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Proteomics Insight into the Biological Safety of Transgenic Modification of Rice As Compared with Conventional Genetic Breeding and Spontaneous Genotypic Variation

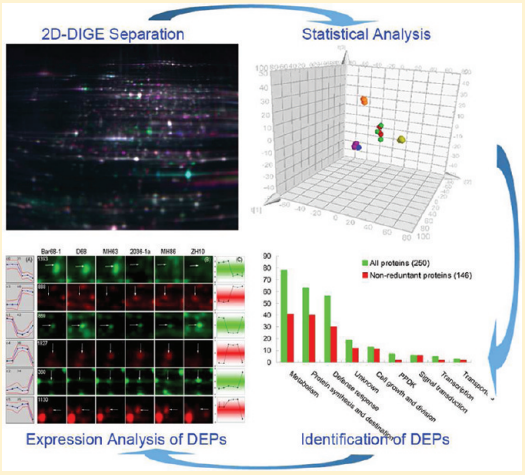
Chun Yan Gong,[†,#] Qi Li,[†,‡,#] Hua Tao Yu,[†,‡] Zizhang Wang,[†] and Tai Wang*[,†]

[†]Key Laboratory for Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences and National Center for Plant Gene Research, Beijing 100093, China

[‡]Graduate School of Chinese Academy of Sciences, Beijing 100049, China

Supporting Information

ABSTRACT: The potential of unintended effects caused by transgenic events is a key issue in the commercialization of genetically modified (GM) crops. To investigate whether transgenic events cause unintended effects, we used comparative proteomics approaches to evaluate proteome differences in seeds from 2 sets of GM *indica* rice, herbicide-resistant Bar68-1 carrying *bar* and insect-resistant 2036-1a carrying *cry1Ac*/*sck*, and their respective controls D68 and MH86, as well as *indica* variety MH63, a parental line for breeding MH86, and *japonica* variety ZH10. This experimental design allowed for comparing proteome difference caused by transgenes, conventional genetic breeding, and natural genetic variation. Proteomics analysis revealed the maximum numbers of differentially expressed proteins between *indica* and *japonica* cultivars, second among *indica* varieties with relative small difference between MH86 and MH63, and the minimum between GM rice and respective control, thus indicating GM events do not substantially alter proteome profiles as compared with conventional genetic breeding and natural genetic variation. Mass spectrometry analysis revealed 234 proteins differentially expressed in the 6 materials, and these proteins were involved in different cellular and metabolic processes with a prominent skew toward metabolism (31.2%), protein synthesis and destination (25.2%), and defense response (22.4%). In these seed proteomes, proteins implicated in the 3 prominent biological processes showed significantly different composite expression patterns and were major factors differentiating *japonica* and *indica* cultivars, as well as *indica* varieties. Thus, metabolism, protein synthesis and destination, and defense response in seeds are important in differentiating rice cultivars and varieties.



KEYWORDS: *Oryza sativa, seed proteome, 2D-DIGE, safety assessment, unintended effects, transgenes, conventional genetic breeding, natural genotypic variation*

INTRODUCTION

Since the commercialization of transgene-based genetically modified (GM) crops in 1996, the accumulated hectares of GM crops reached 148 million by 2010, for an 87-fold increase from 1.7 million hectares in 1996.[1] Thus, GM biotechnology is the fastest-adopted technology in the history of modern agriculture. However, the commercialization of GM crops has been controversial. An important issue is whether unintended effects are caused by the introduction of exogenous genes into host genomes.[2,3] Random insertion of exogenous genes in plant genomes probably results in disruption of endogenous genes and rearrangement of the genome, which could produce modified biochemical processes, new proteins (especially new allergens or toxins), or other secondary or pleiotropic effects.[4,5] These changes, in turn, lead to accumulation of undesired compounds or biochemical modifications that could interact with regulation of other biochemical pathways and alter the plant-derived products.[6,7] Therefore, evaluating whether transgenic events cause unintended changes in molecules and metabolisms, i.e., whether GM and traditional crops have "substantial equivalence", is essential to guarantee the safe use of GM crops and alleviate the fears consumers have about GM food.

