Hydrogen peroxide ($H_2O_2$) plays a dual role in plants as the toxic by-product of normal cell metabolism and as a regulatory molecule in stress perception and signal transduction. However, a clear inventory as to how this dual function is regulated in plants is far from complete. In particular, how plants maintain survival under oxidative stress via adjustments of the intercellular metabolic network and antioxidative system is largely unknown. To investigate the responses of rice seedlings to $H_2O_2$ stress, changes in protein expression were analyzed using a comparative proteomics approach. Treatments with different concentrations of $H_2O_2$ for 6 h on 12-day-old rice seedlings resulted in several stressful phenotypes such as rolling leaves, decreased photosynthetic and photorespiratory rates, and elevated $H_2O_2$ accumulation. Analysis of ~2000 protein spots on each two-dimensional electrophoresis gel revealed 144 differentially expressed proteins. Of them, 65 protein spots were up-regulated, and 79 were down-regulated under at least one of the $H_2O_2$ treatment concentrations. Furthermore 129 differentially expressed protein spots were identified by mass spectrometry to match 89 diverse protein species. These identified proteins are involved in different cellular responses and metabolic processes with obvious functional tendencies toward cell defense, redox homeostasis, signal transduction, protein synthesis and degradation, photosynthesis and photorepiration, and carbohydrate/energy metabolism, indicating a good correlation between oxidative stress-responsive proteins and leaf physiological changes. The abundance changes of these proteins, together with their putative functions and participation in physiological reactions, produce an oxidative stress-responsive network at the protein level in $H_2O_2$-treated rice seedling leaves. Such a protein network allows us to further understand the possible management strategy of cellular activities occurring in the $H_2O_2$-treated rice seedling leaves and provides new insights into oxidative stress responses in plants. Molecular & Cellular Proteomics 7:1469–1488, 2008.
to downstream signaling molecules such as Ca\(^{2+}\)-binding proteins, G proteins, and phosphatidic acid, which together modulate different developmental, metabolic, and defense pathways in plants (2, 5–8). Because the amount of H\(_2\)O\(_2\) required for this process is low, there would be no significant alteration of the overall redox state in the cells and thus no true oxidative stress. Given the dual function of H\(_2\)O\(_2\) as both toxic by-product and crucial regulator, it is clear that the steady-state level of H\(_2\)O\(_2\) as well as other ROS must be tightly controlled in plant cells.

Under steady-state conditions, the excessive H\(_2\)O\(_2\) is efficiently scavenged by various antioxidative defense mechanisms in plant cells. The major ROS-scavenging enzymes include ascorbate peroxidase (APx), catalase (CAT), superoxide dismutase, glutathione peroxidase, and peroxiredoxin (2). Together with the antioxidants ascorbic acid and glutathione, these enzymes provide plant cells with highly efficient machinery for detoxifying H\(_2\)O\(_2\) and other ROS (2). However, the equilibrium between production and scavenging of H\(_2\)O\(_2\) may be perturbed by various abiotic and biotic stresses, leading to a rapid and transient increase of the intracellular H\(_2\)O\(_2\) levels. Eventually plant tissues under such stresses often suffer from serious oxidative damages when the level of H\(_2\)O\(_2\) reaches the threshold value that triggers the disorder of physiological processes and metabolic pathways in plant cells. In plants, the prolonged period of H\(_2\)O\(_2\) generation that results in oxidative stress has been reported during external adverse stimuli such as chilling (9), drought (10), salinity (11), UV irradiation (12), ozone exposure (13), heavy metal (14), wounding (15), phytohormones including absicisic acid (6) and jasmonic acid (15), and elicitors and pathogen challenges (16). In almost all cases, H\(_2\)O\(_2\) seem to be positively used by plants to activate some stress-responsive genes that help them to cope with environmental changes.

Global gene expression profiling experiments have revealed that a large number of genes in Arabidopsis and tobacco plants are responsive to oxidative stress (17–19). More than 170 non-redundant ESTs are found to be regulated by H\(_2\)O\(_2\). Of these, 113 are induced and 62 are repressed under H\(_2\)O\(_2\) stress. A substantial proportion of these ESTs have predicted functions in cell rescue and defense processes. RNA blot analysis of 14 selected genes demonstrates that other stresses such as wilting, UV irradiation, and elicitor challenge also induce the expression of most of these genes, indicating that H\(_2\)O\(_2\) can mediate cross-tolerance toward other stresses (17). Furthermore another survey at the transcriptome level reveals that 349 transcripts are up-regulated and 88 are down-regulated by high light-induced H\(_2\)O\(_2\) in CAT-deficient Arabidopsis plants (18). In this experiment, H\(_2\)O\(_2\) is inferred to play a key role in regulating the transcriptions of two cluster genes that encode heat shock proteins (HSPs) and that participate in the anthocyanin regulatory and biosynthetic pathway, and several transcription factors and candidate regulatory genes responsive to H\(_2\)O\(_2\) stress are also identified. Likewise 713 ESTs are found to be regulated by high light-induced H\(_2\)O\(_2\) in CAT-deficient tobacco plants, and their transcriptional responses mimic those that have been reported during other abiotic and biotic stresses. Expression profiling corroborated by physiological experiments shows that a short term H\(_2\)O\(_2\) exposure of the CAT-deficient plants can trigger an increased tolerance against a subsequent severely oxidative stress, also indicating that the cross-tolerance is mediated by H\(_2\)O\(_2\) (19). Additionally by comparing the publically available transcriptome data sets of H\(_2\)O\(_2\)-stressed Arabidopsis, Mittler et al. (2) established a reactive oxygen gene network in plants. This network is composed of at least 152 genes involving ROS producing and scavenging such as APx, CAT, superoxide dismutase, glutathione peroxidase, peroxiredoxin, thioredoxins, glutaredoxin, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, alternative oxidase, NADPH oxidase, and ferritin and is involved in managing the level of ROS (2). Although large scale transcriptome analysis has documented the transcriptional dynamics of a large number of antioxidative genes, it should be essential to utilize proteomics and even metabolomics strategies to gain the system-level understanding of plant responses to H\(_2\)O\(_2\) stress.

In the present work, we initiated the first functional proteomics investigation of proteins that are responsive to elevated H\(_2\)O\(_2\) in rice. Using two-dimensional electrophoresis (2-DE) in combination with MS/MS analysis, we characterized proteins whose expression was altered upon exposure to different concentrations of H\(_2\)O\(_2\). Such an oxidative stress challenge resulted in a dramatic proteomic response involving at least 144 proteins. Identification of these proteins combined with their abundance changes as well as the impacts of oxidative stress on leaf photosynthesis reveals a close linkage between the changes in specific protein abundance and the overall defense response to oxidative stress, gives a global view of the ubiquitous cellular changes under oxidative stress, and demonstrates the first possibly intimate protein network elicited by H\(_2\)O\(_2\) in rice seedling leaves. These results presented in this study provide the framework for further functional studies of each member of this network in intracellular redox homeostasis and H\(_2\)O\(_2\) metabolism.

**EXPERIMENTAL PROCEDURES**

*Chemicals—* CHAPS, IPG DryStrip, IPG buffer, and iodoacetamide were purchased from GE Healthcare; thiourea and n-octyl glucopyrano side were from Sigma; and trypsin (MS Gold), urea, and acrylamide were from Promega (Madison, WI). Deionized water (Millipore, Bedford, MA) with resistance greater than 18 megaohms cm was used throughout.

*Plant Material and Stress Treatment—* Rice seeds (Oryza sativa L. cv. 93-11, whose genome was sequenced in Beijing Genomics Institute, China) were soaked in distilled water for 24 h and germinated in the dark for 45 h at 37 °C, and then the rice seedlings were grown in a growth chamber at 28/21 °C (16-h day/8-h night), photon flux density of 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), and relative humidity of 70%. To provide whole nutrition to rice seedlings, Hogland solution was sup-
plied every 2 days. 12-day-old seedlings were treated with three H\textsubscript{2}O\textsubscript{2} concentrations (0.6, 3.0, and 15.0 mM) for 6 h in three plastic containers, respectively. The middle portions of the second and third leaves were collected and frozen in liquid nitrogen and then stored at −80 °C for protein extraction. Seedlings immersed in double distilled H\textsubscript{2}O\textsubscript{2} were used as control.

Measurements of Photosynthesis and H\textsubscript{2}O\textsubscript{2} Level—Net photosynthetic rates, stomatal conductance, intercellular CO\textsubscript{2} concentration, and transpiration speed of the second and third rice leaves of each sample were measured using a LI-COR 6400 portable gas analysis system with an light-emitting diode light source (LI-COR Inc., Lincoln, NE). At least nine leaves for each sample were measured.

