National Center for Biotechnology Information. The applied MASCOT search parameters were as follows: (i) taxonomy: *Arabidopsis thaliana*; (ii) potential modifications: carbamidomethyl and oxidation as fixed modifications, myristoylation (N-term G, K); (iii) max missed cleavage: 1; (iv) peptide tolerance: ±0.5 Da; (v) MS/MS tolerance: ±0.2 Da; and (vi) peptide charge: $2^+$ and $3^+$. Proteins detected from peptide fragments with high reliability [MASCOT score >40 ($P$ <0.05)] were selected as identified proteins.

The presence of putative motifs in the identified proteins was analysed using Eukaryotic Linear Motif resource (ELM; http://elm.eu.org/) for destruction-box (D-box) and KEN-box, and GENETYX-MAC software for PEST sequences.

### *Antibody production*

To produce a polyclonal fructose biphosphate aldolase-like (FBA) antibody, the open reading frame (ORF) of *Arabidopsis* FBA (At3g52930) was amplified with primers 'fructose AntiB-F' (5′-GGAATTCCATATGTCTGCCTTCACAAGCAA-3′) and 'fructose AntiB-R' (5′-CGGAATTCTCAGTACTTGTAATCCTTCACG-3′), thereby introducing a *Nde*I site at the 5′ terminus and an *Eco*RI site at the 3′ terminus. The *Nde*I–*Eco*RI fragment of FBA was cloned into the expression vector pET28c (+) carrying 6×-histidine at the N terminus. The resulting plasmid was transformed into BL21 (DE3) cells. The purified histidine-tagged FBA protein was injected into a rabbit as the antigen. The antiserum obtained was used as anti-FBA antibody for immunoblot analysis.

### *Agroinfiltration*

The ORFs of At1g12840 (de-etiolated 3; DET3) and At3g04120 (glyceraldehyde-3-phosphate dehydrogenase C subunit; GAPC) were amplified from RIKEN *Arabidopsis* Full-Length cDNAs (RAFL) using the following primers: 'attB1-At1g12840-5′′ (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACTTCGAGATAT-3′) and 'attB2-At1g12840-3′′ (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCAGCAAGGTTGATAGT-3′), and 'attB1-At3g04120-5′′ (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCTGACAAGAAG-3′) and 'attB2-At3g04120-3′′ (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCCTTTGACATGTG-3′), respectively. Transfer of the PCR products to the entry vector pDONR221 was performed by BP reaction (Gateway; Invitrogen). Each ORF fragment of At1g12840 and At3g04120 on pDONR221 was transferred to the binary vector pGWB14 (Nakagawa *et al.*, 2007) carrying 3× HA by LR reaction (Gateway; Invitrogen).

The resulting constructs (DET3-HA and GAPC -HA) were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Agroinfiltration using *N. benthamiana* leaves was performed as described previously (Katou *et al.*, 2005). Discs were collected from leaves infiltrated with *Agrobacterium* cells after infiltration for 2 d.

### *Degradation assays*

For the degradation assays of DET3-HA and GAPC-HA proteins, total protein was extracted from agro-infiltrated *N. benthamiana* leaf discs with extraction buffer C (50 mM TRIS-HCl, 2 mM ATP, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 20% glycerol) supplemented with 30 μM MG132 or DMSO. For the FBA degradation assay, total protein from *Arabidopsis* seedlings was extracted with buffer C supplemented with 10 μM leupeptin (Peptide Institute) and 30 μM MG132 or DMSO. The protein extracts were incubated at room temperature for 2 h. 3× SDS sample buffer was added to stop the reaction, and the sample was used for immunoblot analysis. Anti-HA antibody was purchased from Abcam plc (Cambridge, UK). The degradation assay for each protein was replicated three times. Signal intensities of proteins detected on immunoblotted membranes were quantitated by digitizing with Image J software (http://rsbweb.nih.gov/ij/). Each quantitated value of HA or FBA signal was divided by the corresponding quantitated value of the control protein. The relative amounts of the remaining proteins (%) after incubation with MG132 or DMSO were calculated.

## Results and discussion

### *Purification and identification of Ub-related proteins from* Arabidopsis *seedlings*

To isolate Ub-related proteins by immunoaffinity chromatography, monoclonal antibody FK2 was applied, which selectively recognizes the Ub moiety but not free Ub (Fujimuro *et al.*, 1994). Approximately 250 mg of total protein was obtained from 50 g of *Arabidopsis* seedlings and applied to the immunoaffinity column under native condition (Fig. 1A). The staining pattern of eluted proteins subjected to SDS–PAGE was reproducible among the samples derived from three independent purification steps (see Supplementary Fig. S1 at *JXB* online). Compared to the mouse serum column, a number of discrete bands on a smeared background were detected in the purified sample eluted with the FK2 column (Fig. 1B), suggesting different mobilities in a gel caused by the heterogeneity of multi-Ub chains, as observed in a study of human cells (Matsumoto *et al.*, 2005).

Numerous Ubs were detected by MS/MS analysis, indicating that they were probably derived from Ub-conjugated proteins. Ubs and E2 proteins were eliminated from the list of proteins isolated with the FK2 column and the proteins isolated with the mouse serum column were further subtracted. In this study, only proteins with a score of over 40 ($P$ <0.05) were selected as candidate proteins with high reliability. Accordingly, 196 proteins, which were represented by 251 distinct genes including possible paralogs, were determined as Ub-related proteins (see Supplementary Table S1 at *JXB* online). Comparing the

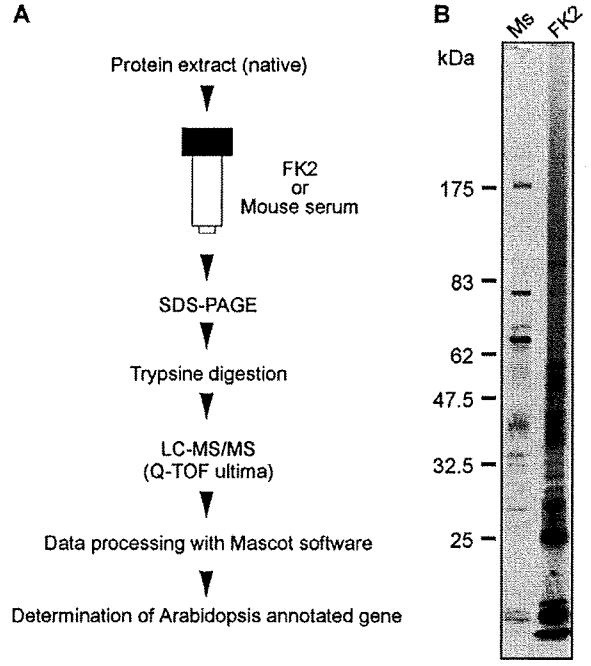


**Fig. 1.** Immunoaffinity purification and identification of Ub-related proteins. (A) Flow chart of purification and identification of Ub-related proteins. (B) Immunopurified proteins with FK2 or mouse serum (Ms) from *Arabidopsis* seedlings were subjected to SDS-PAGE and stained with Flamingo™.

results of this study with *Arabidopsis* ubiquitinated proteomes reported in two studies, 33 proteins were overlapped and only one protein (β-tubulin; protein no. 137 in Supplementary Table S1 at *JXB* online) was common to the three studies (see Supplementary Table S1 at *JXB* online). The low overlapping result compared to the previous studies may be due to the difference in the differentiation state of the protein source. In addition, unstructured threshold settings for protein screening from the MS scores may account for the difference in listed proteins among the three studies. The following reasons are proposed. Immunopurification with FK2 used in this study, enabled recognition of all types of ubiquitinated proteins, whereas each UBA used in the other studies had distinct specificity to ubiquitinated proteins. Therefore, the dominant ubiquitinated proteins were preferentially trapped by FK2. In addition, our dataset included a high proportion of associated proteins of ubiquitinated proteins due to the native condition used in protein purification. In fact, proteins annotated as RING-type E3 (protein no. 109 in Supplementary Table S1 at *JXB* online) and putative Ub receptors, DNA-repair protein RAD23 (protein no. 119 in Supplementary Table S1 at *JXB* online), and UBA-like motif-containing protein (protein no. 196 in Supplementary Table S1 at *JXB* online), were identified in this study, suggesting that non-direct target proteins for ubiquitination were also isolated.

### *Characterization of Ub-related proteins from* Arabidopsis *seedlings*

The identified proteins were categorized on the basis of the biological processes described in 'The *Arabidopsis* Information Resource' (TAIR) (Fig. 2A). The large population of proteins involved in metabolism (23.0%) as well as previous *Arabidopsis* ubiquitinated proteomes (Maor *et al.*, 2007; Manzano *et al.*, 2008) may indicate the significance of ubiquitination for protein regulation in cellular metabolism. Similar to the previous report of Manzano *et al.* (2008), it was found that the proteins involved in stress response (21.4%) were more abundant in seedlings than in cell cultures. This might be due to the differentiation state of the cells, because the growth condition in liquid culture seemed to be more stressful for seedlings than for cell cultures. Indeed, proteins involved in abiotic stress were dominant in this category (Fig. 2A). The majority of translational proteins identified in our study (16.8%) were ribosomes, in agreement with Maor's study (Maor *et al.*, 2007). It has been speculated that ubiquitination might play an important role in the regulation and/or quality control of ribosomal proteins, as reported in human cells (Matsumoto *et al.*, 2005). In addition, the recent report by Kraft *et al.* (2008) of a link between ubiquitination and regulated degradation of mature ribosomes in yeast also supports our results. The percentage of proteins involved in signal transduction (1.5%) was lower than that previously reported for the ubiquitinated proteome from *Arabidopsis* seedlings (Manzano *et al.*, 2008). The difference may depend on the threshold for the screening of identified proteins from MS scores, as described above. Only proteins with a high reliability (95% confidence) were listed in this study, whereas Manzano's list contained proteins with low reliability (Manzano *et al.*, 2008). The proportions of other components were similar to that found in previous *Arabidopsis* ubiquitinated proteomes. One significant difference compared to the previous *Arabidopsis* ubiquitinated proteomes was the high percentage of proteasome subunits (14.3%), which was probably dependent on the native condition for protein purification. This fact indicated that the associated proteins of ubiquitinated proteins were isolated as well, and implied that most proteins were involved in Ub/proteasome-dependent proteolysis.

### *Classification of identified proteins*

Purification under native condition is a feasible technique to identify Ub-related proteins that play major roles in Ub-dependent regulation (Matsumoto *et al.*, 2005). Since the protein population isolated under native condition included not only ubiquitinated proteins but also their associated proteins, they were classified as ubiquitinated proteins and their associated proteins based on the following three criteria.

