Protein conjugation with ubiquitin, known as ubiquitination, is a key regulatory mechanism to control protein abundance, localization, and activity in eukaryotic cells. To identify ubiquitin-dependent regulatory steps in plants, we developed a robust affinity purification/identification system for ubiquitinated proteins. Using GST-tagged ubiquitin binding domains, we performed a large-scale affinity purification of ubiquitinated proteins from Arabidopsis cell suspension culture. High molecular weight ubiquitinated proteins were separated by SDS-PAGE, and the trypsin-digested samples were then analyzed by a multidimensional protein identification technology (MudPIT) system. A total of 294 proteins specifically bound by the GST-tagged ubiquitin binding domains were identified. From these we determined 85 ubiquitinated lysine residues in 56 proteins, confirming the enrichment of the target class of proteins. Our data provide the first view of the ubiquitinated proteome in plants. We also provide evidence that this technique can be broadly applied to the study of protein ubiquitination in diverse plant species. Molecular & Cellular Proteomics 6:601–610, 2007.

Ubiquitin (Ub) is a key regulatory peptide moiety that determines the fate of proteins in eukaryotic cells (1). The covalent attachment of a lysine 48-linked poly-Ub chain to the target protein typically leads to its subsequent recognition and degradation by the 26 S proteasome (1). Polyubiquitination and proteolysis of crucial cellular regulators are often triggered by distinct environmental and developmental cues to control their abundance. Protein ubiquitination is not restricted to proteasome-dependent degradation but also alters protein location. For example, covalent attachment of a single Ub (monoubiquitination), two Ubs (diubiquitination) (2), or a Lys-63-linked Ub chain (non-canonical ubiquitination) is involved in protein trafficking (3). Ubiquitination of many transcription factors is also required for their activation prior to degradation (4).

In plants, genetic analyses have identified many Ub-related genes that regulate various developmental pathways to fine tune homeostasis or that are required for responses to environmental stimuli (5). For instance, Ub ligases are involved in developmental regulations by plant hormones such as auxin (6–8), gibberellin (9, 10), abscisic acid (11), ethylene (12–14), and jasmonic acid (15). Ub/26 S proteasome-related genes are also involved in responses to pathogens (for a review, see Ref. 16), light (17, 18), and circadian rhythm (19). The importance of the Ub-related pathway is also reflected in plant genomes. It is estimated that the Arabidopsis genome encodes more than 1,400 (>5% of the total genome) genes in the Ub/26 S proteasome pathway. Most of these genes encode putative Ub ligases (5), a class of proteins that is also highly represented in the rice genome (20). However, only a few target proteins for these Ub ligases have been identified. Thus, identifying new target proteins that are ubiquitinated is important for many areas of plant biology.

Several large scale proteomics studies to identify targets for ubiquitination have been reported. In yeast, epitope-tagged Ubs were overexpressed, and ubiquitinated proteins were enriched by affinity purification (21–23). Because the purification was performed under fully denaturing conditions this method resulted in low cross-contamination with proteins that bind non-covalently to Ub or to ubiquitinated proteins (23). However, high expression levels of the recombinant protein were required to compete with the high endogenous levels of native Ub. Peng et al. (21) used a yeast strain in which all the native Ub genes had been previously deleted. Large scale immunoprecipitation of ubiquitinated proteins was also performed on proteins extracted from human cell culture using anti poly-Ub monoclonal antibodies (24). This method is quite versatile in that it does not require any genetic manipulation of the cells from which ubiquitinated proteins are enriched. However, the high cost of the antibodies might be limiting for some
laboratories. In another study, Weekes et al. (25) used the commercially available GST-S5a fusion protein (Biomol) to isolate ubiquitinated proteins from human heart tissue.

Proteins recognizing ubiquitinated proteins contain Ub binding domains (UBDs) of 20–150 amino acids that can interact directly with mono-Ub and/or poly-Ub chains. UBDs are present in enzymes that catalyze ubiquitination or deubiquitination or in Ub receptors that recognize Ub conjugants (26). Nine UBDs have been identified so far (26); all of them interact with the hydrophobic isoleucine 44 patch of Ub (27). The affinity of the interaction varies significantly between different domains and is higher for poly-Ub chains than for mono-Ub (27). Two of the UBDs, Ub-associating domain (UBA) and Ub-interacting motif (UIM) are found in a number of proteins. The affinity of the UBA domain toward poly-Ub was reported to be more than 3 orders of magnitude higher than that of UIM (27). The specificity of these UBDs to bind canonical and non-canonical Ub conjugations is less clear.

Here we describe a large scale method for identification of ubiquitinated proteins. Ubiquitinated proteins were enriched by affinity purification using GST-fused UBDs isolated from Arabidopsis. Using a refined LC-MS/MS-based multidimensional protein identification technology (MudPIT) analysis we identified 294 distinct proteins that bind specifically to these domains. From these, we identified 85 ubiquitinated lysine residues, confirming that the strategy enriched for ubiquitinated proteins. The proportions of these proteins in functional categories provide the first assessment of ubiquitination in plant cells.

**EXPERIMENTAL PROCEDURES**

Plant Material—An Arabidopsis (ecotype Landsberg erecta) cell suspension line (28) was used to extract proteins. Cells were grown in Murashige and Skoog medium containing 3% sucrose, 0.5 mg/liter naphthaleneacetic acid, and 0.05 mg/liter 6-benzylaminopurine under a 16-h light/8-h dark cycle. Proteins were extracted from 7-day-old Arabidopsis suspension cultures. When noted, MG132 (Biomol) was added to the cells 4 h before harvesting at a final concentration of 10 μM. Transient expression assays were performed using an Arabidopsis (ecotype Columbia) cell suspension line (29).

Antibodies, Ub Derivatives, and Immunoblotts—Anti-Ub immunoblot analyses were performed with the FK2 monoclonal antibody against ubiquitinated conjugates (Biomol, diluted 1:10,000) or a polyclonal anti-Ub antibody (Biomol, diluted 1:10,000) as primary antibodies and a horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Promega, diluted 1:10,000) as secondary antibody. Anti-HA immunoblots were hybridized with a peroxidase-conjugated anti-rabbit IgG (Promega, diluted 1:10,000) as secondary antibody. Anti-HA immunoblots were hybridized with a peroxidase-conjugated anti-rabbit IgG (Promega, diluted 1:10,000) as secondary antibody.