H\textsubscript{2}O\textsubscript{2} accumulated in treated or control leaves was measured according to Tiwari et al. (20) with modifications. Rice seedling leaves (1.0 g) were ground with a mortar and pestle in liquid nitrogen to a fine powder and added to a 10-ml cuvette containing 8 ml of double distilled H\textsubscript{2}O\textsubscript{2} and 2 ml of 25 mM sodium sulfate and then incubated for 1 h at room temperature. Oxidation of sodium sulfate was recorded by reading A\textsubscript{410}. Readings were converted to corresponding concentrations using a standard calibration plot.

Protein Extraction—Protein extraction was performed according to a method reported recently (21) with modifications. Rice leaves (0.5 g) were ground in liquid nitrogen to a fine powder, and the powder was suspended completely in 10 ml of 10% (w/v) TCA with 0.5% (w/v) DTT at −20 °C overnight. After centrifugation at 14,000 × g for 30 min at 4 °C, the pellets were washed three times with 10 ml of ice-cold acetone. The collected protein pellets were dried with N\textsubscript{2} to remove any remaining acetone. The dried powder was resuspended completely in 2 ml of lysis buffer (9 m urea, 4% (w/v) CHAPS, and 2% amphyolites, pH 3–10). After incubation at 25 °C for 1.5 h, the suspension was centrifuged at 16,000 × g for 30 min at 25 °C to remove the insoluble material. The resulting supernatant with predominantly soluble proteins was reduced by adding 5 mM Tris-(β-carboxyethyl)phosphine hydrochloride. The reduction continued for 1 h at room temperature. Samples were then alkylated by treatment with 16 mM iodoacetamide for 1.5 h at room temperature. This reaction was quenched by the addition of 50 mM DTT. The samples were immediately frozen in liquid nitrogen and then stored at −80 °C in aliquots. Protein contents were quantified according to Yao et al. (22) using BSA as a standard.

2-DE, Gel Staining, and Image Analysis—First-dimensional electrophoresis was performed on an IPGphor IEF system (GE Healthcare). The protein extract was diluted to a final concentration of 2500 μg/ml with an IEF rehydration solution (2% (w/v) CHAPS, 0.5% (w/v) IPG buffer, 2% thiourea, and 6% urea). After centrifugation at 10 min at 10,000 × g, the 450-μl supernatant was loaded onto a commercially available precast IPG strip with a 24-cm linear pH 4–7 gradient and actively rehydrated at 30 V for 12 h at 20 °C. Then focusing was performed on the IPGphor apparatus under the following conditions: 200 V for 40 min, 500 V for 40 min, 1000 V for 1 h, 4000 V for 2 h, and 8000 V for 8 h achieving −73,000 V-h. After the SDS-PAGE, the strips were equilibrated for 15 min in 10 ml of reducing equilibration buffer (6 m urea, 50 mM Tris-HEC at pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS, a trace of bromphenol blue, and 1% (w/v) DTT) and for another 15 min in alkylating equilibration buffer that contained 2.5% (w/v) iodoacetamide instead of 1% DTT. The strips were placed on the top of vertical 12.5% SDS-polyacrylamide self-cast gels. The electrophoresis was carried out at 25 °C and 2.5 watts/gel for 30 min and then at 17 watts/gel until the dye front reached about 1 mm from the bottom of the gel using an EttanTM DALT System (GE Healthcare).

Protein spots in 2-DE gels were detected by a modified colloidal CBB G-250 staining method with blue silver (23). The 2-DE gels were scanned using a UMAX PowerLook 2100XL scanner (UMAX Systems GmbH, Willich, Germany). At least triplicates were applied to each treatment, and a total of 12 CBB-stained 2-DE gels were analyzed using the ImageMaster™ 2-D platinum software version 5.0 (GE Healthcare). Spot detection parameters were set according to the manufacturer’s instructions as follows: sensitivity, 8000; operator size, 45; noise factor, 5; and background factor, 8000. The spots were quantified using the percent volume criterion. The match analysis was performed in an automatic mode, and further manual editing was performed to correct the mismatched and unmatched spots. The relative volume of each spot was assumed to represent its expression level. A criterion of p < 0.05 was used to define the significant difference when analyzing the parallel spots between groups with one-way analysis of variance and Student-Newman-Keuls test using the SAS software package version 8.2 (SAS Institute).

In-gel Digestion, MS Analysis, and Database Searching—Protein spots showing significant changes in abundance during the treatments were selected and excised manually for protein identification. In-gel digestion of protein spots was performed according to Yao et al. (22). All MALDI-TOF/TOF mass spectra were collected with an Ultraflex MALDI-TOF/TOF tandem mass spectrometer and analyzed by the peak list-generating FlexControl™ 2.2 software and the search engine BioTools 2.2 (Bruker Daltonics Inc., Bremen, Germany). The TOF spectra were recorded in the positive ion reflector mode with a mass range from 800 to 4000 Da. Ten subspectra with 30 shots per subspectrum were accumulated to generate one main TOF spectrum. After the search results were assessed automatically by the MASCOT software (Matrix Science, London, UK), the samples not identified by peptide mass fingerprinting (PMF) were automatically submitted to MS/MS analysis based on the potential lift technology. Two of the strongest peaks of the TOF spectra for each sample were chosen for MS/MS analysis.

All of the PMFs were searched in the MASCOT version 2.1 (Matrix Science) with the following criteria: National Center for Biotechnology Information non-redundant (NCBI) (release date September 4, 2006; including 3,658,925 protein entries and 1,257,151,991 residues); species restriction to O. sativa (rice) and Vitis vinifera (green plants) only when no proteins matched in rice; p ≤ 0.05; the coverage of protein by matched peptides was >15%; at least five independent peptides matched; at most two missed cleavage sites; MS tolerance was set as ±100 ppm and MS/MS tolerance was set as ±0.7 Da; fixed modification was carbamidomethyl (Cys); and variable modification was oxidation (Met); and cleavage by trypsin was the C-terminal side of Lys and Arg unless the next residue was Pro. If peptides were matched to multiple members of a protein family or a protein appeared under different names and accession numbers, one entry with the highest score was selected. When protein isoforms were observed, these entries were inspected manually, and the presence of each protein isoform was confirmed by the identification of at least two unique peptides. Additionally the theoretical values of molecular weight (M\textsubscript{r}) and pl of identified proteins were predicted by using the PeptideMass program (ExPasy).

RESULTS AND DISCUSSION

Morphological Changes and Physiological Responses Induced by H\textsubscript{2}O\textsubscript{2} Stress in Rice—The exposure of 12-day-old rice seedlings to exogenous H\textsubscript{2}O\textsubscript{2} results in dramatic changes in morphological and physiological traits. As presented in Fig. 1A, H\textsubscript{2}O\textsubscript{2} treatment with 0.6 mM concentration for 6 h caused the leaf margins to roll inward, whereas 3.0 and 15.0 mM treatments caused the leaves to become cylindrical as the leaf margins rolled inward over the upper surface to the extent that they became completely curled as the H\textsubscript{2}O\textsubscript{2} concentration was increased. To gain more understanding of the adverse
effects of different H$_2$O$_2$ levels, we determined the content of endogenous H$_2$O$_2$ and found increasing H$_2$O$_2$ levels in treated rice leaves (Fig. 1B). The levels of H$_2$O$_2$ in leaves treated with exogenous H$_2$O$_2$ of 0.6, 3.0, and 15.0 mM concentrations were increased by 55.5, 110.7, and 225.5% over control, respectively, thus showing a dose-dependent pattern of accumulation.

To evaluate the adverse effects of oxidative stress, we measured the photosynthetic responses of rice seedling leaves following the stress treatments. The net photosynthetic rate (Pn) declined significantly from 10.09 to 8.03, 5.89, and 3.75 µmol of CO$_2$ m$^{-2}$ s$^{-1}$ upon H$_2$O$_2$ treatments of 0.6, 3.0, and 15.0 mM, respectively (Fig. 1C). Similar patterns of rate decrease were also observed for the stomatal conductance (Gs), the intercellular CO$_2$ concentration (Ci), and the transpiration speed (Ts) (Fig. 1C), suggesting that H$_2$O$_2$ treatment resulted in stomatal closure, further reduced water transpiration, and exoteric CO$_2$ absorption in the treated rice seedling leaves. Similar results were observed in low temperature-treated rice seedlings (24) because adverse stress treatments are often linked to oxidative damage in plants (2, 3).

**Rice Leaf Proteome in Response to H$_2$O$_2$ Stress**—Using the modified TCA/acetone extraction protocol in combination with CBB staining, the average number of detectable spots in this study reached ~2000 on each 2-DE gel (Fig. 2A), sug-
gesting that we could take full advantage of the proteomics strategy to obtain more abundant information about the effects of oxidative stress on plants. Fig. 2B shows four reproducible gel maps in accordance with control and three different treatments. From a spot-to-spot comparison and statistical analysis, a total of 144 stained spots were found to have significant changes ($p < 0.05$). Of these, 65 spots (numbered from 001 to 065) were up-regulated, and 79 spots (numbered from 066 to 144) were down-regulated in response to H$_2$O$_2$ stress (Fig. 2A). Most of these spots had a greater than 1.5-fold change in abundance under at least one of the H$_2$O$_2$ treatments, and 30 of these spots exhibited a more than 2-fold change (Table I). Supplemental Fig. S1 shows examples representing the dynamics of different proteins under oxidative stress conditions. It is noteworthy that some spots (spots 061 and 064) were absent from gels for the control sample but were induced by H$_2$O$_2$ treatments on the basis of our comparison of proteome profiles.