First, previous *Arabidopsis* ubiquitinated proteomes from cell cultures and seedlings, respectively, were examined (Maor *et al.*, 2007; Manzano *et al.*, 2008). Of the proteins identified in our study, 33 have been reported as

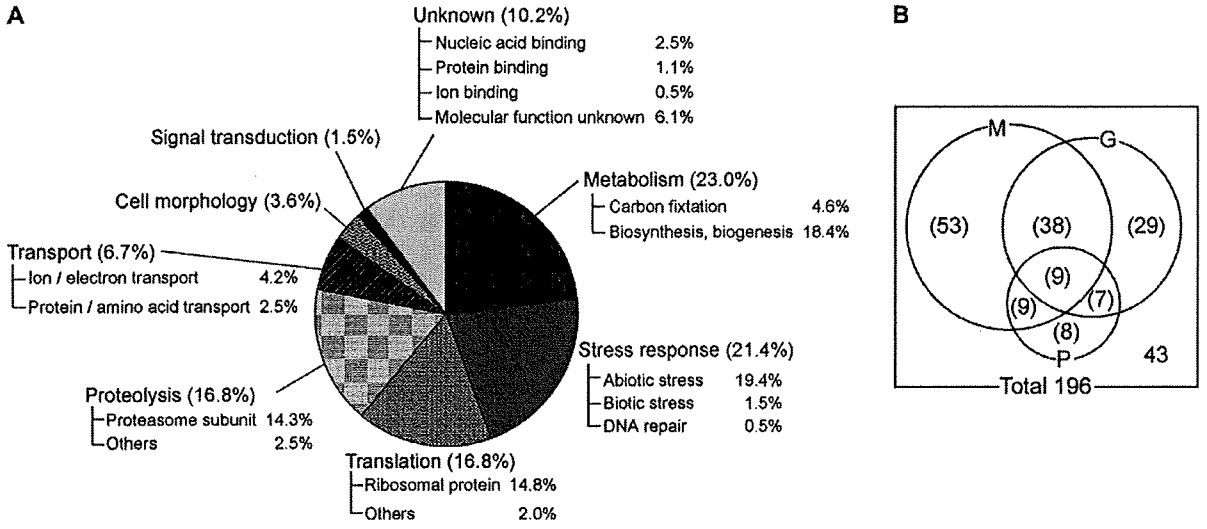


**Fig. 2.** Characterization of proteins identified from *Arabidopsis* seedlings. (A) Proportion of identified proteins categorized according to function. Each category was further subdivided according to specific function. (B) Numbers of potential ubiquitinated proteins and their associated proteins. Number outside the circles indicates the number of associated proteins of ubiquitinated proteins. M, proteins containing at least one motif; G, proteins detected in multiple gel pieces; P, proteins previously reported as ubiquitinated proteins.

ubiquitinated proteins, suggesting that these were the potential direct targets of ubiquitination (Fig. 2B; see Supplementary Table S1 at *JXB* online).

Second, the identified proteins were classified on the basis of potential ubiquitination-targeting signal motifs (D-box, KEN-box, and PEST sequence) to predict the ubiquitinated proteins. D-box and KEN-box are short sequence elements in the substrates of the anaphase-promoting complex/cyclosome (APC/C), which is a multisubunit RING-type E3 (King *et al.*, 1996; Pfleger and Kirschner, 2000), and indeed, RING-type E3 (protein no. 109 in Supplementary Table S1 at *JXB* online) was found in our study. PEST sequences that are rich in proline (P), glutamic acid (E), serine (S), and threonine (T) were found in a number of short-lived proteins controlled by proteolysis, mostly via ubiquitin-mediated degradation. Almost half of the identified proteins (109/196 proteins (55.6%)) contained at least one motif, implying that they are the potential targets of Ub/proteasome-dependent proteolysis (Fig. 2B; see Supplementary Tables S1 and S2 at *JXB* online).

Third, multiple detections from different gel pieces implied multi-ubiquitination of the proteins, since the heterogeneity of multi-Ub chains accounted for the different mobilities in a gel. Of the identified 196 Ub-related proteins, 83 (42.3%) were found in 2–17 gel pieces of different sizes (Fig. 2B; see Supplementary Table S1 at *JXB* online), suggesting that the proteins were probably tagged with heterogeneous multi-Ub chains.

Considering potential ubiquitination-targeting signal motifs, in-gel mobilities of the identified proteins, and previous reports, 153 proteins (78.0%) were predicted as the potential targets of ubiquitination, including 109 potential target proteins (55.6%) of Ub/proteasome-dependent proteolysis, whereas the remaining proteins (21.9%) were potential molecules associated with the ubiquitinated proteins.

### *Degradation assays of proteins predicted as ubiquitinated proteins*

The results suggested that a large proportion of the identified proteins were involved in Ub/proteasome-dependent proteolysis. Therefore, the identified proteins were examined by proteasome degradation assay. According to the prediction described above, three proteins predicted as potential ubiquitinated proteins, At1g12840 (DET3, protein no. 82 in Supplementary Table S1 at *JXB* online), At3g04120 (GAPC, protein no. 1 in Supplementary Table S1 at *JXB* online), and At3g52930 (fructose biphosphate aldolase-like; FBA, protein no. 6 in Supplementary Table S1 at *JXB* online), were chosen for the assay.

DET3 encodes subunit C of vacuolar $H^+$-ATPase (V-ATPase). V-ATPase is one of the major proton pumps that act to acidify intracellular compartments (Sze *et al.*, 2002) and DET3 is significantly responsible for its activity (Schumacher *et al.*, 1999). Since DET3 contains four D-box motifs and was detected in two gel pieces (Supplementary Table S1 at *JXB* online), regulation of DET3 protein by Ub/proteasome-dependent proteolysis was highly expected. DET3 protein tagged with 3× HA was transiently expressed in *N. benthamiana* leaves by agroinfiltration. The protein extract was incubated with MG132, a 26S proteasome inhibitor. As a result, it was found that DET3 protein degradation was inhibited by MG132 treatment but not DMSO treatment (Fig. 3A), indicating that it was conjugated with canonical Ub chains and then underwent the

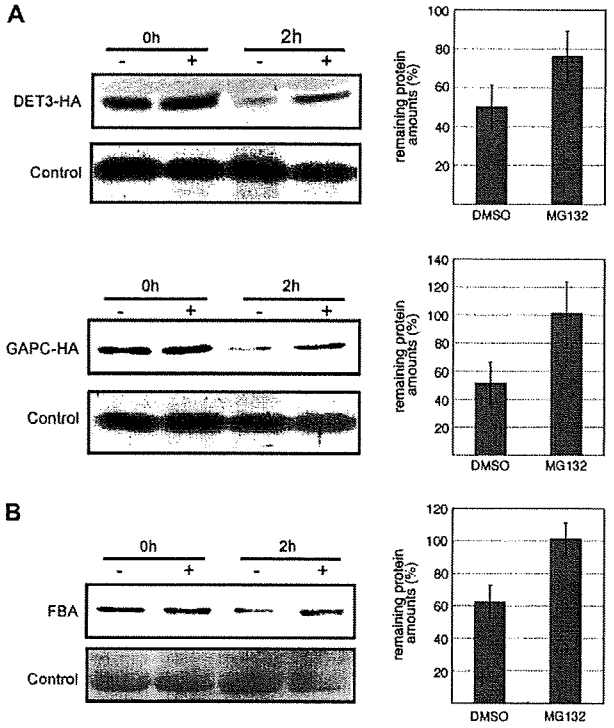


**Fig. 3.** Degradation assays of potential ubiquitinated proteins. Protein extracts were treated with MG132 (+) or DMSO (–). Lower panels of each figure indicate loading controls. Graphs on the right represent the relative amount of remaining protein (%) after treatment with MG132 (+) or DMSO. Error bars indicate standard deviations. (A) DET3-HA and GAPC-HA proteins transiently expressed in *N. benthamiana* leaves were detected with anti-HA antibody. (B) Total proteins were extracted from *Arabidopsis* seedlings. Immunoblot analysis was performed with anti-FBA antibody.

proteasome-dependent proteolysis. Other subunits A and B of V-ATPase were respectively isolated as ubiquitinated proteins in Maor's and Manzano's studies (Maor *et al.*, 2007; Manzano *et al.*, 2008), whereas the anti-Ub antibody used in our study could trap all of these subunits, implying that the difference in the identified subunits of V-ATPase may be attributed to the distinct specificity of the ligands used for affinity purification. It was also suggested that each subunit is ubiquitinated by a distinct ubiquitination pathway, and at least one subunit DET3 of the V-ATPase complex was degraded by the 26S proteasome.

GAPC was detected in four gel pieces, although the ubiquitination-targeting signal motif was absent (see Supplementary Table S1 at *JXB* online). The degradation of GAPC by the 26S proteasome has not been reported to date. Since it has also been reported as a ubiquitinated protein (Manzano *et al.*, 2008), the degradation by the 26S proteasome of the GAPC protein, which was transiently expressed similar to DET3, was examined. As shown in Fig. 3A, the degradation of the GAPC protein was inhibited by MG132 treatment, but not DMSO treatment, indicating that it was regulated by the Ub/proteasome-dependent proteolysis.

FBA was identified in seven gel pieces, although it did not contain any potential ubiquitination-targeting signal motifs (see Supplementary Table S1 at *JXB* online). FBA was also identified as a ubiquitinated protein (Maor *et al.*, 2007). Thus, it was expected that FBA would be degraded by the 26S proteasome. To examine the FBA degradation by the 26S proteasome in *Arabidopsis* seedlings, the protein extract was incubated with or without MG132, and FBA protein was detected with its antibody. As shown in Fig. 3B, the degradation of FBA was inhibited by MG132, demonstrating that FBA was regulated by Ub/proteasome-dependent proteolysis in *Arabidopsis* seedlings.

The degradation of DET3, GAPC, and FBA by the 26S proteasome was demonstrated for the first time in this study. GAPC and FBA are involved in glycolysis and are known to respond to environmental stress. Thus, the turnover of these proteins may be regulated by the Ub/proteasome-dependent proteolysis in the glycolytic pathway and/or stress response. It would be interesting to investigate the contribution of Ub-mediated regulation of these proteins in metabolism and/or stress response as a future work.

## Conclusions

This study showed the Ub-related proteome of *Arabidopsis* seedlings. Protein purification under native conditions with an anti-Ub antibody contributed to the isolation of various Ub-related proteins that mainly comprised proteins involved in Ub/proteasome-dependent proteolysis. The protein population identified contained both the targets of ubiquitination and their associated proteins. Biochemical evidence is required to characterize exactly which protein is the direct target of ubiquitination. Nevertheless, classification of the identified proteins based on the potential ubiquitination-targeting signal motifs, in-gel mobilities, and the previous reports contributed to the prediction of ubiquitinated proteins and their associated proteins. Our results are expected to be of use to the future investigation of Ub-mediated protein regulation in plants.

## Supplementary data

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Respective SDS-PAGE images of proteins immunopurified with FK2 obtained from three independent experiments (Ex1, Ex2, and Ex3). The staining pattern of the purified proteins was reproducible.

**Supplementary Table S1.** Ub-related proteins identified from *Arabidopsis* seedlings.

**Supplementary Table S2.** Proteins containing potential ubiquitination-targeting signal motifs for Ub/proteasome-dependent proteolysis.

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# A simple and high-sensitivity method for analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis

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## Abstract

**Background:** Ubiquitination is mediated by the sequential action of at least three enzymes: the E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) proteins. Polyubiquitination of target proteins is also implicated in several critical cellular processes. Although Arabidopsis genome research has estimated more than 1,300 proteins involved in ubiquitination, little is known about the biochemical functions of these proteins. Here we demonstrate a novel, simple and high-sensitive method for *in vitro* analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis and luminescent detection.