Compared with targeted analysis,[8] "omics" techniques allow for simultaneously measuring and comparing the entire sets of transcripts, proteins, and metabolites in organisms, thus providing unbiased results.[9−12] Transcriptomic analysis revealed little differential expression between maize harboring a *Bt* transgene and control lines.[13,14] The leaf transcriptomic differences were negligible between barley lines carrying a transgene encoding a Chitinase or a β-glucanase and controls.[15]

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Transgenic rice expressing an antifungal protein showed a differential expression of only about 0.40% of the leaf transcriptomes as compared with controls.[16] Metabolomics was considered a replacement for conventional compositional analysis in safety assessment.[17] It has been used widely to assess the safety of barley,[15] maize,[18,19] rice,[20] and other crops.[21−23]

Besides those transcriptomic and metabolomic studies in evaluating transgenic effects, proteomics has emerged as another useful complementary tool in the safety assessment of GM crops. Studies of diverse plant species have demonstrated that changes in transcript levels are not fully followed by the same changes in protein levels.[24,25] Proteins are key players in gene function and are directly involved in metabolism and cellular development or have roles as toxins, antinutrients, or allergens. Thus, comparison of the entire proteomes of GM crops and control lines is of interest and essential to safety assessment. Comparative proteomic studies revealed "substantial equivalence" of transgenic tomato,[6,26] soybean,[27] potato,[28] and maize[29] to their nontransgenic counterparts or large impacts of exogenous genes on the tobacco host proteomic repertoire.[30]

The occurrence of unintended effects is not specific to genetic modification; it also can be found in conventional genetic breeding.[31] Conventional genetic breeding involves natural genetic variations combined with traits-based artificial selection, which induce new variability by artificial means.[4] The plant genetic structure is therefore greatly altered.[32] In addition, spontaneously occurring genetic variation during natural selection and evolution is common in all species and could cause unintended effects as well.[33] Therefore, revealing molecular differences in varieties produced by conventional genetic breeding and natural genetic variation is critical to expand the knowledge about whether GM crops are safe.

Rice (*Oryza sativa*) feeds one-fourth of world population and is one important crop. Asian cultured rice consists of 2 subspecies, *indica* and *japonica*, the classification of which was based mainly on morphological and physiological characteristics, geographic adaptation, and intervarietal hybrid fertility.[34] A variety of *indica* and *japonica* cultivars are derived from natural selection or artificial selection. In this study, we evaluated the effects of transgenes on rice seed proteomes by 2-D differential in-gel electrophoresis (2D-DIGE) combined with mass spectrometry (MS). We used rice seed because they are important sources of nutrients and essential for the human diet and thus the focus in the concern about the safety of GM rice. We used 2 sets of GM *indica* rice and controls: Bar68-1[35] transformed with herbicide-resistant gene *bar* and its nontransgenic control *indica* variety D68, and 2036-1a[36] transformed with insect-resistant genes *cry1Ac/sck* and its nontransgenic control *indica* variety MingHui 86 (MH86). Considering possible molecule changes caused by conventional genetic breeding and natural genetic variation, we also used *indica* rice MH63, which is used as a parental line for breeding MH86, and *japonica* rice ZhongHua 10 (ZH10). Therefore, the experimental design included GM rice and controls, different *indica* varieties (parental and filial), and *indica* and *japonica* cultivars, which covered our concerns about evaluating the biological safety of GM rice. We found that the effects of transgenes on rice seed proteomes were less pronounced as compared with conventional genetic breeding and natural genetic variation. Additionally, the proteins with differential expression between 4 nontransgenic varieties were mainly related to central carbon metabolism (glycolysis and tricarboxylic acid (TCA) cycle), starch synthesis, protein folding and modification, and defense response.

MATERIALS AND METHODS

Plant Materials

Mature seeds were collected from GM rice Bar68-1 and 2036-1a and their respective nontransgenic control *indica* varieties D68 and MH86. Meanwhile, seeds from *indica* cultivar MH63 and *japonica* cultivar ZH10 were also collected. All collected seeds were naturally dried and stored with desiccant at 4 °C until use.