Fig. 3 presents the number of differentially expressed spots under different H$_2$O$_2$ treatments and how these spots overlap using a Venn diagram analysis. Not surprisingly, there were large overlaps between these spots. Of the 65 up-regulated
| Spot no. | NCBI accession no. | Protein name | Average -fold change  |
|---------|--------------------|-------------|----------------------|
|         |                    |             | CF-1 | CF-2 | CF-3 | Score | M<sup>a</sup> | C<sup>c</sup> |
| Up-regulated protein spots | | | | | | | | |
| 001 | ABA95337 | NB-ARC domain-containing protein | ns<sup>d</sup> | 1.42 | ns | 84 | 22 | 23 |
| 002 | XP_490115 | Putative MLA1 | 1.62 | | 1.51 | 64 | 12 | 19 |
| 003 | AAM91872 | Putative nodulin | 1.40 | | ns | 81 | 15 | 35 |
| 004 | XP_470893 | Putative salt-induced protein | 1.90 | | ns | 111 | 11 | 79 |
| 005 | XP_482784 | Putative 12-oxophytodienoate reductase | 1.55 | 1.68 | | 70 | 12 | 22 |
| 006 | AAO72663 | Coproporphyrinogen-III oxidase | 2.18 | | ns | 72 | 13 | 44 |
| 007 | XP_470193 | Putative glutathione S-transferase | ns | 1.73 | | 74 | 10 | 37 |
| 008 | AAX95991 | Protein-disulfide isomerase | ns | 1.45 | | 116 | 19 | 37 |
| 009 | AAX85991 | Protein-disulfide isomerase | ns | 1.37 | | 74 | 12 | 25 |
| 010 | BAD28547 | Putative glyoxalase I | 2.11 | ns | 1.35 | 98 | 17 | 60 |
| 011 | BAD28547 | Putative glyoxalase I | 1.29 | 1.43 | | 103 | 17 | 61 |
| 012 | AAA61831 | Small GTP-binding protein | 2.54 | 1.76 | 1.79 | 63 | 9 | 51 |
| 013 | NP_916988 | G protein β subunit-like protein | 1.62 | 1.63 | 1.62 | 72 | 11 | 53 |
| 014 | AAF11798 | Putative tyrosine-phosphatase | 1.71 | 1.61 | | 90 | 13 | 45 |
| 015 | NP_911336 | Probable photosystem II oxygen-evolving complex protein 2 precursor | 1.44 | 1.36 | 1.37 | 110 | 16 | 59 |
| 016 | NP_911336 | Probable photosystem II oxygen-evolving complex protein 2 precursor | 1.44 | 1.36 | 1.37 | 110 | 16 | 59 |
| 017 | NP_911336 | Probable photosystem II oxygen-evolving complex protein 2 precursor | 1.44 | 1.36 | 1.37 | 110 | 16 | 59 |
| 018 | CAG34174 | Rubisco large subunit | 1.22 | 1.82 | 1.82 | 74 | 13 | 31 |
| 019 | CAG34174 | Rubisco large subunit | 1.44 | ns | ns | 75 | 11 | 27 |
| 020 | CAJ42306 | Rubisco large subunit | 2.56 | ns | ns | 82 | 8 | 58 |
| 021 | AAX95414 | Rubisco activase small isomorf precursor | 1.43 | ns | 1.24 | 61 | 8 | 35 |
| 022 | AAX95414 | Rubisco activase small isomorf precursor | 4.63 | ns | ns | 95 | 15 | 37 |
| 023 | AAS46127 | Rubisco large subunit | 2.08 | 1.54 | 1.64 | 215 | 36 | 47 |
| 024 | NP_052757 | Rubisco large subunit | 2.29 | 2.04 | 2.15 | 83 | 21 | 22 |
| 025 | AA022599 | Sedoheptulose-1,7-bisphosphatase precursor | 1.84 | ns | ns | 87 | 15 | 43 |
| 026 | BAD68170 | Thylakoid lumenal 20-kDa-like protein | ns | ns | 1.52 | 94 | 11 | 58 |
| Energy pathway | | | | | | | | |
| 027 | NP_914553 | Putative acid phosphatase | ns | 1.59 | 2.05 | 86 | 13 | 33 |
| 028 | ABB47307 | ATP synthase CF1, β subunit | 3.22 | 3.01 | 2.43 | 79 | 20 | 52 |
| 029 | ABB47307 | ATP synthase CF1, β subunit | 2.31 | 1.88 | 1.85 | 159 | 28 | 53 |
| 030 | YP_052756 | ATP synthase β subunit | 1.55 | 1.63 | 2.57 | 260 | 23 | 54 |
| 031 | BAD45853 | Putative vacular proton-ATPase | ns | ns | 1.61 | 255 | 41 | 58 |
| Lipid metabolism | | | | | | | | |
| 032 | XP_481639 | Putative enoyl-ACP reductase | ns | ns | 1.86 | 66 | 12 | 30 |
| Carbohydrate metabolism | | | | | | | | |
| 033 | AAT78793 | Putative ADP-glucose pyrophorylase | ns | ns | 1.68 | 69 | 15 | 32 |
| 036 | BAD02294 | Putative phosphoglycerate mutase | 1.60 | 1.51 | | 192 | 31 | 63 |
| 037 | BAB69069 | UDP-glucose pyrophorylase | 1.55 | ns | ns | 70 | 12 | 38 |
| Amino acid metabolism | | | | | | | | |
| 038 | ABA94348 | Cysteine synthase A | 1.55 | 1.78 | 1.67 | 166 | 20 | 81 |
| 039 | ABA94348 | Cysteine synthase A | 1.52 | ns | | 72 | 12 | 52 |
| 040 | XP_469854 | Putative dehydrogenase precursor | ns | ns | 1.53 | 63 | 12 | 43 |
| 041 | XP_467663 | Glutamine synthetase shoot isozyme | ns | ns | 1.49 | 103 | 16 | 56 |
| 042 | AAC05590 | S-Adenosyl-L-methionine synthetase | ns | 2.39 | 2.16 | 80 | 14 | 40 |
| Nucleotide metabolism | | | | | | | | |
| 043 | BAD82147 | Phosphoribulokinase/uridine kinase-like | 2.19 | ns | 1.72 | 193 | 27 | 59 |
| Protein biosynthesis | | | | | | | | |
| 044 | ABA91233 | Putative elongation factor P | 1.37 | ns | ns | 92 | 12 | 38 |
| 045 | S35701 | Translation elongation factor G | ns | ns | 1.45 | 120 | 24 | 38 |
| 046 | XP_466527 | Translation elongation factor Tu | 1.41 | ns | ns | 164 | 29 | 57 |

