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Comparative Proteomic Study Reveals the Involvement of Diurnal Cycle in Cell Division, Enlargement, and Starch Accumulation in Developing Endosperm of *Oryza sativa*

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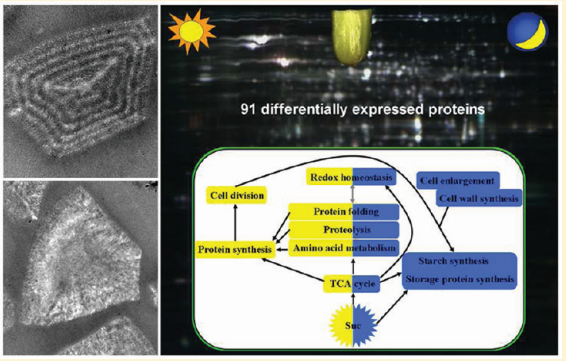
Supporting Information

ABSTRACT: The development and starch accumulation of cereal endosperms rely on the sugar supply of leaves, which is subject to diurnal cycles, and the endosperm itself also experiences a light/dark switch. However, revealing how the cereal endosperm responds to diurnal input remains a major challenge. We used comparative proteomic approaches to probe diurnally affected processes in rice endosperm (*Oryza sativa*) 10 days after flowering under 12-h light/12-h dark. Starch granules in rice endosperm showed a growth ring structure under a normal light/dark cycle but not under constant light. Sucrose showed a high level in light and low level in dark. Two-dimensional (2-D) differential in-gel electrophoresis-based proteomic analysis revealed 101 protein spots diurnally changed and 91 identities, which were involved in diverse processes with preferred distribution in stress response, protein synthesis/destination and metabolism. Proteins involved in cell division showed high expression in light and those in cell enlargement and cell wall synthesis high in dark, while starch synthesis proteins were light-downregulated and dark-upregulated. Redox homeostasis-associated proteins showed in-phase peaks under light and dark. These data demonstrate diurnal input-regulated diverse cellular and metabolic processes in rice endosperm, and coordination among these processes is essential for development and starch accumulation with diurnal input.



KEYWORDS: comparative proteomics, diurnal cycle, endosperm, starch, *Oryza sativa*

INTRODUCTION

The endosperm of cereal seeds represents the main part of the mature seed and is an important storage organ for starch, proteins, and lipids, with a starch content of more than 85%.[1] The endosperm is important food for humans and livestock and functions in nourishing early seedlings of postgermination to support their growth and development.[2] The development of cereal endosperms begins with the fusion of two polar nuclei and one sperm nucleus and involves syncytium formation, cellularization, cell differentiation, endoreduplication, reserve material accumulation, and maturation.[2] Cell division and enlargement, as well as starch accumulation, are key processes determining seed weight. During the accumulation of starch, it is synthesized by the coordinated roles of a group of enzymes and then trimmed and packed into semicrystalline starch granules in amyloplasts.[3,4]

Endosperm development and storage of starch rely on the sucrose (Suc) supply from the source leaves. Sugar level in leaves is subject to diurnal changes in response to the photoperiod and alteration in development.[5,6] Biochemical analysis of sugar levels in leaves from different plant species, including *Arabidopsis thaliana*, spinach (*Spinacia oleracea*), and rice, demonstrated that starch and Suc levels both increase in light and decrease in dark.[5,7−9] In light, leaves produce sugars by photosynthesis; export Suc to nonphotosynthetic organs to support growth, development, and storage of the plant; and synthesize transitory starch in chloroplasts at high rates of photosynthesis.[6,10−12] Transitory starch plays roles in buffering carbon overflow to allow photosynthesis to proceed faster than Suc synthesis.[12] At night, transitory starch is remobilized to support leaf function, as well as synthesis and export of Suc, because the plant is in a status of net-consuming carbohydrates. At the end of night, the leaf has only a little transitory starch.[6,10−13]

The diurnal changes in sugar levels are tightly coordinated with sink demand and activity. For example, plants under short days partition more products of photosynthesis into starch than those under long days, to support metabolism and sugar export during the long night.[5,14,15] In the tuber of potato

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(*Solanum tuberosum*), metabolic processes such as starch synthesis are regulated to be tightly linked to diurnal fluctuation of Suc supply from leaves;[16] more starch is accumulated at the end of the day than at the end of the night, which agrees with the Suc supply, and the transcript level of ADP-glucose pyrophosphorylase (AGPase), which catalyzes the key step of starch synthesis, is higher at the end of the day as well.[16] Cereal endosperms, the most important source of starch worldwide, also experience a light/dark switch and diurnal cycle, as well as diurnal changes in the supply of Suc. Understanding whether and how endosperms respond to the diurnal cycle and diurnal change in sugar supply at the molecular level is essential for insight into the mechanism underlying cellular development and starch synthesis in endosperms.

Several studies have tried to understand the diurnal regulation of development and the starch synthesis of cereal endosperms. Early cytological observation of starch granules of wheat (*Triticum aestivum*) has shown that the growth ring structure occurs under the normal light/dark cycle but disappears under constant light,[17] which indicates possible diurnal regulation of starch synthesis in cereal endosperms. In developing endosperms of sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*), genes encoding starch branching enzymes SBEI, SBEIIA, and SBEIIB expressed in a diurnal fluctuation pattern, with the highest level in light and decreased level in dark.[18] Recent study of diurnal gene expression profiles in developing barley caryopses (seeds) has suggested diurnal regulation in phloem unloading, embryo development, and biosynthesis of storage products in the caryopse,[19] which suggests the importance of diurnal regulation in development and storage materials synthesis of seeds. However, this knowledge is too limited to understand the mechanism underlying diurnal regulation of cellular development and starch synthesis of cereal endosperms. Furthermore, studies of leaves and potato tubers have demonstrated that diurnally changed expression of genes involved in starch metabolism is not followed by the same diurnal fluctuation in protein levels in most cases and that the stimulation of starch synthesis depending on sugar level occurs in a short time window and involves the posttranslational redox activation of AGPase.[10,11] The posttranscriptional mechanism should be key in the regulation of starch synthesis in response to diurnal changes in the sugar level.[10,11]

We aimed to understand the diurnal regulation of cellular development, starch synthesis, and other metabolism processes in cereal endosperms, as well as coordinated interconnections between different processes in response to diurnal regulation. We performed a diurnal proteomic study with endosperms of rice grown under 12-h light/12-h dark alternating conditions, which is an important model plant for plant science research because of its economic value and the available genome database. We first observed the growth ring structure of the starch granule and determined the diurnal change in sugar levels in rice endosperms. Proteomics analysis revealed a set of proteins with diurnal change in levels, with those involved in cell growth, starch and storage protein synthesis, and redox homeostasis. Furthermore, we evaluated transcriptional profiles of genes encoding these diurnally changed proteins.

MATERIALS AND METHODS

Plant Materials and Sampling

The rice cultivar Zhonghua 10 (*Oryza sativa* L. ssp. *japonica*) was planted, and superior caryopses (seeds) of the top three spikelets were labeled at noon of anthesis as described.[20] These rice plants were transferred into a Conviron E8 environmental chamber (model E8, Conviron, Winnipeg, Manitoba, Canada) at 7 days after flowering (DAF) and grown under 12-h/28 °C light (0−12 h, with a light intensity of 100 μE m^{-2} s^{-1}) and 12-h/26 °C dark (12−24 h). After three light/dark cycles of training, labeled seeds at 10 DAF were harvested and dehusked at six successive time points from 0 to 20 h with an interval of 4 h. For transmission electron microscope (TEM) observation, seeds from plants grown under light/dark cycles and constant light were harvested at 10 DAF and dehusked. All seeds were frozen in liquid nitrogen immediately and stored at −80 °C.

TEM Observation of Starch Granules

Endosperms were ground with ice-cold extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 10 mg/L $Na_2S_2O_5$) and then filtered through 150- and 400-mesh cell strainers in sequence. The filtrate was centrifuged at 120*g* for 5 min at 4 °C. The resultant pellet was washed with the extraction buffer four times and then with ice-cold acetone three times; each washing was followed by resuspension and centrifugation at 1000*g* for 10 min at 4 °C. The resulting starch granules were air-dried.

