The accumulation of significant levels of transgenic products in plant cells is required not only for crop improvement, but also for molecular pharming. However, knowledge about the fate of transgenic products and endogenous proteins in grain cells is lacking. Here, we utilized a quantitative mass spectrometry-based proteomic approach for comparative analysis of expression profiles of transgenic rice endosperm cells in response to expression of a recombinant pharmaceutical protein, human granulocyte-macrophage colony stimulation factor (hGM-CSF). This study provided the first available evidence concerning the fate of exogenous and endogenous proteins in grain cells. Among 1883 identified proteins with a false positive rate of 5%, 103 displayed significant changes ($p$-value < 0.05) between the transgenic and the wild-type endosperm cells. Notably, endogenous storage proteins and most carbohydrate metabolism-related proteins were down-regulated, while 26S proteasome-related proteins and chaperones were up-regulated in the transgenic rice endosperm. Furthermore, it was observed that expression of hGM-CSF induced endoplasmic reticulum stress and activated the ubiquitin/26S-proteasome pathway, which led to ubiquitination of this foreign gene product in the transgenic rice endosperm.

Keywords: Quantitative proteomics • Human granulocyte-macrophage colony stimulation factor • Transgenic rice endosperm • Storage protein • Endoplasmic reticulum stress • Ubiquitination

**Introduction**

Transgenic plants, seeds, and cultured plant cells are potentially the most economical systems for large-scale production of recombinant proteins for industrial and pharmaceutical uses. Along with the development of biopharming using cereal grain as a bioreactor, technical concerns regarding the fate of pharmaceutical recombinant proteins and endogenous proteins have generated research interest. In addition to regulatory concerns and the need to develop more efficient methods for downstream processing of recombinant proteins, there are at least other two important issues: (1) Can we engineer plant cells to manufacture amounts that allow cost-effective production? (2) Can we engineer plant cells to post-translationally modify recombinant proteins so that they are structurally and functionally similar to the native proteins? At present, only a few recombinant proteins expressed in plant cell are well-investigated, including phytase,\(^1,2\) human serum albumin,\(^2\) human lysozyme,\(^3\) the B subunit of *Escherichia coli* heat labile enterotoxin,\(^4\) human cytomegalovirus glycoprotein B,\(^5,6\) and recombinant antibodies.\(^7-10\) These studies provide useful information about the cellular localization and trafficking mechanism of recombinant proteins, but the fate of endogenous proteins has rarely been investigated. Batista et al. first reported a global fate of the transcriptome of transgenic rice expressing a recombinant antibody for cancer treatments.\(^11\) Recently, Van Droogenbroeck et al. reported that overproduction of recombinant scFv-Fc disturbs normal endoplasmic reticulum (ER) retention and protein-sorting mechanisms in the secretory pathway, with aberrant localization of the ER chaperone calreticulin and binding protein (BiP) and the endogenous seed storage protein cruciferin in the periplasmic space.\(^12\) However, information regarding the effects on the proteome of transgenic cells is lacking.

The development of systems biology offers new opportunities for appreciating the processes of gene transcription, mRNA stability, processing, translation initiation and efficiency, and protein sorting and trafficking. Whereas microarray technologies provide information about global gene expression with cells, complementary proteomics strategies monitor expression of proteins and their post-translational modifications. Proteomics has been successfully applied to areas as diverse as determining the protein composition of organelles, systematic elucidation of protein–protein interactions and the large-scale mapping of protein phosphorylation in response to stimulus. Relative quantification proteomics using higher sensitivity mass spectrometry techniques is becoming increasingly popular due...
to its high-throughput, reproducibility\textsuperscript{13} and sensitivity. Currently, an isobaric tag-based methodology for peptide relative quantification (iTRAQ) coupled with multidimensional liquid chromatography and tandem mass spectrometry enables the assessment of protein levels where 4 samples can be compared for their common effects.\textsuperscript{14}

Human Granulocyte-Macrophage Colony Stimulation Factor (hGM-CSF) is a pharmaceutical protein widely used in clinical trials of chemotherapy and radiotherapy in various cancers.\textsuperscript{15} Sardana et al. expressed recombinant hGM-CSF in tobacco and rice seeds, but obtained relatively low expression levels.\textsuperscript{16,17} We also found that the expression level of hGM-CSF was much lower than that of other proteins expressed in the same plant production system in our laboratory (unpublished data). This stimulated us to consider using a mass spectrometry-based proteomic approach for the analysis of transgenic rice endosperm in response to the expression of recombinant hGM-CSF.