**H<sub>2</sub>O<sub>2</sub>-responsive Proteins in Rice Seedlings**

**Molecular & Cellular Proteomics 7.8**
| Spot no. | NCBI accession no. | Protein name | Average -fold changea | CF-1 | CF-2 | CF-3 | Score | Mb | Cc |
|---------|--------------------|--------------|-----------------------|------|------|------|-------|-----|-----|
| 066     | AAR26485           | Harpin-binding protein 1 | 1.96 | 1.36 | 1.72 | 81 | 10 | 43 |
| 070     | BAD29554           | Probable Photosystem II oxygen-evolving complex protein 1 precursor | 1.15 | 1.14 | 1.33 | 67 | 9 | 39 |
| 072     | XP_462936          | Putative chelatase subunit | 1.79 | 1.43 | 2.63 | 161 | 26 | 55 |
| 073     | AX39578            | Chlorophyll a/b-binding protein CP26 precursor | 1.64 | 1.72 | 2.27 | 68 | 11 | 43 |
| 074     | XP_464478          | Putative chlorophyll a/b-binding protein type III (PSI) precursor | ns | 2.70 | 2.27 | 62 | 7 | 23 |
| 075     | 2002393A           | Oxygen-evolving complex protein 1 | 1.15 | 1.14 | 1.33 | 67 | 9 | 39 |
| 076     | NP_911136          | Probable Photosystem II oxygen-evolving complex protein 2 precursor | ns | 1.82 | 1.52 | 77 | 8 | 40 |
| 077     | ABB47308           | Ribulose-1,5-bisphosphate carboxylase | -2.27 | -2.13 | -1.85 | 169 | 27 | 44 |
| 078     | CAG34174           | Rubisco large subunit | ns | -1.59 | -2.04 | 185 | 28 | 53 |
| 079     | CAG34174           | Rubisco large subunit | ns | ns | -1.67 | 186 | 31 | 51 |
| 080     | CAG34174           | Rubisco large subunit | ns | ns | -2.38 | 188 | 30 | 53 |
| 081     | CAG34174           | Rubisco large subunit | ns | ns | -1.59 | -1.47 | 185 | 28 | 51 |
| 082     | AAX95286           | Rubisco activase small isomor precursor | -1.30 | -1.19 | -1.14 | 76 | 16 | 32 |
| 083     | AAX95285           | Rubisco activase small isomor precursor | -1.19 | -1.16 | -1.39 | 82 | 20 | 45 |
| 084     | AAX95285           | Rubisco activase small isomor precursor | -1.20 | -1.16 | -1.35 | 134 | 29 | 56 |
| 085     | AAY64542           | Rubisco large subunit | ns | -1.35 | ns | 76 | 9 | 39 |
| 086     | ABA97087           | Rubisco subunit-binding protein a subunit | -1.43 | -1.21 | -1.19 | 135 | 22 | 46 |
| 087     | ABA97087           | Rubisco subunit-binding protein a subunit | -1.49 | ns | -1.30 | 159 | 25 | 47 |
| 088     | ABA97087           | Rubisco subunit-binding protein a subunit | -1.32 | -1.23 | -1.32 | 172 | 23 | 47 |
| 089     | NP_912361          | Fructose-1,6-bisphosphatase | -3.45 | ns | -1.89 | 111 | 17 | 43 |
| 090     | AAX95073           | Fructose-bisphosphate aldolase class-I | -1.47 | -1.47 | -1.30 | 86 | 14 | 38 |
| 091     | XP_467296          | Ribulose-5-phosphate kinase precursor | -1.27 | -1.64 | -1.59 | 128 | 18 | 53 |
| 092     | AAO22559           | Sedoheptulose-1,7-bisphosphatase precursor | -1.30 | -1.20 | -1.22 | 67 | 13 | 39 |
| 093     | AAO22559           | Sedoheptulose-1,7-bisphosphatase precursor | -1.70 | ns | -1.75 | 102 | 21 | 44 |
| 094     | AAA84588           | ATP β gene product | -1.11 | -1.37 | -1.37 | 213 | 33 | 67 |
| 095     | XP_467812          | Putative H⁺-transporting ATP synthase | ns | -1.52 | -1.85 | 67 | 6 | 23 |
| 096     | XP_479756          | Putative fructokinase | -1.45 | ns | -1.43 | 167 | 25 | 59 |
| 097     | XP_479756          | Putative fructokinase | -1.45 | -1.54 | -1.45 | 82 | 13 | 46 |
| 098     | XP_466458          | Putative UDP-glucosyltransferase | -1.54 | -1.30 | -1.54 | 73 | 13 | 37 |
| 099     | XP_477140          | Putative mRNA-binding protein precursor | ns | -1.56 | ns | 77 | 12 | 32 |
| 100     | CAD27458           | Nucleosome assembly protein 1-like protein 1 | -1.52 | ns | ns | 66 | 8 | 33 |
| 101     | AAL79738           | Putative polyprotein | -2.78 | -2.38 | -1.75 | 62 | 16 | 25 |
| 102     | ABA91072           | Putative retrotransposon protein | -1.39 | -1.85 | -1.47 | 64 | 24 | 17 |
H$_2$O$_2$-responsive Proteins in Rice Seedlings

| Spot no. | NCBI accession no. | Protein name | Average -fold change | Score | M$^a$ | C$^c$ |
|----------|-------------------|--------------|----------------------|-------|-------|-------|
|          |                   |              | CF-1 | CF-2 | CF-3 |       |       |
| 103      | BAA02152          | Eukaryotic initiation factor 4A | ns   | -1.61 | -1.72 | 80  | 15   | 39   |
| 105      | S35701            | Translation elongation factor G | -1.56 | -1.56 | -1.43 | 125 | 24   | 30   |
| 106      | XP_466527         | Translation elongation factor Tu | -1.96 | -1.92 | -1.67 | 165 | 24   | 53   |
| 107      | XP_483801         | Putative aminopeptidase N | -1.79 | ns   | ns   | 139 | 30   | 37   |
| 108      | XP_468533         | Oligopeptidase A-like | -1.41 | ns   | ns   | 239 | 35   | 53   |
| 109      | BAA02151          | 21-kDa polypeptide | -1.49 | -1.61 | -1.39 | 116 | 13   | 78   |
| 110      | BAD36074          | Putative chaperonin 21 precursor | -1.75 | ns   | -1.81 | 93  | 12   | 50   |
| 111      | NP_910308         | Putative chaperonin 60 β precursor | -1.22 | -1.32 | -1.39 | 214 | 29   | 53   |
| 112      | NP_910308         | Putative chaperonin 60 β precursor | -1.59 | -1.43 | -1.39 | 151 | 25   | 45   |
| 113      | NP_910308         | Putative chaperonin 60 β precursor | -1.85 | -1.82 | -1.54 | 89  | 16   | 30   |
| 114      | AAB63469          | Endosperm lumenal BiP | -1.52 | ns   | ns   | 114 | 20   | 28   |
| 115      | XP_470141         | Heat shock protein cognate 70 | -2.08 | -1.67 | -2.17 | 98  | 15   | 28   |
| 116      | CAAB82945         | 90-kDa heat shock protein | ns   | -1.70 | 84   | 20   | 27   |
| 117      | ABA97211          | Stromal 70-kDa heat shock-related protein | -1.54 | -1.23 | -1.43 | 79  | 23   | 27   |
| 118      | NP_921687         | Putative endoplasmic reticulum membrane fusion protein | ns   | -1.52 | -1.54 | 204 | 37   | 42   |
| 121      | XP_450482         | Putative plastid-specific ribosomal protein 2 precursor | -1.35 | -1.32 | -1.30 | 87  | 12   | 46   |
| 123      | AAT85778          | Hypothetical protein | -1.37 | ns   | ns   | 63  | 7    | 45   |
| 124      | NP_921715         | Unknown protein | -1.49 | -1.54 | -1.25 | 109 | 20   | 42   |
| 125      | NP_912350         | Unknown protein | -1.41 | -1.70 | ns   | 72  | 10   | 79   |
| 126      | NP_913279         | Unnamed protein product | -2.38 | -1.61 | -2.13 | 67  | 7    | 51   |

$^a$ Spot abundance is expressed as the ratio of intensities of up-regulated (plus value) or down-regulated (minus value) proteins between stress and control. 
$^b$ Fold changes had p values < 0.05. CF-1, CF-2, and CF-3 represent 0.6, 3.0, and 15.0 mM H$_2$O$_2$ treatment concentrations, respectively.
$^c$ Number of mass values matched.
$^d$ Sequence coverage.

Table I—continued

* ns indicates no significant change of spot abundance between stress and control.

spots, 17 spots were up-regulated under all three different treatments, and 15 spots showed increased abundance under any two H$_2$O$_2$ treatments (Fig. 3A). Among the 79 down-regulated spots, 38 spots were down-regulated under all three concentration treatments (Fig. 3B). Taken together, spots for more than 65% of differentially expressed proteins exhibited co-regulation under at least two H$_2$O$_2$ treatment concentrations. Additionally 50 spots showed up- or down-regulation under only one H$_2$O$_2$ treatment concentration (Fig. 3). Of these, 26 spots showed significant changes of protein abundance in response to 0.6 mM H$_2$O$_2$ (the lowest concentration), whereas 16 spots were found to be differentially expressed under 15.0 mM H$_2$O$_2$ (the highest concentration) (Table I). The former might represent a specific adaptation of rice seedlings to low H$_2$O$_2$ concentration, but the latter may reflect cellular damage in rice seedlings exposed to high H$_2$O$_2$ concentration. Therefore, these observations document the changes in abundance of these differentially expressed proteins in response to H$_2$O$_2$ stress and further imply that cells in rice seedling leaves are able to monitor the extent of oxidative stress through modulating the expression of the corresponding proteins.

![Figure 3](image-url)

**Fig. 3.** Venn diagram analysis of the differentially expressed protein spots in rice seedling leaves treated with 0.3, 0.6, and 1.5 mM H$_2$O$_2$. The numbers of differentially expressed protein spots with up- or down-regulation under a given concentration of H$_2$O$_2$ are shown in the different segments. A, the up-regulated protein spots; B, the down-regulated protein spots.

