another example, autophosphorylation involves in brassinosteroids (BR) signaling pathway. The BR receptor-like kinase, BRI1, induces hetero-oligomerization of BRI1 and BAK1 and autophosphorylation of numerous Thr and Tyr residues to initiate BR signal transduction after BR binding (Nam and Li, 2002; Li et al., 2002; Wang et al., 2005; Oh et al., 2009). Another important PK in the BR signaling pathway that is activated by autophosphorylation is the GSK3/shaggy-like PK BIN2 (Kim et al., 2009). The active, phosphorylated, form of BIN2 phosphorylates the transcriptional factors BES1 and BZR which results in their degradation by the proteasome (He et al., 2002; Li and Nam 2002; Kim et al., 2009). Thus, the BIN2 is believed to negatively regulate the BR signaling pathway. These reports indicate that identifying the autophosphorylation activity of plant PKs are an important issue for understanding its regulation mechanism.

However, information about biochemical characterization of PKs has been hindered by the difficulties in obtaining sufficient quantities of functionally active recombinant protein. Conventional recombinant technologies utilizing cell-based protein production systems are well developed and to some extent can serve for both screening and expression. Nevertheless, they possess many intrinsic limitations. Primarily, cannot express proteins that induce cytotoxic effect in cells. Secondly, require time-consuming processes, such as sub-cloning, cell culture and protein purification. Finally, require high quality purification of PKs for functional screening because the host cell has high endogenous phosphorylation activities. Although current cell-free protein expression systems are known to synthesize proteins with high speed and accuracy, the yields of the products are low due to their instability over time. In addition to that, prokaryotic-based systems as represented by *Escherichia coli* cell-free systems are not always suitable for the expression of eukaryotic proteins because of the prokaryotic nature of its translation and folding mechanisms, that is, multidomain proteins found more often in eukaryotes than in prokaryotes, tend to missfold in prokaryotic systems, whether *in vivo* or *in vitro* (Netzer and Hartl, 1997).

A wheat germ cell-free protein production system could synthesize large numbers of proteins with high speed and accuracy, approaching those of *in vivo* translation, and could also express proteins which interfere with the host cell physiology (Kurland, 1982; Pavlov and Ehrenberg, 1996; Madin et al., 2000; Sawasaki et al., 2002a; Endo and Sawasaki, 2004). Using this system, we synthesized over 400 eukaryotic PKs (Sawasaki et al., 2004; Endo and Sawasaki, 2006; Tadokoro et al., 2010). These studies indicated that the wheat germ cell-free system had low endogenous phosphorylation activity. We recently generated a method to label monobiotin proteins that had been synthesized in the wheat cell-free system (Sawasaki et al., 2008). By combining this technology with a commercially available luminescence system, we also developed a high-throughput and high-sensitivity method for protein biochemical analysis. This method was able to specifically detect protease cleavage (Tadokoro et al., 2010), protein ubiquitination (Takahashi et al., 2009), protein–protein interaction (Ryo et al., 2008) and an autoantibody in the serum (Matsuoka et al., 2010), using unpurified (crude) recombinant protein. In this study, we applied this system to analyze *Arabidopsis* PK autophosphorylation activity (the scheme is shown Fig 1a and b).

2. Results

2.1. Autophosphorylation of biotinylated AtCPK3 and AtCPK13 were detected with high sensitivity and specificity using a luminescence system

We adapted the AlphaScreen technology for detecting interactions between autophosphorylated PKs and anti-phosphoserine/phosphothreonine (anti-pSer/pThr) antibody. This principle is illustrated in Fig. 1b. For autophosphorylation analysis of the expressed proteins, the translation mixture was used without any purification. In the AlphaScreen system, autophosphorylation of the biotinylated protein results in a biotinylated protein-anti-pSer/pThr antibody complex that is captured simultaneously by the streptavidin-coated donor beads, which is bound to the biotinylated PK, and the protein A-conjugated acceptor bead, which is bound to the anti-pSer/pThr antibody. The resultant proximity of the acceptor and donor bead generates the luminescent signal upon excitation at 680 nm.

To validate this system, we used the well-characterized calcium-dependent PKs AtCPK3 and AtCPK13 (Kanchiswamy et al., 2010). Biotinylated or non-biotinylated recombinant AtCPK3, AtCPK13 and dihydrofolate reductase (DHFR), serving as a negative control, were synthesized in the wheat germ cell-free system. As a

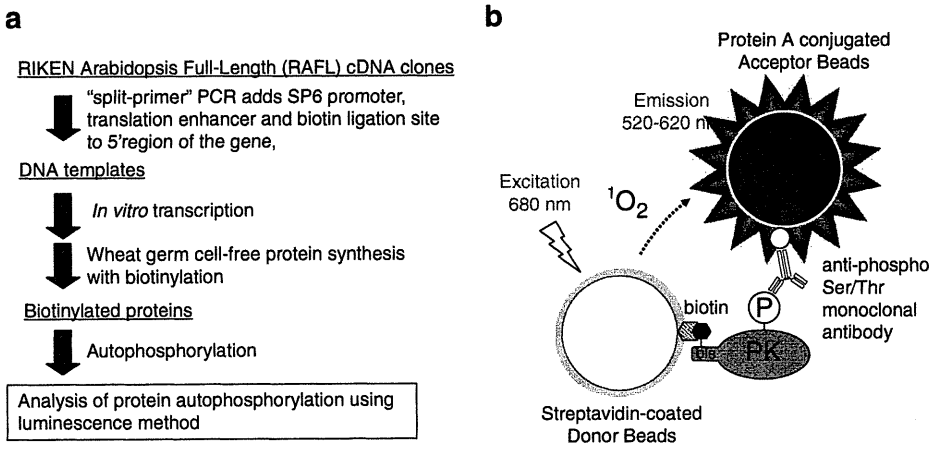

Fig. 1. Schematic diagram for determining autophosphorylation activity using a biotinylated PK library synthesized using the wheat germ cell-free system. (a) Flow chart of the cell-free expression procedures for high-throughput autophosphorylation profiling of *Arabidopsis* PKs. The method begins with generation of DNA templates generated by "Split-primer" PCR, followed by *in vitro* transcription using phage coded SP6 RNA polymerase, and finally translation in a bilayer reaction. All of the steps were carried out in 96-well microtiter plates. (b) Autophosphorylation analysis of the biotinylated PK using a luminescence system. Protein A-conjugated acceptor beads bound to anti-pSer/pThr monoclonal antibody and streptavidin-coated donor beads bound to biotinylated protein are in close proximity. Upon excitation at 680 nm, a singlet oxygen is generated by the donor beads, transferred to the acceptor beads within 200 nm, and the resultant reaction emits light at 520–620 nm.

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result, a high luminescent signal was observed in the presence of biotinylated recombinant AtCPK3, and AtCPK13 (Fig 2a). In contrast, non-biotinylated PKs and DHFR showed very low signals. These luminescent signals were approximately consistent with autoradiography of *in vitro* kinase assay data (Kanchiswamy et al., 2010). These results indicate that the luminescent method can detect autophosphorylation by using an anti-pSer/pThr antibody.

2.2. Construction of a biotinylated protein kinase library using the wheat germ cell-free protein production system

To address high-throughput protein production, we have utilized our wheat germ high-throughput protein synthesis system (Madin et al., 2000; Sawasaki et al., 2002a), which can produce large numbers of recombinant proteins using a fully automated robot (Sawasaki et al., 2005). To create a library of *Arabidopsis* PKs, we selected 768 cDNAs encoding PKs from the RAFL cDNA resource. Since the full-length cDNA was provided in plasmids, the transcriptional template was synthesized by PCR directly from *E. coli* cells carrying cDNA clones, to avoid time-consuming cloning procedure. For biotinylation, a biotin ligation site (bls) was fused onto 5′ end of a target gene using the "split-primer" PCR method, and the reaction was started with *E. coli* cells (Sawasaki et al., 2002a). 759 (98.8%) out of 768 genes were successfully amplified and of those, 759 were transcribed. Protein production was performed automatically using the GenDecoder1000 protein synthesizer with standard 96-well microtiter plates (Sawasaki et al., 2007). After an 18 h incubation at 16 °C, randomly selected 30 proteins were confirmed by SDS–PAGE combined with immunoblot analysis using streptavidin Alexa Fluor 488 conjugate (Invitrogen, Carlsbad, CA) (data not shown). All 759 clones were produced as biotinylated proteins (Supplementary data 1).

2.3. High-throughput analysis for Ser/Thr autophosphorylation activity using a biotinylated protein kinase library

To identify autophosphorylation proteins that react with an anti-pSer/pThr antibody, biotinylated and non-biotinylated PKs libraries were prepared. In each well of a 384-well plate, a translation mixture expressing biotinylated or non-biotinylated PK was incubated for 60 min, and subsequently a mixture of donor and acceptor beads and anti-pSer/pThr antibody were added to each well. After incubation, autophosphorylation of the biotinylated PK was detected by the luminescence assay shown on Fig. 1b. All data are the average of two independent experiments, and the background was controlled for each using the relevant non-biotinylated PK. Fig. 2b graphs the relative luminescent signals (biotinylated PK/non-biotinylated PK) of each PK. As a result, 179 out of 759 PKs demonstrated a relative luminescent signal higher than 5-fold. This assay also showed 67 PKs having a relative luminescence signal higher than 10-fold (Fig 3a). Two of these 67, AtMPK17 (at2g01450) and AtCPK6 (at2g17290) exhibited around 100 relative luminescence signals (Fig 3a). We compared our results with autophosphorylation data from a previous *in vitro* study monitored by autoradiography (Supplementary data 1; Sawasaki et al., 2004a). Of the 179 PKs that demonstrated autophosphorylation in this work, 125 were tested previously. Of these 125 PKs, 81 (64.8%) had autophosphorylation activity in the previous study. Since more than half of 179 PKs had autophosphorylation activity, that a relative luminescence signal higher than 5-fold was defined as autophosphorylation activity. Additionally, 55 out of the high relative luminescence signal ($\geqslant$10-fold) 67 PKs has been analyzed in the previous study, and 45PKs (81.8%) had autophosphorylation activity.

In general, when around 700 PKs are analyzed by autoradiography, it takes at least 2 weeks. In contrast, this method is able to analyze in only 3 h. Therefore, this method is a powerful tool for high-throughput protein phosphorylation analysis.