**Results:** Using wheat cell-free synthesis, 11 E3 proteins from Arabidopsis full-length cDNA templates were produced. These proteins were analyzed either in the translation mixture or purified recombinant protein from the translation mixture. In our luminescent method using FLAG- or His-tagged and biotinylated ubiquitins, the polyubiquitin chain on AtUBC22, UPL5 and UPL7 (HECT) and CIP8 (RING) was detected. Also, binding of ubiquitin to these proteins was detected using biotinylated ubiquitin and FLAG-tagged recombinant protein. Furthermore, screening of the RING 6 subgroup demonstrated that At1g55530 was capable of polyubiquitin chain formation like CIP8. Interestingly, these ubiquitinations were carried out without the addition of exogenous E1 and/or E2 proteins, indicating that these enzymes were endogenous to the wheat cell-free system. The amount of polyubiquitinated proteins in the crude translation reaction mixture was unaffected by treatment with MG132, suggesting that our system does not contain 26S proteasome-dependent protein degradation activity.

**Conclusion:** In this study, we developed a simple wheat cell-free based luminescence method that could be a powerful tool for comprehensive ubiquitination analysis.

## Background

Protein ubiquitination plays a crucial role in numerous cellular processes such as cell growth, regulation of diverse signal transduction and disease [1-3]. The covalent attachment of ubiquitin to protein substrates requires a stepwise cascade of enzymatic reactions. First, ubiquitin is activated by E1 (ubiquitin-activating enzyme, UBA) in an ATP-dependent manner by forming a high-energy thioester-bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of E1. The activated ubiquitin is then transferred to the core-cysteine residue of E2 (ubiquitin-conjugating enzyme, UBC). Together with an E3 ligase enzyme, ubiquitin is attached via its carboxyl-terminus to an e-amino group of a lysine residue in the target protein. Since E3 binds to both E2 and the target protein, and acts as scaffold between E2 and the substrate protein, the E3 ligase is the major determinant for selecting target proteins for ubiquitination. There is large number of genes encoding E3 ligases in all eukaryotes, and the diversity of E3s is thought to contribute to the substrate specificity of numerous target proteins. E3 ligases are structurally divided into three groups: HECT, RING and U-box [4]. The HECT-type E3 ligase is distinct from the other two ligases in that it forms a thioester-bond with ubiquitin prior to the transfer of ubiquitin to target proteins. The RING-type E3 ligase contains a unique domain similar to the zinc finger motif that mediates protein-protein interactions [5] and is further divided into two classes: one that can function alone and another that forms a complex with other E3 components [4].

Recent studies have shown that attachment of polyubiquitin chains on target proteins linked via lysine-48 of ubiquitin typically leads to degradation by the 26S proteasome [6], whereas linkage via lysine-63 mediates different pathways such as internalization of membrane proteins, activation of signal transduction and DNA damage repair [7]. The formation of lysyl-63-linked polyubiquitin chains is generated by specific combinations of E2s and E2 variants, which are similar to E2s except that they lack core cysteine residues required for E2 activity [8,9]. In addition, ubiquitination of substrates without polymerization, mono-ubiquitination, acts as a sorting signal for protein endocytosis and as a regulation factor for diverse proteins, including histones and transcription factors [10].

In plant, genomic research of the model plant *Arabidopsis thaliana* showed that there are two E1s, 37 E2s and more than 1,300 predicted E3s [11]. Although little is known about protein ubiquitination in plants compared with yeast and mammals, recent studies revealed that the plant ubiquitination pathway is involved in the regulation of morphogenesis, the circadian clock and responding to hormone or pathogen signal molecules [12-15]. Despite the importance of ubiquitination in plants, much of the plant ubiquitination cascade is still unknown because of its complexity and the issues inherent to the use of Arabidopsis plants for biochemical analysis. Although several interactions between E2s and RING type E3s have been demonstrated *in vitro* using recombinant proteins expressed in *Escherichia coli*, these efforts are hampered by the inability to obtain functional protein using conventional methods [16].

With this in mind, we sought to develop a novel *in vitro* method to analyze the ubiquitin pathway genome-wide. The two major obstacles hindering the development of an *in vitro* assay for genome-wide screening are the difficulty of efficiently producing recombinant protein and the inability to detect ubiquitination in a high-throughput fashion. To address the first problem we used the wheat cell-free protein synthesis system, which has been previously reported to produce a wide range of functional Arabidopsis and human proteins [17-19]. Moreover, a collection of RIKEN Arabidopsis Full Length (RAFL) cDNA clones covering about 70% of Arabidopsis genes is available [20]. Using these RAFL clones as templates, recombinant proteins involved in the ubiquitination pathway were expressed in the wheat cell-free system and used for several functional analyses. For screening, conventional detection methods such as immunoblot analysis or radioisotope-labeled proteins are not suitable for the detection of a large number of ubiquitination reactions. Recently, a high-throughput luminescence method to detect protein ubiquitination was reported [21], however this method requires purified protein and creation of specialized vectors to produce proteins. In this study, a novel *in vitro* assay to detect polyubiquitin chain formation was developed using wheat cell-free synthesis and a modified luminescence-based detection method. We demonstrate (1) creation of a simple *in vitro* method to detect polyubiquitination using crude recombinant E3s, (2) discovery of the activity of At1g55530 by screening a RING subgroup in the reported assay, and (3) the polyubiquitination assay in the presence of MG132 demonstrated the absence of 26S proteasome-dependent protein degradation activity in wheat cell-free system.

## Results

### *Detection of Polyubiquitin Chains on AtUBC22 E2 enzyme*

Recently, AtUBC22 (At5g05080) E2 protein has been shown to catalyze polyubiquitin chain formation without an E3 ligase, although AtUBC35 (At1g78870) E3-independent polyubiquitination activity could not be detected [16]. We employed AtUBC22 and AtUBC35 as model E2 proteins to develop a novel polyubiquitination assay. We have also demonstrated that addition of biotin ligase (BirA) and biotin to the wheat cell-free protein production system yields a single biotinylation on a target pro-

tein containing a biotin ligation site [22]. Using this method, biotinylated recombinant AtUBC22 and AtUBC35 were synthesized and, without purification from the translation mixture, the polyubiquitination reaction was performed on the crude recombinant protein. After the reaction, biotinylated AtUBC22 and AtUBC35 were purified using streptavidin-conjugated magnetic beads and the polyubiquitin chain was detected by immunoblot analysis. As shown in Fig 1A, AtUBC22 showed polyubiquitination, whereas AtUBC35 showed mainly monoubiquitination. Interestingly, both E2s still had activity in absence of exogenous E1 in polyubiquitin reaction mixture (Fig. 1A, middle lanes), suggesting that wheat cell-free system has high endogenous E1 activity.

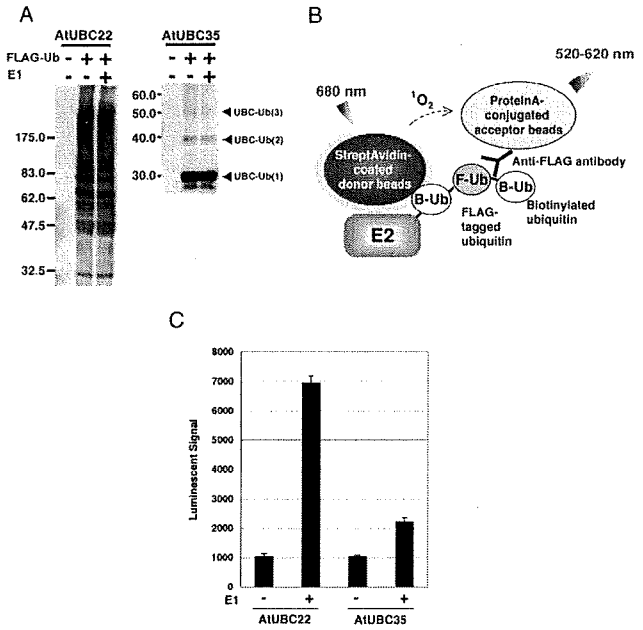


**Figure 1**
**Detection of E3-independent polyubiquitination of AtUBC22 by luminescent analysis.** A, Polyubiquitin chain on AtUBC22 but not on AtUBC35 was detected by immunoblot analysis. In this assay, polyubiquitination reaction was carried out with FLAG-tagged ubiquitin, and detected by immunoblot analysis using anti-FLAG antibody. B, Schematic diagram of detection of polyubiquitin chains by luminescent analysis. Protein A-conjugated acceptor beads and streptavidin-coated donor beads are bound to anti-FLAG antibody bound to FLAG-tagged ubiquitin and biotinylated E2, respectively, and these two beads are in closed proximity when polyubiquitin chain formed. Upon excitation 680 nm, a singlet oxygen is generated from the donor beads, and then transferred to the acceptor beads within 200 nm, and the singlet oxygen reacts the acceptor beads which in turn emits light at 520–620 nm. This light is measured by AlphaScreen kit and change to signal value. C, Polyubiquitin chain on purified recombinant E2 was detected by luminescent analysis in the presence (E1 +) or absence (E1 -) of exogenous E1. Error bars represent standard deviations from three independent experiments.

While immunoblot analysis is an excellent detection method, it is not suitable for high-throughput detection of numerous polyubiquitination reactions. Initially, we attempted to use luminescent analysis, based on the AlphaScreen technology, to detect the polyubiquitination activity of AtUBC22 and AtUBC35. In principle, if a polyubiquitin chain is formed by FLAG-tagged and biotinylated ubiquitins, it will bring into proximity the streptavidin-coated donor bead (bound to biotin) and the protein A-conjugated acceptor bead (bound to anti-FLAG IgG), producing a luminescent signal (Fig. 1B). Considering that the wheat cell-free system has high endogenous E1 activity (Fig. 1A), it may also have endogenous E2 and E3 activity. In order to avoid formation of polyubiquitin chains by an endogenous wheat germ ubiquitin pathway, purified E2s were used in this assay. As shown in Fig 1C, high luminescent signal was observed in the presence of AtUBC22 in E1-dependent manner. In contrast, AtUBC35 showed low signal. The two luminescent signals were approximately consistent with immunoblot data that AtUBC22 and AtUBC35 have high and low polyubiquitination activities respectively, as demonstrated in Fig 1A. These results indicate that the luminescent method can detect polyubiquitin chain formation by using the two types of ubiquitins.