Starch granules and small cubes (1 mm^3) were treated with 8% hydrochloric acid at 38 °C for 30 h and centrifuged at 1000*g* for 10 min at room temperature. After three washes with distilled H_2O, the starch pellet was fixed in 3% glutaraldehyde and 1% osmic acid and the cube pellet in 3% glutaraldehyde, dehydrated in a series of acetone, incubated in a series of mixtures of acetone and Spurr at 2:1, 1:1, 1:2 volume proportion, and embedded in pure Spurr. The sectioning, staining, and TEM observation of specimens were performed as described.[21]

Sugar Measurement

Endosperm samples (70 mg) supplemented with 50 μL internal standard (2 mg mL^{-1} ribitol in water) were homogenized in 1.5 mL of methanol solution (80%) with FastPrep-24 (MP Biomedicals, Irvine, CA, USA). The homogenized endosperm was incubated for 30 min at room temperature. After centrifugation at 10000*g* for 10 min at room temperature, the supernatant was transferred to a new tube, and the pellet was extracted again. A total of 200 μL combined supernatant was dried in the ISS110 SpeedVac concentrator (Thermo Savant, Holbrook, NY), and then mixed with 500 μL of distilled H_2O and 500 μL of chloroform with vigorous vortexing. After centrifugation at 10000*g* for 10 min, the mixture was separated into polar (upper) and nonpolar (lower) fractions, and 50 μL polar fraction was dried in the SpeedVac concentrator.

For gas chromatography−time-of-flight mass spectrometry (GC−TOF/MS; Pegasus IV, LECO, St. Joseph, MI, USA) analysis, the dried sugar was oximated with 50 μL of methoxylamine hydrochloride in anhydrous pyridine (20 mg mL^{-1}) at 30 °C for 90 min, and then silylated with 80 μL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) at 37 °C for 30 min. GC was performed on a VF-5-ms column (30 m × 0.25 mm × 0.25 μm, Varian, Lake Forest, CA, USA). Data were acquired and analyzed by use of Chroma TOF 3.32 (LECO, St. Joseph, MI, USA). Three independent biological replicates were performed for each endosperm sample.

Protein Preparation, 2-D Differential In-Gel Electrophoresis (2D-DIGE), Image Analysis, and Protein Identification

Preparation of endosperm proteins, 2D-DIGE, image analysis, and protein identification were all performed as described.[22]

Briefly, 0.2 g of endosperms was used for protein preparation, and for each endosperm sample, three independent protein preparations were performed. Protein samples were assigned to nine DIGE gels representing three independent biological repeats of each of the six endosperm samples (Supplementary Figure S1, Supporting Information). After 2-D electrophoresis, fluorescent gel images were acquired by the use of a Typhoon 9400 scanner (GE Healthcare) and then analyzed by use of DeCyder 6.5 (GE Healthcare). Spots reproducible in 24 of the 27 (3×9) images were used to identify differentially expressed protein spots. Analysis of variance (ANOVA) was used to determine the differential expression of protein spots ($p < 0.01$). Gels with 1 mg of internal standard proteins were stained with Coomassie Brilliant Blue (CBB) and used for picking the differentially expressed spots. After in-gel digestion of the picked spots with trypsin (Roche), Ultroflex II matrix-assisted laser-desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) (Bruker Daltonics, Germany) was used to reveal peptide mass fingerprints (PMFs). A standard peptide mixture (Bruker) was used for external calibration of MALDI-TOF/TOF-MS to ensure the protein identification accuracy, and peptides from trypsin (Roche) autolysis and keratins were used for internal calibration by FlexAnalysis 2.4 (Bruker Daltonics), with deviations limited to 10 ppm. After removal of peptides produced by trypsin autolysis and keratins, the PMFs were transferred to BioTools 3.0 (Bruker Daltonics) to search against the NCBInr protein database (http://www.ncbi.nlm.nih.gov/; NCBInr 20090314; 8 016 074 sequences; 2 759 887 765 residues) with the Mascot engine 2.2.03 (http://www.matrixscience.com). The taxonomy was *Oryza sativa* (132 558 sequences). Monoisotopic and $[M + H]^+$ were selected for mass values. Mass tolerance was set at 50 ppm, and one missing cleavage was permitted. Carbamidomethyl (C) was set as fixed modification, and Gln → pyro-Glu (N-term Q) and Oxidation (M) as variable modifications. The identified proteins had to meet the three standards to improve identification accuracy: (1) probability-based MOWSE score of the search result > 64 ($p < 0.05$), (2) order of the magnitude of the following nonhomologous protein not significantly different from or less than that of the first one, (3) most abundant peptides in the peak list matched to the identified protein (the first hit).

Bioinformatics Analysis

To confirm the unique proteins (nonredundant proteins), the chromosome loci of genes encoding identified proteins were searched in the Rice Genome Annotation Project database by use of the protein sequences and Blast search procedure (http://rice.plantbiology.msu.edu/blast.shtml), and loci with the highest scores were used. Proteins matched to the same locus were considered 1 unique protein.

Cluster analysis was performed as described,[22] except the cluster range was set at 3−5. After comparing different ranges, four clusters were chosen.

Western Blot Analysis

Protein samples were prepared as described above. Proteins of 5 μg were loaded for each lane and separated by 12.5% SDS-PAGE as described previously.[23] Western blots were performed as described previously[23] with a modified transfer buffer.[24] In brief, proteins in the gel were electrophoretically transferred to a PVDF membrane (Pierce) with a transfer buffer (40 mM Tris, 39 mM glycine, and 20% methanol). The membranes were blocked with 5% skimmed milk in TTBS buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h, incubated with primary rabbit antibodies in TTBS for 1 h, washed three times with TTBS, and then incubated with alkaline phosphatase labeled goat antirabbit IgG (Amresco) in TTBS for 1 h. After being washed, the positive signals were visualized by the use of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma). Signal intensities were quantified by analyzing the membrane images with the Image-Pro Plus 5.1 software, and the correlation coefficients between signal intensities and corresponding protein abundances in 2D-DIGE were calculated. These primary rabbit antibodies used were against chloroplastic superoxide dismutase [Cu−Zn] (Agrisera no. AS06 170, Stockholm, Sweden), stromal 70-kDa heat shock protein (Agrisera no. AS08 348), and UDP-glucose pyrophosphorylase (Agrisera no. AS05 086).

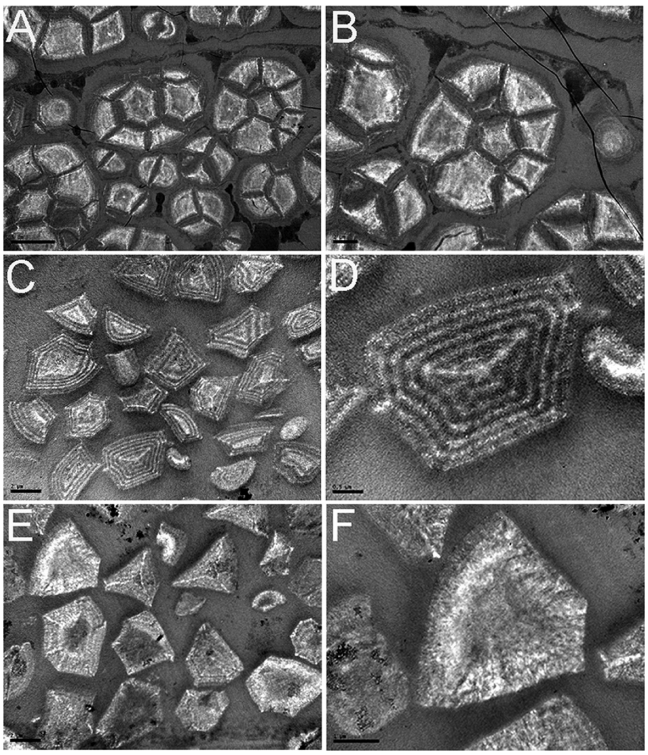

Figure 1. TEM observation of amyloplasts and starch granules of endosperms from rice plants grown under diurnal condition (A, B, C, D) and constant light (E, F). A and B, amyloplasts. C and D, presence of growth rings in starch granules. E and F, absence of growth rings in starch granules. Scale bar = 5 μm in A, 2 μm in B, C and E, 0.5 μm in D, 1 μm in F.