In the study described below, we report the use of a 4-channel relative quantitation mass spectrometry technique to compare the protein expression profiles of the transgenic and the wild type rice endosperm. We identified different classes of proteins that influence the processing machinery of plant cells. For example, our results showed that the major endogenous storage proteins, glutelin, globulin and prolamin, and a majority of carbohydrate metabolism-related proteins were down-regulated, while 26S proteasome-related proteins and molecular chaperones were up-regulated in the transgenic rice endosperm. Distorted protein body morphology was observed in the transgenic endosperm. Interestingly, higher levels of protein ubiquitination were found in the transgenic endosperm cells, indicating that more proteins would be degraded via ubiquitin/26S proteasome pathway.

**Experimental Methods**

**Plant Material Preparation.** A homozygous transgenic rice line 7-24-3 expressing recombinant hGM-CSF in rice endosperm was used for this study.\textsuperscript{18} The T3 seeds of line 7-24-3 and seeds of nontransgenic rice variety TP309 were grown under natural conditions in the summer season in Wuhan, China. The immature endosperms at 10 days after pollination (DAP) and mature endosperms were harvested, ground with liquid nitrogen, and stored at $-80^\circ$C until use.

**Transmission Electron Microscopy.** For microscopic observation, immature endosperms were harvested at 10–14 DAP. The fixation, slice preparation and immunoelectronic microscopic observations were performed as described elsewhere.\textsuperscript{3}

**Western Blot Analysis.** Antibodies against rice storage protein glutelin and globulin were gifts from Dr. Ning Huang, Ventria Biosciences (Sacramento, CA). Ubiquitin and hGM-CSF antiserum were purchased from Bethyl Laboratories, Inc. (Montgomery, TA) and BioSource International, Inc. (Camarillo, CA), respectively. About 100 mg of immature endosperms and 100 mg of mature endosperms were homogenized using 400 $\mu$L of protein extraction buffer (66 mM Tris, pH 6.8, 2% SDS, 1 mM DTT) followed by centrifugation at 10 000g for 20 min. Protein concentration of the supernatant was determined using a DC Protein Assay Kit II (Bio-Rad, Hercules, CA). About 10 $\mu$g of protein in a 10 $\mu$L sample buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) was loaded onto the gel and separated by 12% polyacrylamide gel electrophoresis. Samples were then transferred to nitrocellulose membrane according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). All procedures were followed as described,\textsuperscript{3} except for the image development with 5-bromo-4-chloro-3‘-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride.

