TABLE 1
Characteristics of the new wheat germ cell-free translation system

	Wheat embryos	*E. coli* cells	Rabbit reticulocytes
Productivity (mg per ml)	10	6	μg order
Folding	Co-translation	Post-translation	Co-translation
Quality	High	Low	High
Codon preference	Loose	Tight	Tight
Reaction temperature (°C)	Wide (4–30)	37	30
Post-translational modification	Yes	No	Yes
Protein complex	Yes	No	No
Membrane protein	Yes	Yes	Yes
Disulfide bond formation	Yes	Yes	No
Biohazard	No	Yes	Yes
Ethical issues	No	Yes	Yes
Cost	Low	Low	High

(b) *mRNA's low codon preference:* This permits high-throughput protein production of falciparum malaria of high A/T composition and *Thermus thermophilus* of high G/C composition without any adjustment of codon usage [12,13]. Introduction of disulfide bonds is also possible [14].
(c) *Safety:* Being made from grain, the wheat germ cell-free system is free from biohazard and bioethical issues. Throughout the entire process from the making of the translation solution to synthesized proteins, bio-pollution is controlled to the minimum.

Furthermore, wheat germ extract contains N-terminal methionine excision activity for protein maturation [15] and N-myristoylation activity [16], additional important characteristics of the system to ensure correct folding. By contrast, N-glycosylation requires addition of endoplasmic reticulum of canine pancreas.

Protein production protocols

Integrating the elemental techniques described above, we completed two protocols for protein production with the wheat germ cell-free system, protocol (A) for comprehensive parallel production of a large variety of samples in small quantities for functional analysis, and protocol (B) for large-scale production for structural analysis (Fig. 1). Protocol (A) describes (a) selection of target genes, (b) construction of transcription templates by the Split-PCR method and subsequent mRNA synthesis, and (c) protein synthesis for functional or other kinds of screening. In most cases, 20-μL reaction volumes using a 96-well titer plate produce enough products for measurements of enzymatic activity. Protocol (B) describes (a) transcription using the pEU vectors containing target genes, and (b) translation by the bilayer method or an intermittent reaction method in which the reaction solution is exchanged at prescribed intervals. The stable translation capability of wheat embryo made it possible to automate protein production; a series of robots have been developed and are commercially available (http://www.cfsciences.com/). Among them, GenDecoder can produce 384 proteins in an overnight campaign, Protemist DTII can synthesize and purify 6 protein samples (0.5 mg each) overnight, and Protemist XE can produce 1 g of a protein in a 36-hour campaign.

Application of the system for high-throughput functional protein analysis

Protein factory

To use the reservoir of genomic information, which had been accumulated over years, we constructed for protein studies a platform for high-throughput protein production and functional analysis, which in turn was used to establish a human protein factory. The first step toward the human protein factory was to construct 33,275 Gateway entry clones from full-length human cDNA libraries and Gateway destination vectors containing various fusion tags. [Gateway entry clones are available to all qualified potential users from the National Institute for Technology and Evaluation (Japan) Biological Resource Center (http://www.nbrc.nite.go.jp/e/hgentry-e.html)]. Of those, 13,364 clones were subjected to protein synthesis in the wheat germ cell-free system using mRNA produced with transcription templates prepared by PCR. All the 75 phosphatases selected from the template collection were successfully produced and purified using a GST fusion tag. Of the 75 phosphatases, 58 showed activity to p-nitrophenyl phosphate, an artificial substrate, and two of them in particular showed high substrate specificity to a known phosphoprotein. Cytokines were synthesized in dithiothreitol-free translation solution to promote the formation of disulfide bonds. The synthesized cytokines showed as much biological activity as commercially available counterparts. Furthermore, the wheat germ cell-free protein production technology has been successfully used in combination with 13,277 Gateway destination vectors to develop protein active microarrays to detect antigen–antibody reactions and measure enzyme activity [14]. Protein mass spectrometry has become a powerful tool in proteome research, but there remain some serious limitations, quantification of proteins being one of them. This was recently solved by the absolute quantification method named FLEXIQuant (Full-Length Expressed Stable Isotope-labeled Proteins for Quantification) developed by Singh *et al.* [17]. The internal standards that form

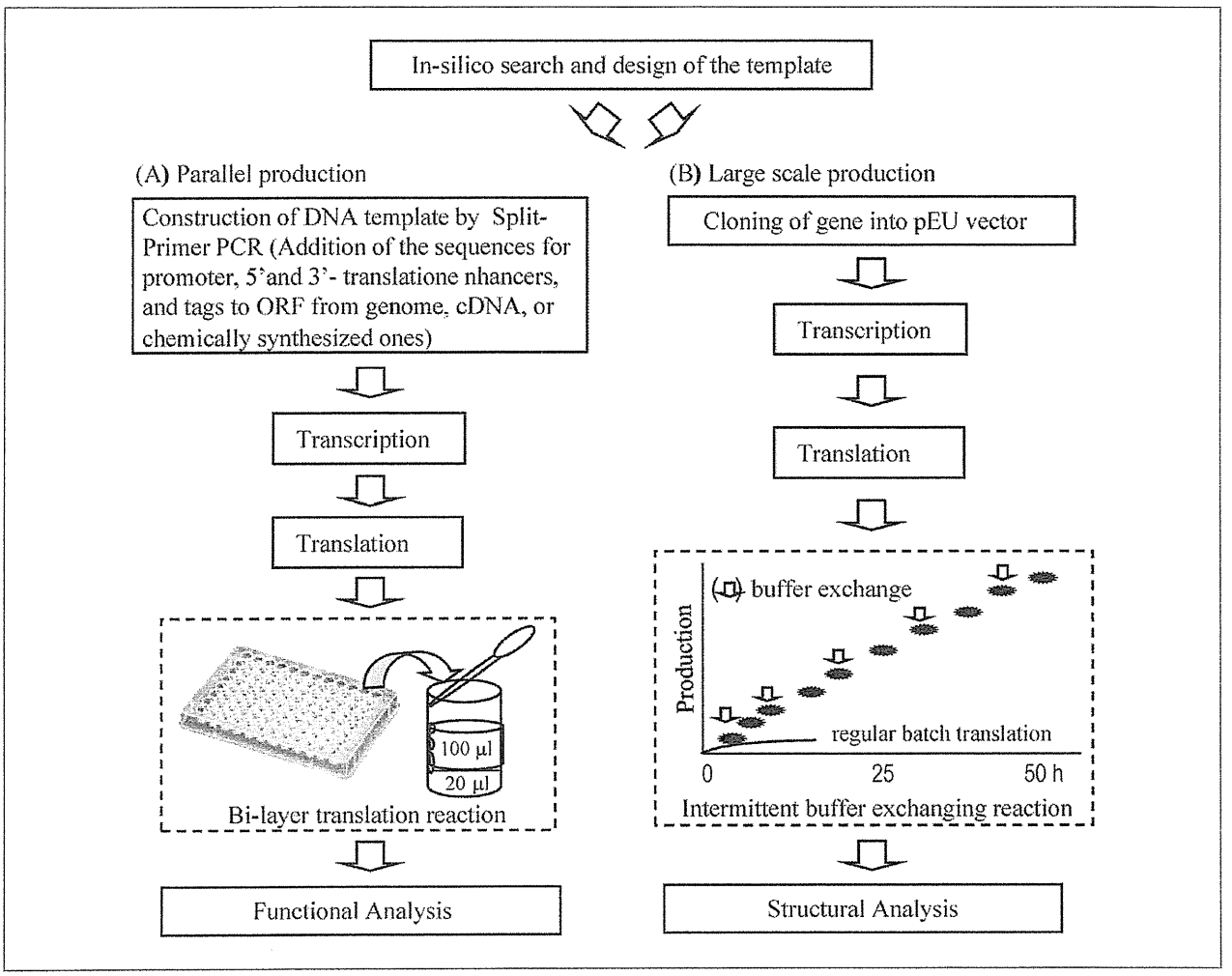

FIGURE 1

Protocols for protein production based on the wheat germ cell-free system. **(a)** Genes of interest are selected from DNA databases and transcription templates are constructed using the Split-PCR method. A typical DNA used for amplification comprises an SP6 promoter, an open reading frame, a reporter, and/or a purification tag (SP6-Tag1-ORF-Tag2). After transcription, the solution is directly used as the mRNA source in the bilayer translation system. **(b)** Suitable gene products selected in (a) can be produced in large quantities. Genes are cloned into a preferred type of pEU plasmid and the mRNA is transcribed. Before translation, the conditions (e.g. ion concentrations and incubation temperature) should be optimized in a small scale, and proteins are produced by intermittent buffer exchange reaction.

the core of FLEXIQuant are ^{15}N- and ^{13}C-labeled full-length proteins of high quality produced in the wheat germ cell-free system.

Method for comprehensive functional analysis

Although a variety of methods using sophisticated instruments to measure protein–protein interaction have been commercialized in recent years, their utility in comprehensive functional analysis is still limited. One issue associated with them is that protein samples are immobilized on beads or other substrates and another is the way samples are prepared and stored. The first issue is exemplified by the protein active microarray described above. It uses beads to immobilize proteins and, as a result, interaction reactions between these immobilized proteins and free proteins take place through the solid phase. The second issue is exemplified by the conventional biochemical method (radioactive isotope labeling) we have used for identifying protein kinases [18] or searching for substrate proteins [19] using the wheat germ cell-free system. This method is not suitable for high-throughput, high sensitivity detection. To circumvent those issues, we developed a method that combines the wheat germ cell-free protein synthesis technology with the AlphaScreen™ (PerkinElmer) detection technique. An example of its application is our ongoing comprehensive search for protein–protein interaction as illustrated in Fig. 2 and is described below.