**Small Scale Purification of Ubiquitinated Proteins**—Either 100 ml of 7-day-old Arabidopsis cell suspension culture or 3–5 g of leaf tissue of 2-week-old Arabidopsis, tobacco, or barley were ground in liquid nitrogen using a mortar and pestle. Proteins were extracted using 10 ml of 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5% glycerol, and 1% Triton X-100 (TBST) supplemented with one tablet of Complete EDTA-free protease inhibitor mixture (Roche Applied Science), 10 mM iodoacetamide, 1 μg/ml DNase I, and 10 μg/ml RNase. Lysates were centrifuged twice at 6,550 × g for 10 min each at 4 °C and filtered through four layers of Miracloth (Calbiochem). Typically 10 μg of total protein were extracted, and protein extracts were mixed with a 0.1-ml packed volume of glutathione-Sepharose with bound GST, GST-UIM, or GST-ISO for 2 h at 4 °C in 15-ml Falcon tubes using a head-over-head rotator. Tubes were centrifuged for 20 s at 3,500 rpm, and the pellet was washed three times in 10 ml of ice-cold TBSGT. Proteins were eluted by boiling in 2× sample buffer, separated by 8% SDS-PAGE, and transferred to nitrocellulose membranes for immunoblot analysis.

**Large Scale Purification of Ubiquitinated Proteins**—This procedure was used for obtaining peptides for MudPIT analysis. Three liters of 7-day-old Arabidopsis cell suspension culture were collected by vacuum filtering over Miracloth and kept at −80 °C until use. Frozen cells were initially broken using a mortar and pestle and subsequently ground to a dry powder with a Waring blender. Broken cells were blended in 50 ml of 8 M urea for 1 min. The mixture was then diluted with 300 ml of TBSGT. Lysates were centrifuged twice at 6,550 × g for 10 min each at 4 °C and filtered through four layers of Miracloth. Lysates were then further diluted using TBSGT supplemented with three tablets of Complete EDTA-free protease inhibitor, 10 mM iodoacetamide, 1 μg/ml DNase I, and 10 μg/ml RNase A to a final urea concentration of 0.4 M. Typically 2–3 g of total protein were extracted, and protein extracts were mixed with a 5-ml packed volume of glutathione-Sepharose with bound GST, GST-UIM, or GST-ISO for 2 h at 4 °C in 15-ml Falcon tubes using a head-over-head rotator. Tubes were centrifuged for 20 s at 3,500 rpm, and the pellet was washed three times in 10 ml of ice-cold TBSGT. Proteins were eluted by boiling in 2× sample buffer, separated by 8% SDS-PAGE, and transferred to nitrocellulose membranes for immunoblot analysis.

**Detection System**—The protein bands were visualized using the ECL Plus Western blotting detection system (GE Healthcare). The concentration of protein was determined by the BCA protein assay (Pierce).
volume of 2× sample buffer for 5 min and loaded onto an 8% SDS gel without a stacking gel. Proteins were resolved at 80 V for 45 min and stained for 10 min in Coomassie Brilliant Blue staining buffer (65% H2O, 25% isopropanol, 10% glacial acetic acid, and 1 g/liter Coomassie Brilliant Blue). Gels were destained thoroughly in 65% H2O, 25% isopropanol, and 10% glacial acetic acid. The entire area above the GST fusion protein band was cut out for in-gel trypsin digestion. Gel slices were first chopped into 1-mm² cube pieces, pooled, and dehydrated with 30% acetonitrile in 50 mM triethyl ammonium bicarbonate for 15 min at room temperature. Proteins were reduced with 10 mM DTT for 35 min at 55 °C and alkylated with 50 mM iodoacetamide for 20 min at room temperature in the dark. Proteins were digested overnight at 37 °C with 2 μg of proteomics-grade trypsin (Sigma) for mass spectrometric analyses (see below). Each trypsin-treated sample was aliquoted and injected 2–10 times into the LC-MS/MS system for independent analyses.

**MudPIT**—Samples were analyzed by multidimensional chromatography coupled in line to nanoelectrospray ionization mass spectrometry on an LTQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) (30). Biphasic columns were packed in house into Self-Pack PicoFrit² columns (uncoated; 75–μm inner diameter; tip, 15 μm; New Objective, Woburn, MA) with 6 cm of reverse-phase resin (BioBasic, C18, 5 μm, 300 Å, Thermo Fisher Scientific) and 3 cm of polysulfoethyl A strong cation exchange (SCX) matrix (5 μm, 300 Å, Poly LC). Samples were initially loaded onto a microprecolumn cartridge (5 μm, 100 Å, LC Packing) at a flow rate of 20 μl/min and desalted for 3 min with buffer A (0.1% formic acid in HPLC-grade water). Samples were resolved from the peptide trap and loaded onto the SCX part of the biphasic column using a 40-min gradient of 0–40% buffer B (0.1% formic acid in HPLC-grade acetonitrile) followed by a 10-min gradient of 40–98% buffer B. The flow rate was adjusted to 250 nL/min with a splitter. Peptides were step-eluted from the SCX phase onto the reverse phase of the biphasic column using a 2-min salt pulses of 7.5, 15, 20, 25, 30, 35, and 40% buffer C (500 mM ammonium acetate, 5% acetonitrile, and 0.1% formic acid), respectively, or a 7-min gradient of 40–100% buffer C. Peptides were resolved from the reverse phase using a 60-min gradient of 2–25% buffer B followed by a 40-min gradient of 25–55% buffer B except for the last salt fraction where peptides were resolved from the reverse phase using a 55-min gradient of 2–60% buffer B for 10 min and desalted for 3 min with buffer A (0.1% formic acid in HPLC-grade water). Samples were resolved from the peptide trap and loaded onto the SCX part of the biphasic column using a 40-min gradient of 0–40% buffer B (0.1% formic acid in HPLC-grade acetonitrile) followed by a 10-min gradient of 40–98% buffer B. The flow rate was adjusted to 250 nL/min with a splitter. Peptides were step-eluted from the SCX phase onto the reverse phase of the biphasic column using a 2-min salt pulses of 7.5, 15, 20, 25, 30, 35, and 40% buffer C (500 mM ammonium acetate, 5% acetonitrile, and 0.1% formic acid), respectively, or a 7-min gradient of 40–100% buffer C. Peptides were resolved from the reverse phase using a 60-min gradient of 2–25% buffer B followed by a 40-min gradient of 25–55% buffer B except for the last salt fraction where peptides were resolved from the reverse phase using a 55-min gradient of 2–60% buffer B for 10 min and desalted for 3 min with buffer A (0.1% formic acid in HPLC-grade water). Samples were resolved from the peptide trap and loaded onto the SCX part of the biphasic column using a 40-min gradient of 0–40% buffer B (0.1% formic acid in HPLC-grade acetonitrile) followed by a 10-min gradient of 40–98% buffer B. The flow rate was adjusted to 250 nL/min with a splitter. Peptides were step-eluted from the SCX phase onto the reverse phase of the biphasic column using a 2-min salt pulses of 7.5, 15, 20, 25, 30, 35, and 40% buffer C (500 mM ammonium acetate, 5% acetonitrile, and 0.1% formic acid), respectively, or a 7-min gradient of 40–100% buffer C. Peptides were resolved from the reverse phase using a 60-min gradient of 2–25% buffer B followed by a 40-min gradient of 25–55% buffer B except for the last salt fraction where peptides were resolved from the reverse phase using a 55-min gradient of 2–60% buffer B followed by a 40-min gradient of 60–80% buffer B.