**Protein Extraction, Trypsin Digestion, and iTRAQ Isobaric Labeling.** Total protein was extracted from 400–500 mg of mature endosperm cell powder using extraction buffer (66 mM Tris-HCl, pH 6.8, 2% SDS, 2% $\beta$-mercaptoethanol) at room temperature followed by centrifugation for 20 min at 40 000g to remove the insoluble fraction. One milliliter of the resulting supernatant was mixed with the solutions in a 1:8:1 ratio in the following order of 1 mL of supernatant, 8 mL of 100% ice-cold acetone, 1 mL of 100% trichloroacetic acid (100% TCA) and 10 $\mu$L of $\beta$-mercaptoethanol. The precipitation was followed by centrifugation at 18 000g for 15 min at 4 $^\circ$C in a microcentrifuge. The supernatant was discarded and the protein pellet was washed with 1 mL of ice-cold acetone twice. The protein pellets were resuspended in 400 $\mu$L of sample preparation buffer (100 mM Tris-HCl, 8 M urea, 0.4% SDS, 5 mM tributylphosphine, pH 8.3) and placed on ice. Sample mixtures were sonicated with five 20-s pulses (50 W), with 1 min intervals on ice, followed by iodoacetamide alkylation at room temperature for 1 h. Protein concentrations were measured using a MicroBCA protein assay kit (Pierce, Rockford, IL) as per the manufacturer’s instructions before reactions were quenched by the addition of dithiothreitol. The protein mixtures were then diluted 8-fold in 50 mM Tris-HCl, pH 8.5. Modified trypsin (Sigma, Saint Louis, MO) was added to a final substrate-to-enzyme ratio of 30:1 and the trypsin digests were incubated overnight at 37 $^\circ$C. The peptides from each digest solution were acidified to pH 3.0 with formic acid (FA) and loaded onto a Discovery DSC-18 Cartridge (Sigma, Saint Louis, MO). The peptides were desalted (5 mL of 0.1% FA) and eluted with 3 mL of a solution composed of 50% acetonitrile with 0.1% FA.

Equal amounts (100 $\mu$g) of sample were labeled with 4-plex iTRAQ reagent (Applied Biosystems, Framingham, MA) according to the manufacturer’s instructions. Briefly, after desalting on a C18 cartridge, the peptide mixtures were lyophilized and resuspended in 30 $\mu$L of 0.5 M triethylammonium bicarbonate (TEAB), pH 8.5. The appropriate iTRAQ reagent (dissolved in 70 $\mu$L of ethanol) was added, allowed to react for 1 h at room temperature, and then quenched with 10 $\mu$L of 1 M Tris, pH 8.5. Replicates were used in the experiment to assess technical variation. The entire experiment (including the generation of cell pellets) was performed twice. The general workflow of the iTRAQ experiment is shown in Figure 1.

**Off-Line Strong Cation Exchange Chromatography.** iTRAQ labeled peptides were then concentrated, mixed, and acidified to a total volume of 2.0 mL, followed by injection into an Agilent 1100 HPLC system with a Zorbax 300-SCX column (4.6 i.d. $\times$ 250 mm) (Agilent, Waldbronn, Germany). Solvent A was 5 mM K$_2$HPO$_4$ and 25% acetonitrile (pH 3.0) and solvent B was 350 mM KCl in solvent A. Peptides were eluted from the column with a 40 min mobile phase gradient of solvent B. A total of 30 fractions were collected and samples were dried by a speed-vac prior to LC-MS/MS analysis.

**Online Nano-LC ESI QqTOF MS Analysis.** A nanobore LC system (Dionex, Sunnyvale, CA) which was interfaced to a QSTAR XL QqTOF mass spectrometer with a NanoSpray ion source (Applied Biosystems, Foster City, CA) was used for tandem mass spectrometry. The Magic C18 100 A pore 75 $\mu$m i.d. $\times$ 150 mm (Michrom Bioresources, Inc. Auburn, CA) was
packed in-house. Solvent A was 3% acetonitrile, 0.1% formic acid FA and 0.01% trifluoroacetic acid (TFA) and solvent B was 85% acetonitrile, 10% isopropanol, 0.1% FA and 0.01% TFA. Peptide mixtures (reconstituted in 125 μL of 5% FA) were injected and eluted from the column with a 90 min mobile phase solvent B gradient (5–11% B in 8 min, 11–13% B in 7 min, 13–14% B in 5 min, 14–36% B in 60 min, 36–92% B in 0.5 min, and 92% for 5 min) at a flow rate of 250 nL/min. The mass spectrometer was operated in an information dependent acquisition (IDA) mode whereby, following the interrogation of MS data (m/z 350–1500) using a 1 s survey scan, ions were selected for MS/MS analysis based on their intensity (>20 cpm) and charge state (+2, +3, and +4). A total of 3 product ion scans (2, 3, 3 s each) were set from each survey scan. Rolling collision energies were chosen automatically based on the m/z and charge-state of the selected precursor ions. The IDA Extensions II script was set to one repetition before dynamic exclusion.