Real-Time Quantitative RT-PCR

All procedures were performed as described previously.[20] Total RNA of endosperm samples was extracted with the RNAprep pure Plant kit (Tiangen). An amount of 1 μg total RNA was used to synthesize the first-strand cDNA with Rever-Tra Ace (Toyobo). Three replicates for each sample were performed with the StepOnePlus Real-Time PCR system (Applied Biosystems) by the use of SYBR Green Realtime PCR Master Mix-Plus- (Toyobo). Primers designed with the Primer Express 2.0 software (Applied Biosystems) were listed in Supplementary Table S5, Supporting Information. The relative quantity of mRNA was evaluated with the $2^{-\Delta\Delta Ct}$ method.

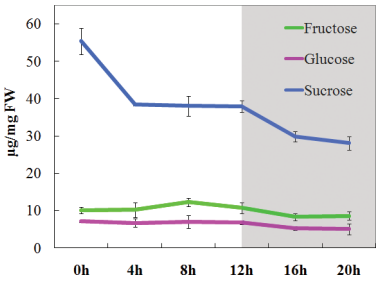

Figure 2. Changed sugar levels in rice endosperms under diurnal conditions. White and gray areas represent the light and dark phases in a diurnal cycle, respectively. Data are means and deviations of three independent biological replicates.

RESULTS

The Presence of a Growth Ring in Starch Granules of Rice Endosperm

Storage starch is synthesized in amyloplasts of nonphotosynthetic storage organs, whereas transitory starch is in chloroplasts of photosynthetic organs. An amyloplast can generate multiple starch granules (compound granules) or only a single granule (simple granule).[2,10,25] Typically, a starch granule has alternate semicrystalline and amorphous regions, with different resistance (high and low, respectively) to acid or degradative enzymes.[17,26] Therefore, acid/enzyme-treated starch granules display a growth ring structure under TEM or scanning electron microscope (SEM). In the rice endosperm, each amyloplast synthesized multiple granules, which indicates that rice granules are of the compound type (Figure 1A,B). TEM observation of acid-treated starch granules revealed growth rings in starch granules from endosperms of rice plants grown under the light/dark cycle (Figure 1C,D) but not constant light (Figure 1E,F), so growth rings or the alternate semicrystalline and amorphous structure is diurnally dependent. Therefore, diurnal cycle-mediated biological processes are involved in regulating starch synthesis and granule packaging in the endosperm.

Diurnal Changes in Sugar Levels in Endosperm

Given the diurnal cycle-dependent growth rings in starch granules of rice endosperms and the diurnal change of sugar synthesis in and export from sources (see Introduction), we further evaluated whether sugars imported into endosperms have diurnal features by assaying the level of glucose (Glc), fructose (Fru), and Suc in rice endosperms (Figure 2). As a whole, Suc was at a much higher level than Glc and Fru in the endosperm. The level of Suc showed a significant diurnal change: high at the start of the light phase, remaining slightly lower and constant in the following light phase, and a decrease when entering the dark phase. However, levels of Glc and Fru did not show a significant diurnal change; only the level of Fru showed a slight increase at the midpoint (8 h) of the light phase. Therefore, Suc was the main form of carbon supply from leaves for reserve synthesis and metabolic activities of endosperms, and the import of Suc changed diurnally.

Identification of Differentially Expressed Proteins During Diurnal Cycle

We previously showed that 2-D gel electrophoresis with pH 4−7 strips had better resolution for rice seed/endosperm proteins than with pH 3−10 strips.[20] Therefore, we profiled endosperm proteins at six time points using 2D-DIGE with pH 4−7 strips and obtained 27 images from nine 2D-DIGE gels with three independent biological repeats for each endosperm sample. About 2300 spots were resolved in each image (Figure 3, Supplementary Figure S1, Supporting Information), and 101 protein spots showed a significant change in expression ($p < 0.01$) (Figure 3, Supplementary Table S1, Supporting Information).

Further MS analysis identified 81 of the 101 spots: 71 contained a single protein each, and the other 10 had 2 proteins each (Supplementary Table S2, Supporting Information). Therefore,

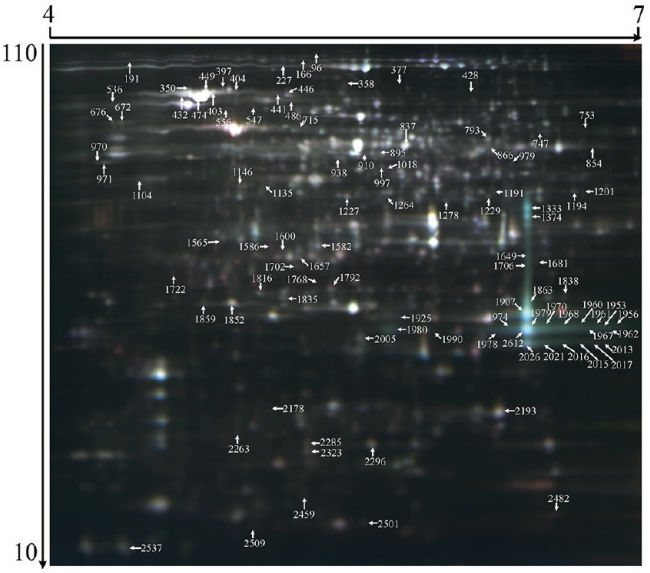

Figure 3. Differentially expressed protein spots in a representative 2D-DIGE image. Endosperm proteins were resolved by 2D-DIGE. The 2D-DIGE images were acquired with a Typhoon 9400 Scanner. These differentially expressed protein spots under diurnal cycle were determined with ANOVA (for details, see Materials and Methods). All images were shown in Supplementary Figure S1, Supporting Information, and all data of these differentially expressed protein spots are in Supplementary Table S1, Supporting Information. Molecular mass (in kilodaltons) and pI of proteins were shown at the left and top of the image, respectively.

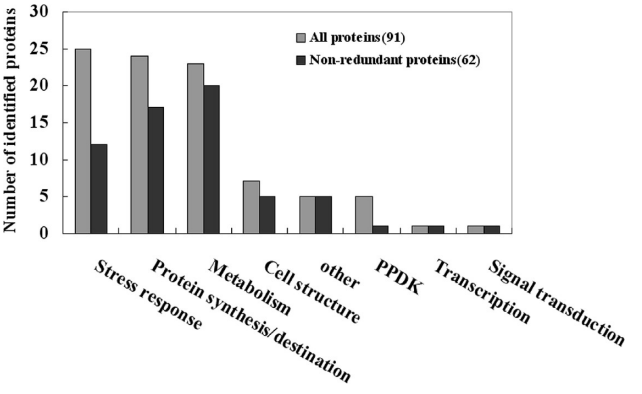

Figure 4. Function classification of identified differentially expressed proteins. Total 91 proteins (gray column) which represent 62 unique proteins (dark) distributed in different function categories. Raw data involving identification of these proteins was listed in Supplementary Table S2, Supporting Information, and detailed information about functional features of these proteins in Supplementary Table S3, Supporting Information.