**Quality Control**—Fragmentation spectra acquired by Xcalibur 1.4 SRF (Thermo Electron Corp.) were searched against the translated open reading frames of the Arabidopsis genome database (Ath1.pep, downloaded from The Arabidopsis Information Resource at www.arabidopsis.org/, version available in April 2004) with Sequest (BioWorks version 3.2, Thermo Electron Corp.). Sequest parameters were: 1) peptide mass tolerance of 2.0 Da, 2) parent ion masses were treated as monoisotopic, 3) fragmentation ion masses were treated as averaged, 4) a 57.0-Da static modification on cysteines accounted for alkylation, 5) a 16.0-Da variable modification on methionine accounted for oxidation, and 6) a 114.1-Da variable modification on lysines accounted for ubiquitination. Sequest results were filtered with the following requirements for peptides and locus identifications to be considered valid. A minimum of two unique (different either in sequence or in post-translational modifications) valid peptides per locus is required, and at least one of the peptides must have a minimum Xcorr of 2.0, 2.5, and 4.0 for singly, doubly, and triply charged ions, respectively. A minimum Xcorr of 2.2 is then accepted for additional doubly charged peptides matching the same locus with a minimum ΔCn of 0.1 and no peptides in the control experiment where the bait protein was GST. Only peptides resulting from trypsin digests were considered, allowing for up to three miscleavages.

**Automatic Filtering of Results**—Two lists of peptides were assembled: a “background” list and a “sample” list. The background list included peptides identified by the pulldown performed with GST as the bait, and the sample included peptides identified by the pulldown performed by either ISO or UIM as the bait. We generated a list of proteins that have at least two matching peptides in the sample list but none in the background list (proteins matching at least two unique peptides). Assembly of this list was automated using a custom PERL script, which is supplied as Supplemental File 1. This script takes as input two comma-delimited text (CSV) files: a background file and a sample file.

**Immunoprecipitation**—A DNA fragment encoding a Golgi SNARE protein (At2g36900) was cloned into a modified version of the pBIN-61 binary vector (31) containing a strong viral promoter (35S), a 3×HA tag, and the 35S terminator. Plasmids were introduced into the GV3101 strain of Agrobacterium tumefaciens and expressed transiently in Arabidopsis cell suspension as described previously (32). Cells were harvested 48–72 h after transformation and ground in liquid nitrogen. Proteins were extracted in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, 50 μM MG132, 10 mM iodoacetamide, and one tablet of Complete protease inhibitor mixture (Roche Applied Science). Cell lysates were cleared by centrifuging twice at 3,500 rpm for 10 min each, and recombinant proteins were immunoprecipitated by the Anti-HA Affinity Matrix (Roche Applied Science). After a 2-h incubation, beads were washed four times in the extraction buffer and boiled for 5 min in SDS sample buffer. The proteins were separated by SDS-PAGE and blotted to a nitrocellulose membrane. Ubiquitinated proteins were visualized with anti-Ub antibodies.

**RESULTS**

For a large scale isolation of ubiquitinated proteins from Arabidopsis cell suspension (ecotype Landsberg), we created a Ub affinity purification system using two distinct Arabidopsis UBDs, UIM and UBA. We utilized a triple UIM domain from the S5a subunit of the proteasome (AIRPN10) and a double UBA domain from the deubiquitinating enzyme isopeptidase (AUBP14) (Fig. 1A). We designated these two domains UIM and ISO, respectively. We expressed these two domains in E. coli as GST fusion proteins and tested their binding specificity using pure forms of mono-Ub and either Lys-48- or Lys-63-conjugated chains (Fig. 1B). Mono-Ub was only pulled down using the ISO fusion protein, whereas the Lys-48- and the Lys-63-linked chains were pulled down by both fusion proteins.

A common problem encountered when enriching ubiquitinated proteins is the presence of highly active deubiquitinating enzymes (23). To inhibit the activity of deubiquitinating enzymes, we added 10 mM iodoacetamide to the extraction buffer. Omitting iodoacetamide resulted in the complete absence of ubiquitinated proteins in the pulldown experiments as detected by immunoblot analysis (data not shown). We also incubated the samples in 8 M urea because a previous report showed a significant decrease in recovery of noncovalent Ub interactions (24). The urea was then diluted to 0.4 M before addition of the Ub-binding fusion protein.

Using this protocol, we isolated ubiquitinated proteins from Arabidopsis suspension cell cultures with GST-UIM or GST-ISO. Because polyubiquitinated proteins are often short lived, we treated the cells with 10 μM MG132, a 26 S proteasome
inhibitor, for 4 h prior to extraction for comparison with extracts from control cells treated with an equal amount of DMSO. We also used GST-glutathione-Sepharose to identify proteins that bound to the column independently of the UBD. From these control experiments, we generated a background dataset from duplicate experiments with GST-glutathione-Sepharose using proteins extracted from MG132-treated cells or the DMSO control (Supplemental Table 1). Putatively ubiquitinated proteins were defined as those represented by at least two unique peptides in GST-ISO or GST-UIM interactions but not represented by a single peptide in the GST dataset. By these criteria, we identified a total of 294 unique proteins that selectively bound to the GST-UIM or GST-ISO system (Table I and Supplemental Table 2). Although many genuine ubiquitinated proteins may have been excluded from the results, we expect that these stringent criteria should greatly reduce the representation of false-positive identifications.