Step 1: Generate DNA templates in either of the two types, one encoding a biotin-ligating peptide at the N-terminus and the other having FLAG-tag at the C-terminus, which are to bind to donor and accepter beads, respectively. After transcription, dispense each of the mRNA-containing solutions into one of the wells of a 96-well titer plate filled with the translation mixture and carry out

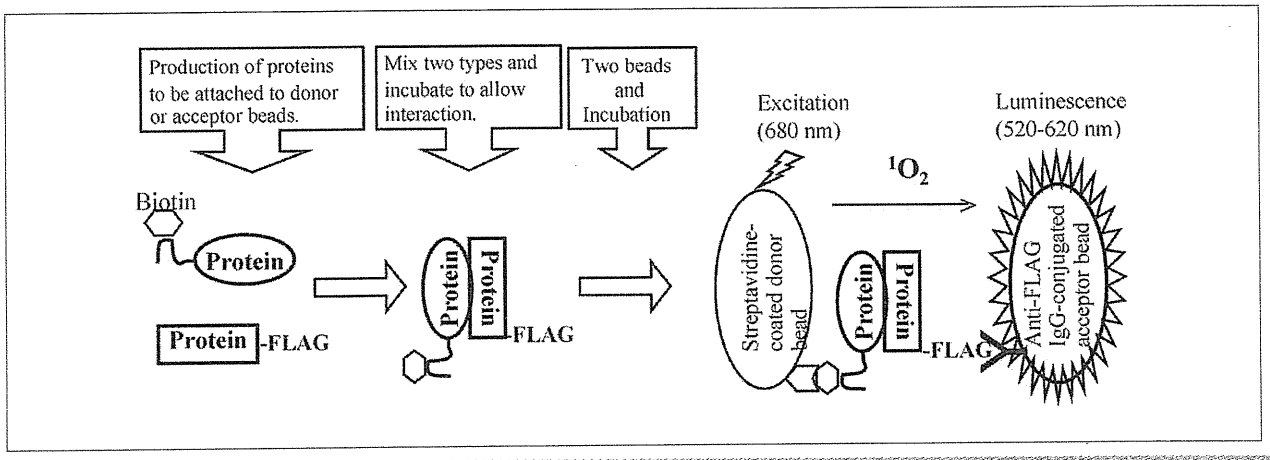

FIGURE 2
Schematic diagram for the functional analysis method based on the wheat germ cell-free protein production system and AlphaScreen™.

protein synthesis. For the production of biotin-labeled protein, carry out translation in the presence of biotin and biotin ligase produced beforehand in the wheat germ cell-free system.

Step 2: Dilute the translation solutions, add samples containing unpurified proteins of interest prepared in the type opposite to the one selected in Step 1, then incubate to allow interaction or reaction between two protein molecules in each well.

Step 3: Add commercially available AlphaScreen™ reagents (Streptavidin-coated donor beads and Protein A-conjugated acceptor beads) and measure the intensity of the fluorescence. When donor beads and acceptor beads come close within 100–200 nm, singlet oxygen is ejected from donor beads by laser, excites the acceptor beads, and releases fluorescence (Refer to the company's catalog).

Although its operating principle allows only semi-quantitative analysis, this method makes it possible to use high quality, unpurified protein samples produced by the wheat germ cell-free system and permits comprehensive detection of interactions between molecules in the liquid phase. It is also adaptable to other analytical methods such as analysis of ubiquitin complex [20] and single molecule fluorescence spectroscopy (Olympus, Japan) [21], because the wheat germ cell-free system enables us to prepare fusion protein samples with tags designed according to the principle of the analytical method chosen.

Applications to substrate discovery for protease

Genome sequencing projects have succeeded in annotating 461 genes for human proteases. Functional analysis of those proteases, however, has been slow, because it has been difficult to produce them using conventional recombinant protein production systems. This difficulty arises from the way proteases are produced in cells, first as inactive precursors which are then processed into mature molecules. In addition, their cleavage sites are generally unique and specific. It is still difficult to predict their mature structures from the genetic information alone, and therefore one has to determine them biochemically, which is a laborious task. Even after the gene of a mature protease could be constructed, its expression in living cells is difficult because expression itself will result in damage to the host cells. The wheat germ cell-free system, by contrast, allows us to produce proteases in mature forms at low temperatures where the rate of protein digestion by produced proteases is sufficiently low. This is because, as the germination of wheat seeds in cold winter demonstrates, the translational machinery of wheat germ functions even at 4°C (Table 1). This system also works well when we search for mature structures: all we have to do is to run parallel translation reactions with a series of DNA templates truncated at their 5′-open reading frame and find a template that gives a smear protein band on SDS-PAGE.

Identifying protein substrates of an enzyme from genetic information alone is difficult and requires biochemical experiments. As an example, we describe below our search for substrates of the protease caspase-3, and its recent results. We first chose the genes of 411 human kinases. We then constructed their DNA templates, which contained a biotin-ligating sequence and a FLAG-tag to produce reporter tags at N- and C-termini of the proteins, respectively. We synthesized mRNAs and used them as translation templates for protein production. We chose 304 high yield samples and, adding caspase-3, incubated them at 37°C. Although we could produce caspase-3 in an active form in the wheat cell-free system, we used for our experiment a commercially available grade made by the *E. coli in vivo* recombinant system, so that we could compare the results of our experiment with previous reports on a consistent basis. We performed the first round of substrate screening with AlphaScreen™ (Streptavidin-coated donor beads, Anti-FLAG-tag IgG-protein A-conjugated acceptor beads), using fluorescence quenching as the indicator of the cleavage of kinase molecular chains. We then took the candidates selected by this preliminary screening, incubated them with caspase-3 and separated the resultant peptide chains by SDS-PAGE. The original N- and C-terminal fragments were detected with Streptavidin-Alexa488 and Anti-FLAG antibody-FITC and, finally, the cleavage site was confirmed by mass spectroscopy. As a result, we identified 43 kinases as candidate substrates of caspase-3 (Sawasaki *et al.*, in preparation), of which 13 were among the 38 caspase-3 substrates that had been known previously and the other 30 were novel

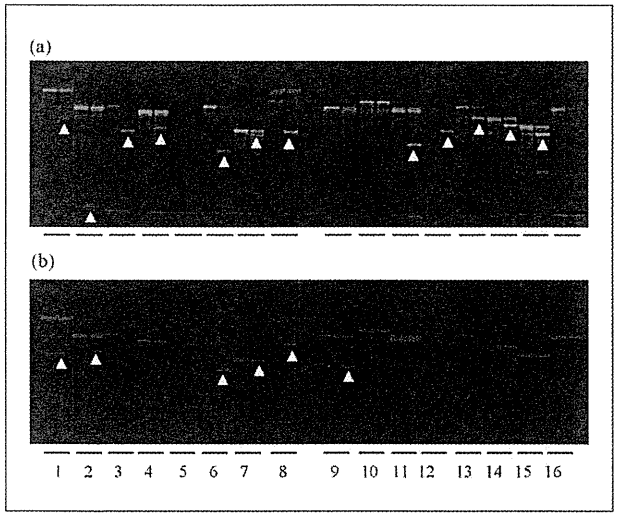

FIGURE 3

Cleavage of substrate kinases by caspase-3. 16 candidates (bars with number) from the first screening using AlphaScreen™ were incubated in the absence (left lane of each substrate underlined) or in the presence (right lane) of caspase-3 for 30 min at 37°C, and were separated by SDS-PAGE. Bands containing the N-terminal **(a)** and C-terminal **(b)** of the original molecules were visualized with Streptavidin-Alexa488 and Anti-FLAG antibody-FITC, respectively. Fragments with molecular weights smaller than 10 kDa were difficult to see. Arrowheads mark the produced fragments.

candidates. This demonstrates the efficacy of *in vitro* screening of substrates, although the results are to be verified directly by cell-level tests.

The second round of screening experiments was carried out as illustrated in Fig. 3. Sixteen candidates out of those identified by the first round of screening using fluorescence were incubated with caspase-3 and separated by SDS-PAGE. Protein or fragment bands with the N-terminal or the C-terminal were then stained and visualized. Although the scope of the experiment was limited to single molecule kinases and did not include complexes with other proteins, we could finally identify 13 new kinases as candidate substrates of caspase-3.

Protein structural analysis

Thanks to its capability to produce large quantities of high quality proteins of both prokaryotic and eukaryotic origin and to the ease of amino acid specific labeling, the wheat germ cell-free protein production system has found application in protein structural analysis. The amino acid metabolic system in wheat germ in hibernation contains only three kinds of transaminase activities, and their inhibitors are well known. With the wheat cell-free system, therefore, protein samples labeled with amino acid residue-specific stable isotopes can be readily prepared in the presence of such inhibitors [22] and used to generate HSQC (heteronuclear single quantum coherence) spectra signals for the location of amino acid residues [23]. The system is also suitable for producing protein samples labeled with SAIL (stereo-array isotope labeling) [24] for the dynamic NMR structural analysis of amino acid residues. In fact, using the wheat germ cell-free system with SAIL amino acids, Hideo Akutsu *et al.* have succeeded in preparing SAIL-labeled membrane proteins in various forms of proteo-liposome for their solid NMR dynamic structural analysis (Akutsu, H., Personal communication, 2010). For the application of the wheat cell-free system to solid NMR analysis, refer to the paper in this issue by Böckmann and colleagues. In X-ray crystallography, the wheat cell-free system facilitates the preparation of selenium-labeled protein samples for MAD (multiwavelength anomalous diffraction) phasing [12]. This is accomplished simply by replacing methionine in the amino acid substrate mixture with selenomehionine. The Center for Eukaryotic Structural Genomics (CESG) at the University of Wisconsin-Madison has successfully adapted the wheat germ cell-free system to build a protein production platform for their NMR and X-ray diffraction structural analyses. Their achievements are presented in their website (http://www.uwstructuralgenomics.org/).

Production of 'difficult-to-express' proteins and their functional and structural analyses

In general, it is difficult to produce a protein complex consisting of hetero subunits in cell-based expression systems. In many cases, it is also difficult to obtain such a complex in an active form in the wheat germ cell-free system, if we synthesize individual subunits separately and then combine them *in vitro* to reconstruct the complex. An easy way to circumvent this difficulty is to co-translate multiple mRNA in the wheat translation solution to produce the protein complex in a folded, active form [25]. An example of recent success is the production of a high quality sample of the human replication protein A complex through co-translation in the wheat germ cell-free system (Makino, S.-I. and Fox, B.G., Personal communication, 2010). This material was subsequently used to produce a crystal, whose structure was then successfully analyzed by X-ray diffraction [26]. Membrane proteins are another group of difficult-to-express proteins. When produced in the wheat germ cell-free system, transmembrane proteins containing single or double transmembrane domains can be recovered in the soluble fraction [13], but those containing multi-transmembrane domains such as GPCR become insoluble aggregates as they are produced. There has been a report of success in producing such a protein in a soluble and active form using the wheat germ cell-free system in the presence of an appropriate surfactant or liposome [27,28].

Conclusion and perspective

Focusing on the functional and structural analyses of eukaryotic proteins, we introduced examples of the application of the wheat germ cell-free protein production system found in recent reports, and some results from our ongoing experiments. We selected those examples to demonstrate the system's characteristics useful for building technology platforms for genome-wide functional and structural analyses.