### *Ubiquitination and Polyubiquitination Analyses of HECT-TypeE3 Ligases*

Polyubiquitination activity of E3 ligases activated by the step-wise E1 to E3 cascade is well documented [3]. We next attempted to reconstruct this cascade *in vitro* and to detect the E3-formed polyubiquitin chains using our luminescent method. Due to the size of HECT-type E3 ligases, ranging from 100 to 428 kDa in Arabidopsis, production of active protein by traditional expression methods may not be easy and biochemical analysis using only truncated recombinant protein has been carried out previously [23]. We attempted to produce full-length Arabidopsis HECT-type E3 ligase proteins using the wheat cell-free system and monitored ubiquitin-conjugation and polyubiquitination by luminescence. Two genes that encode Arabidopsis HECT-type E3 ligase, *UPL5* and *UPL7* [24], were analyzed in this study. We obtained UPL5 and UPL7 cDNA from the RAFL library and produced FLAG-tagged protein in the wheat cell-free system. Ubiquitination of FLAG-labeled UPLs (UPL-FLAGs) was investigated by both the luminescent and immunoblot methods. The successful production of the two recombinant HECT proteins was observed by immunoblot analysis (Fig. 2A) and used in the luminescence assay without purification. To detect ubiquitination of the HECT proteins, UPL-FLAGs

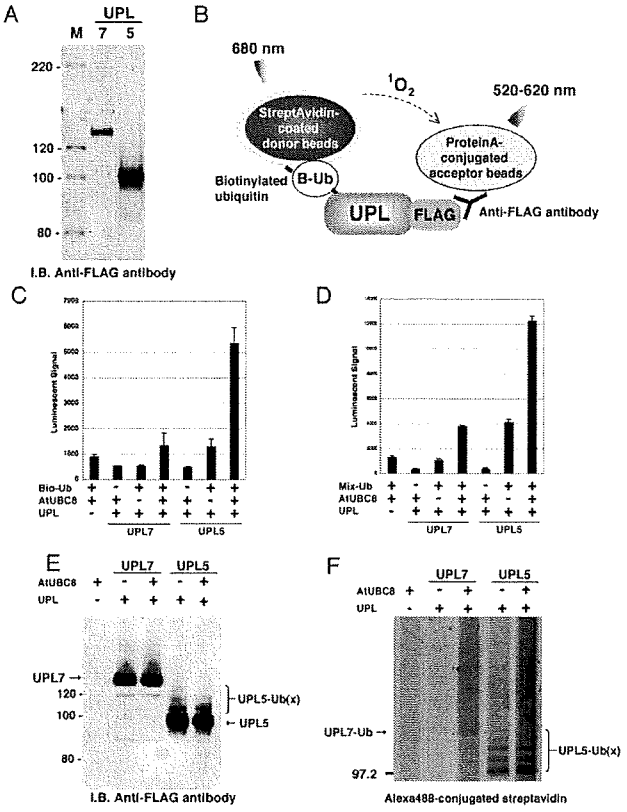


**Figure 2**

**Analysis of recombinant Arabidopsis HECT-type E3 ligases (UPL7 and UPL5).** A, Production of FLAG-tagged recombinant UPL proteins was detected by immunoblot analysis. For analysis, 5 μl of crude recombinant UPL proteins were loaded, and detected by immunoblot analysis using anti-FLAG antibody. B, Schematic diagram of detection of ubiquitin-conjugation of UPLs by luminescent analysis. Protein A-conjugated acceptor beads and streptavidin-coated donor beads were bound to anti-FLAG antibody bound to FLAG-tagged recombinant UPLs and biotinylated ubiquitin, respectively, and detected by same principle and procedure described in Figure 1B. C, The ubiquitination of crude recombinant UPL7 and UPL5 was detected by luminescent analysis described in B. Bio-Ub means biotinylated ubiquitin. D, polyubiquitination of crude recombinant UPL7 and UPL5 was detected by luminescent analysis with anti-His antibody. Mix-Ub indicated the mixture of His-tagged and biotinylated ubiquitin. E and F, Mobility shift of UPLs (E) and formation of polyubiquitin chains (F) were detected by immunoblot using anti-FLAG antibody and Alexa488-conjugated streptavidin, respectively. The polyubiquitination reaction was done with FLAG-tagged recombinant UPLs in presence or absence of crude AtUBC8, and then recombinant UPLs were purified by anti-FLAG antibody-conjugated agarose. Error bars represent standard deviations from three independent experiments.

and biotinylated ubiquitin were used. When biotinylated ubiquitin is conjugated to the UPL-FLAG, a high luminescent signal is obtained (Fig. 2B). As a result of the analysis, ubiquitin-conjugation of UPL5 was observed (Fig. 2C). In addition, polyubiquitin chains formed by UPLs were detected with the luminescence assay using His-tagged and biotinylated ubiquitin. To subtract polyubiquitin chain formation from endogenous E2 and E3 in wheat cell-free system, the assay was performed without recombinant UPL and only low signal was detected (Fig. 2D, "UPL-" lane). As expected, luminescent signal was observed in recombinant UPL5 and UPL7 (Fig. 2D). Although the luminescent signal of UPL7 was lower than that of UPL5, the signal was still two-fold higher than the endogenous background signal. These results were confirmed by immunoblot analysis that showed distinct mobility shifts of UPL5 (Fig. 2E) when detecting FLAG-tagged UPLs, and polyubiquitin chain formation of UPL5 monitoring Alexa488-conjugated streptavidin (Fig. 2F). Comparing the amount of polyubiquitin chain formation in absence of UPLs (Fig. 2F, "UPL-" lane), UPL7 formed weak but distinct polyubiquitin chains in presence of AtUBC8. These luminescent signals were consistent with immunoblot data. Interestingly, polyubiquitin chains were formed by UPL5 without supplementing exogenous E2 protein (Fig. 2D and 2F, "AtUBC8-" lane), suggesting that wheat germ extract has endogenous E2 activity as well as endogenous E1 activity. These data indicate that the wheat cell-free production system is able to produce high molecular weight proteins in functional forms and that our luminescence method can detect activity of HECT-type E3 ligases without purification. This is the first data showing that full length recombinant HECT-type E3s have ubiquitin-conjugating and polyubiquitination activity. Taken together, the luminescent method based on the wheat cell-free system could be useful for biochemical analysis of HECT-type E3 ligases.

### *Detection of Polyubiquitin Chains by RING-Type CIP8 E3 Ligase*

It is reported that at least 469 predicted RING-type E3 ligases are encoded in the Arabidopsis genome [25]. Like the HECT-type E3, we attempted to express and carry out the functional analysis of the RING-type E3 ligases. In this study, we selected CIP8 as a model RING-type E3 ligase, which is reported to possess a RING finger motif and have typical features of an E3 ligase [26]. At first, polyubiquitination activity of purified CIP8 in presence or absence of exogenous E1 and purified E2 (AtUBC8) was investigated by luminescence. As shown in Fig 3A, luminescence analysis using His-tagged and biotinylated ubiquitin showed the polyubiquitination of purified CIP8 only when exogenous E1 and purified E2 were added to the reaction mixture. The CIP8-dependent polyubiquitination was

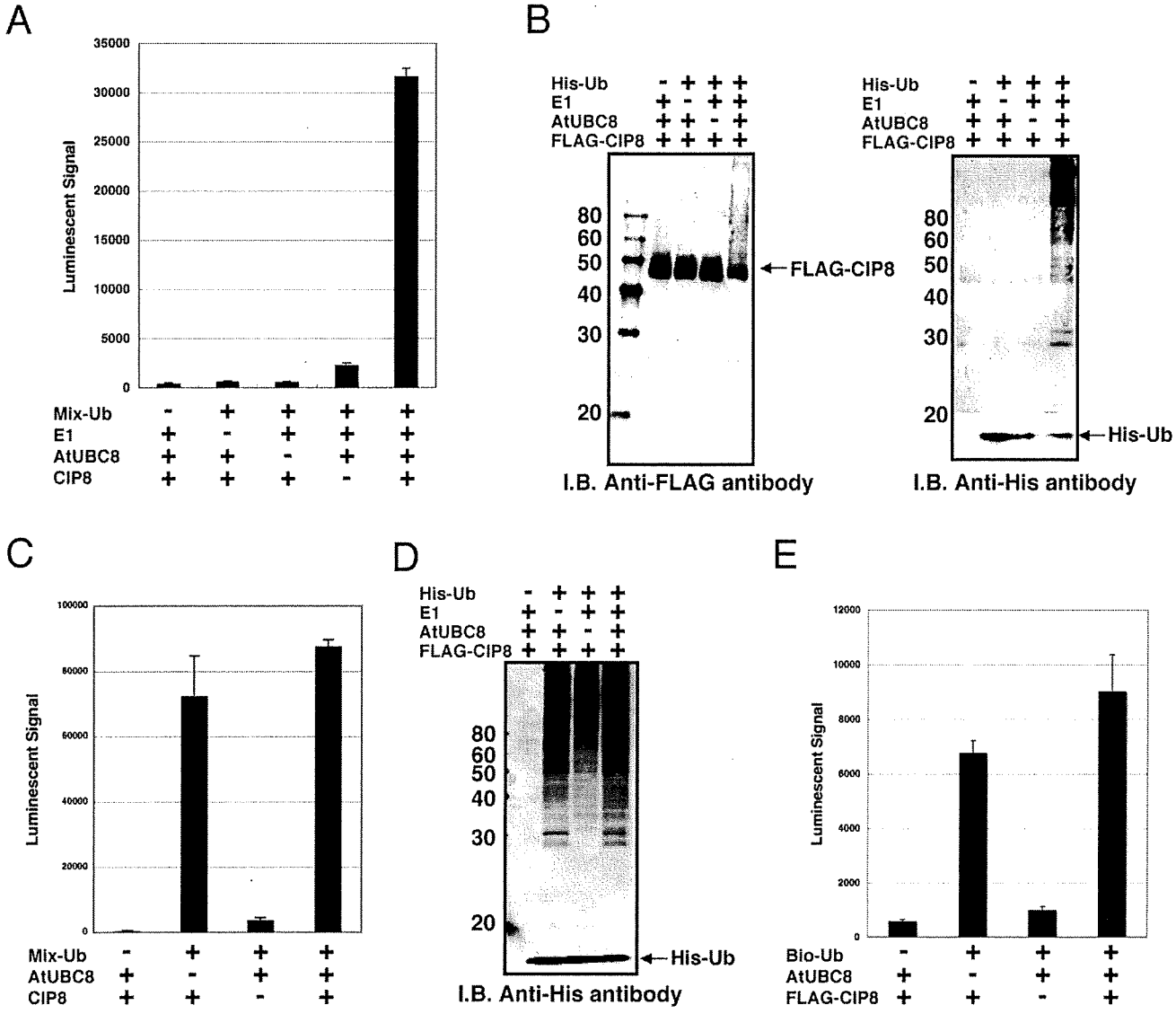


**Figure 3**

**Detection of polyubiquitination and self-ubiquitination of CIP8**. A to D, The polyubiquitination assay was carried out with purified (A and B) or crude recombinant CIP8 (C and D) and detected by luminescent analysis with anti-FLAG antibody (A and C) and immunoblot analysis (B and D). His-Ub or Mix-Ub indicate His-tagged ubiquitin or the mixture of FLAG-tagged and biotinylated ubiquitin, respectively. The polyubiquitination assay using luminescent analysis was carried out with recombinant CIP8 without tag in the presence or absence of ubiquitin related components indicated below each graph. E, Ubiquitination of crude recombinant CIP8 was observed by luminescent analysis with anti-FLAG antibody. The assay was carried out with or without biotinylated ubiquitin and crude AtUBC8 recombinant protein. Bio-Ub means biotinylated ubiquitin. Error bars represent standard deviations from three independent experiments.

confirmed by immunoblot analyses detecting both FLAG-CIP8 and His-tagged ubiquitin (Fig. 3B). On the other hand, luminescent analysis with crude CIP8 protein showed high polyubiquitination activity both in the presence or absence of purified E2 (Fig. 3C), and was confirmed by immunoblot analysis with crude protein (Fig. 3D). These data indicated that, like recombinant UPL5, crude CIP8 also utilized endogenous wheat extract E1 and E2 proteins, and therefore we could carry out the simple polyubiquitination analysis of E3 without addition of exogenous E1 and E2 proteins. Furthermore, immunoblot analysis detecting purified CIP8 (Fig. 3B) showed a mobility shift of FLAG-tagged CIP8 to higher molecular weights due to ubiquitination, whereas the mobility of the E2 was

not altered (data not shown). This result indicates that the CIP8-dependent polyubiquitin chains might be elongated on CIP8 itself. This data is consistent with a recent report showing that TRIM5a, a typical RING-type E3 ligase in human, also undergoes self-ubiquitination, forming polyubiquitin chains on itself [27]. To clarify whether the mobility shift of CIP8 was concomitant with polyubiquitin chain formation resulting from self-ubiquitination, we tried to detect ubiquitination of CIP8 by the luminescent method using crude FLAG-CIP8 protein and biotinylated ubiquitin. The luminescent method clearly detected the binding of biotinylated ubiquitin to FLAG-tagged CIP8 both in the presence and absence of exogenous E2 (Fig. 3E). Similar to polyubiquitin formation, the ubiquitination of CIP8 also occurred without the addition of exogenous E2 protein (Fig. 3E, "AtUBC8-" lane). Taken together, these data demonstrate that the luminescent method could detect formation of RING-type CIP8-dependent polyubiquitin chains and self-ubiquitination of crude CIP8.