2.4. Functional classification analysis of high autophosphorylation protein kinases

We next classify high relative luminescence signal 67 PKs according to both their primary sequence and functions. With the completion of the *Arabidopsis* genome sequencing, a classification of the PKs has been reported and the database is available at PlantP (http://www.plantsp.sdsc.edu/) (Gribskov et al., 2001). Using this database, functional classification of the 67 PKs noted above was analyzed. According to PlantP classification, the largest class of the 67 PKs was non-transmembrane protein kinases [Class IV, 53% (35 PKs)] (Fig. 3b). PKs in this class do not have an identifiable transmembrane domain (TMD), indicating that are not closely related to the transmembrane receptor kinases. Class IV PKs play a central role in the transduction of various extra- and intracellular signals (e.g. MAPK, calcium response kinase). The next class was receptor-like protein kinases (RLK) [Class 1, 23% (13 Pks)]. RLKs are defined by the presence of a signal peptide, an extracellular domain, a TMD and a C-terminal Ser/Thr kinase domain (Shiu and Bleecker, 2001), which are involved in symbiosis (Parniske 2008), disease resistance (Afzal et al., 2008), self-incompatibility (Takayama and Isogai, 2005), BR signaling

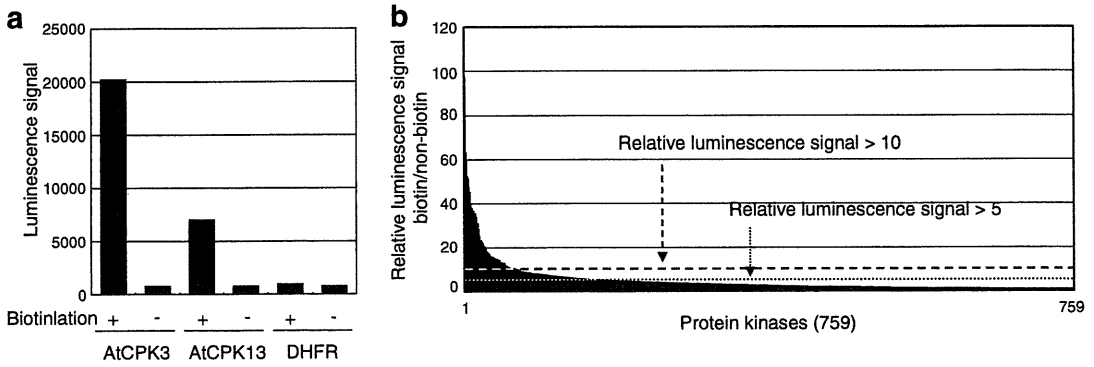

Fig. 2. High-throughput production and autophosphorylation activity profiling of *Arabidopsis* PKs. (a) Autophosphorylation of biotinylated AtCPK3 and AtCPK13 were detected by the luminescence system. (b) Autophosphorylation of 759 biotinylated PKs synthesized using the wheat germ cell-free system, as described in the text, were detected by the luminescence system. All data are the average of two independent experiments, and the background was controlled for each using the relevant non-biotinylated PK.

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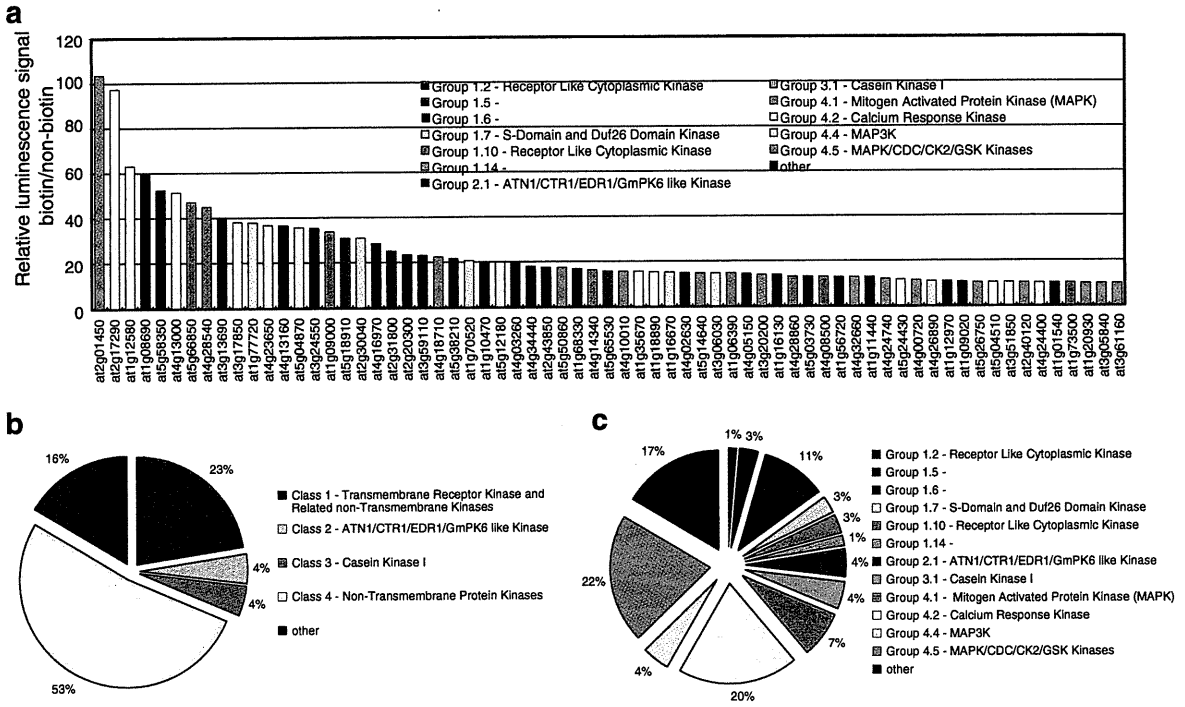

Fig. 3. Functional distribution of 67 high luminescent signal PKs. (a) Sixty-seven highly signal biotinylated PKs were selected based on luminescent signal ⩾10-fold background signal. This data is average of two independent experiments, and the background control for each was the relevant non-biotinylated PK. (b and c) The 67 PKs were grouped by the corresponding PlantP database classification number and classified by (b) Class and (c) Group.

(Belkhadir and Chory, 2006), cell growth regulation (Hematy and Hofte, 2008) and formation of the shoot stem cell niche (Clark, 2001; Stahl and Simon, 2005).

We next annotated the 67 PKs by PK Groups using the PlantP database. Interestingly, the groups represented included MAPK/CDC/CK2/GSK kinases [Group 4.5, (22%)], calcium response kinases [Group 4.2 (20%)], unclassified protein kinases (17%) and Group 1.6 (11%) (Fig 3c). Additionally, MAP3 K [Group 4.4, (50%)], Group 1.6 (35%), MAPK/CDC/CK2/GSK kinases [Group 4.5, (23%)] and receptor like cytoplasmic kinase [Group 1.10 (22%)] had the highest percentage of autophosphorylating PKs (Fig. 4).

Finally, to get a better overview of the annotated 67 PKs, we used the gene ontology annotation search on the TAIR website (Berardini et al., 2004) and classified the 67 PKs into functional category (Supplementary data 2 and Fig. 5). As a result, 19 out of 67 PKs were annotated in localization of plasma membrane, and those more than halves were classified into Groups 1.6 and 4.2. (Supplementary data 2). Moreover, the annotated proteins were involved in diverse biological functions such as hormone biosynthesis and response (6%), response to other stresses (5%), development (4%), response to abiotic or biotic stimulus (3%), signal transduction (3%), biological process unknown (2%), transport (1%), transcription (0.5%), cell organization and biogenesis (0.5%) (Fig. 5b).

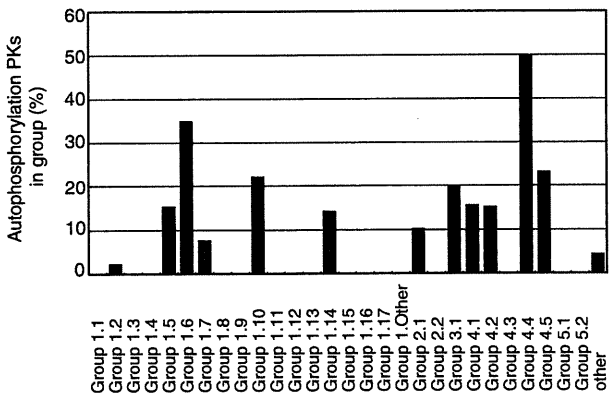

Fig. 4. PK groups compared by the percentage of protein autophosphorylation. Group 1.2; receptor like cytoplasmic kinase, Group 1.4; crinkly 4 like kinase, Group 1.7; S-domain and Duf26 domain kinase, Group 1.8; leucine rich repeat receptor kinase, Group 1.10; receptor like cytoplasmic kinase, Group 1.11; legume lectin domain kinase, Group 1.12; leucine rich repeat receptor kinase, Group 1.13; leucine rich repeat receptor kinase, Group 1.16; receptor like cytoplasmic kinase, Group 1.17; wall associated kinase, Group 2.1; ATN1/CTR1/EDR1/GmPK6 like kinase, Group 2.2; unknown function protein kinase, Group 3.1; casein kinase I, Group 4.1; mitogen activated protein kinase, Group 4.2; calcium response kinase, Group 4.3; unknown function protein kinase, Group 4.4; MAP3 K, Group 4.5; MAPK/CDC/CK2/GSK kinases, Group 5.1; other protein kinase, Group 5.2; other protein kinase, other; unclassified PKs (group unnamed). The classification numbers correspond to the PlantP database.

3. Discussion

Phosphorylation of protein is an important property of protein regulations reported both from the plants as well as animals. Autophosphorylation may also be important not only as a modulator of PK activity (Chehab et al., 2004) but also as a means of altering protein binding properties that can affect a broad range of functional properties from cellular localization (Nayler et al., 1998.) to substrate interactions (Fan et al., 2004.) to macromolecular complex formation (Ikeda et al., 2000; Merkle et al., 2002). Therefore, analysis of the autophosphorylation activity of plant PKs is a key issue for understanding its regulation mechanism. The plant PKs functions have been revealed by primarily genetic approaches such as loss and gain of gene function in plant. Moreover, recent studies

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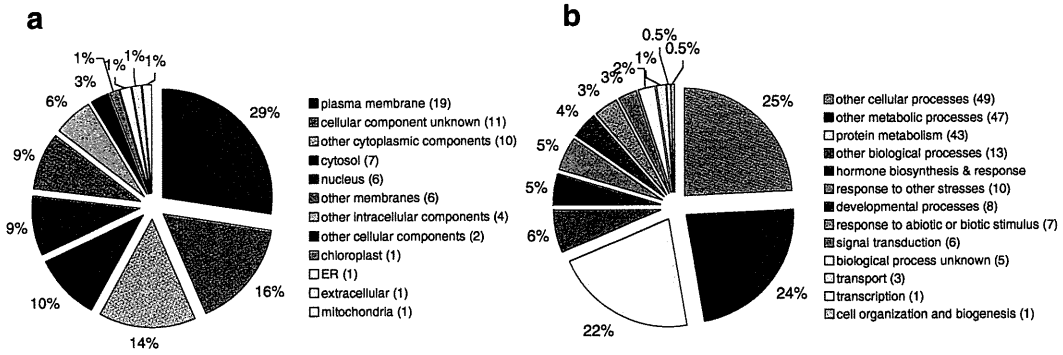

Fig. 5. Gene ontology classification of 67 high luminescent signal PKs. The 67 high luminescent signal PKs were grouped by (a) cellular Component and (b) biological process based on Arabidopsis gene ontology annotation. Gene counts and percentages are listed for each category. Because a single gene may have more than one functional annotation, the gene counts do not add up to the total number of analyzed genes. Numbers in parentheses indicate the number of genes in each annotation group.

found many of phosphopeptides in *Arabidopsis* by MS-based phosphoproteomic screens (Benschop et al., 2007; de la Fuente van Bentem et al., 2008; Sugiyama et al., 2008). However, information about biochemical characterization of PKs has been limited.

To solve this problem, we developed a simple and highly sensitive autophosphorylation analysis method by combination of the wheat germ cell-free protein synthesis system and luminescent detection system (Fig. 1a and b). One advantage of this method is that the wheat germ cell-free protein synthesis system can produce large numbers of quantity and good quality recombinant proteins without time-consuming cloning and protein purification steps (Madin et al., 2000; Sawasaki et al., 2002a, 2005.). In addition, there is no extreme difference in the concentration of the synthesized protein (Nozawa et al., 2009). Second, the luminescence system that combines the biotinylated proteins is able to specifically detect protein modification (Takahashi et al., 2009; Tadokoro et al., 2010) and protein–protein interactions (Ryo et al., 2008; Matsuoka et al., 2010). Using this method, we conveniently detected autophosphorylation of biotinylated AtCPK3 and AtCPK13 (Fig. 2a). Thus, this method represents a simple and quick alternative to conventional laborious kinase assays using [γ-^{32}P]-ATP.