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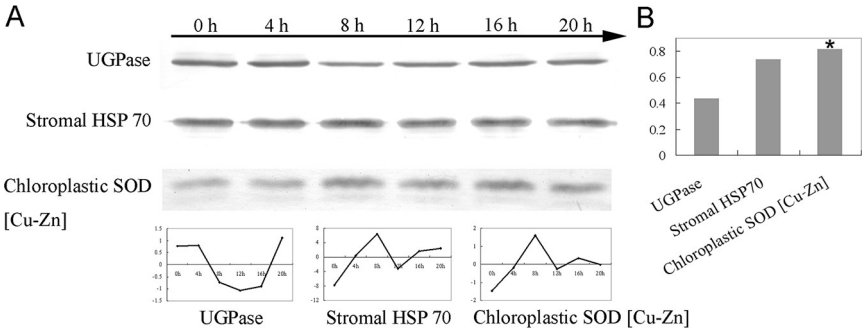

Figure 5. Western blot analysis of proteins. (A) Western blot results of UDP-glucose pyrophosphorylase (UGPase), stromal 70 kDa heat shock-related protein (stromal HSP 70), and chloroplastic superoxide dismutase [Cu−Zn] (chloroplastic SOD [Cu−Zn]). 5 μg proteins were loaded in each lane. Plots established with normalized values received from GeneCluster 2.0 software show expression profiles of the three proteins detected by 2D-DIGE analysis. (B) Correlation analysis between expression profiles of the three proteins determined by Western blot and 2D-DIGE. Signal intensities in the Western blot bands intensities were quantified with the Image-Pro Plus 5.1 software. Gray bars represent the correlation coefficient between the two types of profiles, and * represents significant correlation ($p < 0.05$).

we identified 91 proteins representing 62 unique proteins (unipros) (Supplementary Table S2, Supporting Information). According to database annotation and the scheme for functional category classification used for rice endosperm proteins,[22] the 91 proteins were classified into 7 functional categories, and one "other" group including proteins not clearly assigned (Figure 4, Supplementary Table S2, Supporting Information). In total, 79% of the proteins belonged to three large categories: stress response (27.5%), protein synthesis/destination (26.4%), and metabolism (25.3%) (Figure 4), which may be important processes in rice endosperms in response to the diurnal cycle.

To further understand diurnally changed features of the metabolism and protein synthesis/destination categories, we classified metabolism-related proteins into five subcategories: tricarboxylic acid (TCA) cycle, starch metabolism, amino acid metabolism, nucleotides metabolism, and secondary metabolism (Supplementary Table S2, Supporting Information). Proteins involved in protein synthesis/destination were divided into three subcategories: protein synthesis, protein folding, and proteolysis (Supplementary Table S2, Supporting Information). These subcategories gave more detailed messages about diurnal change in metabolism and protein synthesis/destination in rice endosperms.

To validate the differentially expressed proteins detected by 2D-DIGE, we used Western blot analysis to examine the expression profiles of three identified proteins because of the availability of antibodies against them (Figure 5A). The diurnally changed patterns of all the three proteins detected by Western blot were positively correlated to those by 2D-DIGE (Figure 5B), suggesting the similar diurnally changed expression patterns of these proteins in Western blot and 2D-DIGE analysis. Thus this indicates the reliability of 2D-DIGE-based proteomic results.

Coexpression Patterns of the Differentially Expressed Proteins

We used a SOM clustering tool to examine the coexpression of 71 differentially expressed proteins, excluding 10 spots with 2 proteins each (Supplementary Table S3, Supporting Information) and revealed 4 distinct expression patterns (clusters, c0, c1, c2, c3) (Figure 6A). Proteins in c0 (11 proteins) showed a tendency of increased expression levels in light, with 2 peaks at early (4 h) and end points (12 h) of the light phase, and then decreased levels in dark. The expression pattern of proteins in c3 (16 proteins) was opposite to that of c0 proteins, with the lowest level at the end

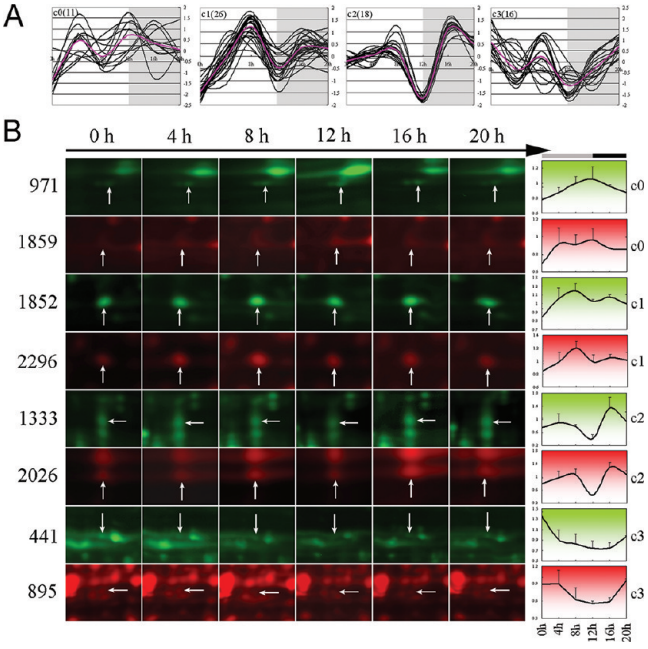

Figure 6. SOM cluster analysis of differentially expressed proteins. Spots containing two proteins were not included in the analysis. (A) All proteins were organized into four clusters. Black lines represent diurnally changed profiles of individual proteins in a cluster, and the pink line represents concentriods of the cluster. (B) Diurnally changed profiles of eight protein spots shown as examples: spots 971 and 1859 in c0, 1852 and 2296 in c1, 1333 and 2026 in c2, and 441 and 895 in c3.

(12 h) of the light phase. Proteins in c1 (26 proteins) and c2 (18 proteins) both had 2 expression peaks, one in the light phase and one in the dark phase. The peaks in light (8 h) and dark (16 h) were significant for proteins in c1 and c2, respectively. The expression patterns were directly observable in 2D-DIGE images and exemplified by spots 971 and 1859 in c0, 1852 and 2296 in c1, 1333 and 2026 in c2, and 441 and 895 in c3 (Figure 6B).

Cluster analysis revealed that most of the proteins in a given function category and subcategory had similar diurnally changed patterns (Table 1, Supplementary Table S3, Supporting Information). For example, most metabolism-related proteins (9/16) were in c3. More than half of proteins in the protein

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Table 1. SOM Clusters of Differentially Expressed Proteins and Distribution of Different Categories Associated Proteins in Clusters[a]

Function Category	c0: 11	c1: 26	c2: 18	c3: 16	*Total*
01 Metabolism	0	6	1	9	16
01.01 TCA cycle	0	2	0	1	3
01.02 Starch metabolism	0	0	0	3	3
01.03 Amino acid metabolism	0	2	1	4	7
01.04 Nucleotide metabolism	0	1	0	0	1
01.05 Secondary metabolism	0	1	0	1	2
02 Protein synthesis and destination	3	12	1	4	20
02.01 Protein synthesis	2	0	0	0	2
02.02 Protein folding	1	9	0	3	13
02.03 Proteolysis	0	3	1	1	5
03 Stress response	1	5	14	0	20
04 PPDK	2	0	0	1	3
05 Cell structure	4	2	0	0	6
06 Transcription	0	0	0	1	1
07 Signal transduction	0	1	0	0	1
08 other	1	0	2	1	4
Total	11	26	18	16	71

[a] The spots in cluster figures represent harvest time (hour; 0, 4, 8, 12, 16, 20 h). 0−12 h, light; 12−20 h, dark.

synthesis/destination group (12/20) and those in stress response group (14/20) were in c1 and in c2, respectively. Cell structure-related proteins (4/6) were mainly in c0. For subcategories in the metabolism group, all starch metabolism-associated proteins (3/3) and most of the amino acid metabolism-related proteins (4/7) had the c3 pattern. For subcategories in protein synthesis/destination, all proteins involved in protein synthesis (2/2) were in c0, and those implicated in protein folding (9/13) and proteolysis (3/5) were mainly in c1. Furthermore, we plotted the expression profiles of proteins in a given category (subcategory) together (Figure 7) and demonstrated coexpressed features of proteins in a given category (subcategory).