**Data Analysis.** Data was processed by a “through” search against a TIGR Rice Genome Annotation Release 5 database (66,710 entries) using the Paragon algorithm within ProteinPilot v2.0.1 software with trypsin as the digest agent, cysteine alkylation, an ID focus of biological modifications and other default settings (Applied Biosystems, Framingham, MA). The software calculates a percent confidence which reflects the probability that the hit is a false positive, so that at 95% confidence level, there is a false positive identification rate of 5%. This software automatically accepts all peptides with a confidence of identification >1%, so only proteins which had at least one peptide with >95% confidence were initially recorded. However, the software may also support the presence of a protein identified using other peptides. Performing the search against a decoy (reversed) database allowed estimation of a false discovery level of 4%. Using the ProteinPilot 2.01 software, quantification was based upon the signature peak areas (m/z: 114, 115, 116, 117) and corrected according to the manufacturer’s instructions to account for isotopic overlap. Relative quantification ratios of the identified proteins were calculated, averaged, and corrected for any systematic errors in the labeling of the iTRAQ peptides. The accuracy of each protein ratio is given by a calculated ‘error factor’ in the software, and a p-value to assess whether the protein is significantly expressed. Error factor expresses the 95% uncertainty range (95% confidence error) for a reported ratio, where this 95% confidence error is the weighted standard deviation of the weighted average of log ratios multiplied by the Student’s t-factor for n – 1 degrees of freedom, where n is the number of peptides contributing to protein relative quantification. P-value is determined by calculating Student’s t-factor by dividing the Weighted Average of Log Ratios-log Bias by the Weighted Standard Deviation, allowing determination of the p-value, with n – 1 degrees of freedom, again where n is the number of peptides contributing to protein relative quantification. To be identified as being significantly differently expressed, a protein had to be quantified in at least three spectra (allowing generation of a p-value), have a p-value < 0.05, and have a ratio fold change >1.1 or <0.9 in both experimental replicates. These fold change limits were selected on the basis of our previous work with iTRAQ reagent.

**Co-Immunoprecipitation and Western Blotting.** Mature endosperm powder (200 mg) from the transgenic plants and the wild-type plants was, respectively, homogenized with 10 mL of RIPA buffer (66 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.01% NP40 v/v) supplemented with a protease inhibitor tablet (Sigma, Saint Louis, MO). The resulting mixture was clarified by centrifugation at 13 000g for 10 min. Protein concentration of the supernatant was determined using a DC Protein Assay Kit II (Bio-Rad, Hercules, CA). Ten microliters of ubiquitin antibody (Bethyl Laboratories, Inc., Montgomery, TX) was added to the supernatant or RIPA buffer (as an empty control). The mixture was rotated at 4 °C for 1 h. Fifty microliters of 50% Protein A (Sigma, Saint Louis, MO) was added to the supernatant or RIPA buffer and rotated again at 4 °C for 1 h.

The Co-IP complex was separated by microcentrifugation for 30 s at 10 000 rpm. The supernatant was discarded and the pellet was washed five times with 500 μL of PBS buffer in the presence of 3 μM MG132. Twenty microliters of 2x reducing sample buffer was added to the pellets. Western blotting was performed with hGM-CSF antibody (BioSource International, Inc., Camarillo, CA).