We synthesized the 759 biotinylated PKs of *Arabidopsis* and applied for the autophosphorylation assay (Fig. 2b, Supplementary data 1). As a result, we identified 179 PKs having a relative luminescence signal than 5-fold. Comparing to the previous study, 179 PKs are expected to have the autophosphorylation activity. This assay also showed 67PKs of high relative luminescence signal (Fig. 3a and Table 1). It is thought that 67 PKs has the high autophosphorylation activity when based on the principle of the antibody detection that the signal value is correlation to the number of phosphorylated peptide. However, the three-dimensional conformation effects of PK against the accessibility and specificity of the antibodies cannot be discounted. Unfortunately, currently available antibodies against phospho-Ser/Thr residues generally may have low affinity and be specific to the surrounding amino acid sequence. Therefore, the anti-pSer/pThr antibody of the independent to the surrounding amino acid sequence should be developed to improve this assay system.

Using PlantP database, functional classification of the 67 PKs was analyzed. Interestingly, non-transmembrane protein kinases [Class IV] accounted for half of the 67 PKs, and MAPK/CDC/CK2/GSK kinases [Group 4.5] and calcium response kinases [Group 4.2] were the most of it (Fig. 3a and b). Besides, MAP3K [Group 4.4, (50%)], Group 1.6 (35%) had the highest percentage of autophosphorylating PKs (Fig. 4). MAP3K involved in the activation of the MAP kinase cascade. This result has been suggested that MAP3K phosphorylation activity is enhanced by autophosphorylation, as is proposed for other eukaryotes MAP3Ks (Nishihama et al., 2002). However, the autophosphorylation activity of other MPKs except AtMPK17 are not admitted. In mammals, MPKs show some Tyr kinase activity and may autophosphorylate on both Thr and Tyr residues (Wu et al., 1991). The autophosphorylation may increase the affinity of MAP2K for MPK (Haystead et al., 1992). In our studies, plant MPKs exhibit little autophosphorylation activity, therefore functional regulation by autophosphorylation might be different from mammals (Roux and Blenis, 2004; Pimienta and Pascual, 2007).

In our studies, the seven of GSK3/Shaggy like PKs showed a high autophosphorylation activity, and other 3 PKs had autophosphorylation activitiy (Supplementary data 1). GSK3/Shaggy like PKs were isolated from *Arabidopsis* as Shaggy (in *Drosophila melanogaster*) and GSK-3 (in mammals) homologues gene (Bianchi et al., 1994; Jonak et al., 1995). Mammalian GSK-3 expressed in bacteria shows evidence of autophosphorylation on Tyr, Ser/Thr residues, which raises the possibility, that Tyr phosphorylation of GSK-3 in mammalian cells is an autocatalytic event (Wang et al., 1994.). As well as GSK-3, BIN2 activity is controlled by autophosphorylation of Tyr residues in T-loop (Kim et al., 2009). However, the functional role of autophosphorylation of Ser/Thr residues is unclear. In mammals, the role of cis/trans-phosphorylation (itself or other kinase) of Ser/Thr residues has been suggested to cause inactivation of enzyme (Wang et al., 1994; Cross et al., 1995). In plants, it might influence the activity and complex formation, localization of GSK/shaggy PKs by autophosphorylation of Ser/Thr residues.

The 19 out of 67 PKs were annotated with localization of plasma membrane, and those more than halves were classified into Groups 1.6 and 4.2. (Table 1). Furthermore, many of Calcium response kinases [Group 4.2] (including of CPKs) were annotated with involved in ABA mediated signaling (Supplementary data 2). Many of the reported CPKs have shown autophosphorylation activity *in vitro* in the presence of Ca^{2+} (Harmon et al., 1987; Binder et al., 1994; Frylinck and Dubery, 1998; Yoon et al., 1999; Anil et al., 2000; Hegeman et al., 2006). Many stress signals, such as wounding, cold, high salinity, and drought, are known to elicit fluctuations in cytosolic Ca^{2+} levels, as well as changes in protein phosphorylation (Bush, 1995; Trewavas, 1999; Knight and Knight, 2001). Several lines of evidence suggest that CPKs mediate abiotic stress signaling pathways (Urao et al., 1994; Monroy and Dhindsa, 1995; Botella et al., 1996; Yoon et al., 1999; Patharkar and Cushman, 2000; Saijo et al., 2000; Chico et al., 2002; Xu et al., 2010). On the other hand, the best-studied *A. thaliana* PERK [Group 1.6], PERK4 is a positive regulator implicated in the early stage of ABA signaling, which modulates root cell elongation, and its effects are mediated by Ca^{2+} (Bai et al., 2009). In the presence of ABA, PERK4 is activated though autophosphorylation, which leads to activation of Ca^{2+} channel and stimulation expression of genes

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Table 1
Summary of autophosphorylation activity of 67 Arabidopsis protein kinases.

No. of Arabidopsis genes	PlantP funciton[a]	PlantP symbol[a]	PlantP no.[a]	PlantP family[a]	Relative luminescence signal[b]	Autophosphorylation[c]
at2g01450	Putative mitogen activated protein kinase 17	AtMPK17	21188	Family 4.5.1 – MAPK family	103.5	Yes
at2g17290	Calcium-dependent protein kinase, isoform 6	CPK06	26192	Family 4.2.1 – calcium dependent protein kinase	97.3	Yes
at1g12580	Phosphoenolpyruvate carboxylase-related kinase	PEPRK1	21496	Family 4.2.1 – calcium dependent protein kinase	63.2	Yes
at1g08690	–	–	–	–	60.1	–
at5g58350	WNK kinase 4	–	37670	–	52.4	–
at4g13000	Putative protein kinase	–	21830	Family 4.2.6 – IRE/NPH/PI dependent/S6 kinase	51.4	Yes
at5g66850	Putative MAP3 K	–	22064	Family 4.1.1 – MAP3 K	47.3	Yes
at4g28540	Putative casein kinase 1	–	21883	Family 3.1.1 – Casein kinase I family	45.1	Yes
at3g13690	Putative protein kinase	–	21717	Family 1.6.2 – Plant external response like kinase	40.3	Yes
at3g17850	Putative protein kinase	–	21210	Family 4.2.6 – IRE/NPH/PI dependent/S6 kinase	38.3	Yes
at1g77720	Putative homolog to msp1 protein kinase	–	21464	Family 4.4.4 – Unknown function kinase	37.8	Yes
at4g23650	Calcium-dependent protein kinase, isoform 3	CPK03	21867	Family 4.2.1 – Calcium dependent protein kinase	36.8	Yes
at4g13160	–	–	–	–	36.6	–
at5g04870	Calcium-dependent protein kinase, isoform 1	CPK01	21951	Family 4.2.1 – Calcium dependent protein kinase	35.7	Yes
at3g24550	Protein kinase	–	21699	Family 1.6.2 – Plant external response like kinase	35.5	Yes
at1g09000	Putative NPK1-related protein kinase 2	–	21067	Family 4.1.1 – MAP3 K	33.9	Yes
at5g18910	Pto kinase interactor 1	–	21302	Family 1.10.1 – Receptor like cytoplasmic kinase VI	31.0	Yes
at2g30040	Putative protein kinase	–	21643	Family 4.4.1 – Unknown function kinase	31.0	nd
at4g16970	Putative casein kinase II	–	21930	-	28.4	–
at2g31800	Putative protein kinase	–	21523	Family 2.1.2 – Ankyrin repeat domain kinase	25.2	nd
at2g20300	Putative protein kinase	–	21557	Family 1.2.2 – Receptor like cytoplasmic kinase VII	23.4	Yes
at3g59110	Receptor like protein kinase	–	21782	Family 1.6.3 – Receptor like cytoplasmic kinase V	23.2	Yes
at4g18710	GSK3/shaggy-like protein kinase eta	ASK-eta	21835	Family 4.5.4 – GSK3/shaggy like protein kinase family	22.6	Yes
at5g38210	Wall-associated kinase	–	21317	Family 1.5.2 – LRK10 like kinase (Type 1)	21.9	Yes
at1g70520	Putative protein kinase	–	21455	Family 1.7.2 – Domain of unknown function 26 (DUF26) kinase	20.8	Yes
at1g10470	Cytokinin-induced response regulator protein	–	32297	-	20.3	–
at5g12180	Calcium-dependent Protein Kinase, isoform 17	CPK17	21973	Family 4.2.1 – Calcium dependent protein kinase	20.0	Yes
at4g03260	Protein phosphatase regulatory subunit	–	10885	-	19.5	–
at4g34440	Putative ser/thr protein kinase	–	21909	Family 1.6.2 – plant external response like kinase	18.1	Yes
at2g43850	Putative protein kinase	–	21516	Family 2.1.2 – ankyrin repeat domain kinase	17.6	Yes
at5g50860	Cyclin-dependent protein kinase like	–	22022	Family 4.5.2 – CDC2 like kinase family	17.5	Yes
at1g68330	–	–	-	-	17.0	–
at4g14340	Putative casein kinase I	–	21925	Family 3.1.1 – casein kinase i family	16.4	Yes
at5g65530	Putative protein	–	22057	Family 1.10.1 – receptor like cytoplasmic kinase VI	15.8	nd
at4g10010	Putative protein kinase	–	21817	Family 4.5.2 – CDC2 like kinase family	15.7	–
at1g35670	Calcium-dependent Protein Kinase, isoform 11	CPK11	20998	Family 4.2.1 – calcium dependent protein kinase	15.7	Yes
at1g18890	Calcium-dependent Protein Kinase, isoform 10	CPK10	21443	Family 4.2.1 – calcium dependent protein kinase	15.5	Yes
at1g16670	Hypothetical protein	–	21169	Family 1.7.1 – S domain kinase (Type 1)	15.3	nd
at4g02630	Putative ser/thr protein kinase	–	21799	Family 1.6.3 – receptor like cytoplasmic kinase V	15.0	Yes
at5g14640	GSK3/shaggy-like protein kinase	–	21976	Family 4.5.4 – GSK3/shaggy like protein kinase family	14.8	Yes
at3g06030	NPK1-related protein kinase 3	–	21692	Family 4.1.1 – MAP3K	14.8	Yes
at1g06390	GSK3/shaggy-like protein kinase iota	ASK-iota	21488	Family 4.5.4 – GSK3/shaggy like protein kinase family	14.7	Yes
at4g05150	–	–	–	–	14.5	–
at3g20200	Putative protein kinase	–	21229	Family 1.14.2 – receptor like cytoplasmic kinase IX	14.1	nd
at1g16130	Similarity to wall-associated protein kinase 1	–	21088	Family 1.5.1 – wall associated kinase-like kinase	14.1	nd
at4g28860	Putative casein kinase I	–	21886	Family 3.1.1 – casein kinase I family	13.2	Yes
at5g03730	Protein kinase CTR1	CTR1	21949	Family 2.1.3 – CTR1/EDR1 kinase	13.2	Yes
at4g08500	Putative MAP3K	–	21814	Family 4.1.1 – MAP3K	13.1	nd
at1g56720	Putative protein kinase	–	21010	Family 1.6.3 – receptor like cytoplasmic kinase V	13.0	Yes
at4g32660	Protein kinase AME3	–	21902	Family 4.5.6 – LAMMER kinase family	12.9	Yes
at1g11440	–	–	–	–	12.9	–
at4g24740	Protein kinase	–	21871	Family 4.5.6 – LAMMER kinase family	12.1	Yes

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Table 1 (*continued*)