Isoforms of Proteins

Isoforms of proteins are generated by posttranscriptional alternative splicing of transcripts of a gene and/or posttranslational modifications (PTMs) of the protein. 2DE-based proteomic studies can reveal isoforms of a protein and have shown isoforms of proteins common in proteomes of diverse tissues/organs such as rice seeds and endosperm.[20,22] We found that 9 unipros had isoforms from 2 to 12 with diurnally changed levels (Table 2, Supplementary Table S4, Supporting Information). In these unipros, six (alanine aminotransferase 2, stromal 70-kDa heat shock-related protein, luminal-binding protein 3, chloroplastic 20-kDa chaperonin, 26S proteasome non-ATPase regulatory subunit 4, and DNA repair protein RAD23) had isoforms with the same molecular mass but different pI values on 2D-DIGE (Figure 8A). Two proteins (dihydrolipoamide *S*-acetyltransferase and pyruvate orthophosphate dikinase 1) had isoforms with different molecular mass and different pI values (Figure 8A). Germin-like protein 3 had 12 isoforms, with some having a similar molecular mass and some similar pI values (Figure 8A). Further data searching revealed three unipro-encoded genes with alternative splicing products in the rice database (http://rice.plantbiology.msu.edu/) and five unipros with post-translational modifications in UniProt database (http://www.uniprot.org/) (Table 2). This information supports our findings of isoforms in these proteins.

Expression profile analysis showed the isoforms of eight of the nine unipros with positively correlated expression profiles (Figure 8B), with significant correlation for isoforms of seven unipros (3 at $p < 0.05$, 4 at $p < 0.01$) (Table 2, Supplementary Table S4, Supporting Information). Only the three isoforms of pyruvate orthophosphate dikinase 1 showed negatively correlated expression patterns (Figure 8B, Table 2, Supplementary Table S4, Supporting Information). This indicates the reliability of our experimental data.

Transcriptional Profiles of Genes Encoding the Identified Differentially Expressed Proteins

To understand the relationship between expression profiles of proteins and their mRNAs in rice endosperms during the diurnal cycle, we evaluated expression profiles of genes encoding the identified differentially expressed proteins using real-time quantitative RT-PCR. Totally, we analyzed expression profiles of 41 in the 48 genes encoding the 71 differentially expressed proteins, which were identified as a single identity in each spot (Supplementary Table S3, Supporting Information), and revealed that the correlation coefficients of expression profiles of protein-mRNA pairs were significantly different, ranging from 0.93 to −0.83 (Supplementary Table S5, Supporting Information). Among the examined 41 genes, 15 displayed expression changes in mRNA levels similar to that of corresponding proteins (Figure 9A, Supplementary Table S5), while 6 had almost reverse

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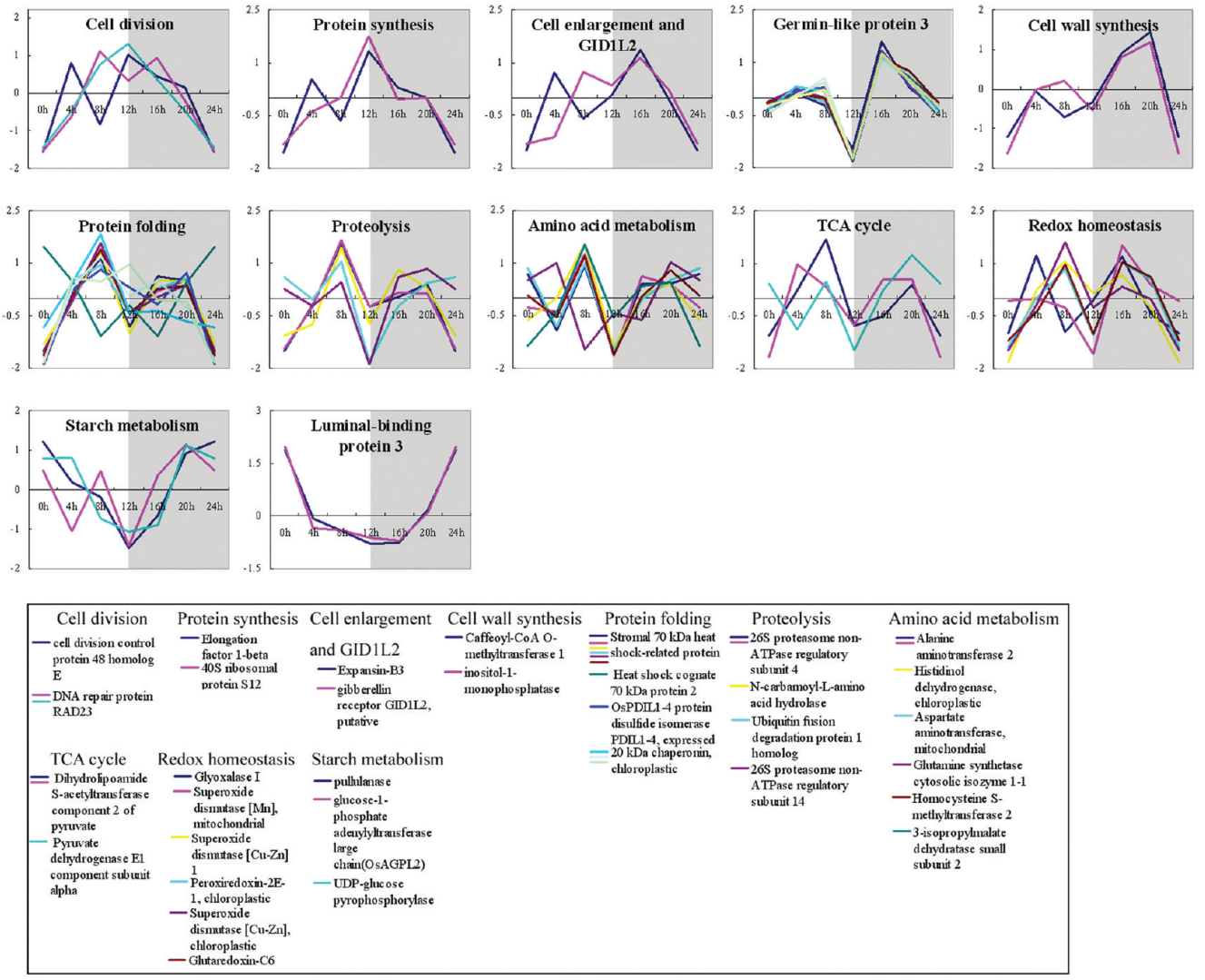

Figure 7. Expression profiles of proteins involved in major categories. The profiles were drawn with normalized relative volume values of proteins (vertical axis) and harvest time (hour; horizontal axis). The normalization was carried out with GeneCluster 2.0 software. White areas representing light phase (0−12 h), gray areas dark phase (12−24 h). The relative volume values of proteins at 24 h were repeats of those at 0 h. Luminal-binding protein 3 was drawn separately and not included in the protein folding category.

expression profiles between mRNAs and proteins (Figure 9B, Supplementary Table S5, Supporting Information). Other 20 showed mRNA and protein expression profiles variable in several phases of the diurnal cycle (Supplementary Table S5, Supporting Information). These data appear be supported by these studies of leaves and potato tubers, which have demonstrated that the diurnally changed expression of genes is not followed by the same diurnal fluctuation in protein levels in most cases.[10,11] This suggested a mechanism beyond transcription is involved in the expression regulation of proteins during the diurnal cycle. Furthermore, our data showed that proteins in different functional categories/subcategories had different relationships with their corresponding mRNAs in expression profiles. For example, in the cell structure, two genes (Os10g30580 encoding cell division control protein 48 homologue E, and Os10g40720 encoding Expansin-B3) had reverse expression profiles of proteins and mRNAs (Figure 9B, Supplementary Table S5, Supporting Information), and the other three genes in the category had these profiles variable in several phases of the diurnal cycle. In the starch metabolism subcategory, one gene (Os09g38030 encoding UDP-glucose pyrophosphorylase) showed a dynamic change in mRNA levels similar to that of the protein (Figure 9A, Supplementary Table S5, Supporting Information), and the other two genes (encoding pullulanase and OsAGPL2) had mRNA and protein expression profiles variable in several phases of diurnal cycle. In the amino acid metabolism subcategory, 4/5 genes (Os10g25130, Os01g13190, Os02g14110, Os02g43830) showed a dynamic change in mRNA levels similar to that of the proteins (Figure 9A, Supplementary Table S5, Supporting Information). These data should be helpful to understand posttranscriptional regulation of genes involved in different cellular and metabolic processes.