Rapid advancement in life science calls for the development of certain core technologies for the future, one of which is the protein microarray. The focus of its development would be the stable storage of proteins incorporated in the microarray. It is clear that conventional design concepts developed for DNA microarrays would not work for protein microarrays. This is because, unlike DNA molecules that share stable properties, every protein molecule with its own characteristics requires a specific condition for its stability, and it is difficult to create such conditions in a minute

space on the microarray. It is actually next to impossible to do so, because the functions and properties of a majority of gene products are unknown. It seems that slow progress in the establishment of protein libraries is also attributable to those technical problems yet to be solved. Aiming at solving such problems, we are developing a technique to produce protein microarrays or protein libraries in the fashion of instant noodles. The idea is to store template DNA, mixture of transcription reaction, and the wheat germ translation system in wells of a micro titer plate and freeze-dry them for storage. When we are ready to use proteins, all we have to do is to add water and subject the plate to incubation for a short time to produce fresh proteins *in situ*. Stored in natural lyophilization, the wheat translation enzymes are thought to be stable in their natural state. In fact, we have ascertained through our preliminary experiments that the lyophilized systems do maintain their transcription and translation activities for several years.

We hope that this new wheat germ cell-free protein production system will help to accumulate knowledge about the functions and structures of gene products and accelerate the advancement of life sciences and pharmaceutical R&D.

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Use of domain enzymes from wheat RNA ligase for *in vitro* preparation of RNA molecules

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ABSTRACT

Wheat RNA ligase can be dissected into three isolated domain enzymes that are responsible for its core ligase, 5′-kinase, and 2′,3′-cyclic phosphate 3′-phosphodiesterase activities, respectively. In the present study, we pursued a practical strategy using the domain enzymes for *in vitro* step-by-step ligation of RNA molecules. As a part of it, we demonstrated that a novel side reaction on 5′-tri/diphosphate RNAs is dependent on ATP, a 2′-phosphate-3′-hydroxyl end, and the ligase domain. Mass spectroscopy and RNA cleavage analyses strongly suggested that it is an adenylylation on the 5′ terminus. The ligase domain enzyme showed a high productivity for any of the possible 16 combinations of terminal bases and a high selectivity for the 5′-phosphate and 2′-phosphate-3′-hydroxyl ends. Two RNA molecules having 5′-hydroxyl and 2′,3′-cyclic monophosphate groups were ligated almost stoichiometrically after separate conversion of respective terminal phosphate states into reactive ones. As the product has the same terminal state as the starting material, the next rounds of ligation are also possible in principle. Thus, we propose a flexible method for *in vitro* RNA ligation.

1. Introduction

In vitro RNA manipulation with cutting and joining of RNA molecules of defined nucleotide sequences has been of limited use in molecular biology in spite of the important roles of RNA molecules in gene expression. One obstacle to routine RNA manipulation has been the general low yields of RNA ligation. Since the pioneering works in which roles of structural elements in natural RNA molecules were analyzed through substitution with other oligonucleotides [1–5], T4 RNA ligase 1 has been most widely used for this purpose. This enzyme can result in low product yields for some substrates due primarily to the biased preference for terminal bases [6,7] and also to the preference of the enzyme to the substrates with a tRNA fold [8].

Possible alternative RNA ligases that are well characterized are yeast tRNA ligase [9] and plant RNA ligases [10,11]. These eukaryotic RNA ligases have the activities to modify terminal phosphate states of the substrate RNA molecules in addition to the core ligase activity in a single polypeptide. As a result, these enzymes can connect either a 5′-hydroxyl or a 5′-monophosphate end to either a 2′,3′-cyclic monophosphate or a 2′-monophosphate end [12,13], whereas 3′-monophosphate and 2′,3′-dihydroxyl ends are not utilized as a substrate [14]. While yeast tRNA ligase is specific for tRNA precursors [9], plant RNA ligases from wheat and *Arabidopsis thaliana* act on various RNA molecules with single-stranded regions [15–18]. Thus, it seemed to us that the plant enzymes could be more useful for general RNA ligation experiments. We have succeeded in dissecting the wheat enzyme into three independent, non-overlapping, fully active domain enzymes [19]. The ligase (L) domain adenylylates the 5′-phosphate terminus of the substrate RNA and links this terminus to the 3′-hydroxyl end with 2′-phosphate to form a 3′-5′ phosphodiester bond. The kinase (K) domain phophorylates 5′-hydroxyl ends. The 2′,3′-cyclic phosphate 3′-phosphodiesterase (P or CPD) domain opens a 2′,3′-cyclic monophosphate ring to leave a 2′-monophosphate group. The activity of each domain enzyme in the isolated form is at least as high as the activity that is embedded in the full-length enzyme. Because the L enzyme catalyzes ligation between a 5′-phosphate end and a 2′-phosphate-3′-hydroxyl end and probably does not utilize a 5′-hydroxyl end nor a 2′,3′-cyclic monophosphate end, it is expected that the enzyme is useful for *in vitro* RNA preparation through step-by-step ligation of smaller RNA molecules into a larger one with controlling the terminal phosphate states with the aid of end-modifying enzymes.

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In the present study, we investigated the L domain enzymes in more detail on their properties related to the practical usage in *in vitro* RNA ligation. We first characterized an unknown side reaction that was found previously to occur during the ligation reaction of an RNA molecule with a 5′-triphosphate terminus [19]. We then performed a systematic analysis of substrate specificity of the full-length and L domain enzymes. We propose a general RNA ligation scheme that integrates the use of the L domain enzyme, in which a 2′,3′-cyclic phosphate group can serve to block unwanted ligation to the end and can be converted on demand into an active end with the P domain enzyme. The form of the 5′ terminus can also be controlled with commercially available enzymes. The method fits well with the general methods for preparation of RNA molecules with a defined nucleotide sequence that generate 2′,3′-cyclic monophosphate ends.

2. Materials and methods

2.1. Preparation of enzymes and substrate RNAs

The full-length wheat RNA ligase and its domain enzymes were prepared as in the previous study [19]. The domain symbols may be used in this paper to denote these enzymes: "F" stands for the full-length enzyme, "L" for the core ligase domain enzyme, "K" for the kinase domain enzyme, "P" or "CPD" for the phosphodiesterase domain enzyme, and "KP" for the domain enzyme with the kinase and phosphodiesterase activity. DNA enzymes [20] shown in Fig. 1C were oligonucleotides from Invitrogen, which were further purified by electrophoresis on a 15% polyacrylamide gel containing 8 M urea.

The methods for RNA preparation were essentially the same as in the previous study [19], RNAs were synthesized by *in vitro* transcription of DNA templates containing an SP6 promoter by SP6 RNA polymerase (Promega). The template DNAs were prepared by PCR with KOD -Plus- DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) and a set of template oligonucleotides. After transcription, RNAs were separated on 8 M urea-polyacrylamide (acrylamide:bisacrylamide = 19:1) denaturing gels, and were extracted from the gels. The names and the sequences of the starting RNA substrates are as follows: 32NT (pppGAA GAG CUU GCA CUU GGA AAC GUG AUC ACG CA>p, 32 nts); 18NTG, 18NTA, 18NTC, 18NTU (pppGAA GAU GUC ACA UAC CAN>p, *N* = G, A, C, and U, respectively, 18 nts); GP1, AP1, CP1, UP1 (*N*AA UAC ACG GAA UUC GAG CUC GUU UUU A, *N* = G, A, C, and U, respectively, 28 nts); and 12NT (pppGAA GAA GAA GAA>p, 12 nts); in which "ppp" denotes 5′-triphosphate, and ">p" denotes 2′,3′-cyclic phosphate. "18NTN" and "NP1" represent any one of the four 18-nucleotide and 28-nucleotide RNA, respectively. The 2′,3′-cyclic phosphate ends of the 12NT and 18NTN RNAs were generated by a *cis*-cleavage of an HDV ribozyme [21] attached to their 3′-ends. The NP1 RNAs have a 5′-hydroxyl end generated by a *cis*-cleavage of a hammerhead ribozyme [22] attached to their 5′-ends. tRNA first half was as described previously [17]. The diphosphate version of 12NT (ppGAA GAA GAA GAA>p, where "pp" represents 5′-diphosphate) was prepared by transcription with 1 mM NTP and 8 mM GDP.

Derivatives of the RNA molecules were prepared by modification of the termini by treatment with one or two of the following enzymes: calf intestine alkaline phosphatase (CIP) (Takara Bio), which removes phosphomonoester, T4 polynucleotide kinase (PNK) (Takara Bio), which phosphorylates the 5′-hydroxyl end to form a 5′-phosphate and also dephosphorylates 2′,3′-cyclic phosphate at the 3′-end to form a 2′,3′-dihydroxyl end, the P enzyme (CPD) [19], which hydrolyzes the 2′,3′-cyclic phosphate to form a 2′-phosphate-3′-hydroxyl end, T4 polynucleotide kinase *pseT1* mutant (*pse*) (Roche) [29], which phosphorylates the 5′-hydroxyl end but does not affect the 3′ end, and the KP enzyme (KPD) [19], which has the 5′-kinase activity in addition to the activity of the P enzyme. These derivatives are denoted in this paper by the RNA name followed by the enzyme symbol(s) shown above in parentheses,