**Identification and Functional Classification of the Differentially Expressed Proteins**—A total of 144 differentially expressed protein spots were analyzed by MALDI-TOF/TOF MS. Of these, 111 spots were successfully identified by PMF as listed in Table I, and 18 spots were identified by MS/MS analysis (Table II). The results for spot 056 are presented in supplemental Fig. S2 as an example. Meanwhile all PMF images and annotated spectra by MS/MS analysis are shown in supplemental Figs. S3 and S4, respectively. Among the 129 identities, 124 (96%) have been deposited in the current da-
| Spot no. | NCBI accession no. | Protein name | Average -fold change<sup>a</sup> | Score | C<sup>b</sup> | Sequence | m/z | z<sup>c</sup> |
|----------|-------------------|--------------|----------------------------------|-------|-------|----------|-----|-------|
|          |                   |              | CF-1   | CF-2   | CF-3   |          |     |       |
| Up-regulated spots | | | | | | | | |
| Cell rescue/defense | 051 | XP_462957 | Putative hydrolase | ns<sup>d</sup> | ns | 1.41 | 47 | 3 | AVSAIVSCLLGPDR | 1457.88 | +1 |
| Redox homeostasis | 052 | AAC60202 | Putative peptide methionine sulfoxide reductase | 1.71 | ns | ns | 54 | 5 | FYPABEYHQR | 1339.65 | +1 |
| Photosynthesis | 053 | NP_916411 | Putative 1,4-benzoquinone reductase | 1.73 | 1.61 | 1.77 | 48 | 6 | VYVYYSMYGHVAK | 1694.96 | +1 |
| 054 | CAA44032 | Rubisco large subunit | 1.48 | ns | ns | 93 | 3 | TFQGPPHGIVER | 1465.88 | +1 |
| 055 | AAR23409 | Rubisco large subunit | 1.34 | 2.17 | 1.60 | 46 | 3 | TFQGPPHGIVER | 1465.83 | +1 |
| 056 | XP_493811 | Glyceraldehyde-3-phosphate dehydrogenase | ns | ns | 1.41 | 72 | 3 | VWAWEYNWYQQR | 1772.63 | +1 |
| Nucleotide metabolism | 057 | XP_477140 | Putative mRNA-binding protein precursor | ns | ns | 1.39 | 55 | 2 | DCEEWFFDR | 1303.55 | +1 |
| Protein biosynthesis | 058 | XP_478772 | Putative ribosome recycling factor | 1.23 | 1.26 | 1.65 | 78, 107 | 4, 7 | AIEIVQANNFNTVR, TIVAANLGVTSPSNGSIR | 1505.78, 1926.02 | +1, +1 |
| Protein degradation | 059 | NP_568427 | Endopeptidase CLPP2 | 1.41 | 1.50 | ns | 40 | 2 | AYDIFSR | 871.51 | +1 |
| Down-regulated spots | | | | | | | | |
| Signal transduction | 127 | XP_483800 | Putative C2 domain-containing protein | -1.37 | -1.27 | -1.35 | 87 | 5 | LPLRDVLDAGVGAR | 1566.94 | +1 |
| Photosynthesis | 128 | AAC62122 | Ribulose-1,5-bisphosphate carboxylase | -1.32 | -1.72 | -1.69 | 92 | 3 | TFQGPPHGIVER | 1465.83 | +1 |
| 129 | AAC28314 | Rubisco activase | -1.47 | ns | -1.21 | 97, 94 | 3, 4 | IVDSFPPQSIDFFGALR, DPIVTQGNDSTLYALPIR | 1868.98, 2089.19 | +1, +1 |
| 130 | AAB82133 | Glyceraldehyde-3-phosphate dehydrogenase subunit | ns | -2.13 | ns | 50 | 3 | VWAWEYNWYQQR | 1786.88 | +1 |
| Protein biosynthesis | 131 | O22386 | 50 S ribosomal protein L12 | -1.25 | -1.37 | -1.43 | 69 | 9 | TEFDVVIEVPSAR | 1677.88 | +1 |
| 132 | ABA96140 | Putative ribosomal protein S15 | ns | -1.21 | -1.43 | 90, 51 | 2, 1 | TFQGPPHGIVER, DTLIAAFR | 1465.75, 1021.50 | +1, +1 |
| Protein folding and assembly | 133 | AAO73241 | Putative ribosomal protein S5 | -1.52 | -1.28 | -1.47 | 43 | 5 | VMLRACPGSGQAVAGVR | 1868.07 | +1 |
| RNA processing | 134 | ABA97211 | Stromal 70-kDa heat shock-related protein | -1.28 | ns | -1.30 | 60 | 1 | QPAEEBADQVLR | 1461.91 | +1 |
|          | 135 | BAD46651 | Putative nucleic acid-binding protein | -1.54 | -1.47 | ns | 89 | 4 | IYVGNLPGQVDSSR | 1661.88 | +1 |

<sup>a</sup> Spot abundance is expressed as the ratio of intensities of up-regulated (plus value) or down-regulated (minus value) proteins between stress and control. -Fold changes had p values <0.05. CF-1, CF-2, and CF-3 represent 0.6, 3.0, and 15.0 mM H<sub>2</sub>O<sub>2</sub> treatment concentrations, respectively.

<sup>b</sup> Sequence coverage.

<sup>c</sup> Precursor charge.

<sup>d</sup> ns represents no significant change of spot abundance between stress and control.
BLASTP (NCBI) was used to search the homologues of the unknown proteins in Table I. The homologues with the highest homology are shown.

| Spot no. | NCBI accession no. | NCBI accession no. | Protein name | Organism | Identities | Positives |
|----------|---------------------|---------------------|--------------|----------|------------|-----------|
| 050 XP_463882 | CAL52837 | AAA⁺⁺-type ATPase | Ostreococcus tauri | 24 | 43 |
| 123 AAT85778 | AAB97805 | Retrotransposon protein, putative, Ty3-gypsy subclass | O. sativa | 68 | 72 |
| 124 NP_921715 | NP_201547 | Hydrolase, hydrolyzing O-glycosyl compounds | Arabidopsis thaliana | 29 | 42 |
| 125 NP_912350 | ZP_01202146 | 3-Oxoacyl-[acyl-carrier-protein] synthase III | Flavobacteria bacterium | 25 | 41 |
| 126 NP_913279 | BAA96146 | Putative heme-binding protein 2 | O. sativa | 100 | 100 |

a The accession number of the unknown proteins in Tables I and II.
b The accession number of the homologues.
c The extent to which two amino acid sequences are invariant.
d The similarities based on the scoring matrix used.

**FIG. 4. Functional classification and distribution of all 129 identified proteins.** Unknown proteins include those whose functions have not been described, but may be deduced based on analysis of sequence homology as listed in Table III.
of the seven RLSs (spots 018–020, 023, 024, 054, and 055) ranged from 19.14 to 30.86 kDa, much smaller than the theoretical 52.79 kDa. Similar results were found in chilled rice seedling leaves and were further confirmed by Western blot analysis (24). Additionally the enhanced partial degradation of photosynthetic proteins such as RLS, RASIP, GAPDH, and SBP (Table I) might result in impaired photosynthesis in rice seedlings (Fig. 1C). Similar findings were also observed in cold stress rice seedlings (24). Likewise a number of partially degraded products of intact proteins participating in the tricarboxylic acid cycle showed up-regulation in Arabidopsis and pea mitochondria under H₂O₂ and other abiotic stresses (25, 26), indicating that these adverse stresses impair aerobic respiration in Arabidopsis and pea plants. Thus, a range of adverse stresses impairs physiological processes and metabolic pathways in plants through the accelerative degradation of many key enzymes that may protect plants from further damage triggered by external adverse conditions.

On the other hand, our results also showed that 20 unipros appeared as 47 identities as marked in Fig. 5A. Interestingly 15 unipros representing 35 identities (isoforms) showed that each set of isoforms had the same up- or down-regulated change patterns in abundance in response to H₂O₂ stress, whereas the other five unipros (ATP synthase CF1, β subunit; RBC, ribulose 1,5-bisphosphate carboxylase; RSBPS, Rubisco subunit binding-protein α subunit; CSA, cysteine synthase A. Con, HT1, HT2, and HT3 represent different H₂O₂ treatments of 0, 0.6, 3.0, and 15.0 mM, respectively.

Fig. 5. Close-up of possible isoforms detected by 2-DE (A) and their expression profile patterns (B). All 47 differentially expressed protein spots matched to 20 unique proteins are shown. The numbered spots correspond to proteins listed in Tables I and II. 33kDaOECP, 33-kDa oxygen-evolving complex proteins of PS II; ATP-SCF1S, ATP synthase CF1, β subunit; IPP, inorganic pyrophosphatase; RBC, ribulose 1,5-bisphosphate carboxylase; RSBPS, Rubisco subunit binding-protein α subunit; CSA, cysteine synthase A. Con, HT1, HT2, and HT3 represent different H₂O₂ treatments of 0, 0.6, 3.0, and 15.0 mM, respectively.
translation elongation factors G (EF-G) and Tu (EF-Tu); oligopeptidase A (OPA); and putative mRNA-binding protein precursor) appeared as 12 isoforms that exhibited opposite expression patterns within each set of isoforms (Fig. 5B and supplemental Table S2). Likewise many similar phenomena were also observed in other previously reported proteomics studies (24, 27–30). Based on changes in isoelectric state of protein spots on 2-DE gels and confirmation of phosphatase treatment, Gallie et al. (31) found that the phosphorylation state of several wheat translation initiation factors and the poly(A)-binding protein is subject to alteration during germination and following heat shock of wheat seedlings. This finding allows us to reasonably speculate that the proteomic responses of some of the latter five unipros in this study (e.g. EF-G, EF-Tu, and mRNA-binding protein precursor) are likely involved in posttranslational regulation, such as phosphorylation and dephosphorylation. The posttranslational modification of these unipros in response to \( \text{H}_2\text{O}_2 \) stress might result in the opposite expression patterns of different isoforms with phosphorylation or dephosphorylation state. These results suggest that isoforms of certain unipros may play the same or different roles in modulating defense responses to \( \text{H}_2\text{O}_2 \) stress in rice seedlings.