DISCUSSION

Our analysis revealed that starch granules of rice endosperms had diurnal cycle-dependent growth rings, and that sucrose, the main form of carbon import from leaves, changed diurnally in

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Table 2. Isoforms of Proteins[a]

NPN	protein name	NI	AS	PTM	CORREL level
1	dihydrolipoamide *S*-acetyltransferase	2	1	unknown	P
9	alanine aminotransferase 2	2	1	the N-terminus is blocked	P*
24	stromal 70 kDa heat shock-related protein	5	1	unknown	P**
25	luminal-binding protein 3	2	1	glycoprotein	P**
30	20 kDa chaperonin, chloroplastic	2	1	unknown	P*
32	26S proteasome non-ATPase regulatory subunit 4	2	2	phosphoprotein	P**
41	germin-like protein 3	12	1	disulfide bond; glycoprotein	P**
50	pyruvate, orthophosphate dikinase 1	3	2	phosphoprotein	N
53	DNA repair protein RAD23	2	2	unknown	P*

[a] NPN, unipro number; NI, number of isoforms of a unipro; AS, alternative splicing forms received by searching in the rice database (http://rice.plantbiology.msu.edu/); PTM, post-translational modification received by searching in the UniProt database (http://www.uniprot.org/); P, expression profiles of isoforms of a unipro positively correlated; N, expression profiles of isoforms of a unipro negatively correlated. *, significantly correlated at $p < 0.05$. **, significantly correlated at $p < 0.01$.

levels in the endosperm. Further proteomics study of the endosperm identified a set of diurnally changed proteins, which were involved in diverse cellular and metabolic processes. A comparison of proteins and their mRNAs expression profiles showed that 36.6% of protein−mRNA pairs concordantly expressed, while the other displayed variable expression, thus clearly indicating the importance of proteomics study in understanding the mechanisms underlying cellular and metabolic processes.

Endosperm Cells Undergo a Proliferation Cycle in the Diurnal Cycle

The mitotic cell division period in cereal endosperm development is responsible for the final endosperm cell population, and cell division and subsequent cell enlargement are key to determine endosperm size.[2] Developing rice seeds (endosperms) reach the size of mature endosperms at 12 DAF.[20] The 10 DAF endosperms used in this study had active starch synthesis and underwent a cellular development process leading to the final endosperm size at 12 DAF.[20] Our proteomic analysis revealed distinct features of proteins with diurnally changed levels associated with cell division and enlargement. Cell division control protein 48 homologue E (CDC48E) and 2 isoforms of DNA repair protein RAD23 showed increased expression in light, which peaked at the switch point from light to dark (12 h), and then decreased in dark (Figure 7). CDC48 is involved in the cell division cycle in yeast and higher plants.[27] Mutation in *RAD23* gene of *Arabidopsis* led to a defect in phyllotaxy by impairing cell division.[28] The expression of these proteins showed positive correlation with those implicated in protein synthesis, such as 40S ribosomal protein S12 and elongation factor 1-beta ($p < 0.05$, Supplementary Table S6, Supporting Information, Figure 7). This finding suggests a possible relation of protein synthesis with cell division and/or enlargement. By contrast, cell wall loosening and synthesis-related proteins showed high abundance in the dark. Expansin-B3, localized in the cell wall, functions in cell elongation by loosening and extending plant cell walls,[29,30] and in the rice endosperm, showed peaked abundance at the early dark phase (16 h) (Figure 7). The expression of the 12 isoforms of germin-like protein 3, another cell wall-localized protein, peaked at the early dark phase (16 h) as well (Figure 7). However, cell wall synthesis proteins caffeoyl-CoA O-methyltransferase 1 and inositol-1-monophosphatase showed peak levels in the late dark phase (20 h) (Figure 7). The former is involved in lignin biosynthesis and the latter in hemicellulose and pectin synthesis by the inositol oxidation pathway.[31,32] Therefore, cell division and enlargement in the rice endosperm may have distinct light and dark features, cell division occurring mainly in the light phase and cell enlargement and wall synthesis in the dark phase.

How diurnal cycles regulate endosperm cell division and enlargement remains unknown. Cytokinin is the hormone promoting cell division.[33] Zeatin and zeatin riboside, the two most abundant cytokinins in higher plants, peaked in maize endosperms at 9 DAF, which is consistent with the maximal rate of cell division in the endosperm.[34] The exposure to red light caused a rapid increase in extractable cytokinins in *Rumex obtusifolius* seeds,[35] and peak cytokinin activity occurred at daybreak in leaves of poplar (*Populus* × *robusta*).[36] Therefore, in rice endosperms, the content and activity of cytokinins may be increased with the switch to the light phase. In addition, gibberellin treatment upregulated expansin-B3 gene expression, which was involved in rapid internode elongation in deepwater rice.[37] Several studies of plant de-etiolation showed light-reduced bioactive gibberellin levels in barley, *Arabidopsis*, and pea (*Pisum sativum* L.), which suggests that the gibberellin level is higher in dark than in light.[38] Our study revealed the coexpression of expansin-B3 and putative gibberellin receptor GID1L2 (Figure 7), both with peaked abundance at the early dark phase. Thus, diurnally regulated changes in cytokinin and gibberellin levels may be involved in diurnal cycles of cell division and enlargement in rice endosperms by affecting the function of currently unknown effects and/or coordinating with proteins described above.

Storage Starch is Synthesized Mainly in Dark in Rice Endosperm

In contrast to wheat endosperms, in which each amyloplast generates only a single granule,[17] rice endosperm synthesize compound-type granules in each amyloplast (Figure 1); however these granules in both rice (compound-type) (this study) and wheat (simple-type)[17] have a diurnal cycle-dependent growth ring structure. Thus, storage starch synthesis may differ in light and dark phases and undergo diurnal change.

Endosperm cells use carbons imported from source organs to synthesize starch, and the main carbon is Suc.[19] In sink organs, mobilization of Suc into starch synthesis and other basic metabolisms is first regulated by invertases and Suc synthases (SuSy). Invertases cleave Suc to generate Glc and Fru, and SuSy use Suc and UDP to produce Fru and UDP-Glc, which is used for starch