Nucleic Acid Extraction

Genomic DNA was isolated from transgenic lines and their nontransgenic controls by the CTAB method.[37] Total RNA was isolated from mature seeds by use of RNAprep pure Plant Kit according to the manufacturer's instructions (Tiangen, China).

PCR and Semiquantitative RT-PCR

PCR was performed to confirm the presence of exogenous genes in the transgenic rice genome. Semiquantitative RT-PCR was used to check the expression of exogenous genes in transgenic rice. The gene-specific primers used were P1F (5′-CCCAAACATCAACGAATGCA-3′) and P1R (5′-CAAAGTAACCGAAATCGCTGG-3′) for *cry1Ac*; P2F (5′-CTCTTTTGTGCCTTCACCACC-3′) and P2R (5′-CTTCATCCCTGGACTTGCAAG-3′) for *sck*; P3F (5′-TTGGATCCATGAGCCCAGAACGACGC-3′) and P3R (5′-TAGAGCTCCTAAAATCTCGGTGACGGC-3′) for *bar*. The *TubA* (accession no. X91806) amplified with primers P4F (5′-TCAGATGCCCAGTGACAGA-3′) and P4R (5′-TTGGTGATCTCGGCAACAGA-3′) was used as an internal control.

Protein Preparation

Dehusked seeds were ground thoroughly in ice-cold extraction buffer (20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM EGTA, 10 mM PMSF). The mixture was centrifuged at 27,000*g* at 4 °C for 15 min to collect supernatant, and the pellet was resuspended in ice-cold extraction buffer and centrifuged. The pooled supernatants were further centrifuged at 37,000*g* at 4 °C for 15 min to remove debris. Then 1/4 volumes of ice-cold 50% trichloroacetic acid were added to precipitate proteins at 4 °C for 1 h. The proteins were collected by centrifugation at 37,000*g* at 4 °C for 15 min, washed with 80% ice-cold acetone twice, and air-dried. Resulting proteins were dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-HCl, pH 8.5), and the pH was adjusted to 8.5. After quantification by the Bradford method[38] by a DU730 Nucleic Acid/Protein analyzer (Beckman), the proteins underwent 2D-DIGE immediately or were stored at −80 °C.

2D-DIGE and Image Analysis

For each of the 6 distinct seed samples, three independent preparations of proteins were performed. Protein samples (Supplemental Figure S1A) were labeled with Cy3 and Cy5 minimal fluorescent dye according to the manufacturer's instructions (GE Healthcare), respectively. The internal standard was prepared by mixing aliquots of all analyzed samples and labeled with Cy2 minimal fluorescent dye (Supplemental Figure S1A). After a mixing with equal volumes of 2x sample buffer (8 M urea, 130 mM DTT, 4% CHAPS, 2% Pharmalyte, pH 3−10), the protein mixture containing Cy3- and Cy5-labeled proteins and the internal standard was

adjusted to a 450-μL volume by adding rehydration buffer (8 M urea, 13 mM DTT, 4% CHAPS, 0.5% Pharmalyte pH 3−10) and then loaded onto a24-cm pH 4−7 linear gradient IPG strips (GE Healthcare). Isoelectric focusing and SDS-PAGE were performed as described.[39]

All samples were separated in 9 DIGE gels, each containing 2 distinct samples and 1 internal standard (sample distribution shown in Supplemental Figure S1). Fluorescence images of proteins in gels were acquired by use of the Typhoon 9400 series Variable Mode Imager (GE Healthcare). Three images were produced from each gel by scanning at 488/520, 532/580, and 633/670 nm excitation/emission wavelengths for Cy2, Cy3, and Cy5 fluorochromes, respectively. This analysis generated 27 (3 × 9) images with similar strengths of total signal.

Statistical Analysis

Images were analyzed by use of DeCyder 6.5 software following the DeCyder User Manual (GE Healthcare) with parameters described previously.[39] Only spots reproducible in at least 21 of 27 images underwent quantification analysis. The log-standardized abundance, where the standardized abundance was derived from the normalized spot volume standardized against the intragel standard, was used for all statistical analysis. The statistical methods Student's *t* test and one way-ANOVA in the DeCyder-BVA module were used to identify proteins with significant difference in expression. Principal component analysis (PCA) involved use of SIMCA-P 12.0 to assess differences in expression of all analyzed proteins, group rice lines with overall similar expression characteristics, and identify proteins responsible for variability between groups. Cluster analysis involved use of GeneCluster 2.0 (http://www.broadinstitute.org/cancer/software/genecluster2/gc2.html) for cluster expression profiling of the identified differentially expressed proteins (DEPs).