### *Screening of RING-Type E3 Ligases Having Polyubiquitination Activity*

Recent papers have reported that the polyubiquitin chain is an important biological regulator. Identification of activity and features of E3 ligases offers important information about the ubiquitin-dependent regulation system. Our luminescent method based on the wheat cell-free system produced a simple and high-sensitivity detection of CIP8-dependent polyubiquitin chains without any purification (Fig. 3C). Using these tools, we screened new E3 ligases for the ability to form polyubiquitin chains like CIP8.

The RING-type E3 ligases in Arabidopsis were divided into 30 subgroups based on domain structure, and CIP8 is categorized into subgroup 6 as it contains a coiled-coil domain [25]. Eight other RING-type E3s from subgroup 6 were selected for screening, and the simple polyubiquitination assay was carried out with FLAG-tagged and biotinylated ubiquitins, and the crude recombinant RING-type E3s without addition of exogenous E1 and E2. The screening result showed significant polyubiquitination activity of At1g55530, whereas other RING-E3 proteins were not active (Fig. 4A). Immunoblot analysis of purified recombinant At1g55530 confirmed the polyubiquitination activity and indicated that At1g55530 was self-ubiquitinated (Fig. 4B). The polyubiquitination activity of At1g55530 suggests that it may have a biological role for proteasome-mediated degradation like CIP8 [26]. These results show that the wheat cell-free protein expression system and the luminescent ubiquitination detection method could support functional high-throughput screening of E3 proteins.

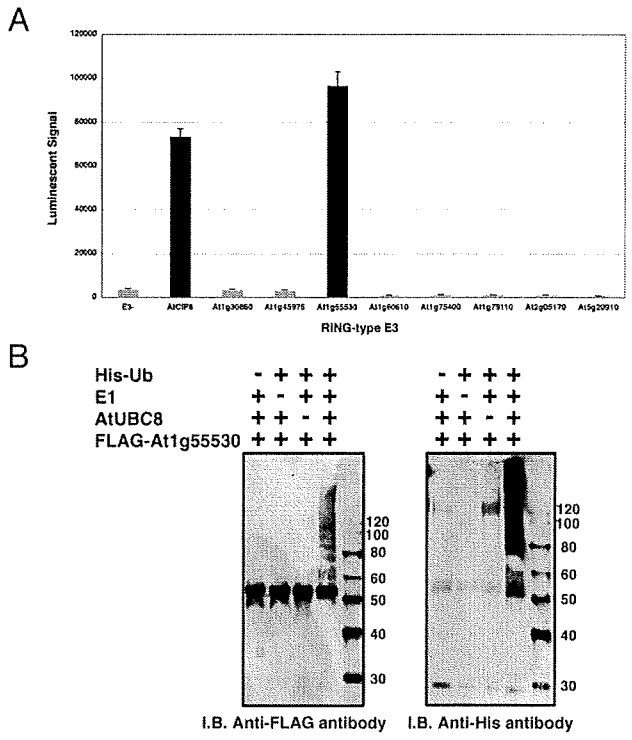


**Figure 4**
**Screening of RING-type E3 ligases having polyubiquitination activity.** A, Polyubiquitination reaction of crude recombinant E3 proteins was carried out with mixture of FLAG-tagged and biotinylated ubiquitins, and investigated by luminescent analysis with anti-FLAG antibody. B, Polyubiquitination activity of At1g55530 was confirmed by immunoblot analysis. The assay was carried out using purified recombinant AtUBC8 and At1g55530, and mobility shift of FLAG-tagged At1g55530 and polymer of His-ubiquitin were detected by immunoblot analysis using anti-FLAG and anti-His antibodies, respectively. Error bars represent standard deviations from three independent experiments.

### *Analysis of the Wheat Cell-free Based Ubiquitination in the Presence of Proteasome Inhibitor*

It is known that some cell extracts, such as rabbit reticulocyte or HeLa S-100 fraction, have 26S proteasome-dependent proteolytic activity [28,29]. Based on the presence of endogenous E1 and E2 ubiquitination and polyubiquitination in the wheat cell-free system, it is expected that the 26S proteasome activity will be very low (Fig. 2, 3 and 4). It was previously reported that the wheat germ extract had little 26S proteasome-dependent protein degradation activity [30]. Thus, we determined whether the wheat cell-free system contains active 26S proteasome. Using the crude recombinant proteins that formed polyubiquitin chains in this study, the polyubiquitination reaction was carried out in presence or absence of MG132, and accrual of the polyubiquitinated recombinant pro-

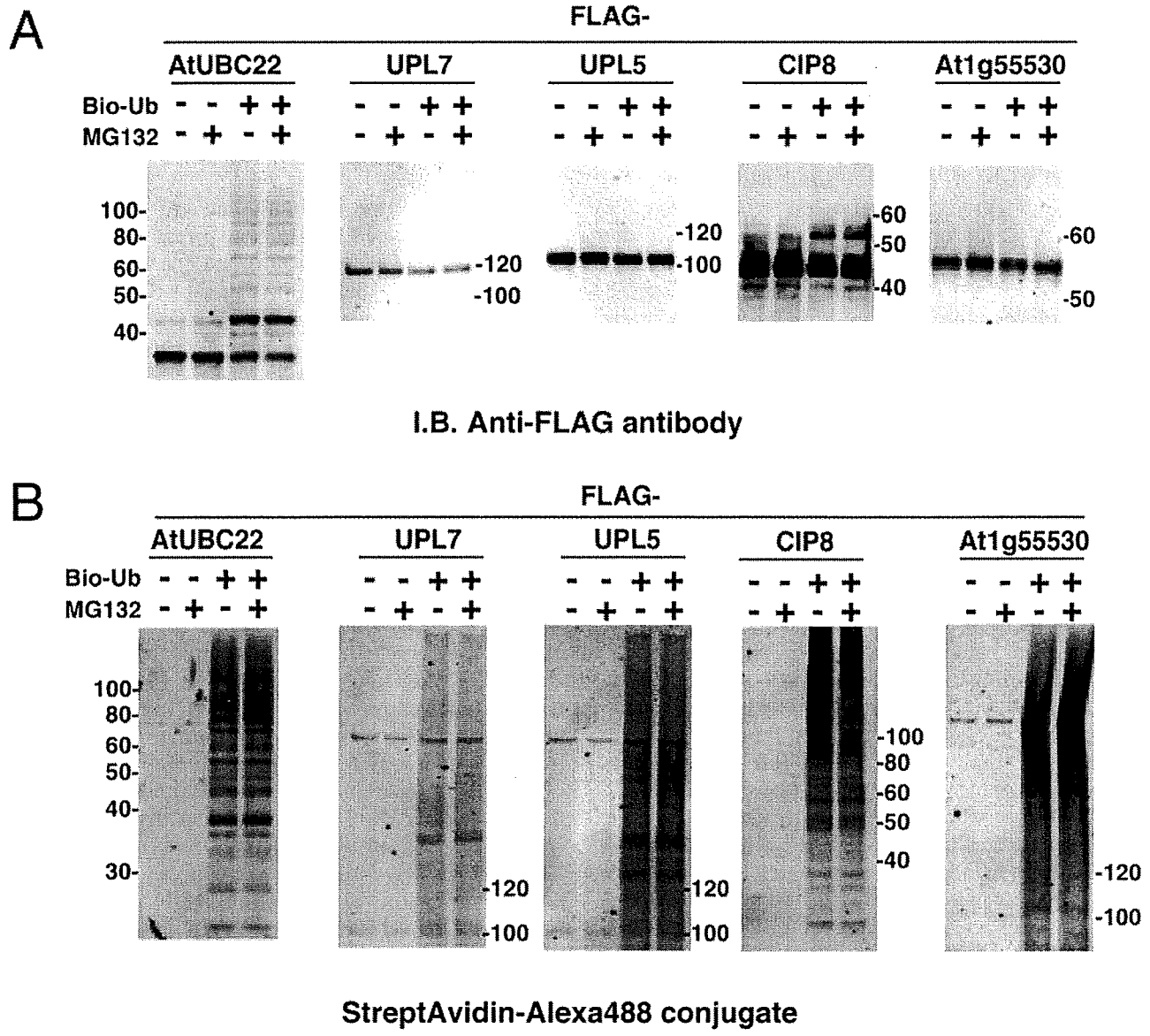


**Figure 5**
**Effect of proteasome inhibitor on stability of polyubiquitinated proteins**. Polyubiquitination assays of crude FLAG-tagged E2s and E3s were carried out in the presence or absence of biotinylated ubiquitin and 20 μM MG132. A, FLAG-tagged recombinant proteins were detected by immunoblot analysis using anti-FLAG antibody. B, Polyubiquitination chain formed by each recombinant protein was detected by Alexa488-conjugated streptavidin.

teins and its polyubiquitin chain was estimated. As shown in Fig 5, the amounts of UBC22, UPL5, UPL7 and At1g55530 (Fig. 5A) and of its polyubiquitin chains (Fig. 5B) were hardly altered by MG132 treatment. This result indicates that the proteolytic activity of the 26S proteasome in the wheat cell-free system was below the detection level. Thus, the wheat cell-free system could be suitable for ubiquitination analysis.