No. of Arabidopsis genes	PlantP funciton[a]	PlantP symbol[a]	PlantP no.[a]	PlantP family[a]	Relative luminescence signal[b]	Autophosphorylation[c]
at5g24430	CDPK-related protein kinase isoform 4	CRK4	21984	Family 4.2.1 – calcium dependent protein kinase	11.7	Yes
at4g00720	GSK3/shaggy-like protein kinase tetha	ASK-tetha	21794	Family 4.5.4 – GSK3/shaggy like protein kinase family	11.5	Yes
at4g26890	Putative NPK1-related protein kinase	–	21878	Family 4.4.1 – unknown function kinase	11.1	nd
at1g12970	–	–	–	–	11.1	–
at1g09020	Activator subunit of SNF1-related protein kinase SNF4	–	10475	–	10.9	–
at5g26750	GSK3/shaggy-like protein kinase ASK-alpha	ASK-alpha	21309	Family 4.5.4 – GSK3/shaggy like protein kinase family	10.6	Yes
at5g04510	3-Phosphoinositide-dependent kinase-1 PDK1	–	21950	Family 4.2.6 – IRE/NPH/PI dependent/S6 kinase	10.5	Yes
at3g51850	Calcium-dependent Protein Kinase, isoform 13	CPK13	21791	Family 4.2.1 – calcium dependent protein kinase	10.5	Yes
at2g40120	Putative protein kinase	–	21546	Family 4.5.8 – unknown function kinase	10.4	nd
at4g24400	SNF1-related protein kinase, subfamily 3	SnRK3.13	21869	Family 4.2.4 – SNF1 related protein kinase (SnRK)	10.3	nd
at1g01540	Hypothetical protein	–	21038	Family 1.6.3 – receptor like cytoplasmic kinase V	10.3	Yes
at1g73500	Putative protein kinase	–	21164	Family 4.1.3 – MAP2K	10.2	Yes
at1g20930	Putative cdc2-like protein kinase	–	21078	Family 4.5.2 – CDC2 like kinase family	10.0	Yes
at3g05840	GSK3/shaggy-like protein kinase, ASK-gamma	ASK-gamma	21676	Family 4.5.4 – GSK3/shaggy like protein kinase family	10.0	Yes
at3g61160	GSK3/shaggy-like protein kinase beta	ASK-beta	21787	Family 4.5.4 – GSK3/shaggy like protein kinase family	10.0	Yes

Abbreviations: Yes, detected; nd, not detected; –, not analyzed.

[a] Their classification were according to PlantP database (http://plantsp.sdsc.edu/).

[b] This works.

[c] Autoradiography of *in vitro* kinase assays (Sawasaki et al., 2004).

related with cell growth (Bai et al., 2009). Furthermore, *Brassica napus* BuPERK (a putative orthologous gene of *Arabidopsis thaliana* At-PERK1) gene expression is induced by wound and fungal pathogens stress (Silva and Goring, 2002). Thus, both of the signaling pathway of CPK and PERK be induced by the stress and ABA, and may have cross-talk via a Ca^{2+}-signaling pathway, or regulation by direct trans-phosphorylation.

The reversible protein phosphorylation on Ser, Thr, and Tyr is a key post-translational modification in eukaryotes with stunning regulatory and signalling potential. We clarified the Ser/Thr autophosphorylating activity of 759 PKs that the biochemical characterization was indistinct. In addition, the autophosphorylation of PKs biologically suggested that it be the important. This study will be a basis for understanding the function of PKs in phosphorylation network for future research. The function of the Tyr phosphorylation has been largely neglected because a mammalian protein Tyr kinase homolog was not found in plants. By using this method, may be able to identify protein Tyr kinase and phosphorylation network in plants.

4. Conclusion

In this study, we found that the wheat cell-free system was an excellent expression system to produce recombinant plant PKs efficiently and to carry out *in vitro* phosphorylation assays without purification, the interference of endogenous PK and phosphatase activity. Using a full-length cDNA library of *A. thaliana* genes encoding PKs, we demonstrated that the wheat germ cell-free system is capable of profiling the autophosphorylation activity of 759 PKs, and also found 67 highly active PKs. The annotation analysis revealed that some of these PKs may be involved in phosphosignaling pathways such as signal transduction, stress response, and the regulation of cell division. Information from this study may shed light on many unknown plant PKs.

5. Experimental

5.1. General

Details of the following procedures were either described or cited previously (Ogasawara et al., 1999; Madin et al., 2000; Sawasaki et al., 2002a,b): isolation of wheat germs and preparation of the extract, generation of DNA template by "split-primer" PCR, synthesis of mRNA, *in vitro* protein synthesis, and the estimation of protein quantity synthesized by densitometric scanning of the Coomassie brilliant blue (CBB)-stained band and autoradiography.

5.2. Construction of DNA templates for transcription

The unique primer for the "split-primer" PCR for each of the 768 cDNAs from the RAFL clones was designed according to the sequence in the database (Seki et al., 2002). The DNA templates for transcription were constructed by "split-primer" PCR technique described in previous reports. (Sawasaki et al., 2002a; Sawasaki et al., 2007) The first PCR product was amplified with 10 nM of each of the following primers: a gene specific primer, 5′-CCACC CACCACCACCAATGnnnnnnnnnnnnnnnnnnn (n denotes the coding region of the target gene), and AODA2303 (5′-GTCAGACC CCGTAGAAAAGA) or AODS (5′-TTTCTACGGGGTCTGACGCT). The second PCR products for protein synthesis were constructed with 100 nM SPu 5′-GCGTAGCATTTAGGTGACACT, 1 nM deSP6E02bls-S1 (5′-GGTGACACTATAGAACTCACCTATCTCTCTACACAAAAC-ATTTC CCTACATACAACTTTCAACTTCCTATTATGGGCCTGAACGACATCTTCG AGGCCCAGAAGATCGAGTGGCACGAACTCCACCCACCACCACCAATG) and 100 nM AODA2303 or AODS. By this "split-primer" PCR, the bls was fused onto the N-terminals of all the genes for protein biotinylation (Sawasaki et al., 2008).

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5.3. Cell-free protein synthesis

In vitro transcription and cell-free protein synthesis were performed as described (Sawasaki et al., 2005). 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 (Sawasaki et al., 2002a) with slight modifications. The translation mixture that formed the bottom layer consisted of 60 A260 units of the wheat germ extract (CellFree Sciences, Yokohama, Japan) 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. SUB-AMIX® (125 μl) was placed on the top of the translation mixture, forming the upper layer. After incubation at 16 °C for 18 h, the synthesized proteins were confirmed by SDS–PAGE. For biotin labeling, 1 μl of crude biotin ligase (BirA) produced by the wheat cell-free expression system was added to the bottom layer, and 0.5 μM (final concentration) of d-biotin (Nacalai Tesque, Inc., Kyoto, Japan) was added to both upper and bottom layers, as described previously (Sawasaki et al., 2008).

5.4. Analysis of autophosphorylation activity by luminescence method

In vitro autophosphorylation assays were carried out in a total volume of 15 μl consisting of 50 mM Tris–HCl (pH 7.6), 100 mM potassium acetate, 10 mM $MgCl_2$, 1 mM DTT, 66 μM ATP, 2 μl biotinylated or non-biotinylated (as control) PK at 30 °C for 1 h in a 384-well Optiplate (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). In accordance with the AlphaScreen IgG (ProteinA) detection kit (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) instruction manual, 10 μl of detection mixture containing 50 mM Tris–HCl pH 7.6, 100 mM potassium acetate, 10 mM $MgCl_2$, 0.1 mM DTT, 5 μg/ml anti-phospho Ser/Thr antibody (Upstate Biotechnology, Lake Placid, NY, USA), 1 mg/ml BSA, 0.1 μl streptavidin-coated donor beads and 0.1 μl anti-IgG acceptor beads were added to each well of the 384 Optiplate followed by incubation at 23 °C for 1 h. Luminescence was analyzed by the AlphaScreen detection program. All data are the average of two independent experiments, and the background was controlled for each using the relevant non-biotinylated PK. The relative luminescence signal of biotinylated PK was calculated by normalizing each signal against that of non-biotinylated PK.

5.5. Functional characterization

As a further annotation of the 67 high luminescent signal PKs, we used the gene ontology system (Ashburner et al., 2000). Comparison of frequencies with Arabidopsis within the Biological Processes and Cellular Component gene ontology categories was done using the whole *Arabidopsis* genome annotation tool on the TAIR website (http://www.arabidopsis.org/) and DAGViz (Yano et al., 2009).

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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.phytochem.2011.02.029.

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Production and partial purification of membrane proteins using a liposome-supplemented wheat cell-free translation system

Akira Nozawa[1,2], Tomio Ogasawara[1,2], Satoko Matsunaga[1], Takahiro Iwasaki[1], Tatsuya Sawasaki[1,2,3*] and Yaeta Endo[1,2,3*]

Abstract

Background: Recently, some groups have reported on cell-free synthesis of functional membrane proteins (MPs) in the presence of exogenous liposomes (liposomes). Previously, we reported synthesis of a functional AtPPT1 plant phosphate transporter that was associated with liposomes during translation. However, it is unclear whether or not lipid/MP complex formation is common to all types of MPs in the wheat cell-free system.

Results: AtPPT1 was synthesized using a wheat cell-free system with or without liposomes. AtPPT1 synthesized with liposomes showed high transport activity, but the activity of AtPPT1 synthesized without liposomes was less than 10% activity of that with liposomes. To test whether co-translational association with liposomes is observed in the synthesis of other MPs, we used 40 mammalian MPs having one to 14 transmembrane domains (TMDs) and five soluble proteins as a control. The association rate of all 40 MPs into liposomes was more than 40% (mean value: 59%), while that of the five soluble proteins was less than 20% (mean value: 12%). There were no significant differences in association rate among MPs regardless of the number of TMDs and synthesis yield. These results indicate that the wheat cell-free system is a highly productive method for lipid/MP complex formation and is suitable for large-scale preparation. The liposome association of green fluorescent protein (GFP)-fusion MPs were also tested and recovered as lipid/MP complex after floatation by Accudenz density gradient ultracentrifugation (DGU). Employment of GFP-MPs revealed optimal condition for Accudenz floatation. Using the optimized Accudenz DGU condition, P2RX4/lipid complexes were partially purified and detected as a major band by Coomassie Brilliant Blue (CBB)-staining after SDS-PAGE.

Conclusion: Formation of lipid/AtPPT1 complex during the cell-free synthesis reaction is critical for synthesis of a functional MP. The lipid/MP complex during the translation was observed in all 40 MPs tested. At least 29 MPs, as judged by their higher productivity compared to GFP, might be suitable for a large-scale preparation. MPs synthesized by this method form lipid/MP complexes, which could be readily partially purified by Accudenz DGU. Wheat cell-free protein synthesis in the presence of liposomes will be a useful method for preparation of variety type of MPs.

* Correspondence: sawasaki@eng.ehime-u.ac.jp; yendo@eng.ehime-u.ac.jp
[1]Cell-Free Science and Technology Research Center and the Venture Business, Laboratory, Ehime University, 3 Bunkyo-Cho, Matsuyama, Ehime 790-8577, Japan
Full list of author information is available at the end of the article

Background

MPs comprise up to 30% of genes in fully sequenced genomes and have critical roles in a variety of biological processes including signal transduction, substrate transport, and energy production [1,2]. However, functional and structural studies of MPs are far behind that of soluble proteins. One of the major bottlenecks in the study of MPs is the difficulty in obtaining sufficient amounts of homogeneous protein. For instance, it is typically not easy to purify MPs in preparative scale, due to their low abundance in natural sources. Overexpression of recombinant MPs in living cells is often unsuccessful due to the inhibitory effect of high MP concentration on host cell physiology [3].

