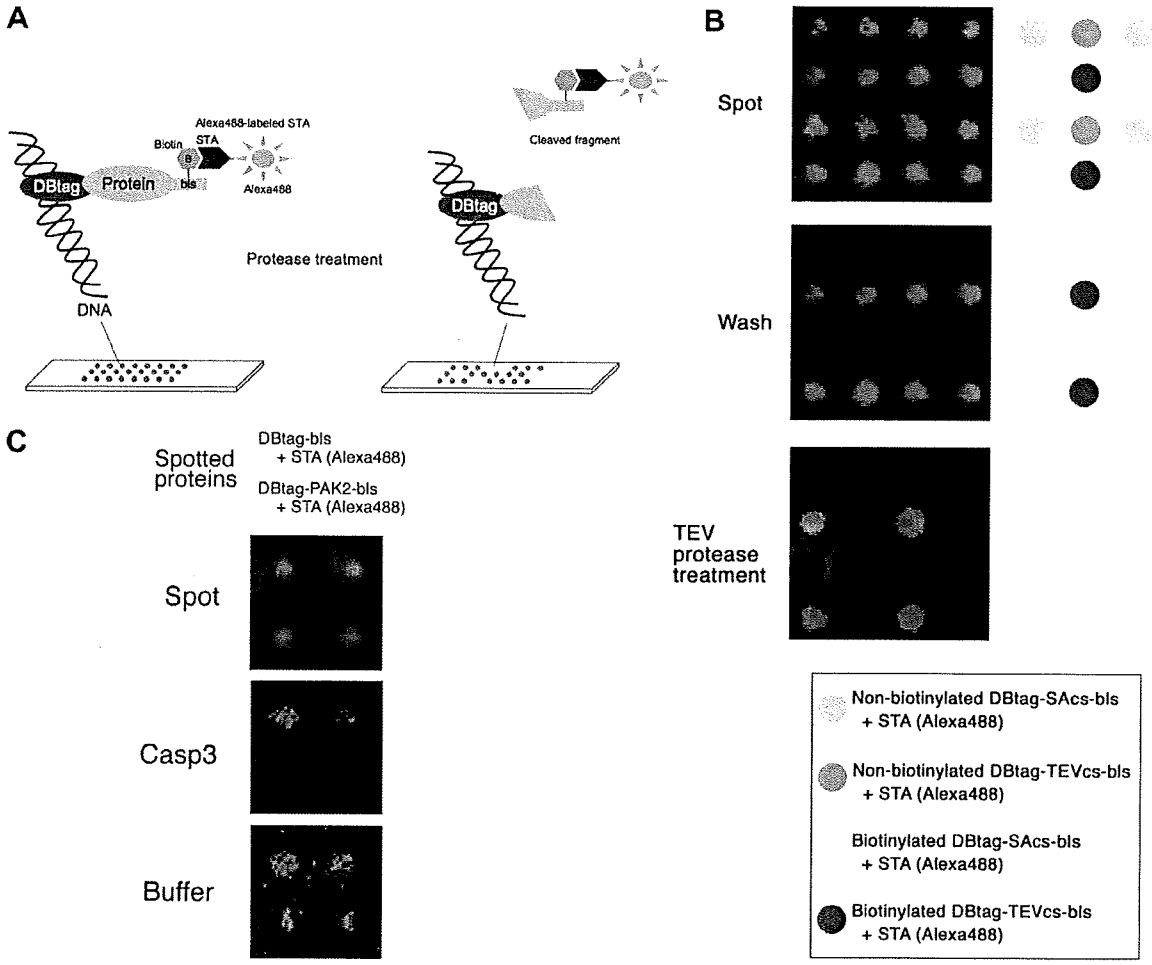

Fig. 4. Specific cleavage of protein substrates by TEV protease and caspase 3 on agarose/DNA microplate. (A) Schematic diagram showing an immobilized DBtagged protein labeled with a fluorescence-labeled streptavidin. The fluorescence-labeled region was released by the protease cleavage, and as a result, the fluorescence was lost upon washing. (B) The biotin-labeled proteins (blue spot: biotinylated-DBtag-TEVcs-bls and light blue spot: biotinylated-DBtag-SAcs-bls) retained fluorescence on the microarray, while the non-biotin labeled proteins (pink spot: DBtag-TEVcs-bls and light pink spot: DBtag-SAcs-bls) lost the fluorescence after washing. Four proteins having TEV protease cleavage site (TEVcs, spots indicated in blue color as biotin-labeled form) lost the fluorescence after the TEV protease treatment. Other proteins, having the cleavage site of SARS virus protease (SAcs), showed fluorescence (spots indicated in light blue) because they were not recognized by the TEV as substrates. (C) DBtag-bls indicates DBtag-TEVcs-bls above. Two proteins including PAK2 (lower spot in each panel: DBtag-PAK2-bls labeled with Alexa488-STA) lost the fluorescence after the caspase 3 (casp3) treatment ("Casp3" panel). DBtag-bls proteins (upper spot in each panel: DBtag-bls labeled with Alexa488-STA) and buffer without the casp3 ("buffer" panel) showed fluorescence because they were not recognized by the casp3 as substrates.