## Discussion

The ubiquitin signal is an important protein modification in eukaryotes. Binding of a single ubiquitin to a target protein, mono-ubiquitination, is essential for membrane trafficking, protein functions and protein-protein interaction [7]. As for polyubiquitination, both Lys-48- and Lys-63-linked polyubiquitin chains have been well characterized in mammals and yeast. Lys-48 linked chains cause proteolysis of target proteins [6], and Lys-63 linked chains reg-

ulate signal transduction such as cellular localization of protein or protein-protein interactions [7]. In mammals, the multi-functional activities of NF-κB are regulated by the Lys-63 linked chain [31]. In plants, the function of the Lys-63 linked chain is still obscure. However, Arabidopsis E2 and its variants promote formation of the Lys-63 linked chain [32], suggesting that the Lys-63 linked chain in plant cells might also function similar to animal cells. Hence, comprehensive analysis of the ubiquitin-related plant proteins would open a door for elucidation of the plant ubiquitin pathway. In this study, we developed a simple and highly sensitive ubiquitination assay method by combination of the wheat cell-free protein synthesis system and luminescent detection. In general, *in vivo* protein production requires many time-consuming steps such as vector construction, cell culture and purification to obtain the recombinant protein. In contrast, this cell-free based luminescence method could analyze a large amount of ubiquitin reactions without these steps.

Using this method, we conveniently detected polyubiquitin chain formation of E2 and E3s by using two tagged ubiquitins (Fig. 1, 2, 3 and 4). The result of polyubiquitination analysis of the E2s obtained from luminescent-based detection method was verified by immunoblot analysis (Fig. 1). Our analysis also produced recombinant protein of HECT-type E3 ligases without truncation and detected their ubiquitin-conjugation and polyubiquitination activity by luminescent analysis (Fig. 2C and 2D). The ubiquitin-conjugation of UPL5 was not observed when a reductant was added to the reaction (data not shown), suggesting that UPL5 formed a thioester bond with ubiquitin. In addition, the model RING-type E3 CIP8 possessed high polyubiquitin formation activity without substrate, consistent with what was reported previously [26]. Crude recombinant CIP8 formed polyubiquitin chains in the absence of exogenous E1 and E2 (Fig. 3C and 3D), suggesting that the wheat cell-free system might include enough endogenous E1 and E2 activity. It was reported that wheat germ extracts have only a partial ubiquitin pathway [30]. Although the process to isolate wheat germ extract is different from the conventional methods [33], this report strongly supports the existence of endogenous ubiquitin pathway in our wheat cell-free system. Indeed, luminescent analysis using crude recombinant protein showed slight polyubiquitin chain formation even in absence of recombinant E3 (Fig. 2D, Fig. 3C and Fig. 4A, "E3-" lane), indicating that wheat cell-free system might include not only E1 and E2, but E3s or other factors that accelerates the polyubiquitin chain formation. Further, quantitative immunoblot analysis using anti-ubiquitin antibody showed that free ubiquitin was also present in wheat germ extract at a concentration of at least 10 nM (data not shown). This is similar to the ubiquitin concentration supplied in the *in vitro* assay. Although we developed a convenient screening method to detect E3 activity in this study, removal of the endogenous ubiquitin and ubiquitin related components such as E1, E2 and E3, would yield a more sensitive assay. However, wheat cell-free system does not have 26S proteasome proteolytic activity (Fig. 5), indicating that using crude recombinant protein is sufficient for *in vitro* ubiquitination assays.

By using this method, we found that a previously uncharacterized RING type E3, At1g55530, possessed high polyubiquitination activity without exogenous E1 and E2 proteins (Fig. 4). This result suggested that the method developed here is expected to find the activity of other unknown E3 ligases such as At1g55530. Despite having only 32% sequence similarity, the E3s CIP8 and At1g55530 showed similar biochemical functions. Polyubiquitin chains formed by CIP8 and At1g55530 elongated on themselves, while another report showed that polyubiquitin chains were formed on E2 before transferring them to substrates [34]. This reflects that the pattern of polyubiquitin chain formation differs between individual E3s and that the detailed mechanisms are still unknown. These studies suggest the importance of functional analysis using active recombinant proteins. Although we developed a simple screen using crude recombinant E3s in absence of exogenous E1 and E2 (Fig. 4), this method could not detect the activity of some E3 ligases that were unable to utilize endogenous ubiquitination components in wheat cell-free system. The polyubiquitination activity of At5g20910 recombinant protein, expressed in *E. coli* in the presence of AtUBC8 [25], was not active in our *in vitro* system (Fig. 4A), suggesting that in some cases exogenous E2 and/or other components are necessary additions. Such modifications to the ubiquitination assays detailed here would help elucidate the biochemical features of E3s (e.g., addition of recombinant E2s to reaction mixture could give us further information about the E2–E3 specificity, and of other E3 components would lead to the elucidation of structure of complex type E3 ligase such as SCF).

## Conclusion

In this study, we found that the wheat cell-free system was an excellent expression system to produce recombinant protein efficiently and to carry out *in vitro* ubiquitination assays without the interference of proteolytic activity. Coupled with luminescent analysis, detection of these ubiquitin reactions in the crude translation reaction mixture was possible. Thus, this method should be helpful for solving the complicated ubiquitin pathway in plant.

## Methods

### *Construction of DNA Templates for Transcription*

We used RAFL as templates. DNA templates of E2s and E3s for transcription were constructed by "Split-Primer"

PCR as described previously [17]. Primers used in this study are summarized in Additional file 1. The first round of PCR was performed on each cDNA template using 10 nM of each of the following primers: a target protein specific primer (5'-CCACCCACCACCACCAatgnnnnnnnnn nnnnnnnn-3'; lowercase indicates the 5'-coding region of the target gene) and the AODA2306 primer. Then, a second round of PCR was carried out to construct the templates for protein synthesis using a portion (5 μl) of the first PCR mix, 100 nM SPu primer, 100 nM AODA2303 primer and 1 nM deSP6E02 primer. GST tags were used according to the methods we described previously [17]. The transcription templates of two HECT-type E3 ligases, UPL7 and UPL5, were generated as C-terminal FLAG-tagged proteins using the Gateway System® (Invitrogen, Carlsbad, CA, USA). Briefly, the ORF sequences of UPL7 and UPL5 were amplified by PCR with sense and antisense primers containing attB1 and FLAG-attB2 sequences, respectively. According to the manufacturer's instructions (Invitrogen), these DNA fragments were subcloned into pDONR221 vector by BP reaction and then inserted into the Gateway-based pEU vector (pEU-E01-GW) by LR reaction. Using these recombinant vectors as templates, PCR was carried out with 100 nM SPu primer and 100 nM AODA2303 primer and used as transcription templates.

### *Cell-free Protein Synthesis*

*In vitro* transcription and cell-free protein synthesis were performed as described [18]. 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 [35] 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 15 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 [22].

### *Purification of E2 and E3 Proteins*

Purification of GST-tagged protein was carried out according to the procedure described previously [36] with slight modification. Crude GST-tagged recombinant protein (450 μl) produced by the cell-free reaction was precipitated with glutathione sepharose™ 4B (GE Healthcare, Buckinghamshire, UK). The recombinant proteins were eluted with PBS buffer containing 0.1 U of AcTEV protease (Invitrogen) in order to cleave the GST tag from the protein.

### *Detection of Polyubiquitination by the Luminescent Method*

*In vitro* polyubiquitination assays were carried out in a total volume of 15 μl consisting of 20 mM Tris-HCl pH 7.5, 0.2 mM DTT, 5 mM $MgCl_2$, (10 μM zinc acetate in the assays for RING-type E3s only), 3 mM ATP, 1 mg/ml BSA, 25 nM biotinylated ubiquitin, 25 nM FLAG-tagged ubiquitin, 1 μl of recombinant E2 (purified or crude) and 1 μl of recombinant E3 (purified or crude) in the presence or absence of 0.05 μM rabbit E1 (Boston Biochem, Cambridge, MA, USA) at 30°C for 1 hr in a 384-well Optiplate (PerkinElmer, Boston, MA, USA). In accordance with the AlphaScreen IgG (ProteinA) detection kit (Perkin Elmer) instruction manual, 10 μl of detection mixture containing 20 mM Tris-HCl pH 7.5, 0.2 mM DTT, 5 mM $MgCl_2$, 5 μg/ml Anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, 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 hr. Luminescence was analyzed by the AlphaScreen detection program.

### *Detection of Ubiquitinated E2 by Immunoblot Analysis*

Crude biotinylated recombinant E2 proteins (40 μl) were used for the ubiquitin-conjugating assay in a total reaction volume of 50 μl containing 20 mM Tris-HCl pH 7.5, 0.2 mM DTT, 5 mM $MgCl_2$, 3 mM ATP and 4 μM FLAG-tagged ubiquitin (Sigma) for 3 hr at 30°C. The reaction products were purified by Streptavidin Magnesphere Paramagnetics particles (Promega, Madison, WI, USA). After washing the beads with PBS buffer, recombinant E2s were boiled in 15 μl of SDS sample buffer containing 50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 0.2% bromophenol blue, and then separated from the magnet beads. The proteins were separated by SDS-PAGE and transferred to PVDF membrane (Millipore Bedford, MA, USA) according to standard procedures. The blots were detected by the ECL plus detection system (GE Healthcare) with anti-FLAG antibody (Sigma) according to the manufacturer's procedure.

### *Detection of Polyubiquitination by the Immunoblot Analysis*

For polyubiquitination of HECT-type E3 ligases, crude FLAG-tagged UPL recombinant protein (20 μl) was ubiq-

uitinated in a total reaction volume of 50 μl consisting of 20 mM Tris-HCl pH 7.5, 0.2 mM DTT, 5 mM $MgCl_2$, 3 mM ATP, 4 μM biotinylated ubiquitin and 20 μl of crude recombinant AtUBC8 for 3 hr at 30°C. Then, recombinant UPL protein was gathered by anti-FLAG M2 agarose (Sigma). After washing the agarose with PBS buffer, the recombinant UPL protein was boiled in 15 μl of SDS sample buffer and then separated from beads by centrifugation. For polyubiquitination of RING-type E3 ligases, the assay was carried out in 10 μl of reaction mixture containing 20 mM Tris-HCl pH 7.5, 0.2 mM DTT, 5 mM $MgCl_2$, 10 μM zinc acetate, 3 mM ATP, 1 mg/ml BSA, 4 μM FLAG- or His-tagged ubiquitin, 1 μl of purified or crude recombinant E2 and 1 μl of purified or crude recombinant E3 at 30°C for 3 hr. Then, 5 μl of three-fold concentrated SDS sample buffer was added to the reaction mixture and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to Hybond-LFP PVDF membrane (GE Healthcare) according to standard procedures. Immunoblot analysis was carried out with anti-FLAG antibody (Sigma) or anti-His antibody (GE Healthcare) according to the procedure described above. When detecting biotinylated ubiquitin, blots were treated with 5 μg/ml Alexa488-conjugated streptavidin (Invitrogen) in PBS buffer. After washing with PBS containing 0.1% Tween-20, the blot was analyzed by a Typhoon Imager (GE Healthcare) using the 532 nm laser and 526 emission filters.

### *Polyubiquitination Assay with 26S Proteasome Inhibitor*

Polyubiquitination reaction was carried out as same procedure described above except addition of MG132 (Calbiochem, San Diego, CA, USA) at a final concentration of 20 μM to reaction mixture. Then, the protein on blot was detected by immunoblot analysis with anti-FLAG antibody or Alexa488-conjugated streptavidin.