**Results**

**Recombinant hGM-CSF Expressed in Rice Endosperm Showed a Smear Pattern.** The expression level of recombinant hGM-CSF in the transgenic endosperm was 13.67 μg/seed, which is much lower than the level of other recombinant proteins using the same expression system in our laboratory (unpublished data). Western blot analysis was done to explore possible reasons for this. As shown in Figure 2, different expression patterns of hGM-CSF were observed in the immature endosperm compared to the mature endosperm of the transgenic plants. In the transgenic immature endosperm, hGM-CSF was present in three different forms. One form with the smallest size is similar to recombinant hGM-CSF derived from *E. coli*; the other two forms could be the products of post-translational modification (Figure 2, lane 3). However, we observed multiple bands with smear pattern ranging from about 18 to 120 kDa in the transgenic mature endosperm (Figure 2, lane 1). Interestingly, similar expression patterns were also observed by others who found that recombinant hGM-CSF ranged in size from 18 to 50 kDa when it was overexpressed in yeast, mammalian and plant cells. There are two potential N-glycosylation sites at Asn27 and Asn37 of hGM-CSF, and the molecular weight of unglycosylated hGM-CSF is 15–18 kDa. Okamoto et al. observed 16–35 kDa hGM-CSF expressed in human lymphoblastoid Namalwa cells. They assumed that the different molecular masses of hGM-CSF are
due to full, partial or no glycosylation at two potential glycosylation sites. Sardana et al. suggested that higher molecular mass versions of the recombinant hGM-CSF could be caused by glycosylation and dimerization in plant cells. Unfortunately, those proposed mechanisms could not explain the extremely high molecular weight of hGM-CSF variants from 50 to 120 kDa observed in this study. Possibly, the cause of this could be associated with our lower production and smear pattern of hGM-CSF in rice endosperm cells. With this in mind, we conducted proteomic analyses to elucidate the effect of overexpression of hGM-CSF in rice endosperm cells.

Proteomic Analysis of the Transgenic Endosperm Cells. To study the effects of overexpressed hGM-CSF on rice endosperm development, we analyzed the protein profile of the transgenic versus the wild-type endosperms at 10 DAP using iTRAQ labeling followed by LC-MS/MS analysis. A total of 1883 unique proteins (>95% confidence intervals) were identified in rice endosperm cell at 10 DAP. After two sets of comparative data (115 versus 114 and 117 versus 116) were processed, 117 proteins were present with \[p\text{-value} < 0.05\] and error factor < 2 (Supplementary Table 1 in Supporting Information). Among them, 7.69% were endogenous storage proteins, 5.13% were molecular chaperones, 6.84% were translation related; 2.56% were from ubiquitin/26S proteasome-related protein degradation pathways, 16.24% were related to carbohydrate metabolism and 61.54% were related to other miscellaneous pathways (Figure 3A). Overall, 103 proteins exhibited significant differences (ratio > 1.1 or < 0.9, \[p\text{-value} < 0.05\]) between the wild-type and the transgenic endosperm cells, composed of 46 up-regulated proteins (39.32%) (ratio > 1.1) and 57 down-regulated proteins (48.72%) (< 0.9). The molecular chaperones and proteins related to ubiquitin-proteasome degradation pathways were up-regulated, whereas the endogenous storage proteins and a majority of carbohydrate metabolism related proteins were down-regulated in the transgenic endosperm cells. Figure 3B shows an example of MS/MS spectrum of a peptide AAPSVADNLNP from a glutelin digestion mixture.

Endogenous Storage Proteins Remarkably Reduced in the Transgenic Endosperm. In rice endosperm, the major storage protein glutelin, which is encoded by a multigene family, accounts for 70–80% of the total protein. All glutelin proteins identified were expressed by eight of 12 known glutelin loci (Os02g15178.1, Os10g26000.1, Os02g16830.1, Os02g25640.1, Os03g31360.1, Os01g55690.1, Os02g15150.1, Os02g15070.1). Compared with the wild-type endosperm, the amounts of total glutelin were reduced 30.69%, ranging from 22.22% to 40.01% (Figure 4A). In addition to the glutelin, another storage protein, cupin (Os03g21790.1), was significantly decreased to 80.91% of the content of the wild-type (Figure 4A). The remarkable down-regulation of glutelin and cupin led us to trace changes in three other storage proteins: globulin, prolamin and albumin. We found that albumin levels were similar to wild-type, but the levels of globulin (Os03g61160.1, Os05g16970.1, Os03g57960.1, Os03g6100.3) were slightly decreased and prolamin (Os07g10570.1,
Os07g10580.1) were significantly decreased in two independent sets of experiments even though the reliability of the ratios did not meet the default significant criteria with the error factor <2 and p-value <0.05 (Figure 4B). To further confirm if these proteins were down-regulated in the transgenic endosperm, Western blot analysis of the two major storage protein glutelin and globulin was performed. In both the immature and the mature endosperm, the contents of glutelin and globulin were decreased in the transgenic endosperm (Figure 4C), which is consistent with our proteomic data.