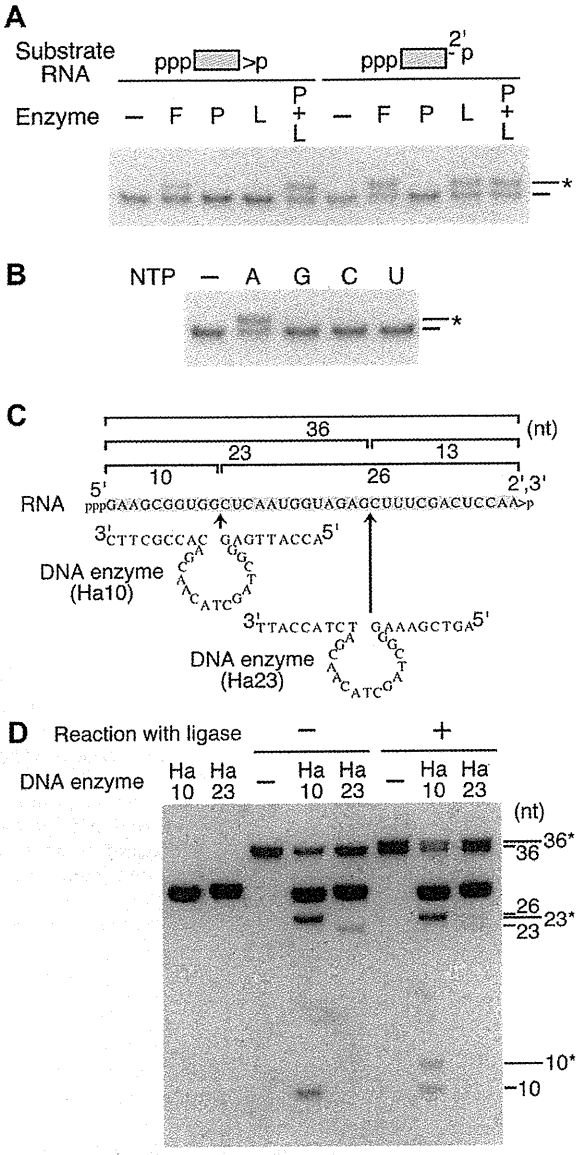

Fig. 1. Analyses of the RNA ligase side reaction. (**A**) A polyacrylamide gel showing that the side reaction is attributed to the ligase domain. The 32NT (left) and 32NTCPD (right) RNAs (3 μM) were incubated with the indicated enzymes at the concentration of 0.86 μM (F), 1.83 μM (P), or 2.1 μM (L), and were separated on the gel. The bands for the side reaction product are indicated by an asterisk. (**B**) A gel showing that ATP, but not the other three nucleoside triphosphates, is required for the side reaction. The 32NT RNA was incubated with the indicated nucleoside triphosphate (1 mM), 1.83 μM P, and 2.1 μM L, and was separated on the gel. (**C**) DNA enzymes [20] and their cleavage sites on the sequence of a target synthetic RNA named previously as tRNA first half [17]. The lengths of the expected RNAs are shown above the RNA sequences. (**D**) A gel separating the RNA products of the DNA enzyme cleavage performed after the side reaction on tRNA first half in order to locate the point of the reaction. 3.4 μM tRNA first half was first incubated with the L and P enzymes and was then incubated with one of the DNA enzymes (8 μM) at 37 °C for 60 min in a buffer containing 50 mM Tris–HCl (pH 7.5) and 10 mM $MgCl_2$. The bands assigned to have the side reaction point are indicated by asterisks. The band for the 13-nt fragment from the reaction with Ha23 was not visible because the reaction was not very efficient with this DNA enzyme.

such as 18NTNCIPCPD, which represents the product of 18NTN treated first with CIP and then with CPD. After the treatment with the enzyme(s), the RNAs were purified with phenol/chloroform extraction, ethanol precipitation, and with the gel separation/extraction method when required. Concentrations of the purified RNAs were quantified by measuring the absorbance at 260 nm.

2.2. Enzymatic reactions

Reactions other than the DNA enzyme reaction were performed in a buffer containing 20 mM HEPES-KOH (pH 7.8), 250 mM NaCl, 80 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.01%(w/v) BSA, and, for the kinase and ligase reactions, 1 mM ATP or another nucleotide triphosphate was also added. The reactions were incubated at 26 °C for 120 min, stopped by adding an equal volume of 2× RNA denaturation buffer containing 97%(v/v) formamide, 10 mM EDTA (pH 8.0), 0.015%(w/v) bromophenol blue, and 0.015%(w/v) xylene cyanol FF, heated at 65 °C for 2 min, quickly chilled on ice, and electrophoresed on an 8 M urea-20%(w/v) polyacrylamide gel with 1× TBE buffer. The gels were stained with methylene blue for detection of RNA bands. For quantitation of the RNA bands, NIH Image 1.62 was used, in which the bands of standard RNAs separated on the same gel were used for calibration.

2.3. MS analysis

The MALDI-TOF analyses of RNAs were carried out with a Voyager DE-PRO (Applied Biosystems). Samples were deionized with ZipTip$_{C18}$ (Millipore) using 0.1 M triethylammonium acetate buffer, and eluted with 50 mg/ml 3-hydroxy-2-picolinic acid, 5 mg/ml ammonium citrate in 50%(v/v) acetonitrile. Mass spectra were obtained in the negative ion mode at an acceleration voltage of 25 kV with a delay time at 300 ns. Typically 100 shots were combined for one spectrum. Reflector mode was chosen for analysis below 10 kDa, otherwise analysis was performed by linear mode. Mass calibration was performed with synthesized oligodeoxyribonucleotides.

3. Results

3.1. Analysis of the side reaction on 5′-triphosphate RNA substrates

Use of different terminal phosphate states during *in vitro* intermolecular RNA ligation is a general strategy for controlling which ends to be ligated with each other. We have recognized that the introduction of the 5′-triphosphate group to the RNA substrate intended to be the phosphate acceptor is useful for limiting the 5′ reaction to the other RNA substrate, in part because 5′-triphosphate ends can easily be generated by *in vitro* transcription. While the 5′-triphosphate RNA did not participate in self-ligation, an unexpected reaction was found to take place giving a recognizable band shift in the electrophoresis as mentioned in the previous study [19]. Thus, we characterized this side reaction (Fig. 1).

When a 5′-triphosphate-2′,3′-cyclic phosphate RNA was used as the substrate, the extra band was seen in the resulting mixture with the F enzyme or with both the L and the P enzymes, but not seen in the case of the individual domains (Fig. 1A). If the substrate RNA was pretreated with the P enzyme to make a 2′-phosphate-3′-hydroxyl terminus, the L enzyme was solely sufficient to catalyze the secondary reaction (Fig. 1A). The reaction was dependent on ATP, which could not be replaced by GTP, CTP, or UTP (Fig. 1B). In order to determine which end, 5′ or 3′, of the substrate RNA is modified, we cleaved the RNA site-specifically by DNA enzymes (Fig. 1C) [20]. This revealed that the modification was on the 5′ part of the RNA (Fig. 1D).

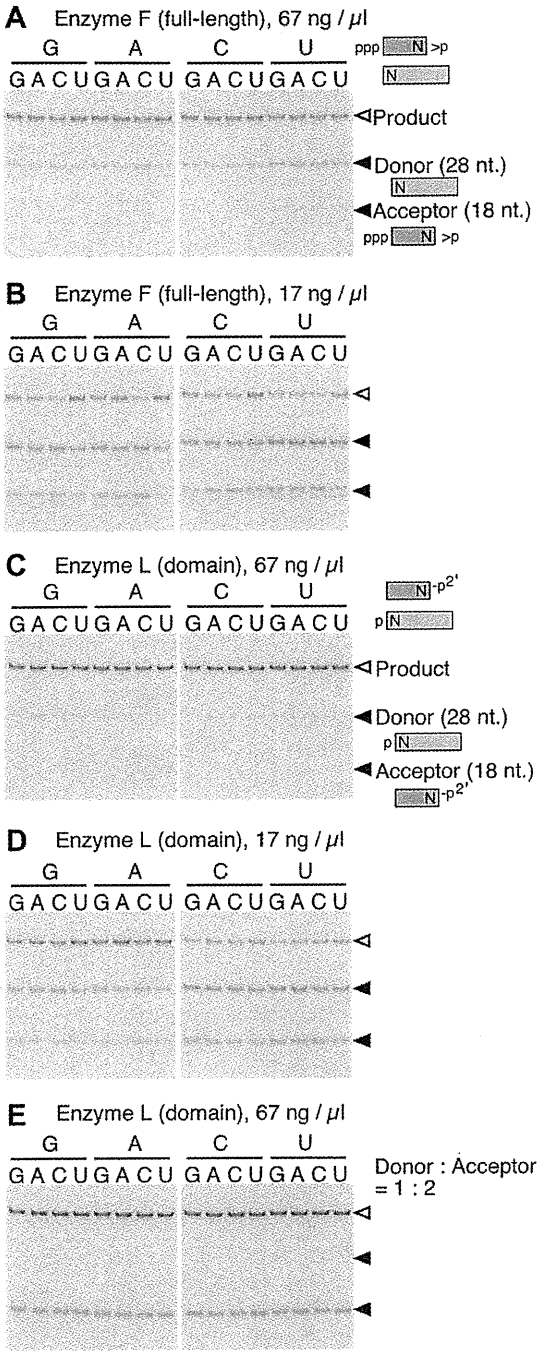

Fig. 2. Terminal base preferences of the full-length and ligase domain enzymes. Polyacrylamide gels resolving the ligation mixtures resulting from the substrates with different terminal nucleotide combinations are shown. A phosphate acceptor substrate (illustrated with an orange box) with the indicated 3′ terminal nucleotide and a phosphate donor substrate (green) with the shown 5′ terminal nucleotide were incubated with the F or L enzyme and separated on the gels. The enzyme used and its concentration, the terminal nucleotides in the acceptor molecules, and the terminal nucleotides in the donor molecules are shown from the top of each panel. The substrate RNAs were 18NTN and NP1 for the F enzyme (**A** and **B**) and 18NTNCIPCPD and NP1PNK for the L enzyme (**C**, **D**, and **E**). The initial concentration of the substrates was 2 μM except for 4 μM 18NTNCIPCPD in **E**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The change in the molecular mass of the RNA was measured to be an increase of 328 Da from the substrate to the product by a

TOF-MS analysis (Fig. S1 in Supplementary material), which coincides with the change for adenylylation. The same phenomenon was seen in the case of a 5′-diphosphate-2′-phosphate-3′-hydroxyl RNA (Fig. S2).

From the above results, the side reaction is likely to be a 5′ adenylylation reaction specific for 5′-triphosphate or 5′-diphosphate RNAs with a 2′-phosphate-3′-hydroxyl end. The ligase domain was able to carry out the side reaction, which seems to proceed by the same mechanism as that of the formation of the 5′ adenylylated intermediate for ligation.

3.2. Terminal nucleotide specificity in the ligation reactions

Although wheat RNA ligase is considered to react with various substrates, there had been no systematic evaluation of its substrate specificity. We executed a systematic analysis by measuring the intermolecular ligation products resulting from the substrates with all the possible combinations of terminal bases (Fig. 2 and Table 1). Although there were slight preferences among the 16 combinations, all of the tested substrates were ligated efficiently. The slightly different pattern between the full-length RNA ligase and the L domain enzyme observed may be due to the altered specificity of the end-modifying activities in the full-length protein. When we used a twofold molar excess of the acceptor over the donor substrate, more than 90 percent of the donor substrate was utilized in the reaction for any combination of the terminal nucleotides (Table 1).

3.3. Terminal phosphate specificity of the L domain enzyme

We next examined the terminal phosphate specificity of the L enzyme. We prepared four substrate RNAs that have an identical nucleotide sequence but differ in the combination of the 5′ and 2′/3′ terminal structures, and investigated whether self-ligation of these substrates are catalyzed by the L enzyme (Fig. 3). In contrast to the full-length enzyme which resulted in self-ligation of all four substrates, the L domain enzyme exclusively ligated the 5′-phosphate-2′-phosphate-3′-hydroxyl RNA (Fig. 3). An efficient intermolecular ligation was also achieved between the 5′-phosphate end and the 2′-phosphate-3′-hydroxyl end (Fig. 3). It is now confirmed that the ligase domain enzyme has a more limited specificity concerning the terminal phosphate states as compared to the full-length protein.