As expected, more unique peptides were identified in protein extracts derived from the MG132-treated cells. Treating with MG132 doubled the number of putatively ubiquitinated proteins identified using the GST-UIM bait. Using GST-ISO as bait, however, over 1,300 additional unique peptides after in vivo MG132 treatment were identified, but only 27 proteins were found with more than two unique peptides. The large number of single peptides from the GST-ISO experiments indicates a much greater sample complexity than that found with the GST-UIM. This may be due to a higher affinity of GST-ISO versus GST-UIM and/or broader substrate specificity of ISO versus UIM. It is also interesting to note that the protein sets from GST-ISO and GST-UIM have only a 14% overlap (Fig. 2). These results may suggest that ISO and UIM domains possess distinct binding specificities to ubiquitinated conjugates (see also below).

All the putative ubiquitinated proteins are listed in Supplemental Table 2. Most of the proteins were not previously identified as targets for ubiquitination. However, some proteins, or their close homologues in other organisms, are known to be ubiquitinated. For example, ubiquitinated forms of a sucrose synthase (33), an annexin (34), the inducible form of heat shock protein 70 (HSP72) (35), chaperonin CCT (36), and prohibitin (37) had been identified already. Thus our data provide a large set of novel ubiquitinated proteins. The predicted functions (based on The Arabidopsis Information Resource annotation) of these proteins are presented in Fig. 3. A large proportion is involved in metabolism (19%) and in protein translation (14%). This trend generally correlates with other large Ub proteomics studies in yeast (23) and in human cell culture (24).

Using MS/MS spectra we directly detected ubiquitination sites. Direct evidence for the ubiquitination of a protein is

![Fig. 1. Use of GST-UIM and GST-ISO for affinity purification of ubiquitinated proteins. A, schematic representation of GST-UIM and GST-ISO from AtRPN10 and AtUBP14, respectively. A domain containing three UIM motifs from AtRPN10 was fused to GST to create GST-UIM. A domain containing two UBA motifs from AtUBP14 was fused to GST to create GST-ISO. VWA, von Willebrand factor type A domain; ZnF UBP, zinc finger, UBP type. B, differential binding capacity of GST-UIM and GST-ISO. 1/100 g of either Ub, Lys-48-, or Lys-63-conjugated Ub chains were incubated with 100/100 g of GST, GST-ISO, or GST-UIM bound with glutathione-Sepharose for 2 h. Sepharose-bound proteins were precipitated by centrifugation, washed with TBSTG, and eluted by boiling in sample buffer. Proteins were separated by 12% SDS-PAGE and blotted onto a nitrocellulose membrane. Ubiquitinated proteins were detected with an anti-Ub polyclonal antibody and an anti-rabbit HRP-conjugated antibody. The experiment was repeated three times with similar results. aa, amino acids.](image-url)

**Table I**

| Affinity column | MG132 | Ubiquitination sites identified by MudPIT | Ubiquitination sites identified by at least two peptides | Ubiquitination sites identified |
|-----------------|-------|----------------------------------------|--------------------------------------------------------|-------------------------------|
| GST-ISO         | −     | 1853                                   | 75                                                     | 10                            |
| GST-ISO         | +     | 3166                                   | 102                                                    | 72                            |
| GST-UIM         | −     | 1131                                   | 54                                                     | 12                            |
| GST-UIM         | +     | 1489                                   | 109                                                    | 6                             |
| Non-overlapping total | NA    | 294                                    | 85                                                     |                               |

*a* includes only peptides with a minimum Xcorr of 2, 2.5, and 4 for +1, +2, and +3 charge, respectively. NA, not applicable.  
*b* indicates the number of proteins identified by at least two unique peptides and by no peptides in the GST control.
possible by identifying the lysine residue(s) that form(s) the isopeptide bond with the C terminus of Ub. Trypsin digestion of ubiquitinated proteins leaves two glycine residues at the conjugated lysine resulting in an increase of 114 Da on this residue. It is also known that trypsin is not able to digest peptides at the ubiquitinated lysine (21). Based on these two criteria, we identified 85 ubiquitination sites within 56 putative ubiquitinated proteins (Table II). We confirmed that one of the proteins listed, SNARE protein membrin 11 (MEMB11) (At2g36900) important for endoplasmic reticulum-Golgi trafficking (38), was indeed polyubiquitinated upon MG132 treatment (Fig. 4). We chose to validate the ubiquitination of this protein as its Xcorr scores are average and therefore representative. It was only identified by two unique doubly charged peptides of which one has a putative ubiquitination site but an Xcorr score of between 2.2 and 2.5 and the other has an Xcorr higher than 2.5. Based on the list we did not find any particular motif as a general ubiquitination site nor did we find particular patterns for differentiating UIM and ISO binding preferences. Nonetheless none of the ubiquitination sites found in this study, except for Lys-48 in Ub, was identified previously in plants. Indeed Ub itself was found to be ubiquitinated on five of its seven lysine residues. The order of relative abundance was Lys-48 > Lys-63 > Lys-11 > Lys-33 > Lys-29 (Table III) as assessed by the overall number of times each signature peptide was identified. Interestingly the GST-UIM column did not yield any Lys-33 conjugation, whereas 19 Lys-33 conjugates were found in GST-ISO. This result might reflect differential binding capacity of UIM and ISO to Ub conjugates.

Given the high sequence conservation of Ub, we investigated whether the UIM and ISO fusion proteins can also be used to enrich for ubiquitinated proteins from other plant species. We tested both fusion proteins using total protein extract from either Nicotiana benthamiana or barley plants. Both baits successfully enriched polyubiquitinated proteins from both plant species (Fig. 5), indicating that the GST-UIM and GST-ISO fusion proteins can enrich ubiquitinated proteins from a wide range of mono- and dicotyledonous plant species.

DISCUSSION

Here we describe a robust method for the large scale isolation and identification of ubiquitinated proteins from plants. We found that UIM and ISO, two different UBDs from Arabidopsis expressed in E. coli as GST fusion proteins, were able to enrich ubiquitinated proteins from complex extracts from Arabidopsis albeit with different apparent affinities toward subsets of proteins.

These studies report the first method for enriching ubiquitinated proteins from plants. Unlike similar methods, which have been reported in other organisms, our method does not require predeleting the native Ub genes (21) or high expression levels of a recombinant Ub gene (21–23). Two methods for identifying Ub proteins from human cells were published recently (24, 25). In common with our approach, these methods do not require any genetic manipulation of the cells used for protein extraction. However, these methods utilized expensive monoclonal antibodies (24) or GST-S5a fusion proteins (25) less suitable for a large scale purification.