Recently, cell-free protein synthesis systems have emerged as a promising tool for MP production [4-6]. In addition to decoupling protein production from the toxic or inhibitory effects on host cell physiology, cell-free systems offer a unique advantage in that protein synthesis can be easily modified by addition of accessory elements, such as detergents and lipids. The addition of detergents and lipids to cell-free systems allows the synthesis of MP/detergent and MP/lipid complexes, respectively, and successful synthesis of functional MPs in this fashion have been reported recently [7-11]. For example, Klammt et al. [12] demonstrated that a G protein-coupled receptor, ETB, can be synthesized in a soluble form using an *Escherichia coli*-based cell-free system supplemented with Brij78, and that the synthesized proteins have ligand binding activity. The ligand binding activity of a human olfactory receptor, hOR17-4, synthesized using a wheat cell-free system in the presence of FC14, has been also reported [13]. Kalmbach et al. [14] reported that *E. coli* cell-free synthesized bacteriorhodopsin in the presence of liposomes was active in black lipid membrane mediated photocurrent measurements. Goren and Fox [15] showed reconstitution of the functional stearoyl Co-A desaturase complex, which consists of three proteins, cytochrome b_5, cytochrome b_5 reductase, and human stearoyl-CoA desaturase 1 (hSCD1) synthesized by wheat cell-free system in the presence of asolectin liposomes. However, the general versatility of this method is unclear as the above examples focus on specific MPs.

In a previous study, we reported functional synthesis of a phosphate trranslocator in a wheat cell-free synthesis system supplemented with liposomes and formation of lipid/MP complexes [16]. The mechanism for production of functional protein in this method is not clear, but association of synthesized MP with liposomes may be an important step. To better understand this, we tested the timing of liposome addition to the cell-free MP synthesis reaction. We also investigated whether other MPs synthesized by the method also associates with liposomes. Moreover, we tried to purify the synthesized MP as a lipid/MP complex by DGU.

Results and Discussion

Timing of liposome-supplementation to wheat cell-free translation system for synthesis of functional MPs

Previously, we reported synthesis and liposome association of functional MPs using a wheat cell-free system supplemented with liposomes [16]. To verify that co-translational association of MP with liposomes is critical for functional synthesis, we tested the synthesis of an *Arabidopsis thaliana* phosphate translocator, AtPPT1, in the presence of, absence of, and after post-translational addition of liposomes. These synthesized proteins were reconstituted into liposomes by freeze-thaw and sonication methods after mixing with substrate-preloaded liposomes and phosphate-incorporation activity was measured. Similar to a previous report [16], AtPPT1 synthesized in the absence of liposomes had only 4% of the activity of AtPPT1 synthesized in the presence of liposomes (Figure 1). AtPPT1 synthesized in the absence of liposomes was mixed post-translationally with liposomes and yielded 6% the activity of AtPPT1 synthesized in the presence of liposomes. The association of synthesized AtPPT1 with liposomes, either co- or post-translationally, was measured after sucrose DGU and

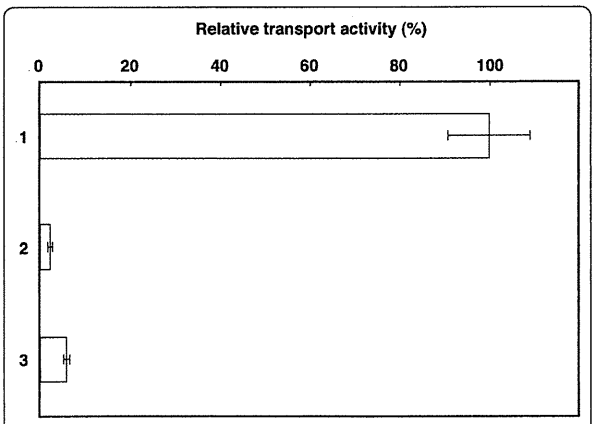

Figure 1 Effect of timing of liposome-supplementation on transport activity of synthesized AtPPT1. Three types of AtPPT1 proteins were prepared, 1: AtPPT1 synthesized with liposomes, 2: AtPPT1 synthesized without liposomes, 3: AtPPT1 synthesized without liposomes and mixed with liposomes post-synthesis. Each type of synthesis was reconstituted in liposomes that had been pre-loaded with 30 mM phosphate. Uptake of [^{32}P] phosphate into the liposomes was measured. The 100% exchange activity of the synthesized protein was 151 n mol/min/mg proteins. Data is reported as the mean±SD of values from three independent experiments.

showed no significant differences (50% co-translational, 62% post-translational). These results indicate that formation of lipid/AtPPT1 complex during the synthesis reaction is an important step for synthesizing functional AtPPT1. Supplementation of liposomes into the cell-free system would prevent aggregation and precipitation of AtPPT1 proteins during synthesis reaction. So, the constitution of AtPPT1/lipid complexes might be effective for formation of functional state MPs in the following freeze-thaw and sonication steps. For preparing MPs in functional state by cell-free system, preventing aggregation and precipitation of MPs during synthesis reaction by supplementation of lipids and/or detergents would be a critical point.

Cell-free synthesis of MPs in the presence of liposomes

Using a wheat cell-free system in the presence of liposomes, a plant MP, AtPPT1, was synthesized as a lipid/MP complex (Figure 1). As MPs account for more than 50% of all human drug targets [17], we wanted to understand if the AtPPT1 membrane association described above was applicable to mammalian MPs. In general, MPs are classified by the number of TMDs and to start we tested five human MPs ranging from 2 to 12 TMD (KCNJ8, 2TMD; GABRD, 4TMD; HTR2B, 7TMD; P2RY11, 7TMD; SLC22A7, 12TMD). The selected mammalian MPs were synthesized in the wheat cell-free system in the presence of asolectin liposomes and their liposome association rates were measured after sucrose DGU. In this experiment, mRNA was prepared from fragments made by split-primer PCR [18,19]. By using a PCR-based fragment as a template for *in vitro* transcription, time consuming steps, such as cloning of a target gene and construction of an expression vector were eliminated. Although the yield of template is low, this step allows for screening large numbers of proteins. As shown in Figure 2, every protein in the test set was associated with liposomes. After sucrose DGU, ^{14}C-labeled proteins were predominantly detected in bands six to eight (Figure 2A), which corresponded with the observed liposome bands [16]. For each fraction, the radioactivity was measured and the relative amount of radioactivity for each fraction is depicted in Figure 2B. The extent of association for these proteins ranged from 52 to 73%, when three fractions, numbers six to eight, were treated as liposome fractions. The observed MP association is similar to that seen AtPPT1 (58%). Also there were no significant differences in the extent of association between the five MPs having two to 12 TMDs. These results suggest that association of synthesized MPs with liposomes during wheat cell-free synthesis is as likely to occur in other MPs as was observed for AtPPT1.

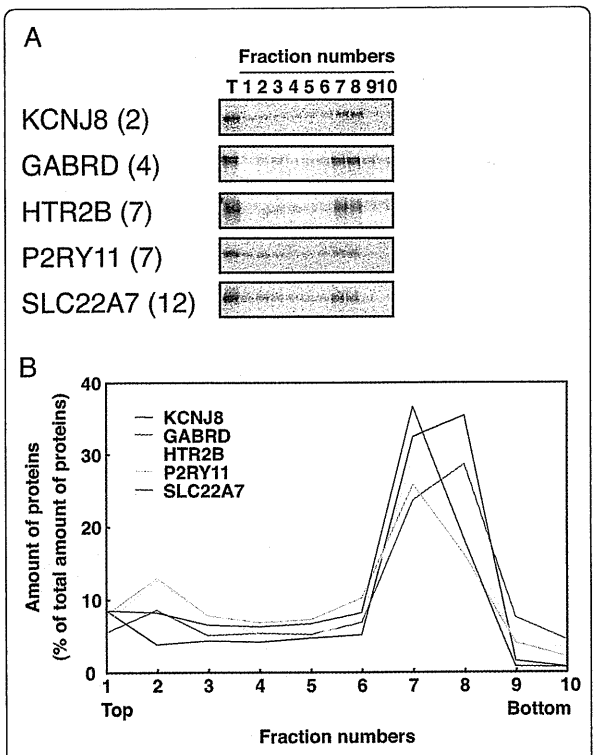

Figure 2 Association of synthesized MPs with liposomes. **(A)** SDS-PAGE analysis of liposome association of MPs. MPs were synthesized using a wheat cell-free system supplemented with liposomes and [^{14}C]-leucine. After the synthesis reaction, the reaction mixture was subjected to sucrose DGU and fractions were collected from the top of the tube. Synthesized proteins in each fraction were detected by SDS-PAGE and autoradiography. The number of TMDs of each protein is shown in parentheses. T means total fraction of reaction mixture. **(B)** Yield of synthesized proteins in each fraction. After fractionation, synthesized proteins were precipitated by 10% trichloroacetic acid and radioactivity of each fraction was measured by liquid scintillation counter.

Next we further analyzed membrane association using a larger set of proteins, which consisted of 29 human and 6 mouse MPs. While a majority of the test proteins were chosen at random, we ensured that there were multiple representatives of each tested MP family to examine synthesis yield and extent of liposome association within a family. As shown in Figure 3A, the 29 MPs tested showed better synthesis efficiency than GFP (not shown, 4.4 μg/150 μL reaction). The mean value of yield for the set of MPs was 5.9 μg/150 μL reaction. All tested proteins belonging to KCNJ, P2RX, GABR and SC5A families were well synthesized in the wheat cell-free system, whereas production of CACNG family proteins was very low (Figure 3B and Table 1). The remaining proteins, belonging to ENDR, P2RY, SLC6A and SLC22A families had both poorly and well synthesized proteins. (Figure 3B and Table 1).