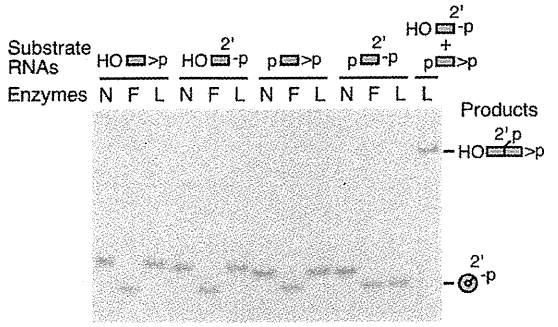

Fig. 3. Selectivity of the ligase domain enzyme for the 5′-phosphate and 2′-phosphate-3′-hydroxyl ends. A gel separating the resulting RNAs from the substrates with different terminal structures is shown. The RNA substrates, shown schematically on the top, were incubated with nothing (N) or with the indicated enzyme at 0.86 μM (F) or 1.05 μM (L) and were analyzed. 4 μM of 12NTCIP, 12NTCIPCPD, 12NTCIP*pse*, or 12NTCIPKPD, or 2 μM each of 12NTCIPCPD and 12NTCIP*pse* were used. Ligated products are schematically shown on the right.

4. Discussion

In vitro RNA cutting and joining makes it possible to prepare hybrid RNA molecules comprising of natural and synthetic molecules that may be useful for biotechnology and for probing the molecular mechanisms involving the RNA molecules at the nucleotide and atomic resolutions. The natural parts may have post-transcriptionally modified nucleotides that may be required for its function. The synthetic parts can be prepared either through chemical methods or enzymatic methods, and they can also have modified or labeled nucleotides. Specific positions of RNA molecules can be cut with ribozymes and DNA enzymes. In our experience, fusion to a hammerhead ribozyme and an HDV ribozyme during *in vitro* transcription is useful for generating a specific 5′-hydroxyl and 2′,3′-cyclic phosphate ends of the transcript. In fact, we adopted this method for preparation of substrate RNA molecules for the assays of the enzymes. DNA enzymes may also be useful for cleaving a specific position to generate the 5′-hydroxyl and 2′,3′-cyclic phosphate ends. In spite of these techniques for preparation of the parts of the hybrid RNA molecules, the methods for joining the parts have so far depended on inefficient ligases.

We observed that the ligase domain catalyzes a reaction that is likely to be an adenylylation of 5′-tri- or diphosphate termini. This reaction is likely a side reaction accompanying the formation of the 5′-adenylylated intermediate during ligation. Both reactions are dependent on the 2′-phosphate and 3′-hydroxyl groups in the substrate RNA. When a 5′-monophosphate terminus is adenylylated as a ligation intermediate, it will be subsequently used for the ligation reaction with a 2′-phosphate-3′-hydroxyl RNA terminus, whereas adenylylated products of 5′-tri- or diphosphate termini will not participate in the ligation reaction. It is now very likely that the hydrolysis of the cyclic phosphate precedes the adenylylation on the 5′ phosphate by the native full-length enzyme, and that the L domain itself is responsible for the specificity to the 2′-phosphate-3′-hydroxyl ends. Similar adenylylation side reactions on nucleoside 5′-tri- or diphosphate are seen in other ligases that share conserved motifs with wheat RNA ligase, including T4 DNA ligase [23] and T4 RNA ligase [24]. These ligases have homology to guanylyltransferase, which guanylylates the 5′-diphosphate ter-

Table 1
Ligation efficiencies for different substrates.

Enzymes	Enzyme concentrations (μM)	Substrate RNAs (μM)		Conversion (%)															
		Acceptor		G				A				C				U			
			Donor	G	A	C	U	G	A	C	U	G	A	C	U	G	A	C	U
F	0.57	2	2	59	63	59	68	59	65	56	67	60	64	64	67	53	55	50	56
	0.14	2	2	32	33	27	50	35	40	25	51	35	34	27	44	22	23	18	33
L	0.70	2	2	79	72	77	76	78	80	86	83	81	73	81	78	80	75	79	83
	0.18	2	2	47	46	42	55	51	58	48	60	25	30	27	34	23	24	28	31
	0.70	4	2	>90, in any combination															

minus of mRNA precursors to form a cap structure [25]. It is therefore conceivable that these ligases also have the capacity for modifying the 5′-tri- and diphosphate termini. Whether this side reaction plays any role in cellular system remains to be investigated.

We also confirmed that the F and L enzymes have broad substrate sequence specificities (Fig. 2 and Table 1). It is well known that ligation reactions with T4 RNA ligase may be inefficient depending on the nucleotides around the ligation site [6,7]. The dependence of the efficiency on the substrate sequences has been systematically investigated, and the yields could be in some cases less than several percents [7]. Although a method for avoiding very low yields have been devised [26] in which the yield is around 50%, the method still requires design of a splint DNA molecule on a case-by-case basis. T4 DNA ligase is also used for splinted ligation of RNA molecules at a double-stranded region, while this method requires very large amounts of the enzyme [27,28]. Although we have observed some difficulty in the ligation of structured RNAs with our F and L enzymes (unpublished data), these enzymes have a desirable sequence-specificity for general ligation.

The data in Fig. 3 confirm the specificity of the L enzyme for the 2′-phosphate-3′-hydroxyl ends and demonstrate that the ends to be joined can be controlled strictly. We propose a flexible method for joining RNAs with defined sequences facilitated by the use of the domain enzymes, in which, once RNA units having a 5′-hydroxyl and a 2′,3′-cyclic phosphate ends are prepared, they can be linked together specifically in any order with a good yield. The starting RNA units can be easily prepared with the use of ribozymes and/or DNA enzymes. RNase T1 digests of natural RNAs may also be useful. The 3′ molecule to be joined at the 5′-end can be activated through the reaction catalyzed either by the K enzyme or the *pseT1* mutant of T4 polynucleotide kinase [29]. The 5′ molecule to be joined at the 3′-end can be converted to have 5′-hydroxyl, 2′-phosphate ends with the P enzyme. The two substrates can now be joined specifically by the L enzyme to generate a new RNA molecule with 5′-hydroxyl and 2′,3′-cyclic phosphate ends. Both substrates and the product neither self-ligate nor concatenate. Although the 2′-phosphate group at the ligation junction remains after the reaction, this issue can be resolved by using a 2′-phosphotransferase, an enzyme playing a role in the splicing of tRNA [30]. The yeast Tpt1 enzyme has already been used for removing the 2′-phosphate group of a wide variety of substrates [14]. We also have tested this enzyme with a substrate RNA and have obtained a good result. The strategy is advantageous also in that the ligation product has the same terminal state as the starting RNA units, because some applications of *in vitro* RNA manipulation require two or more consecutive rounds of ligation. Further investigation may be necessary for establishing the versatility of the ligation scheme with these enzymes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.108.

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Short Communication

Ca^{2+}-dependent protein kinases and their substrate HsfB2a are differently involved in the heat response signaling pathway in Arabidopsis

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Abstract Little is known about the mechanisms by which Ca^{2+}-binding sensory proteins direct the plant heat shock (HS) response. Since two Ca^{2+}-dependent protein kinases (CPK3 and CPK13) were recently shown to phosphorylate the heat shock transcription factor HsfB2a, we assessed in the current study whether these kinases are also involved in HS signal transduction, by monitoring the transcriptional profile of HS protein (Hsp) family genes in Arabidopsis Col-0 plants (WT) and the corresponding mutants. Both with and without HS, the gene transcript levels of *Hsp70*, *Hsp101*, *Hsp17.4-CIII* and *Hsp15.7-CI* were found to be lower in *cpk3* and *cpk13* mutants compared to WT, resulting in the impairment of basal thermotolerance in the mutants. To determine the *in vivo* function of CPKs, CPK3/13 and their substrate HsfB2a (heat shock transcription factor) were co-expressed as cofactors for the transient expression of a reporter (GUS) gene under the control of heat shock element (HSE) in *Nicotiana benthamiana* leaves. However, CPK3/13-phosphorylated HsfB2a did not function in the suppression/activation of HSE-promoted expression in the transient expression system. Implications for possible signal trafficking via CPKs and Hsfs are discussed.

Key words: Arabidopsis, Ca^{2+}-dependent protein kinase (CPK), heat shock response, HsfB2a.

Abiotic stresses, such as drought, salinity or extreme temperatures, are serious threats to agriculture and result in deterioration of the environment. Elucidating the various mechanisms of plant responses to stress and their roles in acquired stress tolerance is thus of great practical and basic importance (Wang et al. 2004). Various stress effects result in the appearance of partly denatured proteins in cells that activate a stress response system. Many molecular chaperones are stress proteins and many of them were originally identified as heat shock (HS) proteins (Hsp) (Wang et al. 2004). Most Hsps begin to be expressed as a result of the HS-induced trimerization of an HS transcription factor (Hsf), which enables Hsf to bind to the HS element (HSE) in the promoter region of Hsp genes (Baniwal et al. 2004; von Koskull-Döring et al. 2007), eventually resulting in the potential acquisition of thermotolerance by plants (Montero-Barrientos et al. 2010).