Differentially Expressed Proteins Are Preferentially Associated with the Photosynthetic Process—Exposure to exogenous \( \text{H}_2\text{O}_2 \) stress led to increasing endogenous \( \text{H}_2\text{O}_2 \) accumulation in rice leaves and impaired the photosynthetic function of rice seedlings (Fig. 1, B and C). Meanwhile 41 differentially expressed identities were found to be associated with the photosynthetic process (Tables I and II), and their dynamics showed the effects of \( \text{H}_2\text{O}_2 \) stress on photosynthesis at the protein level as shown in Fig. 6. These proteins are implicated in four functional subgroups: 1) chlorophyll-binding proteins, 2) proteins participating in the Calvin cycle, 3) Rubisco activases or -binding proteins, and 4) oxygen-evolving complex proteins of PS II (Fig. 6, A–D).

In the first subgroup, two precursors of chlorophyll \( a/b \)-binding proteins (spot 073, CP26 in PS II; and spot 074, type III in PS I) were down-regulated in response to \( \text{H}_2\text{O}_2 \) stress (Fig. 6A). CP26 is a component of the light-harvesting complex of PS II in plants and facilitates light absorption and transfer of the excitation energy to reaction centers for charge separation (32). Type III in PS I is a pigment-protein subunit component of the antenna system in plants and combines chlorophyll molecules in sites 1013 and 1023 (33). Therefore, the decreases in abundance of the two proteins might limit the light absorption and energy transfer of PS in the \( \text{H}_2\text{O}_2 \)-treated rice seedling leaves and further affect the photosynthetic process.

In the second subgroup, there were 22 proteins participating in \( \text{CO}_2 \) assimilation, including 12 RLSs, three SBPs, two GAPDHs, two ribulose 1,5-bisphosphate carboxylases, a ribulose-5-phosphate kinase precursor, a fructose-bisphosphate aldolase class-I, and a fructose-1,6-bisphosphatase.
Among the 22 identities, 13 proteins showed down-regulation, whereas the other nine proteins increased in abundance and were partially degraded products (Fig. 6B). As mentioned above, seven of the nine degraded fragments are RLSs. Rubisco is a very inefficient enzyme with expression levels of 30–55% in excess under normal conditions (34), and there should be a dynamic balance between intact Rubisco and its degraded fragments in rice leaves (35). Otherwise ROS accumulation in chloroplasts induces Rubisco modification and further facilitates subsequent degradation of Rubisco (36). The data presented here show that the down-regulation of the 13 proteins and the up-regulation of the nine degraded fragments together slowed down the Calvin cycle in rice seedling leaves (Fig. 6B) and thus impaired the photosynthetic process (Fig. 1C).

The third subgroup consists of 10 proteins engaged in the activation of Rubisco (five RASIPs, three Rubisco-binding protein α subunits, a Rubisco activase, and a putative chlorate subunit) (Fig. 6C). Besides the two RASIPs, the other eight proteins were down-regulated in response to H2O2 stress. Interestingly the two up-regulated RASIPs were partially degraded products. The fourth subgroup is composed of six oxygen-evolving proteins and a thylakoid lumenal protein. Of them, the five intact proteins, including two 33-kDa oxygen-evolving complex proteins of PS II, two OECP2, and a thylakoid lumenal protein, were up-regulated, whereas the two other degraded products (OECP1 and OECP2) decreased in abundance (Fig. 6D). Remarkably similar results are also observed in proteome analysis of rice seedling leaves in response to salt and low temperature stresses (24, 27, 37), suggesting that abiotic stresses can enhance the expression of oxygen-evolving proteins of PS II. Additionally two isoforms of phosphoglycolate phosphatase precursor (PGP) participating in photosynthesis were down-regulated, implying that H2O2 stress might reduce photosynthesis in rice seedling leaves (Table I).

Taken together, our results suggest that H2O2 stress seems to impair not only the light absorption and energy transfer of PS, CO2 assimilation, and Rubisco activity but also photospiration by virtue of the abundance changes and the partial degradation of proteins. These results mirror the conclusion that H2O2 stress ultimately inhibits the photosynthesis and photospiration in rice seedling leaves and are supported by the photosynthesis measurement (Fig. 1C). These data also provide new insights into the relationship between the impacts of oxidative stress on photosynthetic responses and the regulated expression of proteins (their abundance changes and breakdown) in H2O2-treated rice seedling leaves.

**Differentially Expressed Proteins Are Largely Implicated in Inhibition of Biosynthesis and Enhanced Degradation of Proteins**—A total of 27 identified proteins (21% in total) in response to H2O2 stress were found to be attributed to protein metabolism (Tables I and II) and could be divided into three functional groups (Fig. 7A). The first group consists of 10 identities functioning in protein biosynthesis with six down-regulated proteins (Fig. 7A), including putative ribosomal protein S15, L12, S5, eukaryotic initiation factor 4A, EF-G, and EF-Tu, which are directly involved in initiation and elongation of the newly growing peptide chains. One of the four up-regulated identities (Fig. 7A) is putative ribosome recycling factor, which has been documented to function in the disassembly of the posttermination complex in protein biosynthesis (38). The three other up-regulated identities include putative elongation factor P and isoforms of EF-G and EF-Tu, which are involved in elongation of the growing peptide chains. Remarkably the opposite change patterns between the two isoforms of both EF-G and EF-Tu may implicate their different roles or compartmentation in the H2O2-treated rice leaves. The second group involves 12 identities related to protein folding and assembly (chaperonin 21, chaperonin 60 with three isoforms, HSP70 with three isoforms, 90-kDa HSP, putative dnaK-type molecular chaperone BiP, endosperm lumenal BiP, 21-kDa polypeptide, and putative endoplasmic reticulum membrane fusion protein). These chaperonins, HSPs, and BiPs have been well studied and are known to be responsible for protein refolding and assembly. Additionally the 21-kDa polypeptide is predicted to function as a molecular chaperone, whereas the endoplasmic reticulum membrane fusion protein is implicated in intercellular protein transport (Gene Ontology). As illustrated in Fig. 7A, our results show an interesting trend in the expression of these proteins (11 in group 2 together with six in group 1) responsible for protein synthesis, folding, and assembly whereby the expression levels are remarkably decreased in response to H2O2 stress, suggesting that protein biosynthesis may be inhibited under oxidative stress conditions.

Among five degradation-related proteins, three identities (26 S proteasome subunit α, OPA, and endopeptidase CLPP2) were up-regulated in response to H2O2 stress, whereas putative aminopeptidase N and another isoform of OPA were decreased in abundance (Fig. 7A). The 26 S proteasome subunit α is a component of the large proteasome complex that selectively degrades various cellular proteins with specific degradation signals such as a multiubiquitin chain (39). OPA is the major soluble enzyme able to hydrolyze the lipoprotein signal peptide and further degrades the partially degraded portions of the signal peptide (40). CLPP2 is an ATP-dependent enzyme and functions as a master protease (41). Thus, the up-regulation of these three proteins indicates that protein degradation is enhanced in H2O2-treated rice seedling leaves. Additionally aminopeptidase N is known as a proteinase inhibitor and also as an exopeptidase (42). Thus, its down-regulated expression might release the activity of other proteinases and facilitate protein degradation. Taken together, the regulated expression response patterns of all the proteins in the three groups readily mirror the fact that the inhibition of novel protein biosynthesis as well as folding and assembly and the enhancement of protein degradation are
required for the survival and growth of rice seedlings under H$_2$O$_2$ stress and that an active quality control system of proteins inside cells plays a crucial role in modulating the accommodation of rice seedlings to oxidative stress.