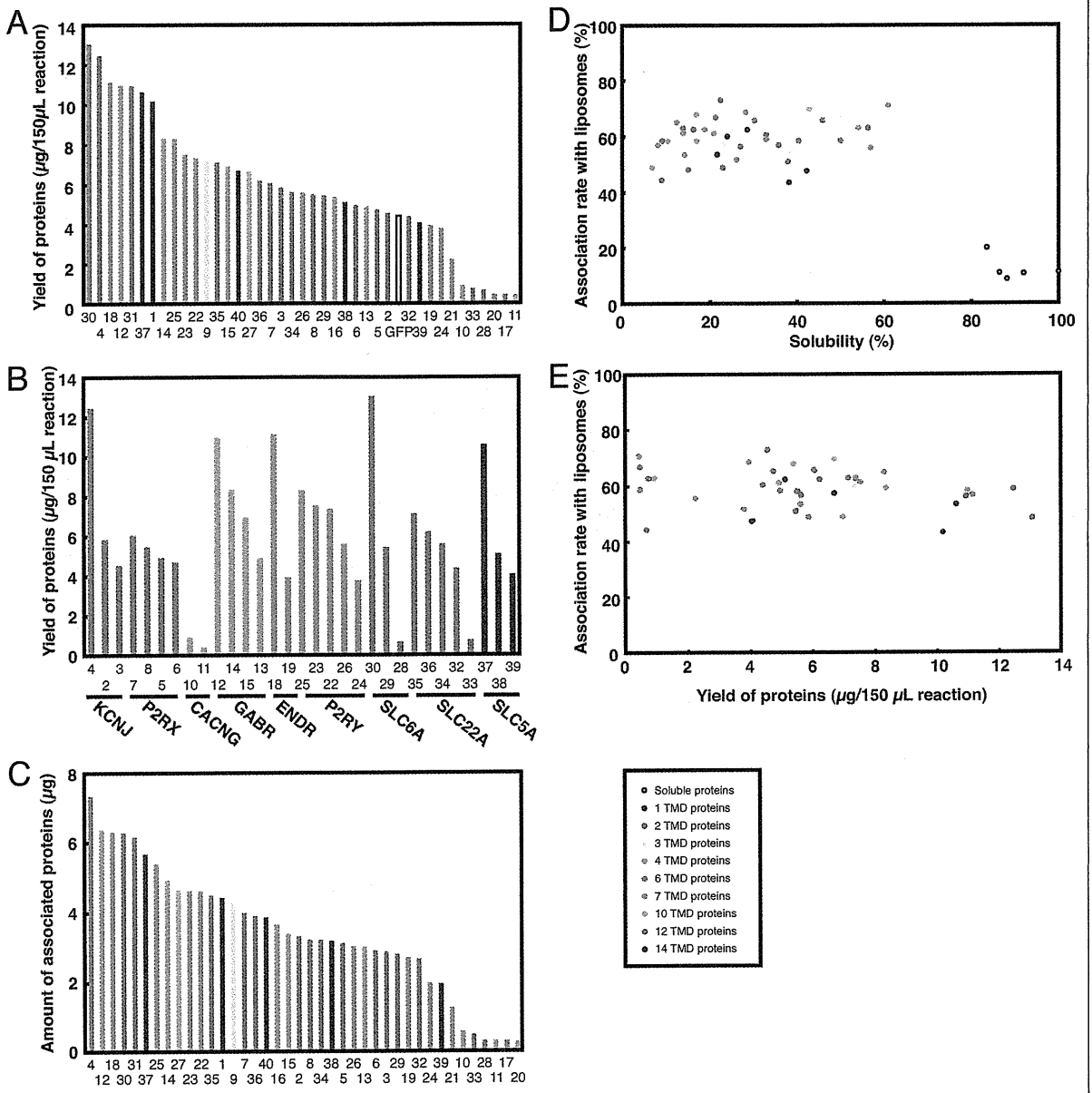

Figure 3 Yield, extent of liposome association and solubility of each protein. (A) Yield of MPs. MPs were synthesized using the wheat cell-free system supplemented with [^{14}C]-leucine and liposomes. Proteins were precipitated by 10% trichloroacetic acid and radioactivity was measured by liquid scintillation counter and the yield of each protein was calculated. The numbers on the x-axis correspond to the proteins in Table I. **(B)** Yield of MPs in each protein family. **(C)** Amount of liposome-associated proteins. MPs were synthesized using the wheat cell-free system supplemented with [^{14}C]-leucine and liposomes. After synthesis, the translation reaction was separated by sucrose DGU and fractions were collected from the top of the tube. The protein content of each fraction was estimated through radioactivity measurements of proteins precipitated by 10% trichloroacetic acid. The extent of association was calculated by combining the liposome fractions six through eight. For each protein, the specific amount of association with liposomes was calculated from the synthesis yield and extent of association. **(D)** The relationship between the extent of association and solubility of each protein. Membrane and soluble proteins were synthesized using the wheat cell-free system supplemented with or without liposomes and [^{14}C]-leucine. Proteins synthesized without liposomes were separated into soluble and insoluble fraction by centrifugation. The unprocessed synthesis reactions and the insoluble fractions were precipitated by 10% trichloroacetic acid and radioactivity was measured by liquid scintillation counter to determine the solubility for each protein. The extent of association for soluble proteins was calculated using the same method as for MPs. The extent of association and solubility of membrane and soluble proteins are plotted. **(E)** Relationship between extent of association and yield for each protein. Values for the extent of association and yield of each protein are plotted.

Table 1 Yield, association rate and solubility of synthesized membrane proteins

	Proteins	Anotation	Number of TMD	Molecular weight (kDa)	Yield (μg/150 μl reaction)	Solubility (%)	Association rates with liposomes (%)	Yield × Association rates (μg)
1	Itga1#	Integrin	1	131	10.2	38.3	43.3	4.4
2	KCNJ8	Potassium inwardly-rectifying channel	2	48	4.6	22.6	72.6	3.3
3	KCNJ13	Potassium inwardly-rectifying channel	2	41	5.9	23.1	48.7	2.9
4	KCNJ15	Potassium inwardly-rectifying channel	2	43	12.5	10.5	58.8	7.3
5	P2RX1	Purinergic receptor	2	45	4.7	46.0	65.4	3.1
6	P2rx2#	Purinergic receptor	2	55	5.0	40.4	58.3	2.9
7	P2RX4	Purinergic receptor	2	43	6.1	30.5	65.4	4.0
8	P2RX5	Purinergic receptor	2	47	5.5	50.0	58.2	3.2
9	GRIA2	Glutamate receptor	3	99	7.3	24.1	59.8	4.4
10	CACNG3	Voltage-dependent calcium channel	4	36	0.9	54.1	62.7	0.6
11	CACNG4	Voltage-dependent calcium channel	4	37	0.4	61.0	70.8	0.3
12	GABRA3	GABA receptor	4	55	11.0	17.1	58.1	6.4
13	GABRB1	GABA receptor	4	54	4.9	21.0	60.9	3.0
14	GABRD	GABA receptor	4	51	8.4	12.5	58.8	4.9
15	GABRG1	GABA receptor	4	54	7.0	6.9	48.6	3.4
16	Glra1#	Glycine receptor	4	52	5.4	17.1	67.6	3.6
17	AQP3	Aquaporin	6	32	0.4	21.4	66.6	0.3
18	EDNRA	Endothelin receptor	7	49	11.1	8.3	56.6	6.3
19	EDNRB	Endothelin receptor	7	50	3.9	28.3	68.3	2.7
20	GPR37	G protein-coupled receptor 37	7	67	0.5	33.0	58.8	0.3
21	HTR2B	5-Hydroxytryptamine receptor	7	54	2.2	57.0	55.8	1.2
22	P2RY2	Purinergic receptor	7	42	7.4	18.9	62.4	4.6
23	P2RY10	Purinergic receptor	7	39	7.5	14.0	61.2	4.6
24	P2RY11	Purinergic receptor	7	40	3.8	26.3	51.7	2.0
25	P2RY13	Purinergic receptor	7	38	8.3	12.7	64.8	5.4
26	P2RY14	Purinergic receptor	7	39	5.6	14.3	53.5	3.0
27	SLC1A7	Glutamate transporter	10	61	6.7	42.9	69.4	4.6
28	SLC6A3	Dopamine transporter	12	69	0.7	9.1	44.4	0.3
29	SLC6A13	GABA transporter, GABA	12	68	5.5	38.1	51.1	2.8
30	SLC6A18	Solute carrier family 6, member 18	12	71	13.1	15.3	48.1	6.3
31	Slc18a2#	Vesicular monoamine transporter	12	56	10.9	27.1	56.3	6.2
32	SLC22A4	Organic cation transporter	12	62	4.4	33.1	60.2	2.6
33	SLC22A7	Organic anion transporter	12	60	0.8	56.2	62.7	0.5
34	SLC22A8	Organic anion transporter	12	60	5.6	36.0	56.9	3.2
35	SLC22A11	organic anion/cation transporter	12	60	7.1	14.1	62.7	4.5
36	SLC22A12	Organic anion/cation transporter	12	60	6.2	16.4	62.3	3.9
37	Slc5a1#	Sodium/glucose cotransporter	14	73	10.6	21.8	53.4	5.7
38	SLC5A6	Sodium-dependent vitamin transporter	14	69	5.1	28.7	62.1	3.2
39	SLC5A10	Sodium/glucose cotransporter	14	62	4.1	42.2	47.5	1.9
40	Slc7a1#	Cationic amino acid transporter	14	67	6.7	23.3	57.3	3.8

Mouse clone.

The extent of association of all 40 proteins tested, containing between one and 14 TMDs, were from 43 to 73% and the mean value was 59% (Table 1). The amount of proteins associated with liposomes was calculated from the yield and extent of association for each protein (Figure 3C and Table 1). The mean value of the association yield was 3.4 μg/150 μL reaction. When analyzing association by the number of TMDs, the lowest extent of association was 43% for 1 TMD (Itga1), whereas the mean value of association for proteins having more than 2 TMDs was approximately 60% (Table 1). These results indicate that efficiency of association of proteins having only 1TMD might be lower than that of proteins having more than 2TMD.

Figure 3D shows the relationship between solubility and association rate of tested proteins. Solubility of MPs synthesized using the wheat cell-free system in the absence of liposomes ranged from eight to 61%. The extent of association of these proteins ranged from 40 to 70% regardless of their solubilities. On the other hand, soluble proteins showed more than 80% solubility and their association rates were less than 20%, indicating that the extent of liposome association for soluble proteins is much lower than that of MPs. In comparison to the tested soluble proteins, the MPs examined had a wide variety of sizes, functional roles and topologies, and all of them appeared to be efficiently associated with liposomes during cell-free synthesis. Moreover, there were no significant relationship between the extent of protein association and their respective yields (Figure 3E).

In this experiment, we found that a variety of MPs make complexes with lipids during wheat cell-free synthesis in the presence of liposomes. It is not yet clear whether these MPs are integrated into liposomes or attached to surface of liposomes with their hydrophobic regions. For replying to this question, further experiments for evaluating function and/or structure of each synthesized MP are needed. However, as association of MPs with liposomes prevents aggregation and precipitation of synthesized MPs during synthesis reaction, formation of lipid/MP complex would be an important step for preparing MPs.

Katzen et al. [20] recently reported that insertion of EmrE into a discoidal membrane scaffold in correctly folded state during cell-free synthesis by analysis of binding activity of its substrate, tetraphenylphosphonium. Moritani et al. [21] demonstrated that connexin-43 synthesized by an *E. coli* cell-free system, PURE system containing minimum protein synthesis factors [22], in the presence of liposomes is directly integrated with a uniform orientation into liposome membrane. The connexin-43 synthesized into liposomes was shown to deliver a hydrophilic and bioactive oligo-peptide to cells through gap junctions [23]. They speculate that liposomes may have chaperone-like function because their system consists of only protein synthesis factors and liposomes [21]. Although it is still not clear any kinds of MPs can receive such a chaperone-like effect from liposomes, the addition of artificial membranes to cell-free reactions appears to be effective in synthesizing membrane-stabilized MPs.

Partial purification of synthesized MPs by density-gradient ultracentrifugation

In this study, we demonstrated that a variety of mammalian MPs are efficiently associated with liposomes during wheat cell-free synthesis supplemented with liposomes. Next, we tried to partially purify the synthesized lipid/MP complexes from the endogenous wheat germ proteins. For this experiment we used P2RX4 as a model protein, because both the yield (6.1 μg) and extent of association (66%) are very close to the mean value for both of these parameters in the full mammalian MP dataset above (5.9 μg and 59%, respectively).

A GFP-P2RX4 fusion protein was synthesized using the wheat cell-free system supplemented with liposomes and the resultant lipid/MP complexes were subjected to Accudenz DGU. Accudenz is a non-toxic medium used for fractionation of proteins, organelles, and cells [15,24-26]. The wheat cell-free synthesis reaction was brought to 40% Accudenz by addition of an 80% Accudenz solution, placed at the bottom of an ultra-centrifugation tube, and overlaid with 35% Accudenz solution, 30% Accudenz solution and DGU solution. It is expected that after Accudenz DGU, liposomes float to the top of the centrifuge tube owing to their low density [15,24]. As shown in Figure 4A, the fluorescence from GFP-P2RX4 fusion proteins was observed at the top of the gradient after Accudenz DGU, while the majority of the fluorescence from a soluble GFP control remained at the bottom. After fractionation, the fluorescence of each fraction was measured with a spectrofluorometer (excitation 480 nm/emission 510 nm). High fluorescence intensity at the top fraction, associated with floated liposomes, was detected in GFP-P2RX4 sample synthesized by liposome supplemented wheat cell-free synthesis (Figure 4B). In the other samples, GFP synthesized with or without liposomes and GFP-P2RX4 synthesized without liposomes, fluorescence was mainly detected in the lower fractions (Figure 4B). The extent of association of GFP-P2RX4 in this experiment (66%: Fraction 1 and 2) was similar to the association of P2RX4 calculated by sucrose DGU (66%). Although we also tested association of P2RX2-GFP with liposomes with same procedure, there were no significant differences in association rate (data not shown).