Plants possess several classes of Ca^{2+}-binding sensory proteins, including calmodulins, calmodulin-like proteins, calcineurin B-like proteins, and Ca^{2+}-dependent protein kinases (CPKs) (Sanders et al. 2002). Several Ca^{2+}-binding sensory proteins [e.g., Ca^{2+}/calmodulin-binding protein kinase (AtCBK3), a member of the PPP family (AtPP7)], are known to play roles in heat-shock signal transduction in which Hsf is phosphorylated in both constitutive and HS-induced manners (Li et al. 2004; Liu et al. 2005; Liu et al. 2007; Liu et al. 2008). Moreover, the CPKs are of special interest, since they represent a novel class of Ca^{2+} sensors, having both a protein kinase domain and a calmodulin-like domain (including an EF-hand calcium-binding site) in a single polypeptide (Klimecka and Muszyńska 2007). CPKs constitute a large family of serine/threonine protein kinases that are broadly distributed in the plant kingdom. In a previous study we demonstrated with *in vitro* kinase assays that

Abbreviations: CPK, Ca^{2+}-dependent protein kinase; HS, heat shock; HSE, heat shock element; Hsf, heat shock factor; Hsp, heat shock protein

This article can be found at http://www.jspcmb.jp/

CPK3 and CPK13 proteins phosphorylate the heat shock transcription factor HsfB2a and with *in vivo* agroinfiltration assays that the CPK-mediated phosphorylation of HsfB2a promotes the transcriptional activation of the plant defensin gene *PDF1.2* in a defense response (Nagamangala Kanchiswamy et al. 2010). Moreover, CPK3 kinase activity appeared to be induced by heat stress treatment (Mehlmer et al. 2010). These results prompted us to assess whether the CPK3 and CPK13 cascades are also involved in HS signal transduction. The expression of various Hsp family genes (*Hsp70*, *Hsp101*, *Hsp17.4-CIII* and *Hsp15.7-CI*) was assayed by quantitative reverse transcription (RT)-PCR in Arabidopsis Col-0 plants (WT) and two T-DNA insertion lines: *cpk3* (SALK_022862) and *cpk13* (SALK_057893) that were subjected to heat stress treatment at 40°C for 3 h. Disruption of CPK mRNA expression in leaves of the respective *cpk* mutants was confirmed previously (Nagamangala Kanchiswamy et al. 2010). Compared to the levels in WT seedlings, unheated *cpk3* seedlings contained significantly lower transcript levels for the *Hsp101* and *Hsp15.7-CI* genes, whereas *cpk13* seedlings contained low transcript levels for all of the Hsp genes analyzed (Figure 1A). In both T-DNA insertion lines, however, no visible phenotypical changes were observed in the unheated condition (data not shown).

WT plants reacted to HS by increasing the transcript levels of all Hsps, compared to the respective levels in unheated plants. In contrast, the transcript levels in *cpk3* and *cpk13* plants were significantly lower compared to those in WT plants, with the sole exception of *Hsp15.7-CI* expression in *cpk3* seedlings (Figure 1B). Both *cpk3* and *cpk13* seedlings were impaired, compared to WT, in thermal tolerance at 40°C for 3 h (Figure 1C). Genetic analysis has shown that Hsp101 is absolutely essential for thermal tolerance in Arabidopsis (Tonsor et al. 2008), and this Hsp has been reported to play a role in preventing oxidative stress (Zhang et al. 2009). Hsp70 is one of the major classes of chaperone molecules and is involved in a variety of tasks in eukaryotic cells, and increased Hsp70 synthesis results in a marked increase in stress tolerance (Hu et al. 2010; Montero-Barrientos et al. 2010). Although plants generate an array of high molecular weight Hsps, most of the translation capacity is devoted to the synthesis of the small Hsps (e.g., *Hsp15.7-CI* and *Hsp17.4-CIII*) which also play an important role in the acquisition of thermal tolerance (Yildiz and Terzi 2008). Accordingly, the drastically decreased levels of expression of Hsps in *cpk3* and *cpk13* seedlings, especially, with HS treatment, very likely caused impairment of the plants' basal thermotolerance.

As described above, it was found that CPK3 and CPK13 phosphorylate HsfB2a (heat shock transcription factor) (Nagamangala Kanchiswamy et al. 2010). This

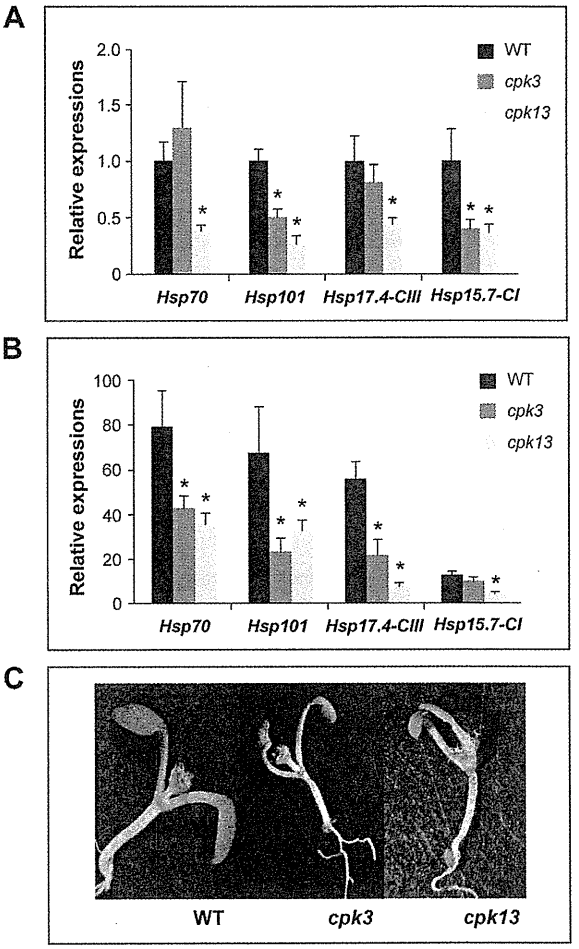

Figure 1. Effects of loss of CPK function on heat response signaling pathway in Arabidopsis seedlings. Arabidopsis seedlings (Col-0) were grown on MS medium containing 2% sucrose and 0.8% agarose in a growth chamber at 22°C. Transcript levels of Hsp genes in seven-day-old seedlings of WT, *cpk3* and *cpk13* were assessed before (A) and after (B) heat stress treatment at 40°C for 3 h. Quantitative reverse transcription (RT)-PCR was done on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems). Primers used for this study are shown in Table 1. Transcript levels of genes were normalized by those of *ACT1* (At2G37620) measured in the samples and expressed relative to the normalized transcript levels in the leaves of unheated WT plants. Data represent the mean+SE ($n>5$). An asterisk (*) indicates that the mutant was significantly different from WT for the indicated gene and treatment ($P<0.05$, ANOVA). (C) Impaired thermal tolerance at 40°C for 3 h both *cpk3* and *cpk13* seedlings with respect to compared to WT phenotype is shown.

Hsf appears to be induced by HS treatment (Li et al. 2009), and belongs to the Hsf class B transcription factors (B-Hsfs). The function of class B-Hsfs differs from that of class A-Hsfs due to a structural variation within the oligomerization domain and the lack of an AHA-motif, which is required for the transcriptional activation function of class A-Hsfs (von Koskull-Döring et al. 2007). Since class B-Hsfs have the capacity to bind to similar or the same sites in the heat shock gene promoters as class A-Hsfs, most of them may act as

Table 1. Primers used for this study

Gene	Purpose	Sequence (5' to 3')
ACT1	Real-time PCR (F)	TGCACTTCCACATGCTATCC
(At2G37620)	Real-time PCR (R)	GAGCTGGTTTTGGCTGTCTC
Hsp15.7-CI	Real-time PCR (F)	TCAACGGCTCTGATTGATTG
(At5g37670)	Real-time PCR (R)	ACTTCCACCACCGGAAAAAG
Hsp17.4-CIII	Real-time PCR (F)	CCCGGAATTTCAAAATCAGATA
(At1g54050)	Real-time PCR (R)	GCCGTTACAGAAGCCATATCA
Hsp101	Real-time PCR (F)	AATTGAACTTCACGCCTTGG
(At1g74310)	Real-time PCR (R)	CTGCCTCCTGCAAAGAAAAC
Hsp70	Real-time PCR (F)	TAAGGTTCTTTCCGGTCCAG
(AT3G12580)	Real-time PCR (R)	CTTGACGCTGAGAGTCGTTG
CPK3	pRE8(XVE) (F)	GAGACTCGAGATGGGCCACAGACACAGCAAG
(At4g23650)	pRE8(XVE) (R)	GAGAACTAGTCTAAATCCACGGATGATTTAGCAC
CPK13	pRE8(XVE) (F)	GAGACTCGAGATGGGAAACTGTTGCAGATCTCC
(At3g51850)	pRE8(XVE) (R)	GAGAACTAGTCTAAGCACTTGCTTTGCAGTCAGC
HsfB2a	pGreen0229 (F)	GAGATTCGAAATGAATTCGCCGCCGGTT
(At5g62020)	pGreen0229 (R)	GAGAGGATCCATTACAAACTCTCTGATT

(F) and (R) indicate the forward and reverse primers, respectively.

repressors of target gene expression (Czarnecka-Verner et al. 2000; Czarnecka-Verner et al. 2004). This assumption was, in fact, supported by our findings that *hsfB2a* mutants showed significantly higher transcript levels of *Hsp101* and *Hsp15.7-CI* but not other Hsp genes (*Hsp70* and *Hsp17.4-CIII*) in heated seedlings, compared to heat-stressed WT seedlings, (Figure 2B). In contrast, in unheated plants, *hsfB2a* mutants showed slightly lower gene expression for *Hsp101* and *Hsp15.7-CI*, compared to WT (Figure 2A). No phenotypic changes were, however, found for the mutant seedlings, irrespective of heated or not-heated conditions, when compared to WT (data not shown).

To confirm the *in vivo* function of the CPK-HsfB2a cascade, constitutively active forms of CPK and HsfB2a were co-expressed as cofactors for the transient expression of a reporter (GUS) gene under the control of HSE (six inverted repeats of nGAAn units) in *Nicotiana benthamiana* leaves, in *Agrobacterium tumefaciens*-mediated transient expression (agroinfiltration) assays. As shown in Figure 3, the activity of the reporter gene was increased when it was co-expressed with HsfB2a as effector (20-fold). Moreover, the activation by HsfB2a was not additionally enhanced when a constitutively active form of CPK3 or CPK13 protein, which lacks the junction and calmodulin-like domains and thus no longer shows Ca^{2+} dependency, was co-expressed. According to these results, we concluded that the CPK3/13-phosphorylated HsfB2a does not function in the suppression/activation of Hsp expression and enhancement of thermotolerance. As described above, since class B-Hsfs generally interact antagonistically with A-Hsfs by binding (or competing for binding) to the HSE consensus sequence, A-Hsfs should be investigated as possible additional cofactors in further studies. It should also be examined whether a suite of A-Hsfs, of which we have not been tested for CPK substrate targeting (i.e., HsfA1d,

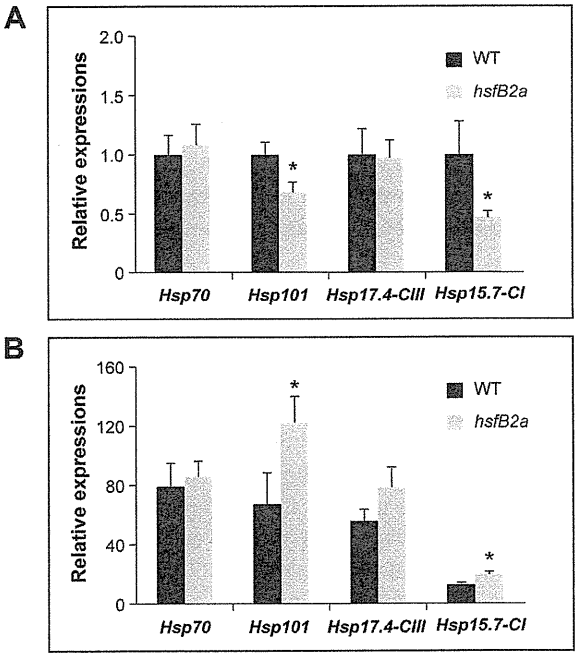

Figure 2. Effects of loss of HsfB2a function on heat response signaling pathway in Arabidopsis seedlings. Transcript levels of Hsp genes in WT and HsfB2a T-DNA insertion mutant (Salk_027578) seedlings before (A) and after (B) heat stress treatment at 40°C for 3 h. Transcript levels of genes were normalized by those of *ACT1* measured in the samples and expressed relative to the normalized transcript levels in the leaves of unheated WT plants. Data represent the mean+SE (n>5). An asterisk (*) indicates that the mutant was significantly different from WT for the indicated gene and treatment (P<0.05, ANOVA).