**Differentially Expressed Proteins Were Involved in Leaf Antioxidative Reactions** — A total of 29 identified proteins (22% in total) in response to H$_2$O$_2$ stress were obviously related to leaf antioxidative reactions and could be classified into four functional categories, including cell rescue/defense, redox homeostasis, signal transduction, and amino acid as well as lipid metabolism (Fig. 7B). Of nine cell rescue/defense-related proteins, five (NB-ARC domain-containing protein, putative MLA1, putative nodulin, putative salt-induced protein, and putative hydrolase) were increased, and four (putative chitinase, harpin-binding protein 1, hydroxyproline-rich glycoprotein, and putative temperature stress-induced lipocalin) were decreased in abundance in the H$_2$O$_2$-treated rice leaves (Tables I and II and Fig. 7B). The NB-ARC domain is a component of the disease-resistant nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins and functions as a molecular switch in plant defense systems (43). The MLA1 protein is known to confer race-specific resistance to the powdery mil-
dew fungus in barley (44), and the putative MLA1 identified in this study shares 37% sequence identity with that in barley. Nodulin is also found to be induced in the resistant *Medicago truncatula* line upon inoculation with *Colletotrichum trifolii*, indicating a strong correlation between the number of up-regulated genes and the resistance phenotype (45). Otherwise chitinase, harpin-binding protein 1, and hydroxyproline-rich glycoprotein are known to play important roles in plant disease resistance (46), whereas the putative temperature stress-induced lipocalins play a possible biological role in membrane biogenesis and repair as well as in the transport of sterol molecules to the membrane under adverse stress conditions (47). It was not expected that these four proteins were down-regulated in response to H$_2$O$_2$ stress, but a possible explanation may be that there was no ample scope for their abilities especially under chemical oxidative stress. However, the question how these cell rescue/defense-related proteins are implicated in rice antioxidative reactions still remains to be addressed.

Among 10 identities implicated in redox homeostasis, nine were up-regulated in response to H$_2$O$_2$ stress, including GST, protein-disulfide isomerase (PDI) with two isoforms, glyoxalase I with two isoforms, and three reductases (Tables I and II and Fig. 7B). GST is an antioxidative protein, and its expression can be strongly enhanced by abiotic and biotic stresses (48). PDI functions in the thioredoxin-based redox pathway and forms part of the antioxidative defense system (49). Glyoxalase I can convert toxic 2-oxoaldehydes into less reactive 2-hydroxyacids using glutathione as a cofactor (50). The three reductases catalyze the reductions of methoxyalted quinine, methionine sulfoxide, and 12-oxophytodienoic acid, respectively (51–53). Thus, the up-regulation of these proteins implies that the antioxidative defense system is provoked in H$_2$O$_2$-treated rice seedling leaves, and such a consistent induction is a likely consequence of antioxidative reactions under oxidative stress in plants. Unexpectedly an APx in this study was found to be down-regulated in response to H$_2$O$_2$ stress (Fig. 7B). Nevertheless similar results are also observed in tobacco and barley plants with different conditions of H$_2$O$_2$ stress (4, 54). Although APx is an important H$_2$O$_2$-scavenging enzyme located in the cytosol, chloroplasts, mitochondria, and peroxisomes of higher plants, the decrease of APx abundance in chemically treated tissues is likely to contribute to an accumulation of H$_2$O$_2$ and the eventual death of plant cells (4, 54).

Furthermore, of four signal transduction-related proteins, three identities (G protein β subunit, small GTP-binding protein, and putative protein-tyrosine phosphatase (PTP)) showed an up-regulated expression pattern, whereas the C2 domain-containing protein (C2-P) was decreased in abundance (Tables I and II and Fig. 7B) under H$_2$O$_2$ stress. The G proteins are mediators that transmit the external signals via receptor molecules to effector molecules and participate in multiple signaling pathways such as those involving sphingo-

lipid, jasmonic acid, and pathogen infection in plants (55, 56). PTP is a signaling enzyme that mediates protein phosphorylation events, playing a particularly important role in maintaining ROS balance (57). Remarkably C2-P has multiple roles in negatively regulating defense responses and cell death in *Arabidopsis*. The loss of the C2-P gene confers an enhanced resistance to pathogens (58). Therefore, the up-regulation of the G proteins and PTP, together with the down-regulation of C2-P, suggests that the stress defense system is up-regulated in H$_2$O$_2$-treated rice seedling leaves. On the other hand, all six identities associated with amino acid and lipid metabolism were increased in abundance (Table I and Fig. 7B), including glutamine synthetase shoot isozyme (GSSI), S-adenosyl-L-methionine synthetase, dehydrogenase, cysteine synthase A with two isoforms, and putative enoyl-ACP reductase. GSSI plays an important role in enhancing rice tolerance to salt and chilling stresses (58). S-Adenosyl-L-methionine synthetase catalyzes the biosynthesis of S-adenosyl-L-methionine and closely correlates with tolerance to cold, salt, and abscisic acid stresses (37, 60). Cysteine synthase A is responsible for the final step in cysteine biosynthesis, the key limiting step in producing glutathione, which is involved in resistance to adverse stresses (61). Enoyl-ACP reductase is a subunit of the fatty-acid synthase complex that catalyzes de novo synthesis of fatty acids, and the lack of this gene in the mol1 mutant of *Arabidopsis* leads to premature cell death in multiple organs (62). Taken together, the findings reported above largely indicate an intimate balanced correlation between the changes in specific protein abundance and the overall antioxidative defense responses in H$_2$O$_2$-treated rice seedlings.

**Preferential Representation of Some H$_2$O$_2$-induced Proteins and Their Cross-talk to Other Abiotic Stresses**—By comparison with previously published rice proteomics data sets, the 12 H$_2$O$_2$-responsive proteins identified in this study (Table IV) were detected in rice seedling leaves under many external stimuli such as cold, O$_2$, SO$_2$, salt, drought, heavy metal, and plant hormones (21, 24, 27–29, 37, 63–69), suggesting their important roles in the defense responses of rice seedlings to various environmental stresses. Some of them show the same expression patterns between H$_2$O$_2$ stress and other adverse stimuli, whereas others do not (Table IV). For example, the expression patterns of eight proteins including GAPDH; GST; EF-Tu; HSP70; PDI; GSSI; ATP synthase CF1, β subunit; and fructose-bisphosphate aldolase (Table IV) in H$_2$O$_2$-treated rice seedling leaves were observed to be similar to those in *Arabidopsis* mitochondria and yeast subjected to H$_2$O$_2$ stress (25, 30), implying the similar response of different species and/or tissues under H$_2$O$_2$ stress. A particular case was GST: its abundance of protein expression was up-regulated not only by H$_2$O$_2$ stress in this study but also by other abiotic stresses such as cold, drought, jasmonic acid, and brassinolide in rice seedling leaves (29, 37, 64, 65). GST is known to function as a pivotal component in plant defense systems and catalyzes the reduction reaction of toxic organic hydroperox-
H_{2}O_{2}-responsive Proteins in Rice Seedlings

**Table IV**

| Protein names                  | Treatments                                                                 |
|-------------------------------|---------------------------------------------------------------------------|
|                               | Rice seedling leaves | Arabidopsis and yeast: |
|                               | This study | Cold | Hormones | Air pollutants | Salt | CuSO_{4} | Drought |
| ATP synthase CF1, β subunit   | 3.22 | >1.50 | (ABA)  | (O_{3})  | 1.90 | ↑       | >3.00 |
| Fructose-bisphosphate aldolase| -1.47 | >3.00 | (ABA)  | (O_{3})  | 1.50 | 1.45    | -1.51 |
| Rubisco activase              | -1.47 | <-1.50 | (ABA)  | (O_{3})  | -1.61 |         |        |
| Rubisco large subunit         | -2.38 | <-1.50 | (ABA)  | (SO_{2},O_{3}) | -2.00 | ↓       |        |
| Glyceraldehyde-3-phosphate dehydrogenase | -2.13 | >1.50 | (BL)   | (O_{3})  | -0.50 |         | -1.34 |
| Ascorbate peroxidase          | -1.72 | >1.50 | (JA)   | (SO_{2},O_{3}) |       |         |        |
| Glutathione S-transferase     | 1.73  | 1.87  | (JA, BL) |         | 1.67 |         | >3.00 |
| Translation elongation factor Tu| -1.96 | 1.63  |         |         | 1.96 | -1.54   |        |
| Heat shock protein 70         | -1.70 | 2.48  |         |         | 1.60 |         | -1.67 |
| Protein-disulfide isomerase   | 1.45  | <-2.00 |         |         |       |         | >3.00 |
| Glutamine synthetase shoot isozyme | 1.49 | >1.50 |         |         | 3.39 |         |        |
| PSII oxygen-evolving complex protein | 1.51  | 1.89  |         |         | 1.30 |         |        |

*References are cited in parentheses.

† and ‡ represent up-regulated and down-regulated expression of proteins but without the values of -fold change listed.

**Table IV**

H_{2}O_{2}-induced proteins and their responses to other abiotic stresses in rice seedling leaves and other species such as Arabidopsis and yeast

ABA, abscisic acid; BL, brassinolide; JA, jasmonic acid; O_{3}, ozone; SO_{2}, sulfur dioxide.


des by using glutathione as a cosubstrate or coenzyme (48). Thus, these consistent results demonstrate that the expression level of GST is remarkably enhanced under various stress conditions, implying that novel GST synthesis is required for plant tolerance to those stresses and that GST plays a pivotal role in plant stress defense responses.