ER Stress Found in the Transgenic Endosperm. Up-regulation of molecular chaperones such as heat shock proteins is in general related to the ER stress.29 The iTRAQ analysis showed that six molecular chaperones were up-regulated in the transgenic endosperm, including heat shock protein 81-3 (Os09g30439.1), luminal-binding protein 3 precursor (Os02g02410.1), stromal 70 kDa heat shock-related protein (Os12g14070.1), heat shock cognate 70 kDa protein 2 (Os11g47760.1), PDI-like protein (Os03g29240.1) and heat shock 70 kDa protein 4 (Os01g08560.2). The contents of these proteins were significantly augmented from 11.25% to 61.65% in the transgenic endosperm with an average of 31.03% compared to the wild-type endosperm (Figure 5A). Up-regulation of the chaperone proteins, especially the luminal binding protein,30 might suggest that the ER stress could occur in the transgenic endosperm. From our previous observation of morphologically different protein bodies in the transgenic rice endosperm overexpressing a human lysozyme,3 we postulated that morphological changes of the protein bodies could probably occur in the transgenic endosperm cells expressing hGM-CSF. To test this possibility, we used transmission electron microscopy to observe the morphology of the transgenic endosperm cells. As expected, the morphology of the protein bodies was remarkably distorted. A large number of nascent protein bodies with a smaller size were observed in the cordial area of the transgenic endosperm, but not in the wild-type endosperm (Figure 5B). Most of the protein bodies were surrounded by a clear halo-like structure (Figure 5B-a and B-c, arrow). Some were found to attach to the ER lumen to form structure similar to a multivesicular body (MVB)31 (Figure 5B-a and B-c, arrowhead). These structures were not observed in the wild-type endosperm.
subunit beta type 4 precursor (Os09g33986.2) and 26S protease regulatory subunit 4 (Os07g49150.1), were significantly up-regulated in the transgenic endosperm. Proteasome subunit beta type 2 and type 4, which belong to subunits of the 20S protease core particle, were up-regulated 61.28% and 16.82%, respectively. 26S protease regulatory subunit 4, which belongs to the 19S regulatory particle, was up-regulated 28.38% (Figure 6A). The up-regulation of those proteins suggested that more ubiquitin-dependent protein degradation events occur in the transgenic endosperm. To evaluate this possibility, we performed Western blot analysis with a plant specific anti-ubiquitin antibody. No obvious difference was observed at 10 DAP between the transgenic and the wild-type immature endosperm. However, obvious smear patterns were found in the mature transgenic endosperm but not in the wild-type (Figure 6B). To verify whether the unusual electrophoresis pattern of hGM-CSF resulted from ubiquitination, we carried out a co-immunoprecipitation assay. As shown in Figure 6C, proteins immunoprecipitated with ubiquitin contained hGM-CSF immunoreactivity, indicating hGM-CSF was indeed ubiquitinated. The molecular weight of ubiquitinated hGM-CSF ranged from about 50 kDa to more than 100 kDa, which fits with our previous observation. This evidence demonstrates that hGM-CSF was ubiquitinated in the transgenic endosperm, suggesting hGM-CSF could be degraded through the ubiquitin—proteasome pathway during the endosperm development. This phenomenon was not found in other transgenic rice endosperm expressing other pharmaceutical proteins (unpublished data). It does, however, explain why hGM-CSF exhibited a lower expression level than other recombinant proteins expressed in rice endosperm using the same system. This is the first report to reveal recombinant protein ubiquitination in the field of pharmaceutical protein production. This finding suggested to us that preventing the degradation of hGM-CSF through the ubiquitin/26S proteasome pathway could be an effective approach to improve the accumulation of hGM-CSF in the rice endosperm.