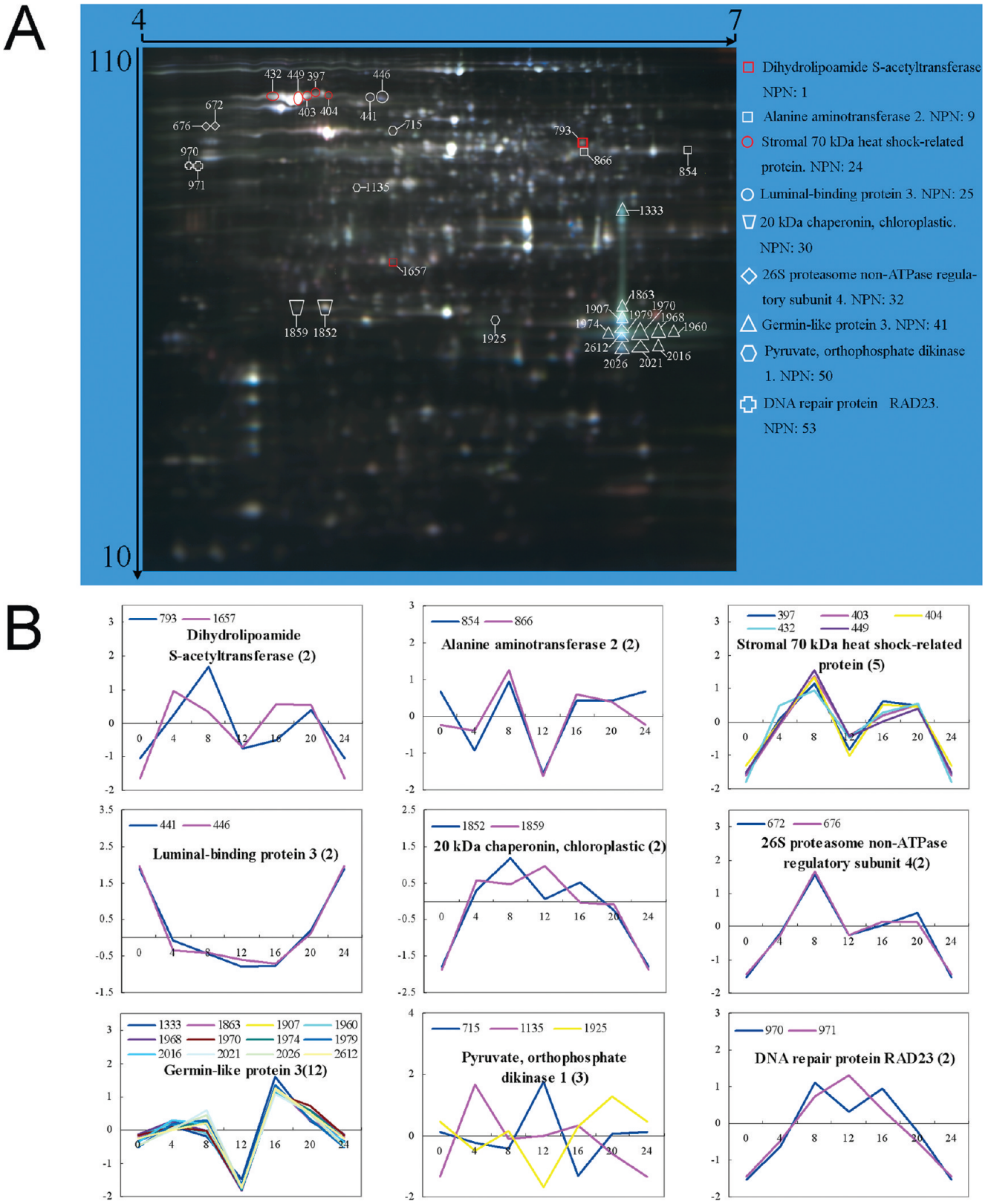

Figure 8. Isoforms of proteins. (A) Isoforms of proteins shown in 2D-DIGE image. Isoforms of a unique protein were labeled with the same denotation. (B) Expression profiles of isoforms. The profiles were drawn with normalized relative volume values of isoforms (vertical axis) and harvest time (hour; horizontal axis). The normalization was carried out with GeneCluster 2.0 software. The numbers of isoforms of a unique protein and protein spot number for each isoform are shown in parentheses and the top of the figures, respectively. The relative volume values at 24 h are repeats of those at 0 h.

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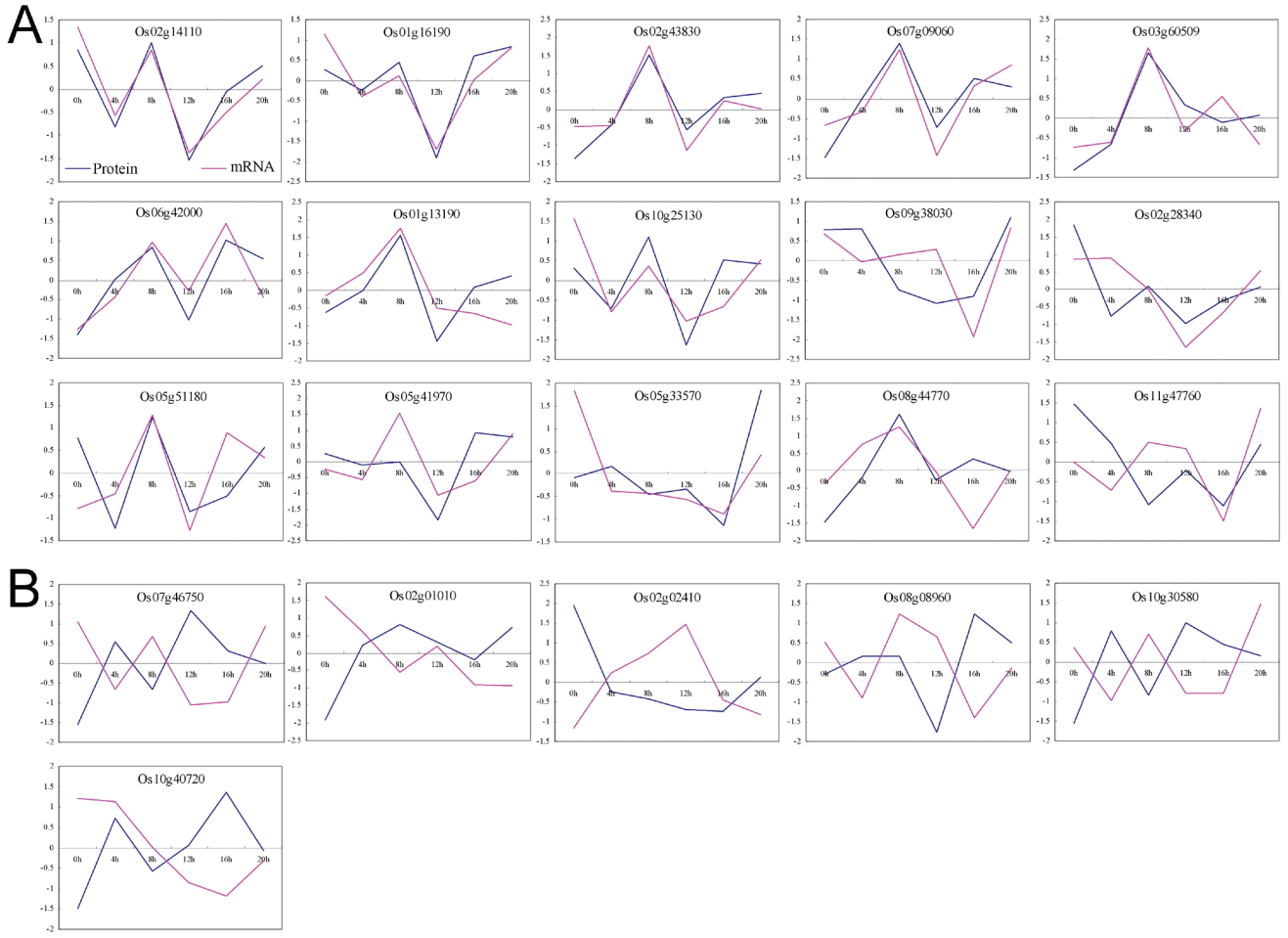

Figure 9. A comparison of expression profiles of protein−mRNA pairs. (A) Protein−mRNA pairs in which the expression profiles of proteins and mRNA were similar. (B) Protein−mRNA pairs in which the expression profiles of proteins and mRNAs were reverse. Expression levels of mRNAs were evaluated with real-time quantitative RT-PCR and 2(-ΔΔCt) method. For proteins with isoforms, the total relative volume values of isoforms were used to represent the expression of the protein. Expression values of proteins and mRNAs were normalized in GeneCluster 2.0 software, and the normalized values were used to establish the graphs.

or cell wall synthesis.[39−41] In fact, SuSy-mediated Suc mobilization is closely associated with sink strength and storage starch synthesis in the sink.[39,42,43] The SuSy-produced UDP-Glc is reversely converted to Glc-1-P by UDP-Glc pyrophosphorylase (UGPase). Then AGPase reversely converts Glc-1-P to ADP-Glc, the substrate of starch synthesis.[4,44,45] Therefore, AGPase-mediated reaction is the first key regulatory step in the starch biosynthetic pathway.[4,44,46,47] In higher plants, AGPase is a teterotetrameric enzyme composed of two small subunits (AGPS) and two large subunits (AGPL).[48,49] In rice, OsAGPS2b and OsAGPL2 are the endosperm-specific isoforms of AGPase.[50] Mutation in OsAGPS2b or OsAGPL2 reduced starch synthesis, thus leading to shrunken endosperms.[50] Our study revealed diurnally changed expression of UGPase, OsAGPL2, and pullulanase, an enzyme involved in yield and structure modification of starch.[4,20,51] All three proteins showed increased levels in dark and decreased abundance in light (c3 in Figure 6, and Figure 7). Consistently, in barley caryopses, SuSy transcripts showed higher accumulation in dark than in light.[19] The diurnal changes in these starch synthesis proteins were temporally consistent with our observation of diurnally changed Suc levels, which demonstrated in the rice endosperm that Suc content rapidly increased with the switch from dark to light, remained at a high level until the end of light, and thereafter greatly decreased in dark (Figure 2). Therefore, carbons probably mainly flows into a starch synthesis process in dark. Thus, starch synthesis is diurnally regulated, and active synthesis should mainly occur in dark in rice endosperms. In addition, luminal binding protein (spots 441 and 446) (Supplementary Table S3, Supporting Information), an endoplasmic reticulum chaperone that mediates the deposition of storage proteins into protein bodies,[52,53] coexpressed with starch synthesis-related proteins (c3; Figures 6 and 7) with significantly positive correlation (r = 0.728, Supplementary Table S6, Supporting Information), which suggests coordinated storage starch and protein accumulation in the diurnal cycle.