To create a reliable list of putative ubiquitinated proteins, we used a stringent selection scheme. We first created a comprehensive dataset of proteins that bind to GST control, including proteins identified even by single peptides. We postulate that incorporating proteins identified by single peptides might result in including many proteins that were falsely identified in the GST dataset. In fact, had the GST dataset been filtered by the same criteria as the UBA/ISO datasets, more than 95% of the proteins currently present on the list would have been eliminated. We chose to have the GST dataset as comprehensive as possible to increase the stringency of our filter criteria even further and reduce the chances of proteins binding to the GST being falsely identified as putative ubiquitinated proteins.

Proteins present in the GST dataset were automatically removed from our list of putative ubiquitinated proteins. This
### Proteins with identified ubiquitination sites

| AGI number | Description | No. of sites | Peptide sequence | Source |
|------------|-------------|-------------|-----------------|--------|
| At1g03490.1 | No apical meristem (NAM) family protein | 1 | K.KEIK#DGHMQKGDLRL.A | GST-ISO |
| At1g04380.1 | Oxoglutarate-dependent dioxygenase | 1 | K.NSIKFNSQERK#SLEETK.V | GST-ISO |
| At1g10880.1 | Expressed protein | 3 | K.QK#IFTVK#ELNL.R.M, K.TENEIK#SMRKTER.I, | GST-ISO |
| At1g23410.1 | Ubiquitin | 5 | K. TL TGK#TITLEVESSTIDNVK.A, K.AK#ODKEGIPPPDQR.L, K.IQDK#EGIPPPDQR.L, R.LIFAGK#QLEDGR.T, R.TLADYNIQK#ESTLHVLRL.R | GST-UIM, GST-ISO |
| At1g30010.1 | Intron maturase, type II family protein | 1 | R.K#KAVGFCAVIR.S | GST-ISO |
| At1g32150.1 | bZIP transcription factor | 1 | K.RSK#GSLGSLMN*IIGKNNEAGK.N | GST-UIM |
| At1g54280.1 | Haloacid dehalogenase-like protein | 2 | K.ILNLLETSK#E.R, K.TSVGADRDEMLEK#VSDMMEK.E | GST-UIM |
| At1g55050.1 | Expressed protein | 1 | R.K#KAVGFCAVIR.S | GST-ISO |
| At1g55325.1 | Expressed protein | 1 | K.R#KLGLAK#SEDNR.L | GST-ISO |
| At1g58807.1 | Disease resistance protein | 1 | K.YALGIGGLSNRISK#VIR.D | GST-ISO |
| At1g62610.1 | Short-chain dehydrogenase/reductase | 1 | K.GGVDTMTRMA*MAIELAVYK#IR.V | GST-ISO |
| At1g68790.1 | Expressed protein | 2 | K.EK#DFAEMAKAVDK.I, R.LAQRREM*EEMOYEK#EALER.E | GST-ISO |
| At1g72050.1 | Zinc finger family protein | 1 | K.EKSGSGGCEK#ENEGNGGS.G | GST-ISO |
| At1g78010.1 | tRNA modification GTPase | 1 | R.EFTNDIVEK#IGVER.S | GST-UIM, GST-ISO |
| At2g15620.1 | Ferrodoxin-nitrite reductase | 1 | K.SM#EELDSEK#SKEK.D | GST-UIM |
| At2g24030.1 | Expressed protein | 1 | R.EK#EQRQIEIIAAEAK.R | GST-UIM |
| At2g28130.1 | Expressed protein | 1 | K.WTLNLILYSD#EKL.R | GST-UIM |
| At2g30560.1 | Glycine-rich protein | 1 | K.GGSUGCIGG#KGGGGGSGGR.G | GST-UIM |
| At2g31100.1 | Putative lipase | 1 | K.EKSGSSEKWK#DDLR.L | GST-ISO |
| At2g31580.1 | Expressed protein | 2 | K.K#ESELYK#RQSSK.I | GST-ISO |
| At2g35340.1 | RNA helicase, putative | 1 | R.DDIEDELYFQDEK#FALTER.E | GST-ISO |
| At2g36490.1 | HhH-GPD base excision DNA repair family protein (ROS1) | 1 | K.EK#SGM#N#AHKL.L | GST-ISO |
| At2g36570.1 | Leucine-rich repeat transmembrane protein kinase | 1 | R.LGTVSPLTNCK#NLRL.R | GST-ISO, GST-UIM |
| At2g36900.1 | Golgi SNARE protein membrin 11 (MEMB11) | 1 | K. M#EAL#ERADLGLR.A | GST-ISO |
| At2g40880.1 | Cytochrome P450 1A9, putative (CYP1A9) | 2 | R.K#YLYAVAFNIN#LAFI#K.R, K.K#QI#KLTG#NEFKI#LAFI#K.R | GST-ISO |
| At2g40955.1 | Hypothetical protein | 1 | K.PK#QRLAPK.S | GST-UIM |
| At2g43900.1 | Endonuclease/exonuclease/phosphatase family protein | 1 | K.TNKE#VRAVNNLDR.Y | GST-UIM |
| At3g01090.1 | Snf1-related protein kinase | 1 | R.SG#VFLYFYK#FLLR.T | GST-ISO |
| At3g03940.1 | Protein kinase family protein | 1 | R.LP#WQOGYQGDK#SFV#C#M.K | GST-ISO |
| At3g06030.1 | NPK1-related protein kinase | 2 | K.O#LSKSAK#EKTQGIR.H.E, K.O#LSP#SASK#EKTQGIR.H.E | GST-ISO |
| At3g18700.1 | Hypothetical protein | 3 | K.K#QI#GLK#NF#E#K.T, K.NIKT#NMI#KEK.A, | GST-ISO |
| At3g23150.1 | Ethylene receptor, putative (ETR2) | 1 | R.K#PV#LRA#M#ESEL.R.V | GST-ISO |
| At3g23810.1 | Adenosylhomocysteinase, putative | 1 | R.EK#V#L#Q#K#ADLGR.A | GST-ISO |
| At3g25270.1 | Hypothetical protein | 1 | K.GN#T#EYK#F#EYK#K#T#Q#E | GST-ISO |
| At4g07960.1 | Glycosyltransferase family | 2 | K.DEK#IK#H#Q#R#V#S#AP#E#A#E#K.K | GST-ISO |
| At4g10730.1 | Protein kinase family protein | 2 | R.VK#E#L#A#L#A#K#D#A#Q#L#A#L#A#K#L.G | GST-ISO |
| At4g11420.1 | Eukaryotic translation initiation factor 3 subunit 10 | 1 | K.PE#L#K#M#K#F#K#LY#L#C#L#V#L#D#K#F#K#R#G | GST-ISO |
| At4g15350.1 | Pyruvate, phosphate dikinase family protein | 1 | R.VFT#G#R#S#E#G#K#M.K.S | GST-ISO |
| At4g16820.1 | Lipase class 3 family protein | 2 | K.TN#E#H#L#R#N#L#V#L#R.T | GST-ISO |
| At4g16970.1 | Protein kinase family protein | 1 | R.LEEK#ND#L#P#S#Q#D#E#Y#Y.P | GST-ISO |
| At4g19530.1 | Disease resistance protein | 3 | K.LYQ#VK#AM#GK#F#N#L#W#E#V#M#G#G#R.G, K.E#M#G#L#T#G#F#G#P#E#K#L#K#F#K#T#E.E#K.N | GST-ISO |
| At4g23890.1 | Expressed protein | 1 | R.LSFL#K#D#T#E#T#S#R#K#Q#T#A.A | GST-ISO |
| At4g24170.1 | Kinesin motor family | 3 | K.LSFL#K#D#T#E#T#S#R#K#Q#T#A.A | GST-ISO |