**Discussion**

**Overexpression of Recombinant Protein in Endosperm Could Promote Unfolded Protein Response (UPR).** A cellular unfolded protein response (UPR) will be elicited when defective proteins accumulate and aggregate in the ER. UPR induces several mechanism to rescue cells from the ER stress, including up-regulation of molecular chaperones to facilitate protein folding and an increase of the ER-associated degradation (ERAD) activity to remove unfolded proteins. In our study, these two mechanisms could clearly be traced. First, many molecular chaperones were up-regulated in transgenic endosperm cells; these are assumed to play a role in facilitating protein folding and preventing aggregation. This result was consistent with the report of Gasser and his co-worker, who discovered that several molecular chaperones were up-regulated in a yeast strain that overexpressed a recombinant protein compared with a wild-type strain. Similarly, the up-regulation of molecular chaperones was also observed when the ER suffered stress in transgenic plant cells. The second mechanism of increased ERAD activity was observed in transgenic endosperm cell. Under normal conditions, basal ERAD activity is sufficient to remove unfolded proteins. However, the UPR is required to increase ERAD activity when the ER is stressed. ERAD is an important response to alleviate the ER stress, so that unwanted proteins that are misfolded, unfolded, or

in the wild-type endosperm cells (Figure 5 B-b and B-d). Distortion of protein body II was less than that of protein body I. These results suggested that serious ER stress takes place in the transgenic endosperm cells.

**More Proteins Were Degraded by Ubiquitin—Proteasome Pathway in the Transgenic Endosperm.** The ubiquitin/26S proteasome complex consists of a 19S regulatory particle (RP) for directing ubiquitinated polypeptide into the proteasome lumen and a 20S protease core particle (CP) for breaking down the polypeptide in the lumen. Recognition and degradation of ubiquitinated substrates by the 26S proteasome is tightly regulated to maintain normal cell growth. From iTRAQ analysis, three proteins related to the 26S proteasome pathway, proteasome subunit beta type 2 (Os03g48930.1), proteasome...
metabolically unnecessary are recognized and returned to the cytosol by a process called retrotranslocation or dislocation, then degraded by the ubiquitin/26S proteasome pathway. In this process, a series of proteins including molecular chaperones, ubiquitin, components of the 26S proteasome and other cofactors are involved. The ER luminal chaperones, such as BiP, play important roles in facilitating protein folding as well as in the recognition of the ERAD substrates by recognizing the exposed hydrophobic patch of unfolded proteins. Polyubiquitin is covalently conjugated with the ERAD substrates and leads the substrate through the degradation pathway, while the 26S proteasomes break down the substrates. Our data showed the molecular chaperones and the components of 26S proteasome were up-regulated, and protein ubiquitination was enhanced, suggesting overexpression of hGM-CSF could promote the ERAD response in the transgenic rice endosperm. In addition, we hypothesized that the down-regulation of storage proteins (glutelin and globulin) was induced by the ubiquitin/26S-proteasome degradation pathway, but further study indicated that there was no ubiquitination of those proteins (data not shown), suggesting other mechanisms could be involved. Taken together, the observation that ubiquitinated proteins accumulate in the mature endosperms rather than immature endosperms suggests that the ubiquitin/26S proteasome-mediated ERAD pathway could be promoted at the later stages of endosperm development. Ning et al. reported that hGM-CSF was biologically active and soluble, suggesting that at least some hGM-CSF in endosperm could be properly folded. But the high level of polyubiquitination of hGM-CSF going through the ERAD pathway suggests that there was a great deal of unfolded or misfolded hGM-CSF accumulating in the ER. Mattanovich et al. have reviewed that different kinds of stress, including not only the ER stress, but also osmotic stress and pH stress, would be induced when overexpressing a recombinant protein in yeast. This report suggested that the ERAD and other changes in the transgenic endosperm cells are due to the cascades of overexpression of the foreign protein hGM-