microplate. For this assay, UBE2N and UBE2v1, a heterodimer of ubiquitin-conjugate enzyme (E2) [27], were used. The DBtag-UBE2N, biotinylating bls-UBE2v1 or DHFR were mixed with Alexa488-STA and then spotted on the microplate (Fig. 5, the third from the top). As negative controls, DBtag protein or bls-DHFR was mixed instead of DBtag-UBE2N or bls-UBE2v1 respectively (first, second and fourth from the top). After washing, spots of DBtag-UBE2N and bls-UBE2V1 mixtures indicated the fluorescence of Alexa488-STA, whereas the fluorescence of DBtag/bls-UBE2v1, DBtag/bls-DHFR, and DBtag-UBE2N/bls-DHFR mixtures lost. This data showed that a protein–protein interaction between UBE2N and UBE2v1 was detected on the microplate. The interaction was reduced with high salt condition (>200 mM). These results suggested that this microarray system could also be used for screening of a protein–protein interaction.

4. Discussion

Several tags such as GST, MBP and polyhistidine have been widely used as efficiently affinity purification. Although many transcription factors are known, their use as a DNA-binding protein tag, however, have not been put into practice [28,29]. The current study demonstrated that the Arabidopsis transcription factor HY5 (and other proteins that bind strongly to DNA) functions as an affinity tag for the purification and immobilization of proteins on the newly designed agarose/DNA microplate.

A method for recombinant protein production includes several time-consuming processes such as construction of DNA template, transformation into the cell. High-throughput protein production is a key issue for making the protein microarray. Although the existing methods using the microarray