Although fluorescence was seen at the top of the gradient in the soluble GFP control after Accudenz DGU

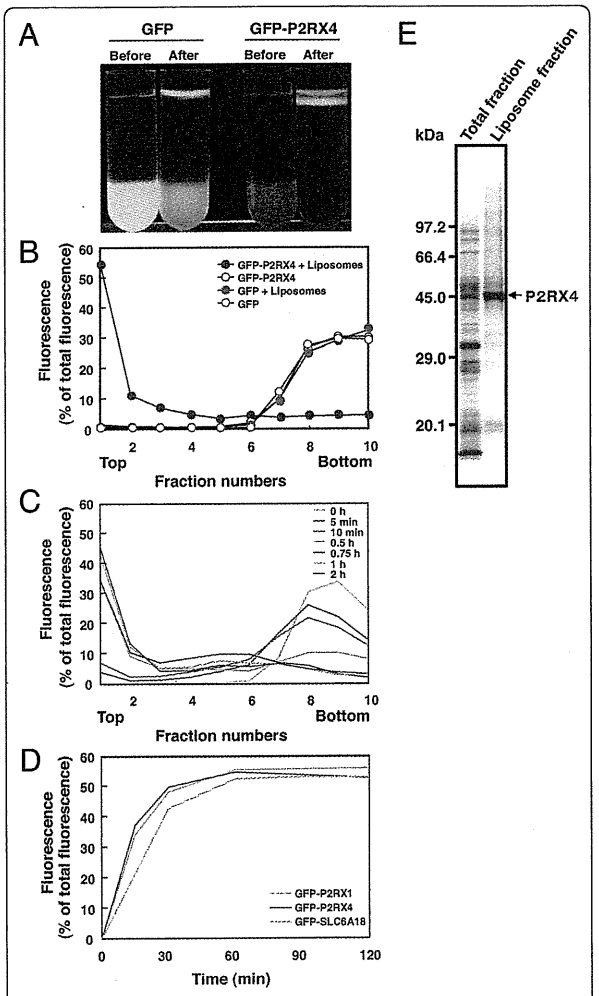

Figure 4 Partial purification of P2RX4 proteins by Accudenz DGU. (A) Floatation of GFP-P2RX4/lipid complex by Accudenz DGU. GFP and GFP-P2RX4 were synthesized using the wheat cell-free system supplemented with liposomes. After the synthesis reaction, the reaction mixture was subjected to Accudenz DGU. Fluorescence images were visualized using a transilluminator. **(B)** Yield of synthesized protein in each fraction. After DGU, fractions were collected from the top of the tube and the fluorescence intensity of each fraction was measured with a spectrofluorometer. **(C)** Effect of centrifugation time on floatation of lipid/MP complex. Synthesized GFP-fusion MPs were subjected to Accudenz DGU at varying centrifugation times. After DGU, fractions were collected from the top of the tube and the fluorescence intensity of each fraction was measured using a spectrofluorometer. **(D)** The effect of centrifugation time on the floatation of liposomes containing different MPs. GFP-P2RX4, -P2RX1, and -SLC6A18 fusion proteins were used in this experiment. Fraction No. one was treated as the liposome fraction and its fluorescence intensity in each condition was plotted. **(E)** Partial purification of P2RX4 proteins. P2RX4 was synthesized using the wheat cell-free system supplemented with liposomes. After the synthesis reaction, the reaction mixture was concentrated and subjected to Accudenz DGU. The synthesis mixture and the top lipid/MP complex fraction after DGU were applied to SDS-PAGE and stained by CBB.

by using a transilluminator (excitation: 400-500 nm) with a filter for removing blue light (Figure 4A), the fluorescence in the top fraction was not detected with a spectrofluorometer using a condition for detection of fluorescence from GFP (excitation 480 nm/emission 510 nm) (Figure 4B). These results indicate that the fluorescence in the top fraction is not attributed to GFP but probably to the liposomes. In comparison to sucrose DGU, Accudenz DGU makes lipid/MP complex recovery easier due to the fact that liposomes float in Accudenz as opposed to sinking as in sucrose. Additionally, Accudenz is a preferred DGU solution because it is non-toxic.

The GFP-fusion P2RX4 is an ideal construct to determine the optimal conditions for Accudenz DGU as the liposome-associated protein can be readily monitored by fluorescence. We tested several centrifugation times for Accudenz DGU. As shown in Figure 4C, the fluorescence in the top fraction gradually increased in proportion centrifugation time. One-h centrifugation appears to be sufficient for lipid/MP complex floatation. The results from Accudenz DGU of two additional MPs, GFP-P2RX1 and GFP-SLC6A18, also showed that the floatation rates of lipid/MP complex plateaus at a one-h centrifugation (Figure 4D).

As shown in Figure 4B, GFP and GFP-P2RX4 could be clearly separated by DGU. The result suggests that lipid/MP complexes can be separated from endogenous proteins derived from the wheat germ extract by Accudenz DGU. Nomura et al. also reported that cytochrome b5 synthesized by the wheat cell-free system in the presence of liposomes was easily purified by simplified discontinuous DGU [27]. In addition, Goren and Fox showed that a MP, hSCD1, synthesized by the wheat cell-free system supplemented with liposomes could be separated from endogenous wheat germ proteins by Accudenz DGU [15]. We next tried to purify P2RX4, without a GFP fusion, by this method. After synthesis supplemented with liposomes, lipid/MP complexes were concentrated by centrifugation and applied to Accudenz DGU. After ultracentrifugation, the top fraction was recovered and applied to SDS-PAGE. As shown in Figure 4E, P2RX4 was detected as a major band by CBB-staining after SDS-PAGE. This result indicates that lipid/MP complexes synthesized by our wheat cell-free system supplemented with liposomes can be partially purified by Accudenz DGU. To test whether proteins purified by this method are functional, we tested the activity of AtPPT1 partially purified as lipid/MP complexes. We detected transport activity of AtPPT1 purified with Accudenz DGU (data not shown). In addition to functional analysis, this partially purified protein could be used for structural analysis. However, some contaminating proteins from wheat germ extracts do

float with the lipid/MP complexes. Goren and Fox [15] reported that, along with other unclassified contaminants, HSP70, elongation factor 1 α and 16.9 kDa heat shock protein were seen in wheat cell-free synthesized and floated lipid/MP complexes. For functional and structural analysis, further purification steps would be required including affinity-tag purification and gel-filtration.

Conclusion

We have developed a production method for lipid/MP complexes using a wheat germ cell-free system supplemented with liposomes. Using this method, a variety of mammalian MPs were efficiently associated with liposomes co-translationally. The resultant lipid/MP complexes are easily separated from other proteins in wheat germ extract by DGU. This synthesis method is useful in the preparation of MP for structural and functional analysis.

Methods

Wheat Cell-free protein synthesis

Details of the wheat cell-free reaction were described in previously [28-30]. The 40 MPs and five soluble proteins in this study were selected from the Mammalian Gene Collection and FANTOM collection (Danaform, Yokohama, Japan). The unique primers for each protein (Additional file 1 Table S1) were designed and templates for transcription were made by the split-primer PCR method as described previously [18,19]. The first PCR was performed with 10 nM of the gene specific primer (Additional file 1 Table S1) and 10 nM of the AODA2303 primer (5'-GTCAGACCCCGTAGAAAAGA) or 10 nM AODS primer (5'-TTTCTACGGGGTCTGACGCT). The second PCR was amplified with 100 nM SPu primer (5'-GC GTAGCATTTAGGTGACACT), 1 nM deSP6E01-S1 primer (5'-GGTGACACTATAGAACTCACCTATCTCC CCAACACCTAATAACATTCAAT CACTCTTTCCAC-TAACCACCTATCTACATCACCAACCACCCACCAC-CACCAATG), and 100 nM AODA2303 primer or 100 nM AODS primer. mRNA was prepared by *in vitro* transcription in a reaction volume of 100 μl and was purified by ethanol precipitation. The mRNA pellet was resuspended in 30 μl of water. The translation reaction was performed using the bilayer method, supplemented with [^{14}C-leucine (50 μCi/ml, GE Healthcare, Tokyo, Japan) [31], in which a 25 μl translation layer was overlaid with a 125 μl substrate feeding buffer. Asolectin liposomes were prepared as described previously [16,32] and added to both layers (10 mg/ml final concentration). The bilayer reaction was incubated at 26°C for 16 h. After the reaction, the amount of [^{14}C]-leucine incorporation into synthesized proteins, an indicator of synthesis yield, was determined by 10% trichloroacetic acid precipitation and liquid scintillation spectroscopy.

Sucrose density gradient ultracentrifugation

Lipid/MP complexes were separated from proteins in the wheat germ extract by sucrose DGU. Synthesized proteins (100 μl) were loaded onto a discontinuous sucrose gradient consisting of 1,300 μl of 10% (w/v) and 600 μl of 30% (w/v) sucrose in DGU solution containing 140 mM NaCl, 5.4 mM KCl and 10 mM Tris-HCl (pH 8.0). The gradient was centrifuged at 105,000 *g* for 4 h at 4°C in a Hitachi S55S rotor (Hitachi High-Technology, Tokyo, Japan). Fractions were collected from the top of the tubes, and the protein content of each fraction was estimated by measurement of the amount of radioactivity associated with proteins precipitated by 10% trichloroacetic acid.

Transport assay of phosphate translocator

Liposomes were prepared from acetone-washed asolectin (Sigma-Aldrich, Tokyo, Japan) by sonication for 5 min at 4°C in water. AtPPT1 was synthesized by wheat cell-free system with (10 mg/ml final concentration) or without liposomes. Half of the AtPPT1 synthesized without liposomes was mixed with liposomes (10 mg/ml final concentration) after the synthesis reaction and incubated for 30 min at 26°C. These reaction mixtures were desalted by gel filtration with a Micro-Spin G-25 column (GE Healthcare) that had been equilibrated with 10 mM Tricine-KOH (pH 7.6).

The amount of protein synthesized in the cell-free system was estimated from the incorporation of [^{14}C]-leucine. After the reaction, the amount of [^{14}C]-leucine incorporation into synthesized proteins, an indicator of synthesis yield, was determined by 10% trichloroacetic acid precipitation and liquid scintillation spectroscopy.

Substrate-including liposomes (80 mg/ml final concentration) were prepared from acetone-washed asolectin by sonication for 5 min at 4°C in a solution containing 200 mM Tricine-KOH (pH 7.6), 40 mM potassium gluconate and 60 mM potassium phosphate (substrate). Desalted reaction mixtures were mixed with the substrate-preloaded liposomes, frozen in liquid nitrogen, thawed at room temperature and sonicated for 18 sec. Substrate that remained outside of the liposomes was removed with a Dowex AG-1X8 column (Bio-Rad, Tokyo, Japan) that had been equilibrated with a solution containing 100 mM sodium gluconate, 40 mM potassium gluconate and 10 mM Tricine-KOH (pH 7.6). The liposome mixture was applied to the column and eluted with the equilibration solution.