HsfA8 (Hsf5), HsfA7a, HsfA7b, HsfA5, HsfA1a (Hsf1) and HsfA3), are directly phosphorylated by CPK3 or CPK13, and then potentially activate the Hsp transcription in an HsfB2a- independent manner.

Our results show that CPK3 and CPK13 function as positive regulators of Hsp gene up-regulation involved in

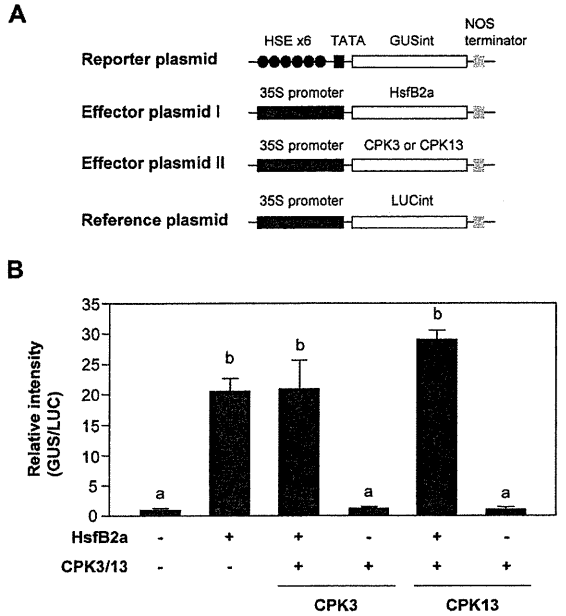

Figure 3. Agroinfiltration assay of a GUS reporter gene under the control of HSE in *Nicotiana benthamiana*. (A) Schematic diagram of the reporter and effector plasmids used in transient assay. An HSE (six inverted repeats of nGAAn units, see underlined sequences) fragment fused to a minimal TATA box: aGAAg cTTCc aGAAc gTTCg aGAAc gTTCg ccc ttc ctc tat ata agg aag ttc att tca ttt gga gag gac tcc ggt) fragment was fused to a minimal TATA box and a GUS reporter gene including the intron (GUSint). Transient activation of the reporter gene according to co-expressed effector(s), HsfB2a, or truncated variant of CPK3 or CPK13, in *N. benthamiana* leaves was assessed according to a modification of the protocol from (Nagamangala Kanchiswamy et al. 2010). *Agrobacterium*, carrying 35S promoter::LUC including intron (LUCint), was used to normalize for the efficiency of agroinfiltration. (B) Transactivation of a GUS reporter gene under the control of HSE. Data represent the mean+SE (n=5). Means followed by different small letters are significantly different (P<0.05, ANOVA followed by Fisher's PLSD test).

HS tolerance in Arabidopsis. In fact, CPK3 (together with CPK6) is also known to play a role in guard cell ion channel regulation that transduces stomatal abscisic acid signaling (Mori et al. 2006). Moreover, CPK3 has been reported to be associated with the plasma membrane and vacuoles, both depending on its N-terminal myristoylation in the salt stress responses, and thus is able to phosphorylate predominantly membrane-associated proteins (Mehlmer et al. 2010). Since the heat-induced withering of *cpk3* seedlings seem to be due to water-loss (see Figure 1C), a significant portion of CPK3 that function in the heat response signaling pathway may be associated with drought/salt stress-related membrane proteins rather than Hsfs (Arimrua and Sawasaki, 2010). Therefore, it is very likely that CPKs contribute to a wide range of central signal transduction responses in plants, whereas their substrate targets are differently involved or function differently.

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ARTICLE ADDENDUM

Plant Signaling & Behavior 5:10, 1-3; October 2010; © 2010 Landes Bioscience

Arabidopsis CPK3 plays extensive roles in various biological and environmental responses

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Plant Ca^{2+}-dependent protein kinase (CPK) signaling is involved in a wide array of intracellular signaling pathways involved in stomatal movement and plant adaptation to various environmental challenges including drought, salt and cold stress. Arabidopsis CPK3 appears to be extensively involved in such a wide range of aspects, and has been shown to function in mediating the signaling following Ca^{2+} influx after insect herbivory. The results reveal the involvement of CPK3 in the herbivory-induced signaling network through phosphorylating the substrate target HsfB2a (heat shock transcription factor) for transcriptional activation of the plant defensin gene *PDF1.2*. Proteomic studies based on the cell-free protein production system allowed us to mine CPK3 targets more extensively and clarify the nature of multifunctional CPK3.

Key words: Arabidopsis, Ca^{2+}-dependent protein kinase (CPK), herbivore

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Plants possess several classes of Ca^{2+}-binding sensory proteins, including calmodulins (CaMs), calmodulin-like proteins, calcineurin B-like proteins and CPKs.[1] Especially, CPKs constitute a large family of serine/threonine protein kinases that are broadly distributed in the plant kingdom; for instance, the Arabidopsis genome is predicted to have 34 different CPKs.[2] Subcellular localization analysis for CPK isoforms from Arabidopsis demonstrated, for example, that AtCPKs 1, 7, 8, 9, 16, 21 and 28 are membrane-associated and other isoforms (AtCPKs 4 and 32) are localized with a nuclear/cytosolic, nuclear and peroxisomal distributions, respectively.[3,4] Hence, a variety of features make CPKs optimally adapted to control diverse responses to elevated Ca^{2+}, including differences in Ca^{2+} responsiveness, localization and substrate preference.[5]

Arabidopsis CPK3 is of special interest, since this kinase is able to act in a manner that depends on a sizable array of (sub)cellular localizations and potentially specific developmental and environmental conditions (Fig. 1). This CPK is associated with the plasma membrane and vacuoles, both depending on its N-terminal myristoylation in the salt stress response, and thus is able to phosphorylate predominantly membrane-associated proteins.[6] In guard cells, CPK3 appears to act in the phosphorylation of plasma membrane S-type anion channels for the Ca^{2+}-reactive stomatal closure response controlled by abscisic acid (ABA) signaling.[7] Moreover, our recent research showed that CPK3 also phosphorylates three nuclear/cytosolic transcription factors [jasmonic acid/ethylene-inducible APE/ERF domain transcription factor 1 (ERF1),[8] heat shock transcription factor HsfB2a, and the wound-inducible CZF1/ZFAR1 transcription factor].[9,10] The CPK3-derived phosphorylation of HsfB2a promotes *PDF1.2* transcriptional activation in the defense response against a feeding generalist herbivore, *Spodoptera littoralis*. ATL2, a member of a multigene family of highly related RING-H2 zinc finger proteins that function as E3 ubiquitin ligases and a potent regulator of *PDF1.2* transcription,[11-13] is also phosphorylated by CPK3. Since herbivory responses in Arabidopsis is not mediated strikingly by the ABA signaling network, additional branches in the herbivory signal transduction network via CPK3 are most likely different from those

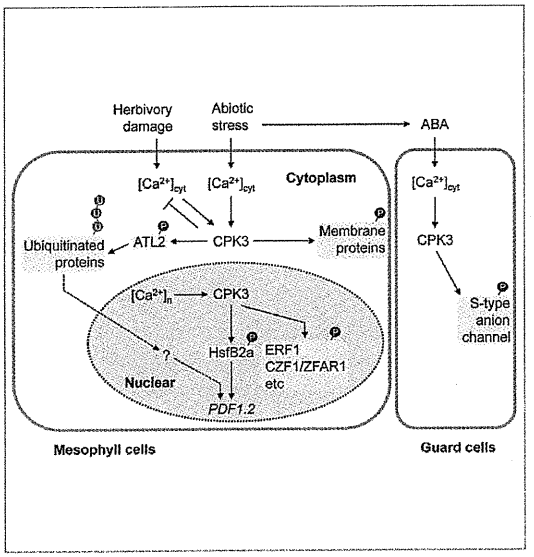

Figure 1. The multifunctional CPK3 signaling required for biotic/abiotic responses.

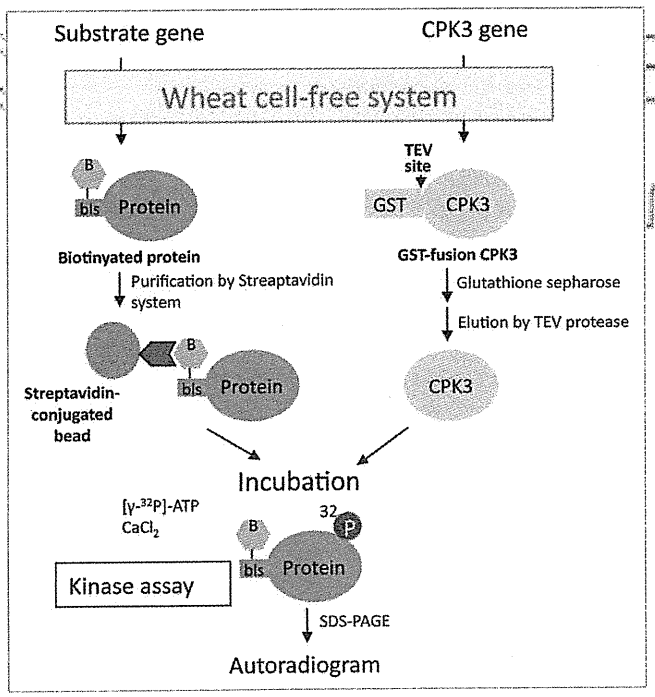

Figure 2. Screening method for the CPK3 substrate identification using the wheat cell-free system.

of the salt stress acclimation as well as the stomatal closure response that are intensively controlled by ABA signaling.[6,7] In addition, the herbivory-induced cascade is also independent from the jasmonic acid/ethylene-related signaling pathways.[10]

Meta Substrate Targeting of CPK3

The identification of protein kinase substrates is important to assess the biological roles of kinases. In addition, recent advances in proteomic technology allow to make use of mass spectrometry MALDI/TOF for identification of the significant phosphorylation sites of substrates,[14] but this method still leaves us far from understanding the functional nature of kinases. Hence, we still need to rely on a wide array of methods to approach the roles of kinases.