Protein Identification

In-gel digestion and mass spectra acquisition were performed as described previously,[40] except that the matrix assisted laser-desorption ionization (MALDI) mass spectra were acquired on an Ultroflex II MALDI time-of-flight/time-of-flight mass spectrometer (MALDI-TOF/TOF MS) (Bruker Daltonics, Germany) by use of FlexAnalysis 3.3 software. Peptide mass fingerprintings (PMFs) were searched against the NCBInr protein database (http://www.ncbi.nlm.nih.gov/; NCBInr 20111105; 16,245,521 sequences) after mass spectra were transferred to BioTools 3.0 interface (Bruker Daltonics). *Oryza sativa* (134,719 sequence entries) was chosen as the taxonomic category, except that the proteins specific for Bar68-1 and 2036-1a were searched against all entries. The other search parameters were set as follows: mass accuracy 100 ppm, allowing 1 missed cleavage, fixed modification carbamidomethyl (C) and variable modifications oxidation (M) and pyro-glu (N-term Q). Protein scores >64 were significant ($p < 0.05$).

RESULTS

Integration and Expression Identification of Exogenous Genes in Transgenic Rice

Bar68-1 had two copies of herbicide-resistant gene *bar* under control of 35S promoter,[35] and 2036-1a carried insect-resistant genes *cry1Ac*/*sck* that were arranged in a T-DNA construct with maize ubiquitin promoter for *cry1Ac* and rice actin promoter for *sck*.[36] Bar68-1 line was derived directly from transgenic rice (T1) via three generations of self-pollination, and 2036-1a line was the descendant of two backcrosses to MH86 and two self-pollinations.[35,36] We further examined integration and expression of these transgenes in GM rice. PCR with specific primers (see Materials and Methods) revealed Bar68-1 had one detectable DNA fragment with a size of 568 bp, which corresponded to *bar* gene. 2036-1a line had 2 detectable DNA fragments of 1709 bp and 358 bp corresponding to *cry1Ac* and *sck* genes, respectively. These DNA fragments were undetected in their nontransgenic controls (data not shown). Thus, the two GM lines contained corresponding exogenous genes in their genomes. Furthermore, we checked the expression of exogenous genes at the transcript levels by semiquantitative RT-PCR. All 3 exogenous genes could be transcribed at different levels in mature seeds of transgenic rice (Figure 1).

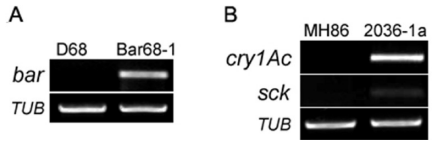

Figure 1. RT-PCR of exogenous genes expression of genetically modified (GM) Bar68-1 transformed with *bar* and 2036-1a transformed with *cry1Ac*/*sck*. (A) *bar* gene expression; (B) *cry1Ac* and *sck* genes expression. The amplified rice *tubulin A* mRNA was used as a constitutive control. *Tubulin A* was amplified for 25 cycles, and *bar*, *cry1Ac*, and *sck* were amplified for 30 cycles, respectively.

The transcript level of *cry1Ac* was higher than that of *sck* in 2036-1a (Figure 1B), and the levels of *bar* mRNA were moderate in Bar68-1 (Figure 1A). The different expression levels of these genes may be due to the difference in promoters used to control these genes.

2D-DIGE Analysis of Rice Seed Proteomes and Statistical Analysis

2-DE separation of rice seed proteins showed that strips with pH 4−7 had higher resolution than those with pH 3−10.[41] In this study, we analyzed seed proteomes from different rice lines using 2D-DIGE with pH 4−7 strips and detected about 2250 protein spots in each image (a representative image in Figure 2; all images in Supplemental Figure S1B). Statistical analysis was performed for all spots reproducible in at least 21 of 27 images (Supplemental Table S1).