**Ubiquitinated Proteome in Arabidopsis**

**Table II**

Proteins with identified ubiquitination sites.

#, ubiquitination; *, oxidation; bZIP, basic region/leucine zipper; HhH, helix-hairpin-helix; TIR, Toll/interleukin 1 receptor; NBS, NB site; AGI, Arabidopsis Gene Index.
rule was enforced even when a protein was identified by a higher number of peptides in the experimental pulldown than in the GST pulldown. The algorithm used for the automated screening is presented here as supplemental data (Supplemental File 1). It can be easily applied for other Arabidopsis proteomics studies. We removed proteins that were represented only as a single unique peptide even when these peptides were identified with high Xcorr values. The final list contains 294 proteins of which 56 proteins were identified with peptides with modified Lys residues. Although certain “genuine” ubiquitinated proteins may have been excluded from the results, we believe that using these stringent criteria greatly improved the signal-to-noise ratio of our data. Even with such high filtering stringency we could not exclude a possibility that some proteins identified as putative ubiquitinated proteins are in fact contaminating proteins that bind to GST. These may include several proteins that localize to the chloroplast or the mitochondria, two organelles that do not

![Fig. 4. Validation of ubiquitination of a selected protein. A putative ubiquitinated Golgi SNARE protein was expressed in Arabidopsis cell suspension culture as an HA fusion. A vector expressing an HA-tagged β-glucuronidase (GUS) was used as a control. When noted, Arabidopsis cell suspension cultures were treated with 10 μM MG132 for 4 h. Proteins were immunoprecipitated using anti-HA affinity matrix, and immunoblots were hybridized with either anti-HA antibodies (A) or anti-Ub monoclonal antibodies (B). Asterisks mark nonspecific bands caused by hybridization of the IgG and the HRP-conjugated anti-mouse antibody. The experiment was repeated twice with similar results.](image)

![Fig. 5. Use of the GST-UIM and GST-ISO to enrich for ubiquitinated proteins from different plant species. 10 mg of total soluble proteins were extracted from Arabidopsis, N. benthamiana, or barley leaves using the method described under “Experimental Procedures.” Ubiquitinated proteins were detected with the FK2 monoclonal antibody and an anti-mouse HRP-conjugated antibody. The experiment was repeated twice with similar results.](image)
Ubiquitinated Proteome in Arabidopsis

contain a proteasome. Final proof of a protein being ubiquitinated can only be obtained by immunoprecipitating a specific target and demonstrating that the protein cross-reacts with an anti-Ub antibody as shown on Fig. 4. It should also be noted that proteins were extracted from ecotype Landsberg, and Sequest analyses were conducted using the genome sequence of ecotype Colombia whose genome sequence is not identical to that of Landsberg. Thus it is possible that some ubiquitinated proteins from these experiments were not identified because of the sequence variation between ecotypes.

The specificities of the two domains were compared using pure forms of mono-Ub, Lys-48-conjugated chains, or Lys-63-conjugated chains. Our observation that mono-Ub could only be pulled down using the ISO fusion protein contrast with a previous report showing binding of mono-Ub to the UIM domain of the human proteasome subunit S5a (39). However, others have reported differential affinities between UIM-containing proteins toward various lengths of Ub chains (40). It is possible that these isolated domains bind differently from their native full-length proteins. We also found a relatively small overlap between proteins identified by the different Ub-binding bait proteins. This result may be attributed to differences in binding specificity of different ubiquitinated proteins or to incomplete coverage of the proteins present in the samples by the mass spectrometer.

Ubiquitination is a highly dynamic process and is balanced by deconjugation of Ub by deubiquitinating enzymes (DUBs). Briefly treating protein samples with 8 M urea and 10 mM iodoacetamide was found to be crucial for reducing the activity of DUBs (data not shown). DUB activity was probably not completely blocked by these chemicals as prolonged incubations of bait proteins with total protein extract resulted in reduction of the signal detected by anti-poly-Ub immunoblot analysis (data not shown). Optimal signal was detected after 1.5–2 h of incubation with significant reduction after 4 h.

The majority of identified proteins for which a function is predicted are involved in metabolism, translation, and RNA processing. In particular, we isolated a number of ribosomal subunits (At1g18540, At1g22780, At1g33120, At1g43170, At1g58380, At2g01250, At2g19730, At2g34480, At2g37190, At2g41840, At3g04230, At3g04840, At3g05590, At3g09630, At3g25520, At4g16720, At4g27090, At5g02870, and At5g20290). Although we did not identify any ubiquitination sites in three of the four R proteins, confirming that these proteins were indeed ubiquitinated (Table II). Another class of proteins identified contains two F-box-containing E3 Ub ligases (CO11: At2g39940 and At5g27750). E3 ligases are often self-ubiquitinated and are short lived (44). As the peptides we identified did not contain modified Lys residues, ubiquitination sites may be at other parts of the proteins.