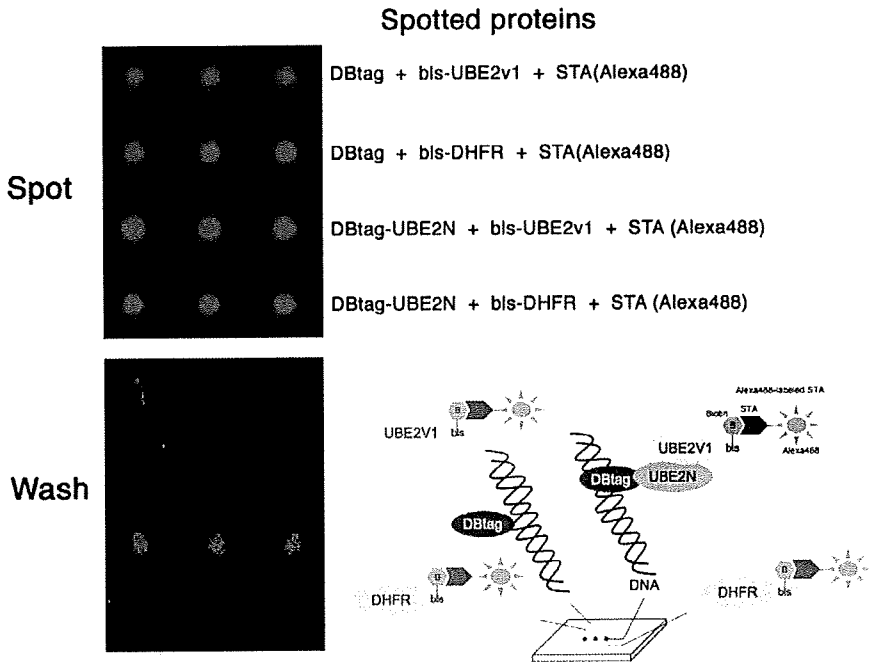

Fig. 5. Detection of a protein–protein interaction between UBE2N and UBE2v1 on the agarose/DNA microplate. Four samples [DBtag + biotin-labeled bls-UBE2v1 + STA(Alexa488), DBtag + biotin-labeled bls-DHFR + STA(Alexa488), DBtag-UBE2N + the bls-UBE2v1 + STA(Alexa488) and DBtag-UBE2N + the bls-DHFR + STA(Alexa488) from the top in "spot" panel] were spotted with three spots on the microplate. The first, second and fourth samples lost the fluorescence after washing ("wash" panel). The third sample showed the fluorescence in "wash" panel, indicating a heterodimer as a protein–protein interaction between UBE2N and UBE2v1 proteins. An image from this result was shown in the right panel.

have mainly used the cell-based method such as yeast or *E. coli* cells [2–4], a large number of protein productions by the cell-based method will definitely be of substantial works. Recently, the cell-free protein production system using extracts from the wheat germ or *E. coli* cells has been used for high-throughput synthesis of proteins because of the simple and effective methodology (see the review [6]). Furthermore, a fully automated robot based on the wheat cell-free system, shown in this paper, could operate a considerable number of protein productions. In addition, the biotin-labeled protein was used for detection of the protein–protein interaction and the cleavage of substrate proteins. Like the biotin labeling with the tag, the reconstruction of protein design allows wide range applications for the microarray-based functional analysis. However, using the cell-based methods, the time-consuming steps such as new vector construction are needed with every redesign of protein. In contrast the cell-free system accepts a linearly-rearranged DNA template produced by the PCR method without the new vector construction, indicating no requirement of the time-consuming steps. Thus, the cell-free system seems to be especially suitable as a protein production method for the microarray-based functional analysis.

To make practical protein microarray or for analysis by a surface plasmon resonance (SPR), the polyhistidine tag has been used for protein immobilization [2,30]. However, the immobilization carried out after purification of proteins, but not direct use of crude lysates or mixtures. The requirement of purification step seems to prevent the protein chip or SPR from considerable background caused by contaminations of undesired proteins. On-chip purification based on conventional affinity tags and microarray designs may be tough works. In addition, amount of the immobilized protein is important for highly sensitive detection of the functional analysis. The proteins on solid surface using the polyhistidine tag were immobilized at below picogram per a spot [2]. Our protein microarray system was available for on-chip purification and high amount of immobilized proteins (150 pg per a spot) because of high affinity of DBtag to genomic DNA and high-salt condition that dramatically reduced the background proteins undesired.

One of the most important attributes of a functional protein microarray is the directionality of proteins immobilized on it [2]. With today's technology, however, it is very difficult to control the directionality, because it requires a molecular-level control on the microplate. On the other hand, columns or beads traditionally used for protein purification have been widely used for functional assays like a pull-down assay, because they could preserve a three-dimensional space for proteins to react with molecules. Here, we explored a way to purify proteins on the microplate by mildly holding the freshly synthesized proteins in a three-dimensional space. This led to a protein microarray design in which the agarose gel formed a scaffold housing the DNAs (DNA–agarose matrix), which in turn were used for immobilizing the DBtagged proteins. This design was analogous to a flexible framework known as the nuclear matrix inside the nuclei [31]. The DNA framework in the agarose gel on a microplate was expected to keep a space around the immobilized proteins in the same way as the columns and beads do. Being a polymer, agarose could also insulate the freshly prepared proteins from the air, which we believe is important for the preservation of protein activity.