## Authors' contributions

HT conceived the study and performed the experiments, and contributed to writing the manuscript. MS and KS provided RAFL cDNA clones. AN conceived the study. YE conceived the study and supervised the work. TS conceived and designed the study, supervised the work and contributed to writing the manuscript.

## Additional material

**Additional file 1**

AGI code of Arabidopsis genes and primer sequences used in this study.

*AGI code of Arabidopsis genes and primer sequences used in this study.* Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2229-9-39-S1.xls]

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# Practical cell-free protein synthesis system using purified wheat embryos

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**Biochemical characterization of each gene product encoded in the genome is essential to understand how cells are regulated. The bottleneck has been and still is in how the gene products can be obtained. The wheat cell-free protein synthesis system we have developed is a powerful method for preparation of many different proteins at a time and also for preparation of large amounts of specific proteins for biochemical and structural analyses. Here, we show a method for preparation of the wheat embryo extract useful for the cell-free reactions, by which 5 ml of a high-activity extract is obtained in 4–5 d. We also describe the methods for small- and large-scale protein synthesis by hands-down operations with the use of mRNAs prepared by transcription of PCR products and pEU plasmids harboring the target cDNAs, which need 2–4 d excepting the time required for plasmid preparation.**

## INTRODUCTION

With much information on the genome and cDNA sequences of many different organisms, we can now obtain and deduce a lot of information about the gene products and their interactions with the aid of bioinformatics, genetics and cell biology. However, such pieces of information are in many cases a result of presumption, or an extrapolation from known facts. To understand specific biochemical phenomena at the molecular level, *in vitro* analyses of biochemically characterized samples are strictly required. Conventionally, biochemists had to purify their samples, i.e., proteins and other biomolecules, from living organisms. The recombinant expression technologies that emerged in the late twentieth century helped them greatly. However, although there is a lot of information and a lot of potential targets to be analyzed, the preexisting technologies do not meet the need to prepare sufficient sized samples of many different proteins.

Cell-free protein synthesis was developed in the middle of the last century. It entered the limelight as a method for the preparation of proteins when the continuous-flow cell-free method was developed by Spirin *et al.*[1] in 1988, in which fresh substrates were supplied into and byproducts were removed from the reaction chamber continuously during the reaction with the extract from *E. coli* or wheat embryos. Our group found that the instability that had been observed for the translation with wheat extracts prepared by conventional methods arose from intrinsic factors that catalytically inhibited translation[2], and developed a method to eliminate the catalysts. Here, we describe the method for preparation of the extract as well as the basic techniques for parallel preparation of many different proteins for functional analyses and those for a large-scale preparation that may be applicable to structural analyses and antigen preparation. These technologies are based essentially on the following three elemental technologies: a method for eliminating the contaminants from the endosperm in the wheat embryo extract, which keeps up the inherent robustness of the natural translation apparatus[2], the PCR-based high-throughput method for preparation of DNA templates (the 'split-primer PCR' method)[3] and a reaction format that fit to highly parallel operation (the 'bilayer' method)[4]. A more detailed history of the development of the wheat cell-free system has been reviewed elsewhere[5,6]. The most prominent advantage of the wheat cell-free method, as recognized by our group through the collaborations with many other groups, is the high quality of the produced proteins, particularly when cytosolic proteins from eukaryotic origins are produced[7] (see below in the 'Applications of the method' section).

The cell-free protein synthesis system from *E. coli* is also capable of both highly parallel protein production and mass production. The wheat system has an advantage over the *E. coli* system in the probability of producing human proteins in soluble forms as clearly demonstrated[7]. This is probably due to the eukaryotic nature of the wheat system. In addition, machines for production of proteins are commercially available for the wheat system, as below. On the other hand, the productivity per reaction time may be higher in the *E. coli* system than in the wheat system. In addition, the method for preparation of the cell extract at the laboratory level is less laborious in the *E. coli* system. As a result, the cost of the extract is lower in the *E. coli* system. Thus, if one wishes to produce polypeptides in insoluble forms, the *E. coli* system has a clear advantage both in the cost and the productivity. The *E. coli* system also has an advantage in the production of bacterial soluble proteins that can fold properly in bacterial cells, as the cost of the extract is lower. Although we do not have clear statistical data, it seems that the codon usage bias in the open reading frame (ORF) sequence to be translated affects the productivity much less severely in the wheat system[8].

The wheat cell-free protein synthesis system has been commercialized by CellFree Sciences (CFS). The products of CFS, including the wheat embryo extract (cat. nos. CFS-TRI-1240/1240H/1240G), have highly controlled qualities, and they come with detailed protocols when purchased. Thus, we start with a method for preparation of the extract, for those who are hesitant to purchase the extract. As the extract is stable at −80 °C for years, it is less convenient to prepare small amounts of the extract at a time. In addition, we have already described the small-scale method several times elsewhere[9,10]. Thus, we show here a large-scale method[11]. The extract prepared by this protocol fits to the CFS protocols. We then describe the 'bilayer' methods for small-scale high-throughput parallel protein synthesis in a microtiter plate and those for larger scale preparation, which are essentially the same as that in the CFS protocols and are most convenient at present. A diagram showing the procedure is in **Figure 1**.

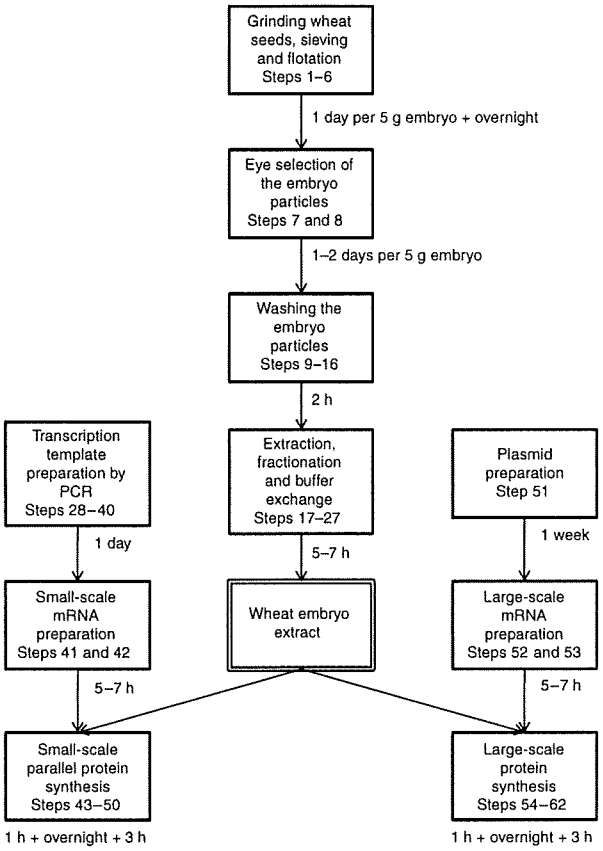


**Figure 1** | Summary of steps involved in the procedure.

## Experimental design

**Preparation of unwashed embryo particles.** The primary (unwashed) embryo particles are prepared by crushing wheat seeds with a mill followed by sieving and selection. Typically, 1 kg of wheat seeds gives around 1 g of crude embryo particles.

The most important step in the preparation of a good extract is to obtain an ensemble of good embryo particles that attach minimal amounts of endosperm to be washed out in the next step. This is possible at present only by selecting the particles apparent to human eye. This eye selection is the toughest step for laboratory workers. Thus, we use conventional methods to select the particles crudely by flotation before selection by eye. However, this raises the problem of organic-solvent waste containing carbon tetrachloride. Therefore, we now use the extract supplied by CFS for ordinary experiments. Nevertheless we describe here the method including the flotation selection step, expecting that the researchers who can use carbon tetrachloride in the laboratory may be able to perform the experiments themselves. The flotation steps may be omitted if more time and effort can be invested in the eye selection. We have not tested if other liquids could be used for the purpose, such as pure methylene chloride that has a density close to the one used in the present procedure and high-density aqueous solutions of polymers and/or salts.

**Preparation of the extract.** The embryo particles should be washed extensively before being crushed. This will eliminate translation inhibitors that come from endosperm. Conventionally, we crushed up to several grams of the washed embryo particles with mortar and pestle under liquid nitrogen in a cold room[2,9,10]. Here, we describe a patented method using a food processing mill/mixer, which has facilitated a larger-scale preparation of the extract with higher activities and has eliminated the risk of choking in the cold room associated with the use of liquid nitrogen[11]. While we used a popular food processor, a conventional Waring Blender can also be used. Although we usually start with 60 g of unwashed embryo particles, the amount of the starting material can be reduced down to several grams. The smallest amount that could be crushed properly is dependent on the size of the blender cup. A machine that can grind green tea may be useful for smaller-scale experiments, although we have not tested any. Once a large amount of the extract is prepared, it can be stored at −80 °C for at least a year.

Conventionally, we prepared the extract in the extraction buffer (EB, see below). However, we found that the buffered substrate mixture as below (BSS) is useful and more convenient than the EB, because preparation of the translation reaction mixture can be simpler, which is important for high-throughput applications.

**The translational activator sequences.** The mRNA molecules to be translated in the wheat cell-free system should have a translational enhancer sequence in the 5′-untranslated region (UTR)[3,12]. We have observed that the introduction of a cap structure at the 5′ end of mRNA molecules by a standard transcription method does not work well unless the mRNA concentration to be used for translation is optimized for each preparation. The 3′-UTR of the mRNA molecules should be at least as long as 500 nt, while no sequence preferences have been observed. Thus, 3′-UTR can contain the sequence from the vector in which the ORF to be translated is subcloned.