The diversity of polyubiquitination sites implies a complicated regulation system governed by Ub. Ubiquitination using each lysine residue of Ub has been described previously in budding yeast (21). In other organisms, polyubiquitination on lysine 63 is known to be involved in DNA damage tolerance (45), the inflammatory response (46), protein trafficking (47), and ribosomal protein synthesis (48). Polyubiquitination on Lys-29 was implicated in protein degradation (49). The recent isolation of Arabidopsis UBC13 encoding an E2 Ub-conjugating enzyme, whose yeast homologue is known to be capable of forming Lys-63 poly-Ub chains, suggests that Lys-63 polyubiquitination also plays an important role in plants (50). To understand the biological significance of these non-canonical poly-Ub chain formations in plants, the Lys-63-linked proteins should be identified. Our method presented here will certainly facilitate further isolation of targets for such non-canonical ubiquitination.

The purification method presented here successfully enriched ubiquitinated proteins from not only Arabidopsis but also other dicot and monocot plants, proving the versatility of the system. Apart from cell suspension cultures, young seedlings are suitable materials as they easily take up MG132 and have been demonstrated to be useful for studies of ubiquitinated proteins (51). With the completion of the rice genome and the sequencing of additional plant genomes in progress, proteomics research in plants is likely to increase in the near future. Given that this method does not require genetic manipulation of the sample plants and that GST-ISO and GST-UIM columns can be produced in a large scale at relatively low cost, we anticipate that the present strategy will find utility in studying protein ubiquitination across plant science. Similar strategies can also be used for identifying proteins conjugated with ubiquitin-related molecules such as small ubiquitin-like modifier and NEDD. Combination of our strategy with recently developed quantitative proteomics methods such as ICAT (52) or isobaric tags for relative and absolute quantification (iTRAQ) (53) will be a powerful tool to identify target proteins conjugated with Ub-related molecules in response to specific stimuli.
REFERENCES

1. Glickman, M. H., and Ciechanover, A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428

2. Hicke, L. (2001) Protein regulation by monoubiquitin. Nat. Rev. Mol. Cell. Biol. 2, 195–203

3. Aguilar, R. C., and Wendland, B. (2003) Ubiquitin: not just for proteasomes anymore. Curr. Opin. Cell Biol. 15, 184–190

4. Conaway, R. C., Brower, C. S., and Conaway, J. W. (2002) Emerging roles of ubiquitin in transcription regulation. Science 296, 1254–1258

5. Smallle, J., and Vierstra, R. D. (2004) The ubiquitin 26S proteasome proteolytic pathway. Annu. Rev. Plant Biol. 55, 555–690

6. Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2003) The F-box protein TIR1 is an auxin receptor. Nature 435, 441–445

7. Kepinski, S., and Leyser, O. (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature 435, 446–451

8. Woodward, A. W., and Bartel, B. (2005) Auxin: regulation, action, and interaction. Ann. Bot. (Lond.) 95, 707–735

9. Thomas, S. G., Rieu, I., and Steber, C. M. (2005) Gibberellin metabolism and signaling. Vitam. Horm. 72, 289–338

10. Sun, T. P., and Gubler, F. (2004) Molecular mechanism of gibberellin signaling in plants. Annu. Rev. Plant Biol. 55, 197–223

11. Lopez-Molina, L., Mongrand, S., Kinoshita, N., and Chua, N. H. (2003) AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. Genes Dev. 17, 410–418

12. Poter, M. T., Lerner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., and Genschik, P. (2003) EIN3-dependent regulation of plant ethylene hormone signaling by two Arabidopsis F box proteins: EBF1 and EBF2. Cell 115, 679–689

13. Gagne, J. M., Smallle, J., Gingerich, D. J., Walker, J. M., Yoo, S. D., Yanagisawa, S., and Vierstra, R. D. (2004) Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proc. Natl. Acad. Sci. U. S. A. 101, 6803–6808

14. Guo, H., and Ecker, J. R. (2004) The ethylene signaling pathway: new insights. Curr. Opin. Plant Biol. 7, 40–49

15. Liechti, R., Gfeller, A., and Farmer, E. E. (2006) Jasmonate signaling pathway. Sci STKE 2006, cm2

16. Devoto, A., Muskett, P. R., and Shirasu, K. (2003) Role of ubiquitination in the regulation of plant defence against pathogens. Curr. Opin. Plant Biol. 6, 307–311

17. Sajio, Y., Sullivan, J., Wang, H., Yang, J., Shen, Y., Rubio, V., Ma, L., Hoecker, U., and Deng, X. (2003) The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HYS activity. Genes Dev. 17, 2642–2647

18. Sek, H., Yang, J., Ishikawa, M., Bolle, C., Ballesteros, M., and Chua, N. (2003) LAFT1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. Nature 424, 995–999

19. Han, L., Mason, M., Risseeuw, E. P., Crosby, W. L., and Somers, D. E. (2004) Formation of an SCF(ZTL) complex is required for proper regulation of circadian timing. Plant J. 40, 291–301

20. Goff, S. A., Ricke, D., Lan, T. H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeler, P., Varma, H., Hadley, D., Hutchison, D., Martin, C., Katagiri, F., Lange, B. M., Moughamer, T., Xia, Y., Budworth, P., Zhong, J., Miguel, T., Paszkowski, U., Zhang, S., Colbert, M., Sun, W. L., Chen, L., Cooper, B., Park, S., Wood, T. C., Mao, L., Quail, P., Wing, R., Dean, R., Yu, Y., Zarkikh, A., Shen, R., Saharabudde, S., Thomas, A., Carrings, R., Gutin, A., Pruss, D., Reid, J., Tavtigian, S., Mitchell, J., Eldredge, G., Scholl, T., Miller, R. M., Bhatnagar, S., Adey, N., Rubano, T., Tsunei, N., Robinson, R., Feldhaus, J., Macalma, T., Oliphant, A., and Briggs, S. (2002) A draft sequence of the rice genome (Oryza sativa L. ssp. japonica). Science 296, 92–100

21. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsichky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003) A proteomics approach to understanding protein ubiquitination. Nat. Biotechnol. 21, 921–926

22. Nakamura, T., and Deshaies, R. J. (2005) Two-step affinity purification of multimerized proteins from Saccharomyces cerevisiae. Methods Enzymol. 399, 385–392

23. Tagwerker, C., Flick, K., Cui, M., Guerrero, C., Dou, Y., Auer, B., Baldi, P., Huang, L., and Kaiser, P. (2006) A tandem affinity tag for two-step purification under fully denaturing conditions: application in ubiquitin profiling and protein complex identification combined with in vivo crosslinking. Mol. Cell. Proteomics 5, 737–748