![Figure 6. Comparison of protein expression of 26S proteasome and ubiquitination between the transgenic and the wild-type rice endosperm, and evidence of ubiquitination of hGM-CSF. Panel A shows the up-regulation of 26S proteasome proteins in the transgenic rice endosperm. The relative ratios are shown on the figure. The bars shown represent the mean ± SD. The upper image in panel B is a Western blot analysis with anti-ubiquitin displaying a high level of ubiquitination in mature endosperm cells. The lower image in panel B shows Coomassie Brilliant Blue (CBB) staining gel as a loading control corresponding to Western blotting. Lanes 1 and 3 represent protein extracts from mature and immature endosperm cells (10 DAP) of the transgenic plants, respectively; lanes 2 and 4 represent protein extracts from the wild-type mature and immature endosperm cells (10 DAP). Panel C shows ubiquitination of hGM-CSF. The cell lysates were subjected to immunoprecipitation (IP) with anti-ubiquitin and the IPs were analyzed by Western blotting for hGM-CSF and ubiquitinated hGM-CSF. The input sample is 5% of total protein used in each immunoprecipitation. The result shows that hGM-CSF was ubiquitinated and its size was shifted to a higher molecular weight range.](image-url)
CSF. However, there is still a lack of direct evidence to determine whether the unfolded or misfolded hGM-CSF was the original signal that triggered the ERAD pathway in the transgenic endosperm cells.

A Crowded and Disordered Environment of ER Affects Normal Protein Trafficking in Rice Endosperm. Goossens et al. and Tada et al. reported that repression of endogenous storage protein genes could improve exogenous gene expression in endosperm cells.47,48 In our study, the protein levels of endogenous storage proteins in the transgenic endosperm cell are remarkably decreased. Taken together, this suggests that the competition within the ER subdomains or translational machinery could occur between recombinant proteins and endogenous storage proteins. Overexpressing an exogenous protein in endosperm cells could cause a crowded environment on the ER surface and in the ER lumen that could activate intracellular signal transduction pathway and initiate signal integration in the ER for the unfolded protein response.49 Because storage protein mRNA is translated on the ER through specifically bound subdomains on the ER surface,50,51 the mRNAs of recombinant proteins and endogenous proteins could deplete the subdomains on the ER surface and the translational machineries of endogenous proteins. Furthermore, this crowded environment on the ER surface or in the ER lumen could promote and enhance the ER stress that induces up-regulation of a number of the molecular chaperones, disturbs the normal protein trafficking route, and subsequently affects the biogenesis of the protein bodies. Hunter et al. analyzed the transcriptomic profiles of several maize opaque endosperm mutations with altered protein bodies, discovering that the increased expression of genes associated with physiological stress and unfolded protein response are common features of protein body distortion and opaque mutants.52 Published data3 and our unpublished data on the protein body distortion in the transgenic endosperm cell indicate that normal vacuolar biogenesis is largely affected. However, the mechanisms affecting native protein trafficking routes and vacuolar biogenesis remain to be elucidated in the future.

Acknowledgment. We would like to thank Dr. Ning Huang for kindly providing the antibodies against the rice storage proteins, glutenin and globulin. This work is supported by the Project of the National “863” high technology Program in China (No. 2007AA100505); the Project of the National Natural Science Foundation of China (No. 30671286) and the Key grant Project of the Chinese Ministry of Education (No. 307018).

Supporting Information Available: Supplementary Table 1 contains proteins identified in the endosperm cells by iTRAQ analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(22) Ernst, J. F.; Mermod, J. I.; DeLamarter, J. F.; Mattaliano, R. J.; Moonen, P. O-Glycosylation and novel processing events during
Proteomic Analysis of Rice Endosperm Cells

research articles