In contrast, six of the 12 co-detected proteins exhibited different expression patterns between H_{2}O_{2} and other abiotic stresses (Table IV). APx was down-regulated in response to H_{2}O_{2} stress but was up-regulated under treatments of cold, salt, drought, and brassinolide (Table IV). These results suggest that protein and carbohydrate metabolic reactions may be impaired in rice seedling leaves exposed to high doses of H_{2}O_{2} but are promoted during abiotic stresses with weakly oxidative intensity. Taken together, the findings presented here suggest that although the cellular responses to abiotic and biotic challenges are rather similar with regard to production of ROS (3, 9–16), the detailed stress defense mechanisms might be different. Therefore, two extremely different types of metabolic changes are involved in the adaptation of plants to different intensities of oxidative stress. Under strongly oxidative situations experienced by plants, the overall reduced substance metabolism may be the result of a series of events in gene expression and oxidative damage that would lead to cell death (70, 71). Nevertheless it may play crucial roles in improving cell fitness and protecting plants from further damage to the continuous ROS production and ultimately maintain plant survival (19). On the other hand, weakly oxidative stresses influence only part of the antioxidative network and modify gene expression in such a way as to strengthen plant defense responses and enhance metabolic systems to maintain homeostasis (70, 71). This finely modulated response strategy in plant cells has evolved over a long period to guarantee plant survival and growth against ever changing environmental threats with a combination of multiple stresses.

**A Possible H_{2}O_{2} Stress-responsive Protein Network in Rice Seedling Leaves**—A tight regulation of the steady-state levels of ROS (particularly H_{2}O_{2}) in plants is necessary to avoid cellular injury and to maintain a basic level of ROS upon which different developmental and environmental signals can be perceived and transmitted. However, no systemic investigation into how plants maintain survival and growth under oxidative stress through adjustment of their metabolic network
and antioxidative system has been conducted to date. In the present study, based on our proteomics and physiological data, an oxidative stress-responsive protein network was proposed with most of the 129 H2O2-responsive proteins identified in rice seedling leaves (Fig. 8 and Tables I and II). This network consists of several functional components, including imbalance between ROS production and scavenging, accelerated degradation and reduced biosynthesis of proteins, impaired photosynthesis and photorespiration, and an enhanced antioxidative defense system (Fig. 8). These changes of metabolic reactions and redox balance eventually lead plants to a new homeostasis that can adapt to and/or resist external adverse stresses. Such a protein network allows us to further understand and describe this possible management strategy of cellular activities occurring in H2O2-treated rice seedling leaves.

When rice seedlings are exposed to high doses of H2O2, the exogenous H2O2 as a stable small molecule can diffuse into cells by cross-membrane transport (1) and also activate some enzymes such as membrane-bound NADPH oxidase and apoplast-located amine oxidase (3) to amplify endogenous H2O2, thus resulting in an increase of intracellular H2O2 level in rice leaves (Fig. 1B). The excessive H2O2 levels in rice leaves cause an imbalance of the original redox homeostasis with the characteristics of elevated oxidative intensity, which would lead to changes of biomolecular metabolism.

Synthesis and degradation of biomolecules (particularly proteins) are the most important links in the life processes. Under the above mentioned oxidative stress, the expression of most biosynthesis-, folding- and assembly-related proteins, including eukaryotic initiation factor 4A, EF-G, EF-Tu, putative ribosomal protein S15, 50 S ribosomal protein L12, CP26, chlorophyll a/b-binding protein CP26 precursor; EIF 4A, eukaryotic initiation factor 4A; ERMF2, putative endoplasmic reticulum membrane fusion protein; FBA, fructose-bisphosphate aldolase class-I; Glyox, putative glyoxalase I; G-P1, small GTP-binding protein; G-P2, G protein β subunit; MSR, methionine sulfoxide reductase; RP-S15, putative ribosomal protein S15; Type III, putative chlorophyll a/b-binding protein type III (PS I) precursor; OX, oxidation; RED, reduction; PB, protein biosynthesis; PD, protein degradation.

The excessive H2O2 in rice leaves not only strongly affects the protein metabolism-related proteins but also acts intensively on carbohydrate metabolism-associated proteins. On the one hand, it is clear from the 2-DE gel images that the abundances of many intact key enzymes involved in the Calvin cycle declined under oxidative stress (Fig. 6, B and C). On the other hand, the protein degradation system provoked by oxidative stress might accelerate the partial degradation of these key enzymes participating in the Calvin cycle in rice leaves, thus increasing protein abundances of the partially degraded products (Fig. 6, B and C). These key enzymes include RLS, SBP, RASIP, fructose-bisphosphate aldolase class-I, and GAPDH (Fig. 8). As a result, the photosynthetic rate occurring in rice seedlings is impaired by H2O2 stress.
(Fig. 1C). Moreover the elevated H2O2 levels still inhibit the expression of light-harvesting antenna proteins such as CP26 and Type III but increase the abundance of PS II oxygen-evolving proteins like 33-kDa OECP and OECP2 (Figs. 6, A and D, and 8). The down-regulation of CP26 and Type III, together with the up-regulation of 33-kDa OECP and OECP2, leads to the reduced light harvesting capability of rice leaves and eventually impaired photosynthesis (Fig. 1C).

Physiological analysis showed the attenuation of photorespiration in H2O2-stressed rice leaves (Fig. 1C). The photorespiratory reactions are involved in a cooperative interaction between three subcellular organelles: chloroplasts, peroxisomes, and mitochondria. Besides the enhanced partial degradation of the photosynthetic components and the relative attenuation of the light harvesting capability, two key enzymes, PGP1 and PGP2, were also found to be down-regulated under oxidative stress (Fig. 8 and Table I). The changes of these critical proteins eventually result in a reduction of the photorespiration rate in H2O2-treated rice seedling leaves (Fig. 1C). In addition, some proteins related to glycolysis (putative fructokinase with two isoforms) and aerobic respiration (inorganic pyrophosphatase with two isoforms) (Table I) were down-regulated under H2O2 stress. Taken together, these basic metabolisms such as photosynthesis, photorespiration, aerobic respiration, glycolysis, and protein biosynthesis in rice seedling leaves were without exception inhibited by H2O2 stress. It is thus believed that plants must be required to make economical use of their substance and energy to adequately deal with severely adverse environments.

On the other hand, the gradually elevated H2O2 is sensitively perceived by cells of rice seedlings via different proteins, enzymes, or receptors and modulates different stress defense pathways. The up-regulated G proteins (G-P1 and G-P2), together with the down-regulated negative regulatory factor (C2-P protein), may cooperatively induce a number of genes with antioxidative functions such as GST, PDI, putative 12-oxophytodienoate reductase, putative glyoxalase I, methionine sulfoxide reductase, and 1,4-benzoquinone reductase in this study (Fig. 8). The abundances of these gene products were significantly increased on 2-DE gel images (Fig. 2 and supplemental Table S1) and led to the enhancement of the cellular antioxidant levels. The activated antioxidative systems in cells of rice seedlings possess a stronger capability for removing H2O2, which can reduce the intracellular H2O2 levels and attenuate the oxidative damage, thus ultimately establishing a new redox homeostasis. Such antioxidative systems play an important role in maintaining the survival and growth of rice seedlings under strong and sustained oxidative stress (Fig. 8).

Conclusions—To investigate changes of global proteins under oxidative stress, we performed a comparative proteome analysis of rice seedling leaves using H2O2 as a model for oxidative stress. The present proteomics study revealed 144 H2O2-responsive spots on the 2-DE gel image containing about 2000 reproducible spots from which 129 differentially expressed proteins were successfully identified in H2O2-treated rice seedling leaves. These proteins not only include many well known H2O2-induced proteins such as HSP70, GST, PDI, APx, and ATP synthase but also 42 novel H2O2-responsive proteins, especially some proteins related to redox homeostasis and signal transduction (supplemental Table S3). It should be noted that these novel proteins did not appear in previous proteomics data sets obtained from H2O2-stressed Arabidopsis mitochondria (25) and yeast (30) as well as other abiotic-stressed rice seedling leaves (21, 24, 27–29, 37, 63–69). The discovery of such new proteins from 2-DE gels provides new insights into the oxidative stress defense mechanism in plants.

Based on the putative functions and abundance changes of the 129 identified proteins, together with previously published results, we proposed an H2O2 stress-responsive protein network including a series of events occurring in rice seedling leaves under higher doses of H2O2 (Fig. 8). These results depict a panoramic view of the adaptation strategies of rice seedlings to oxidative challenge and deepen our understanding of the sophisticated functional network for adaptation to oxidative stress in plants. It is also expected that this network can mirror the management of cellular activities in plants under oxidative stress and provide a basis for further functional research of each member of this network in intracellular redox homeostasis and H2O2 metabolism.

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