Transport reactions were initiated by the addition of 13 μl of [^{32}P] Pi (GE healthcare) to 300 μl of liposomes

(final phosphate concentration, inside: 30 mM, outside: 0.5 mM). The assay was performed at 25°C for 2 min, and the reaction was terminated by application of the reaction mixture to a Dowex AG-1X8 column that had been equilibrated with 150 mM sodium acetate. The radio-activity associated with the eluted liposomes was measured with a liquid scintillation spectrometer.

Plasmid construction

GFP fragments were amplified by PCR with the primers EcoRV-GFP-5' (5'-GAGAGATATCATGGGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAG TGGCACGAAGGTGGAGGTGGAATGGTGAGCAAGGGCGAGGA-3') and GFP-NotI-3' (5'-TCTCGCGGCCGCTCCACCTCCACCCTTGTACAGCTCGTCCATGC-3). The PCR fragments were digested with *Eco*RV and *Not*I and then cloned into the corresponding sites in the pEU-E01-MCS vector (Cellfree Sciences, Matsuyama, Japan). The resultant plasmid was designated pEU-E01-GFP-N. P2RX4, P2RX1, and SLC6A18 were obtained from cDNA clones and amplified by PCR with the primer sets NotI-P2RX4-5' (5'-GAGAGCGGCCGCTGAAAACCTGTATTTTCAGGGCATGGCGGGCTGCTGCGCCGC) and P2RX4-SalI-3' (5'-AGAGGTCGACTCACTGGTCCAGCTCACTAG), NotI-P2RX1-5' (5'-GAGAGCGGCCGCTGAAAACCTGTATTTTCAGGGCATGGCGGGCTGCTGC GCCGC) and P2RX1-SalI-3' (5'-AGAGGTCGACTCACTGGTCCAGCTCACTAG), and NotI-SLC6A18-5' (5'-GAGAGCGGCCGCTGAAAACCTGTATTTTCAGGGCATGGCTCATGCCCCAGAACC) and SLC6A18-SalI-3' (5'-AGAGGTCGACTCAGCGCATGTCCGTGTCCG), respectively. These PCR fragments were digested with *Not*I and *Sal*I and then cloned into the *Not*I-*Sal*I sites of pEU-E01-GFP-N. The resultant plasmids were designated pUE-E01-GFP-P2RX4, pUE-E01-GFP-P2RX1, and pUE-E01-GFP-SLC6A18, respectively, and used for production of GFP-fusion proteins. The P2RX4 ORF was amplified by PCR using the primer pair P2RX4-EcoRV-5' (5'-GAGAGATATCATGGCGGGCTGCTGCGCCGC) and P2RX4-NotI-3' (5'-CTCTGCGGCCGCTCCACCTCCACCCT GG CCAGCTCACTAGCAA). The PCR product was digested with *Eco*RV and *Not*I and then inserted into *Eco*RV-*Not*I sites of pEU-E01-MCS. The resultant plasmid was designated pEU-E01-P2RX4. The nucleotide sequences of each DNA fragment amplified by PCR was confirmed by DNA sequencing.

Detection and quantification of fluorescence from GFP proteins

Fluorescence images were visualized with a transilluminator (excitation: 400-500 nm, Dark Reader DR45M, Clare Chemical Research, Dolores, CO). Fluorescence intensity from GFP proteins was measured by a, Wallac 1420 Multilabel Counter spectrofluorometer (Perkin-Elmer Japan, Chiba, Japan).

Partial purification of P2RX4

mRNA was prepared by *in vitro* transcription using pEU-E01-P2RX4 as the template. The translation reaction was performed in the presence of liposomes (10 mg/ml final concentration) using the bilayer method with a bottom layer of 500 μl and an upper layer of 5500 μl. After the synthesis reaction, the reaction mixture was concentrated to 300 μl with a concentrator (Amicon Ultra-15, 30,000 MWCO, Millipore-Japan, Tokyo, Japan). For Accudenz (Accurate Chemical and Scientific, Westbury, NY) DGU, Accudenz was dissolved into DGU solution to make 30, 35, and 80% (w/v) Accudenz solutions. Three hundred μl of concentrated sample was mixed with 300 μl of 80% (w/v) Accudenz solution. The resultant 40% Accudenz solution containing the synthesized protein was placed in the bottom of a centrifuge tube, and overlaid with 650 μl of 35% (w/v) Accudenz solution, 650 μl of 30% (w/v) Accudenz solution, and 100 μl of DGU solution. The gradient was centrifuged at 105,000 *g* for 4 h at 4°C in a Hitachi S55S rotor (Hitachi High-Technology, Tokyo, Japan).

Additional material

Additional file 1: Table S1.

Abbreviations

CBB: Coomassie Brilliant Blue; DGU: density gradient ultracentrifugation; hSCD1: human stearoyl-CoA desaturase 1; Liposomes: exogenous liposomes; MP: membrane protein; PCR: polymerase chain reaction; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMD: transmembrane domain

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Author details

[1]Cell-Free Science and Technology Research Center and the Venture Business, Laboratory, Ehime University, 3 Bunkyo-Cho, Matsuyama, Ehime 790-8577, Japan. [2]RIKEN Systems and Structural Biology Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. [3]Proteo-Medicine Research Center, Ehime University, Toon, Ehime 791-0295, Japan.

Authors' contributions

YE and TS designed the experiment. AN, TO, SM, and TI performed the experiments.AN and TS wrote the manuscript. All authors read and approved the final manuscript.

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ELSEVIER

Wheat germ cell-free protein production system for post-genomic research

Review

Masaki Madono[1], Tatsuya Sawasaki[2], Ryo Morishita[1] and Yaeta Endo[2,]

[1] CellFree Sciences Co., Ltd., 75-1, Ono-cho, Leading Venture Plaza 201, Tsurumi-ku, Yokohama 230-0046, Japan
[2] Cell-Free Science and Technology Research Center, Ehime University, Matsuyama 790-8577, Japan

Genomic information becomes useful knowledge only when the structures and functions of gene products are understood. In spite of a vast array of analytical tools developed for biological studies in recent years, producing proteins at will is still a bottleneck in post-genomic studies. The cell-free protein production system we developed using wheat embryos has enabled us to produce high quality proteins for genome-wide functional and structural analyses and at the same time circumvent almost all the limitations, such as biohazards and costs, that have hampered conventional cell-free protein synthesis systems. In the present article, we introduce examples of our new wheat germ cell-free protein production system and its application to functional and structural analyses, with the focus on the former.

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Introduction

The use of genomic information for life science studies and industrial applications requires that the information be translated to produce proteins for functional and structural analyses. Producing proteins at will, however, remains a serious bottleneck. Conventional protein production methods using living cells are, by nature, unable to produce proteins that disturb physiological functions of host cells. In fact, there are many proteins that the conventional methods cannot handle. Furthermore, cell culture and gene expression in conventional *in vivo* systems require trial-and-error by skillful hands and are not therefore suitable for comprehensive parallel protein production. By contrast, organic

Corresponding author: Endo, Y. (yendo@eng.ehime-u.ac.jp)

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synthesis is impractical because of the extremely low yields and low qualities of synthesized proteins. The advent of cell-free methods that combined biological organisms' inherent capabilities with chemical technology raised expectations overcoming such limitations. They actually proved to be a powerful tool for tracer experiments at the dawn of life sciences, but the low yield resulting from their unstable translation reactions made them inadequate for protein production [1,2]. Our study on the cause of translation instability that had hampered the conventional cell-free systems resulted in the development of a new wheat germ cell-free system. We found that a set of translation machinery extracted from purified wheat embryos permitted stable translation, and we succeeded in creating a new cell-free protein synthesis method which was as efficient as living cells [3]. We then developed various elemental techniques to transform the new cell-free method into a practical *in vitro* protein production system [4]. The resultant wheat germ cell-free system met regulatory requirements related to biohazards and bioethics. Subsequent development of the system included full automation of protein synthesis, which has facilitated the adoption of the system worldwide.

In this article, we review the new wheat cell-free protein production technology and introduce examples of its application to functional and structural protein analyses, including part of our recent progress in comprehensive functional analysis of gene products. The details of the development of this technology and the protein production protocols for its use have been presented in previous reports [5,6].

Development of a practical wheat germ cell-free protein production system

Preparation of highly efficient cell-free translation solution made from wheat embryos

We succeeded in resolving the common problem of conventional cell-free protein synthesis systems, namely, instability. The key to the success was the discovery of the cause of such instability, which followed our findings [7] with respect to the molecular mechanisms of protein synthesis inhibition by plant poisons including ricin, an infamous poison used for assassination. Those findings are summarized as follows:

(1) Although the translation machinery is a robust system by nature, living organisms contain translation auto-inactivation factors that target their own translation apparatus, some of which might work as self-defense agents against pathogens. We found that such factors consisted of ribosome inactivating proteins [8], translation initiation factor modifying enzyme, deoxyribonucleases, ribonucleases, and proteases.

(2) When cells are damaged by homogenization, those translation inactivation factors contaminated from endosperm of wheat seeds during isolation of embryos are triggered to inactivate the translation machinery. This may be attributable to a glitch of the defensive response. Following these findings, we searched for a raw material whose translation machinery was highly stable and rich in content and whose translation inactivation factors were easy to remove. As a result, we found that wheat embryos fully satisfied those requirements, and subsequently succeeded in producing a stable translation solution from the embryos [3]. This work also confirmed that the activation of the translation auto-inactivation mechanism, which may be called a suicide mechanism, was the main cause of the low protein yields that may be a general effect in conventional cell-free protein synthesis systems.

Development of elemental techniques and completion of the wheat germ cell-free system

The translation machinery obtained by removing the translation-inhibiting contaminants from wheat embryos enabled us to optimize the translation constituents and the reaction conditions. We then developed elemental techniques, which in combination completed a practical cell-free protein production system. They included the Split-PCR method for constructing transcription templates, the transcription reaction method, 5′- and 3′-untranslated regions of mRNA that enhance translation, the pEU vector dedicated to the wheat germ cell-free system, and the translation reaction method [4]. Among the pEU vectors we have developed so far, BISHOP-vector is especially useful for genome-wide preparation of tag-free, highly purified proteins [9].

Advantages and characteristics of the wheat germ cell-free protein production system

Overcoming the barrier against conventional cell-free protein synthesis systems, the wheat germ cell-free protein production system has proved to be effective both in comprehensive parallel protein synthesis and in large-scale protein production at a gram level. In manual synthesis using a dialysis bag as a reaction vessel adapted to a principle of continuous flow cell-free translation method [10], the reaction solution containing the translation machinery from purified embryo was able to produce as much as 10 mg of a protein per 1 mL of the reaction volume [4]. One of the most important qualification criteria for a protein production system is that it can produce active proteins in their correct conformation. In this regard, the wheat germ cell-free system outperforms other well-known cell-free systems, such as those based on *Escherichia coli* cells and rabbit reticulocytes (Table 1) with the following advantages:

(a) *Capability to produce high quality eukaryotic multi-domain proteins:* In eukaryotic translation systems, the measured polypeptide growth rate is 3–5 peptide bonds per second. At this rate, folding of the growing polypeptide is co-translational and takes place as it is ejected from the hydrophobic ribosome tunnel into hydrophilic environment. In prokaryotic translation systems, by contrast, polypeptides grow almost ten times faster. Because of this, folding to produce higher-order structures is thought to be post-translational and, with the help of chaperones, take place after elongation of the polypeptide chain is completed and the chain leaves the ribosome [11]. In an *E. coli*-based translation system, whether it be a cell-based recombinant system or a cell-free system, synthesized eukaryotic multi-domain proteins often come out as insoluble aggregates. This is because, in this prokaryotic system, elongation of the polypeptide chains of those proteins occurs too fast for their correct folding.