As described above, DBtag-fusion proteins synthesized using the wheat germ cell-free system could be immobilized on the microplate, and subsequent simple washing with a high

salt buffer reduced the amount of contaminating endogenous proteins to a very low background. Under this washing condition, the desired proteins were recovered almost as single bands on the CBB-stained SDS–PAGE (Fig. 1). In contrast, when the microplate was washed at a low salt concentration (<50 mM NaCl), many contaminating endogenous proteins were observed (data not shown). This high salt washing was one of the keys to the production of a low-background protein microarray. In the post-genomic era, comprehensive protein analysis is one of the most important approaches to the understanding of the function of the genomic code. The method we describe here would allow analyzing target proteins on protein microarrays without time-consuming protein purification steps. After protein synthesis, researcher can make their own protein microarrays. Therefore, we believe that the protein microarray, which was developed using the wheat germ cell-free system and the DBtag technique described in this study, is a powerful tool for the comprehensive analysis of proteins.

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Wheat Germ Cell-Free System-Based Production of Malaria Proteins for Discovery of Novel Vaccine Candidates▽†

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One of the major bottlenecks in malaria research has been the difficulty in recombinant protein expression. Here, we report the application of the wheat germ cell-free system for the successful production of malaria proteins. For proof of principle, the Pfs25, PfCSP, and PfAMA1 proteins were chosen. These genes contain very high A/T sequences and are also difficult to express as recombinant proteins. In our wheat germ cell-free system, native and codon-optimized versions of the Pfs25 genes produced equal amounts of proteins. PfCSP and PfAMA1 genes without any codon optimization were also expressed. The products were soluble, with yields between 50 and 200 μg/ml of the translation mixture, indicating that the cell-free system can be used to produce malaria proteins without any prior optimization of their biased codon usage. Biochemical and immunocytochemical analyses of antibodies raised in mice against each protein revealed that every antibody retained its high specificity to the parasite protein in question. The development of parasites in mosquitoes fed patient blood carrying *Plasmodium falciparum* gametocytes and supplemented with our mouse anti-Pfs25 sera was strongly inhibited, indicating that both Pfs25-3D7/WG and Pfs25-TBV/WG retained their immunogenicity. Lastly, we carried out a parallel expression assay of proteins of blood-stage *P. falciparum*. The PCR products of 124 *P. falciparum* genes chosen from the available database were used directly in a small-scale format of transcription and translation reactions. Autoradiogram testing revealed the production of 93 proteins. The application of this new cell-free system-based protocol for the discovery of malaria vaccine candidates will be discussed.

Plasmodium falciparum is the protozoan responsible for the widespread return of malaria to tropical countries, particularly in Africa. This reemergence is generally credited to two causes: the development of multidrug-resistant parasites and the development of insecticide-resistant mosquitoes (10). Through decades of work, scientists have learned that vaccination could be a potent curative, but efforts to develop a successful vaccine have not yet succeeded (25). One of the bottlenecks in vaccine development is at the malaria protein production step and is mainly due to the lack of a methodology to enable preparation of quality proteins in an efficient manner. *P. falciparum* genes have a very high A/T content (average, 76% per gene), and a number of them encode repeated stretches of amino acid sequences (8); these features have been proposed as the major factors limiting *P. falciparum* protein expression in cell-based systems. Moreover, the presence of glycosylation machinery in eukaryotic cell-based systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses (9, 21, 26). In fact, the three pioneering genome-wide studies on the production of *P. falciparum* proteins in cell-based systems faced serious problems. For instance, Aguiar et al. (1) were able to obtain expression in *Escherichia coli* cells of only 39 of 292 malaria genes cloned into the glutathione *S*-transferase (GST) fusion vector. Mehlin et al. carried out an even more challenging trial in which 1,000 genes encoding relatively small (<450 amino acids) malaria cytosolic proteins were expressed in *E. coli* (24). In that study only 30% of the genes were expressed and only 6.3% of the proteins were soluble, yielding 0.9 mg to 406 mg of protein per liter of culture medium. The other approach used an engineered *E. coli* strain with tRNAs genetically supplemented to allow reading of the high number of A/U codons in malaria mRNA (31). A significant improvement in protein solubility, up to 20.9%, was observed (38 out of 182 proteins tested were soluble). However, although the *E. coli* translation system is known to support folding of prokaryotic and small eukary-

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