Diurnally Changed Redox Homeostasis in Rice Endosperms

Reactive oxygen species (ROS) can be generated in normal metabolism processes and/or under diverse internal and external conditions, such as internal and external stress, and mitochondrial respiration.[54−56] Their levels are controlled by ROS scavenging enzymes, and the resultant redox homeostasis is involved in the regulation of diverse cellular or metabolism processes.[54−57] In leaves and potato tubers, redox is the preferred mechanism

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regulating various metabolic processes, including starch metabolism, lipid synthesis, and amino acid synthesis in response to light and sugars.[58] However, under diurnal cycles, the involvement of redox in cellular and metabolism processes remains to be identified. Our study identified diurnally changed scavenging enzymes, including glyoxalase I, mitochondrial superoxide dismutase (SOD) [Mn], SOD1 [Cu−Zn], chloroplastic SOD [Cu−Zn] isoform, peroxiredoxin-2E-1 (Prx), and glutaredoxin-C6, (Supplementary Table S3). All these proteins but glyoxalase 1 showed two peaks of abundance, one in light and one in dark (c1 and c2 in Figure 6A, Figure 7, Supplementary Table S3, Supporting Information). SOD converts O_2^- to H_2O_2. Prx and glutaredoxin are both involved in scavenging H_2O_2.[56] In rice, H_2O_2 upregulates glyoxalase I level.[59,60] Glyoxalase I catalyzes methylglyoxal (MG) and GSH to *S*-D-lactoylglutathione (S-LG), and S-LG is further converted to nontoxic D-lactate and GSH by glyoxalase II.[59,61] MG, which is a potent cytotoxic byproduct mainly from triose-phosphates under normal conditions and shows increased concentration under stresses, is the primary physiologic substrate of glyoxalase.[59,62] Both mRNA and protein levels of glyoxalase I are increased in plants under stresses.[59] The coordination of diurnal changes in levels of these proteins suggests diurnally changed redox homeostasis in rice endosperms.

Numerous studies have shown the cereal endosperm is subject to a classic oxidative process, and redox regulation is essential for the synthesis and stability of storage proteins in seeds.[22,55,63] Aberrantly reduced expression of SOD in *viviparous* 1 kernels of maize was accompanied by defective endosperm development.[64] In the rice endosperm, several or most proteins involved in various function groups showed coexpressed profiles with these antioxidative proteins, such as those in the TCA cycle, protein folding and proteolysis, and amino acid metabolism (Table 1). Proteins implicated in protein folding were mainly Hsp70 and other chaperones (Figure 7, Supplementary Table S3, Supporting Information), which act as cellular stress sensors to maintain protein structure and function,[65] and those in proteolysis were components of 26S proteosome and protein hydrolase (Figure 7, Supplementary Table S3, Supporting Information). In rice, exogenously increased level of H_2O_2 enhanced protein degradation.[60] Most of the identified six proteins involved in amino acid metabolism (Figure 7, Supplementary Table S3, Supporting Information) have been found associated with stress response. Alanine aminotransferase is upregulated in response to hypoxia stress in barley and is a marker of inflammation and oxidative stress response in humans,[66−68] and aspartate aminotransferase is upregulated by oxidative stress.[69] Histidinol dehydrogenase is implicated in growth-limiting stress response in *Bacillus subtilis*.[70] Overexpression of glutamine synthetase enhanced tolerance of rice plants to salt stress.[71] Furthermore, our results showed that pyruvate dehydrogenase (E1) and dihydrolipoamide acetyltransferase (E2) (Figure 7, Supplementary Table S3, Supporting Information), two important components of the pyruvate dehydrogenase complex in the TCA cycle, had peaked in-phase abundance both in light and dark in the rice endosperm. This diurnally changed expression pattern of TCA cycle proteins in the rice endosperm differed from that in rice leaves, where TCA cycle proteins showed a decreased level in light and increased expression in dark,[9] which is consistent with the observation in bean leaves, where TCA cycle activity was inhibited in light as compared with that in dark.[72] These lines of evidence suggest that the high abundance of these proteins in-phase in light and dark may be associated with diurnal changes in the TCA cycle. Considering that the endosperm is preferred for cell development in light and for starch synthesis in dark (see Discussion above), the diurnally changed redox homeostasis, along with coordinated protein stability and turnover and metabolism, may be important for efficient starch synthesis under diurnal cycles.

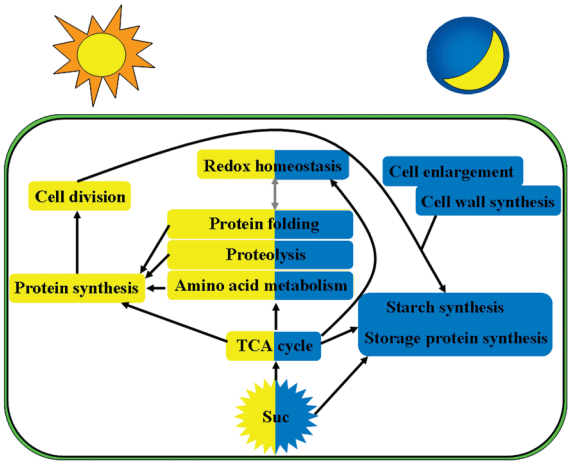

Figure 10. Schematic overview of diurnally regulated cellular and metabolic processes in rice endosperms. Processes in gold and blue areas mainly proceeded in light and dark phases, respectively.

CONCLUSION REMARKS

In cereal endosperms, starch accumulation is the most important process and requires coordinated action of diverse metabolism and cellular processes.[20,22] This study provides the first proteomic evidence of the involvement of diurnal regulation in diverse cellular and metabolic processes in cereal endosperms. Our data clearly showed a diurnal switch of cell division/enlargement and starch synthesis, which are crucial processes for endosperms to accumulate storage starch. These main outputs require coordinated actions of basic metabolisms and biochemical processes such as redox homeostasis, protein folding and proteolysis, TCA cycle, and amino acid metabolism. The coordination of diverse processes guarantees efficient synthesis and accumulation of storage starch under regulation of the diurnal cycle (Figure 10). These diurnal proteomic data provide novel insights into understanding the mechanism underlying starch accumulation and development of cereal endosperms in response to the diurnal cycle. The combination of these diurnally changed and previously published developmental proteomic data[20,22] provides integrative knowledge for further research on starch synthesis, cell proliferation, and diurnal signal transduction of cereal endosperms.

ASSOCIATED CONTENT

Supporting Information

Figure S1, experiment designs for CyDye label and DIGE images. Figure S2, PMFs-based protein identifications. Table S1, expression profile data of the 1200 spots which are present in at least 24 of the 27 images. Table S2, identification of differentially expressed proteins by MALDI-TOF/TOF-MS. Table S3, protein distribution in SOM clusters. Table S4, correlation analysis of isoform pairs. Table S5, comparison of expression profiles between identified differentially expressed proteins and

corresponding mRNAs. Table S6, correlation analysis of function groups. This material is available free of charge via the Internet at http://pubs.acs.org.

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