**Template preparation by PCR.** We describe a method for preparation of transcription-ready PCR fragments with a 5′-enhancer sequence and a long 3′ sequence[3], by which many different cDNA clones could be amplified in parallel. The PCR method comprises two steps. In the first PCR, a 5′ gene-specific primer and the AODA2306 primer (see **Table 1** and **Fig. 2a,b**) are used. The gene-specific primer should be designed for each gene to be expressed so that it hybridizes with the first 20 bases of the ORF to be amplified as in **Table 1**. It should introduce a short leader sequence. The

**TABLE 1** | Oligodeoxyribonucleotides.

| Name | Sequence | Convenient stock concentration (μM) |
|---|---|---|
| AODA2303 | 5′-GTCAGACCCCGTAGAAAAGA-3′ | 1 |
| AODA2306 | 5′-AGCGTCAGACCCCGTAGAAA-3′ | 0.1 |
| deSP6E01 | 5′-GGTGACACTATAGAACTCACCTAT CTCCCCAACACCTAATAACATTCAAT CACTCTTTCCACTAACCACCTATCTAC ATCACCACCCACCACCACCAATG-3′ | 0.1 |
| SPu | 5′-GCGTAGCATTTAGGTGACACT-3′ | 1 |
| Gene-specific primer | 5′-CCACCCACCACCACCAATGNNN NNNNNNNNNNNN-3′ | 0.1 |

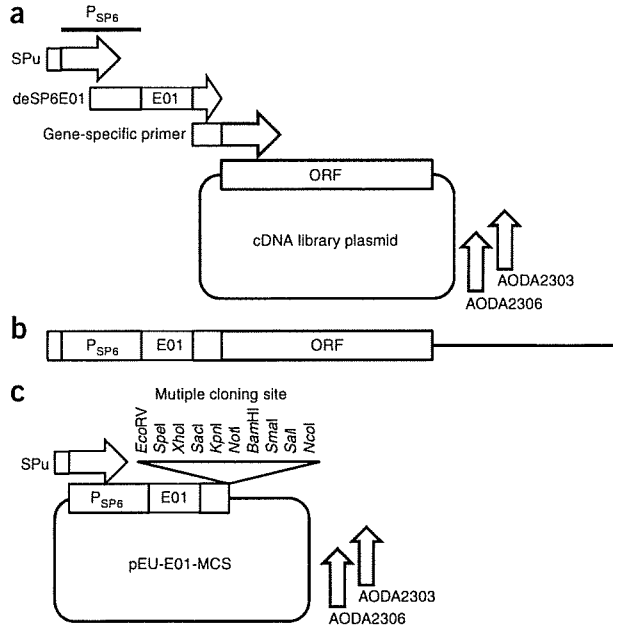


**Figure 2** | Schematic representations of DNA molecules. (**a**) Positions of the primers used for the two PCR amplifications are indicated with a schematic representation of a typical cDNA library plasmid clone. The two downstream primers hybridize near the plasmid origin, and thus the library vector should have the same sequence, and the ORF of the library clone should be inserted in this direction. The gene-specific primer and AODA2306 are used for the first PCR, and the other three primers are used for the second PCR. (**b**) An illustration of the transcription-ready template DNA that should be generated after the second PCR. (**c**) An illustration of pEU-E01-MCS, which is provided in the CFS kits and is useful for cloning of the cDNA sequence to be expressed in the wheat cell-free translation system.

AODA2306 primer is designed to hybridize within the replication origin region of the pUC plasmids, which is present in many cDNA library vectors. In the second PCR, AODA2303, deSP6E01 and SPu are used as primers. AODA2303 hybridizes three bases closer to the ORF than the position for AODA2306. deSP6E01 is a long oligonucleotide containing a partial SP6 promoter sequence lacking the 5′ five bases, the E01 sequence, and the leader sequence that is also in the 5′ region of the gene-specific primer. This primer is used in a lower concentration. The E01 5′ enhancer sequence used here can be substituted with the E02 sequence[12] or by the Ω sequence from tobacco mosaic virus. SPu contains the 5′ 14 bases of the SP6 promoter sequence in the 3′ part (the 3′ 9 bases overlaps with the 5′ region of deSP6E01). It is important to split the promoter sequence so that no primer has a complete promoter sequence because this dramatically reduces the possibility of generating nonspecific amplification of transcribable sequences. This 'split-primer' method is not required if the cDNA clones are inserted into pEU as described below.

**Small-scale parallel protein synthesis.** The PCR products can be transcribed into mRNA by a simple enzymatic reaction. The transcription products can be transferred directly into the translation mixtures. The bilayer method described here is suitable for parallel translation of many different mRNA samples, as it can be performed in microtiter plate wells and is much more efficient than a simple batch reaction. The reaction mixture containing the extract is slightly heavier than the substrate solution, and these two solutions can form a bilayer. Translation starts within a small space with concentrated initiation factors and ribosomes, forming polysomes. As the reaction proceeds, the byproducts are gradually diluted into the upper substrate solution, and the fresh substrates gradually diffuse into the reaction site. Thus, it is very important not to mix the two layers. Robots performing this procedure by parallel operations are available from CFS.

Conventionally, we adjusted the concentrations of the ingredients in the starting reaction mixture that should be layered under the substrate mixture to those of the components in the BSS substrate mixture. However, we have found that this is not necessary, probably because low-molecular-weight compounds will diffuse rapidly into the reaction mixture from the substrate mixture. In addition, it has been found to be unnecessary to remove the white insoluble material generated during the transcription reaction. The protocol shown here is thus quite simple: just mixing three solutions, including the extract, the transcription product and the creatine kinase solution. This simplicity has made this protocol more useful for high-throughput parallel production of many different proteins.

The concentrations of magnesium and potassium ions can affect the translation efficiency, whereas the transcription buffer contains a higher concentration of magnesium ions and no potassium ions, which may cause inefficient translation. However, we have observed no problem in the efficiency of translation. This may be because small ions can exchange rapidly between the reaction and substrate mixtures. It is also possible that the magnesium pyrophosphate precipitate may be serving as a buffer of magnesium concentration.

**The pEU expression vector.** The pEU vector contains an SP6 promoter, a translational enhancer and a multiple cloning site (**Fig. 2c**). This vector is suitable for large-scale expression of the subcloned ORF in the wheat cell-free system. Control pEU plasmids, such as that harboring the GFP cDNA, are available on request. Various pEU plasmids with inserted tags are also available. We are also ready to distribute a Gateway destination vector of pEU on request: various entry vectors for the human cDNA clones are also available from the National Institute of Technology and Evaluation, Japan[7].

**Large-scale protein synthesis.** We describe here the method for the bilayer mode large-scale protein synthesis with an mRNA solution prepared by direct transcription of the pEU plasmid template harboring the ORF sequence to be translated. For large-scale synthesis, more productive methods are available (see below). However, the present method is the simplest to be performed and thus fits with automation. The transcription template can also be prepared by PCR amplification of the plasmid sequence with the SPu and AODA2303 primers. We recommend the direct transcription method here just because the method is simpler than that including a PCR step.

**Control reactions that should be added.** pEU-E01-DHFR, which is available from CFS and from our laboratory, can be used for a positive control reaction for large-scale protein synthesis. For the small-scale experiment, an aliquot from the large-scale transcription product from pEU-E01-DHFR can be used. When only one sample is tested, the sample with no mRNA (water should be

added instead of the mRNA solution) may be used as a negative control, which will help to identify the band of the synthesized protein on the electrophoresis gel among the other bands arising from the components of the extract. If plural different mRNA samples are translated, we usually perform no negative control reaction because each band can be identified by comparison with the other lanes.

### Applications of the method

**Examples of expressed proteins.** We have already tested many different proteins for the productivity in the wheat cell-free system. An encompassing list of successful proteins would be so large that we could not show here. Lists of the successful examples at the time point of 2006 are elsewhere[5]. In such examples, the details of the methods for translation were different from the exact one presented here: as far as we know, success in production of a protein does not depend on the details and the reaction modes described below. Recently, 13,361 human cDNA clones were tested if they were translated in the wheat cell-free system by a bilayer protocol. It was found that 12,996 produced a detectable amount of the polypeptide and that 12,682 were detectable in the soluble fraction, of which 3,040 contain at least one predicted transmembrane domain[7]. Therefore, we believe that most cDNA sequences can be translated into the polypeptide sequences, unless the polypeptide interferes severely with the translation machinery. We have observed that many of the eukaryotic protein kinases can be produced in the active forms in the wheat translation system, while this group of proteins is recognized to be generally difficult to be produced in bacterial systems[5]. In particular, human calcium/calmodulin-dependent protein kinase II delta produced by this method readily phosphorylated novel natural substrates within a HeLa cell extract[13]. We have also produced plant RNA ligase, production of which has been reported to be difficult using bacterial expression systems[14]. It has been suggested that the wheat system has an advantage over bacterial systems in proper folding of multidomain proteins from eukaryotic origins, in particular those with a domain with a high β-strand contents[15]. This may be a result of coevolution of protein sequences and the protein synthesis apparatus, which might have been the origin of various protein functions in eukaryotic cells that confer the dazzling complexity of the eukaryotic organisms. On the other hand, some prokaryote-specific proteins might be produced in an inactive form in the wheat system: we have observed that a bacterial protein with a deep trefoil knot structure is not produced in its fully active form[16]. cDNAs with highly biased codon usages are also difficult to be expressed in bacterial systems. We have already expressed many *Plasmodium* cDNAs that have very high A/T contents successfully[8]. Proteases are generally difficult to be produced *in vitro* because of their activity. However, the wheat cell-free system sustains the translation activity even at 4 °C, and we have already confirmed that some proteases can be produced successfully (our unpublished data). Formation of proper quaternary structures has been observed for several proteins. The crystalline particles of polyhedrin were observed when its mRNA was translated[17]. A heterodimer enzyme was found to fold properly only when synthesized simultaneously in a reaction mixture containing the mRNA molecules for both subunits[18], whereas another heterodimer enzyme had its activity even when each subunit was synthesized separately and mixed with each other after purification[19]. There are only a limited number of examples of disulfide containing proteins and membrane proteins that were expressed efficiently as below. It is very difficult to introduce sugar modifications onto proteins, in part because the endoplasmic reticulum is absent from the extract and, in part, because the extract contains enzymes that degrade sugars.

**The PCR method, fusion proteins and high-throughput production.** The PCR method presented here can be modified in many ways. In fact, the templates for the human proteins above were amplified from *in vitro* recombinants produced in the Invitrogen Gateway system without transformation[7]. The templates for fusion proteins can be produced easily by PCR, and more than 500 different fusion proteins have been tested for their solubility and activities virtually by one person[15]. This was possible because proteins produced in the wheat system were generally quite stable. The stability is due to the lack of the 26*S* proteasome-dependent protein degradation activity[20]. It was also possible to manually produce many different proteins with different N-terminal sequences for a systematic analysis of the N-end rule in the wheat cell-free system[21]. N-terminal small tags, such as 6× His tag, can be fused to any protein by simply changing one primer used in the present PCR protocol. Larger tags such as glutathione-*S*-transferase can also be fused by including a small amount of the DNA fragment encoding the tag. These fusion technologies were successfully used for a high-throughput parallel assay of many different transcription factors encoded in cDNA library clones[22]. This type of rapid parallel assay and screening of many different proteins, we believe, will become very important for the post-genomic researches. A basic technology for construction of a protein chip with the proteins fused to a DNA-binding protein has also been developed[23]. The use of PCR for rapid template preparation was also shown to have a potential to accelerate protein engineering[24]. Most of these high-throughput applications have been performed according to older and more complicated protocols than the one that we show here. The present small-scale protein synthesis protocol can be applied to the parallel production of hundreds of different proteins just by performing it in parallel, using a multichannel pipette or the machine mentioned below.

**Reaction modes for the cell-free translation.** There are several reaction formats for translation. Although we present here the method for translation in the bilayer mode, the other formats are also possible with the extract prepared by the present procedure. Different reaction formats have been summarized elsewhere[9,10]. The batch mode translation, in which the reaction is performed in a homogeneous solution, is useful for testing the activity of the extract and mRNA preparations. For the batch mode synthesis, a fourfold concentrated solution of the substrates (4× BSS, see below) is useful for preparation of the starting mixture in 1× BSS with creatine kinase. In this case, the test can be more sensitive and quantitative if a radiolabeled amino acid is included in the reactions. The dialysis mode translation, in which the reaction is performed within a dialysis bag or a dialysis cartridge with continuous dialysis against the substrate solution, is generally more efficient than the bilayer method. The discontinuous batch (or 'repeat-batch') method[10], in which the buffer/substrate is forcibly exchanged during the translation reaction repeatedly, is very productive, although it needs a machine to be performed. A machine that performs the discontinuous batch mode translation reaction