24. Matsutani, M., Hatakeyama, S., Oyama, K., Oda, Y., Nishimura, T., and Nakayama, K. I. (2005) Large-scale analysis of the human ubiquitin-related proteome. Proteomics 5, 4145–4151

25. Weekes, J., Morrison, K., Mullen, A., Walt, R., Barton, P., and Dunn, M. J. (2003) Hyperubiquitination of proteins in dilated cardiomyopathy. Proteomics 3, 208–216

26. Hicke, L., Schubert, H. L., and Hill, C. P. (2005) Ubiquitin-binding domains. Nat. Rev. Mol. Cell. Biol. 6, 610–621

27. Haglund, K., and Dikic, I. (2005) Ubiquitylation and cell signaling. EMBO J. 24, 3353–3359

28. May, M. J., and Leaver, C. J. (1993) Oxidative stimulation of glutathione synthesis in Arabidopsis thaliana suspension cultures. Plant Physiol. 103, 621–627

29. Forreiter, G., Kirschner, M., and Nover, L. (1997) Stable transformation of an Arabidopsis cell suspension culture with firefly luciferase providing a cellular system for analysis of chaperone activity in vivo. Plant Cell 9, 2171–2181

30. Washburn, M. P., Wolters, D., and Yates, J. R., III (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. 19, 242–247

31. Bendahmane, A., Farnham, G., Moffett, P., and Baulcombe, D. C. (2002) Constitutive gain-of-function mutants in a nucleotide binding site-leucine rich repeat protein encoded at the Rx locus of potato. Proc. Natl. Acad. Sci. U. S. A. 101, 3051–3056

32. Dikic, I. (2003) Ubiquitin-dependent protein degradation in the proteasome. Cell 112, 711–722

33. Hardin, S. C., and Huber, S. C. (2004) Proteasome activity and the post-translational control of sucrose synthase stability in maize leaves. Plant Physiol. Biochem. 42, 197–208

34. Lauvrak, S. U., Hollas, H., Doskeland, A., Aukrust, I., Flatmark, T., and Vedeler, A. (2005) Ubiquitinated annexin A2 is enriched in the cytoskeleton fraction. FEBS Lett. 579, 203–206

35. Qian, S. B., McDonough, H., Boellmann, F., Cyr, D. M., and Patterson, C. (2004) A yeast model of CHIP-mediated stress recovery by sequential ubiquitination of CHIP-associated proteins. Mol. Cell 19, 195–204

36. Aoyama, K., Oyama, K., Oda, Y., and Nishimura, T. (2003) High-throughput protein localization in Arabidopsis using Agrobacterium-mediated transient expression of GFP-ORF fusions. Plant J. 41, 162–174

37. Hardin, S. C., and Huber, S. C. (2004) Proteasome activity and the post-translational control of sucrose synthase stability in maize leaves. Plant Physiol. Biochem. 42, 197–208

38. Kurokawa, K., Ito, M., and Kondo, K. (2003) Two new ubiquitin-proteasome inhibitors from the marine sponge Axinella sp. J. Biochem. 133, 553–559

39. Fujikawa, K., Tenno, T., Sugasawa, K., Lee, J. G., Okhi, I., Kojima, C., Tochio, H., Hiroaki, H., Hanaoka, F., and Shirakawa, M. (2004) Structure
of the ubiquitin-interacting motif of S5a bound to the ubiquitin-like do-
main of HR23B. J. Biol. Chem. 279, 4760–4767
40. Miller, S. L., Malotky, E., and O’Bryan, J. P. (2004) Analysis of the role of
ubiquitin-interacting motifs in ubiquitin binding and ubiquitylation. J. Biol.
Chem. 279, 33528–33537
41. Takahashi, A., Casais, C., Ichimura, K., and Shirasu, K. (2003) HSP90
interacts with RAR1 and SGT1 and is essential for RPS2-mediated
disease resistance in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. 100,
11777–11782
42. Hubert, D. A., Tomero, P., Belkhadir, Y., Krishna, P., Takahashi, A., Shirasu,
K., and Dangl, J. L. (2003) Cytosolic HSP90 associates with and modu-
lates the Arabidopsis RPM1 disease resistance protein. EMBO J. 22,
5679–5689
43. Schulze-Lefert, P. (2004) Plant immunity: the origami of receptor activation.
Curr. Biol. 14, R22–R24
44. Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K. I.
(2001) U box proteins as a new family of ubiquitin-protein ligases. J. Biol.
Chem. 276, 33111–33120
45. Ulrich, H. D. (2002) Degradation or maintenance: actions of the ubiquitin
system on eukaryotic chromatin. Eukaryot. Cell 1, 1–10
46. Sun, L., and Chen, Z. J. (2004) The novel functions of ubiquitination in
signaling. Curr. Opin. Cell Biol. 16, 119–126
47. Hicke, L., and Dunn, R. (2003) Regulation of membrane protein transport by
ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19,
141–172
48. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M., and Finley, D.
(2000) Cell cycle-regulated modification of the ribosome by a variant
mult ubiquitin chain. Cell 102, 67–76
49. Pickart, C. M., and Fushman, D. (2004) Polyubiquitin chains: polymeric
protein signals. Curr. Opin. Chem. Biol. 8, 610–616
50. Wen, R., Newton, L., Li, G., Wang, H., and Xiao, W. (2006) Arabidopsis
thaliana UBC13: implication of error-free DNA damage tolerance and
Lys63-linked polyubiquitylation in plants. Plant Mol. Biol. 61, 241–253
51. Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and
Coupland, G. (2004) Photoreceptor regulation of CONSTANS protein in
photoperiodic flowering. Science 303, 1003–1006
52. Blonder, J., Yu, L. R., Radeva, G., Chan, K. C., Lucas, D. A., Waybright, T.
J., Issaq, H. J., Sharom, F. J., and Veenstra, T. D. (2006) Combined
chemical and enzymatic stable isotope labeling for quantitative profiling
of detergent-insoluble membrane proteins isolated using Triton X-100
and Brij-96. J. Proteome Res. 5, 349–360
53. Jones, A. M., Bennett, M. H., Mansfield, J. W., and Grant, M. (2006)
Analysis of the defence phosphoproteome of Arabidopsis thaliana using
differential mass tagging. Proteomics 6, 4155–4165