For identification of target substrates of protein kinases, a protein library composed of recombinant proteins is a useful platform. Recent protein production technology using a wheat cell-free system allowed the preparation, for example, of 13,000 human proteins as a protein library.[15] Through the screening of Arabidopsis CPK substrates, four CPK3-phosphorylated targets were successfully mined from 100 nuclear and cytosolic proteins that were conveniently prepared with the cell-free system using 96-well plates (**Fig. 2**).[10] These results further confirm that this system is a powerful tool to discover the nature of multifunctional protein kinases such as CPK3.

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Characterization of a caspase-3-substrate kinome using an N- and C-terminally tagged protein kinase library produced by a cell-free system

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Caspase-3 (CASP3) cleaves many proteins including protein kinases (PKs). Understanding the relationship(s) between CASP3 and its PK substrates is necessary to delineate the apoptosis signaling cascades that are controlled by CASP3 activity. We report herein the characterization of a CASP3-substrate kinome using a simple cell-free system to synthesize a library that contained 304 PKs tagged at their N- and C-termini (NCtagged PKs) and a luminescence assay to report CASP3 cleavage events. Forty-three PKs, including 30 newly identified PKs, were found to be CASP3 substrates, and 28 cleavage sites in 23 PKs were determined. Interestingly, 16 out of the 23 PKs have cleavage sites within 60 residues of their N- or C-termini. Furthermore, 29 of the PKs were cleaved in apoptotic cells, including five that were cleaved near their termini *in vitro*. In total, approximately 14% of the PKs tested were CASP3 substrates, suggesting that CASP3 cleavage of PKs may be a signature event in apoptotic-signaling cascades. This proteolytic assay method would identify other protease substrates.
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Subject Category: Immunity

On the basis of the corresponding genetic sequences, >500 human and mouse proteolytic enzymes have been predicted.[1] This number is comparable with that found for protein kinases (PKs), which are the main signal-transduction enzymes.[2,3] Proteases are involved in the maturation, localization, stabilization, and complex formation of proteins, and in many biological processes, for example, normal development,[4,5] cancer,[6,7] infectious diseases,[8] and cell death.[9] Therefore, it is important to be able to identify protease substrates using simple assays.

Apoptosis requires the action of many different proteins that participate in apoptotic cell-signaling pathways.[10] Caspases and PKs are critical components of growth and apoptosis signaling pathways.[2,10] Large-scale analyses of the biological networks involving PKs and caspases are vital for the elucidation of apoptosis signaling pathways. Recent whole-cell proteomic studies that used mass spectrometry attempted to identify substrates of caspases that are involved in apoptosis and have shown that the percentage of PKs found as caspase substrates during apoptosis is 3–6% of ~300.[11,12] However, cellular protein expression levels may have biased the results.[13] Furthermore, it is difficult to identify specific pairs of proteases and substrates because numerous cleavage events occur simultaneously in cells. Therefore, an *in vitro* approach that could identify specific proteases and their corresponding substrates would complement cell-based approaches. A diagram, derived from a comprehensive *in vitro* study, that illustrates the relationships between caspases and their PK substrates would help clarify the signal-transduction events that occur during apoptosis.

A collection of recombinant proteins, that is, a protein library, is needed to screen a large number of protein substrates. In addition, to screen a protein library comprehensively two *in vitro* high-throughput methods – one for protein synthesis and one for the detection of the targeted biochemical reaction – are required. Recently, we developed an automated protein synthesis system that uses a wheat cell-free system.[14–16] Using this system, we were able to synthesize many human and Arabidopsis PKs.[17,18] Recent work by others suggested that the wheat cell-free system could produce 13 364 human proteins, which, because of the large number of proteins involved, represents an *in vitro*-expressed proteome.[19] We also recently developed a method to label monobiotin proteins that had been synthesized in the wheat cell-free system.[20] These monobiotin-labeled proteins were then used directly – without purification – to detect protein ubiquitination[21] and an autoantibody in the serum.[22] As the procedures used with many commercially available detection kits depend on biotin–streptavidin interactions, our purification-free, synthesis/biotin-labeling method provides a simple and highly specific system that can be used for biochemical analyses.

Caspase-3 (CASP3) cleaves many different proteins,[23,24] and its action *in vivo* irreversibly induces apoptosis. For the study reported herein, we delineated a CASP3-substrate kinome using a simple luminescent-based detection method

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to screen an N- and C-terminally tagged (NCtagged) PK library produced in the wheat cell-free system. This comprehensive characterization of a CASP3-substrate kinome is a resource that can be used to understand the roles of PKs in apoptosis.

Results

Generation of an NCtagged PK library used to identify CASP3 PK substrates. To identify PKs that are substrates of CASP3, we first made a library consisting of 248 human and 56 mouse PKs (Supplementary Table S1). The nucleotide sequences for the Flag-tag and the biotin ligation site (bls) were added upstream and downstream, respectively, of the *PK* open-reading frame by PCR incorporation of Gateway recombination tags. Each PCR product (attB1-Flag-*PK*-bls-attB2) was inserted into a pDONR221 vector using the Gateway BP Clonase II system (upper panel, Figure 1). The Flag-*PK*-bls nucleotide sequences from the *Escherichia coli* cultures were used without purification to construct, by split-primer PCR, the DNA templates for protein synthesis.[14] The NCtagged PK library (304 PKs) was produced using an automated protein synthesizer (GenDecoder 1000; CellFree Sciences Co., Ltd., Matsuyama, Japan), with biotin and biotin ligase added into the synthesis mixtures for monobiotin labeling at the bls.[20,21] That the members of the protein library were NCtagged was confirmed by immunoblotting with anti-Flag antibodies and Alexa488-labeled streptavidin.

To assess the suitability of the designed PKs to act as CASP3 substrates, we used NCtagged p21-activated kinase 2 (PAK2), which is a known CASP3 substrate,[25] as the test case. The biotinylated NCtagged-PAK2 (Flag-PAK2-bls~biotin) was treated with CASP3 and cleavage of PAK2 was confirmed by immunoblotting with Alexa488-conjugated streptavidin (Figure 2a). In addition, the cleavage site ($_{319}$DELD↓S$_{323}$), determined by amino-acid sequencing, was found to be the same as that reported previously.[25] (The arrow indicates the hydrolytic bond.)

A luminescent assay to detect PK substrates of CASP3. A schematic of the assay used to monitor cleavage of the NCtagged PKs by CASP3 is shown in Figure 1. The PK construct is first incubated with CASP3. If the construct contains a sequence that can be cleaved by CASP3, cleavage occurs. Acceptor and donor beads are then added. The Flag-tag binds a protein A-conjugated acceptor bead via an anti-Flag antibody, and the biotin bound to the C-terminus of the PK construct binds a streptavidin-conjugated donor bead. If an acceptor bead is in close contact with the donor bead, as is the case when the construct is not a CASP3 substrate and both beads are therefore bound intramolecularly, the system luminesces. However, if CASP3 had cleaved the NCtagged PK, luminescence is suppressed because the beads are no longer in close contact. As a proof-of-concept experiment, cleavage of the test PK, NCtagged PAK2, was assessed

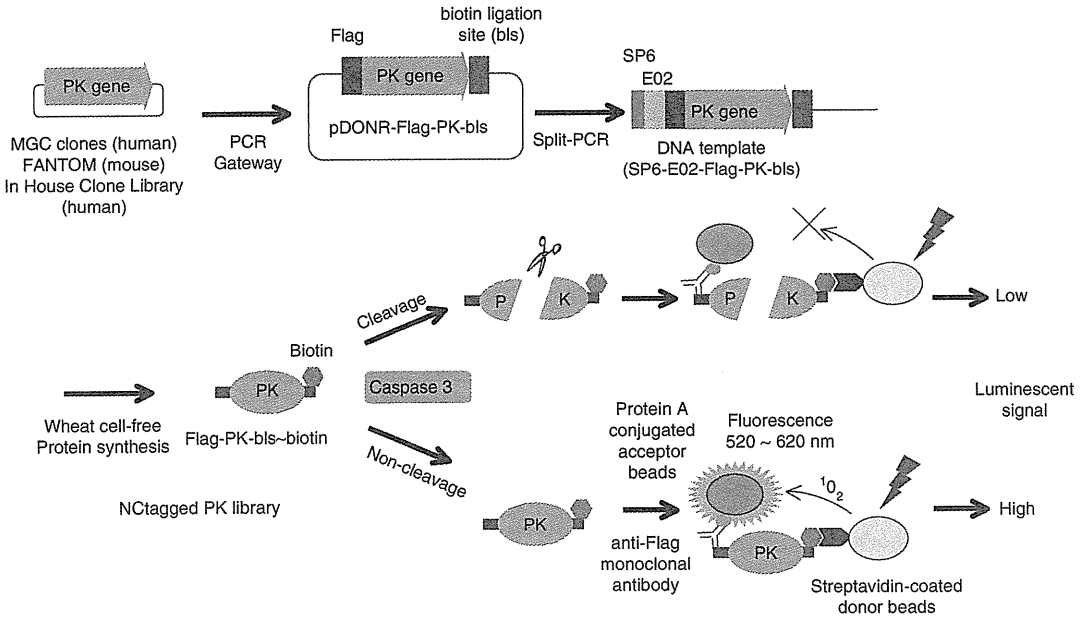

Figure 1 Schematics of the DNA template construction and the CASP3-substrate-screening assay. *Protein kinase* (*PK*) genes were obtained from the human MGC and mouse FANTOM libraries, and from a library of *PK* genes that we had cloned. The *PK* genes were PCR amplified with the Flag and the biotin ligation site (bls) tags added to the upstream and downstream ends, respectively. The modified genes were each inserted into a Gateway pDONR221 vector (pDONR-Flag-PK-bls) and DNA templates (SP6-E02-Flag-*PK*-bls) were constructed by split-primer PCR and then expressed in the wheat cell-free protein synthesis system that included biotin ligase and D-biotin to give Flag-PK-bls~biotin constructs. The Flag and biotin tags were bound to protein A-conjugated acceptor beads via an anti-Flag antibody and streptavidin-conjugated donor beads, respectively. An intact complex luminesced strongly, whereas after CASP3 cleavage and dissociation of the protein fragments, the luminescence was abolished or reduced