PCA to investigate the similarities in the proteomes of the 6 rice lines revealed all nontransgenic varieties clearly separated from each other in principal components 1 to 3 (PC1 to PC3), but separation between transgenic lines and their controls was not obvious (Supplemental Figure S2). Thus, there was much less variation in proteomes between transgenic lines and their controls than between different *indica* varieties or between *indica* and *japonica* cultivars.

We analyzed differentially expressed proteins (DEPs) from the seed proteomes of the 6 lines by Student's *t* test and ANOVA and revealed 423 and 443 protein spots, respectively, with statistically significant differences in expression ($p < 0.01$) (Table 1). We found the largest numbers of spots with changed expression between *indica* (varieties MH63, D68, and MH86) and *japonica* (ZH10) cultivars, and a lower number between the 3 *indica* varieties, an even lower number between MH63 and MH86, and the least between transgenic lines and controls (Bar68-1 vs D68; 2036-1a vs MH86) (Table 1). Although we found several spots with changed expression between

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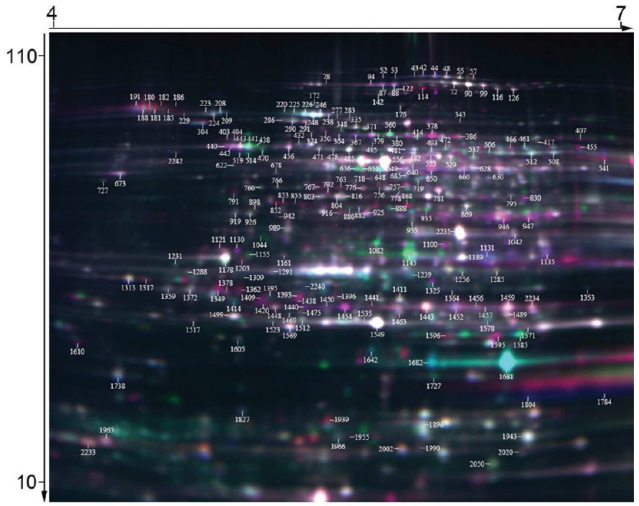

Figure 2. Representative 2D-DIGE image of rice seed proteins. Internal standard was labeled with Cy2, and proteins from ZH10 and D68 were labeled with Cy3 and Cy5, respectively. All proteins with differential expression are marked, and their identities are in Supplemental Table S2. Molecular mass (in kilodaltons) and p*I* of proteins are on the left and top of the image, respectively.

Table 1. Numbers of Proteins with Significant Differential Expression by *t* test and ANOVA in Rice Seed Proteomes[a]

	no. of spots with significant differences between varieties analyzed by *t* test ($p < 0.01$)		no. of spots with significant differences between varieties analyzed by ANOVA ($p < 0.01$)
	all	specific	
Bar68-1 vs D68	12	5	
2036-1a vs MH86	17	6	
MH63 vs ZH10	161	16	
D68 vs ZH10	159	16	443
MH86 vs ZH10	158	17	
MH86 vs MH63	99	7	
D68 vs MH63	123	17	
D68 vs MH86	152	13	

[a]"All" represents the number of all differentially expressed protein spots for each pair-wise comparison. "Specific" represents the number of differentially expressed protein spots specific to each pair-wise comparison.

transgenic lines and controls, most of them (7/12 in Bar68-1, 11/17 in 2036-1a) showed nontransgenic varietal differences (Supplemental Table S1). After eliminating overlapped proteins spots, we identified only 5 and 6 specific spots with changed expression between Bar68-1 and D68, and 2036-1a and MH86, respectively (Table 1). Together with result from PCA results, rice seed proteomes were largely unchanged with transgenic modification as compared with conventional genetic breeding and natural genetic variation.

Identification of Proteins

We used MS to analyze 264 DEP spots selected on the basis of (1) 1.2-fold changes in expression and (2) significant difference ($p < 0.01$) by both Student's *t* test and ANOVA. Among 234 successfully identified spots, 218 had a single protein each, and the other 16 had 2 proteins each. Therefore, this analysis generated 250 identities representing 146 unique proteins (Figure 3, Supplemental Table S2).

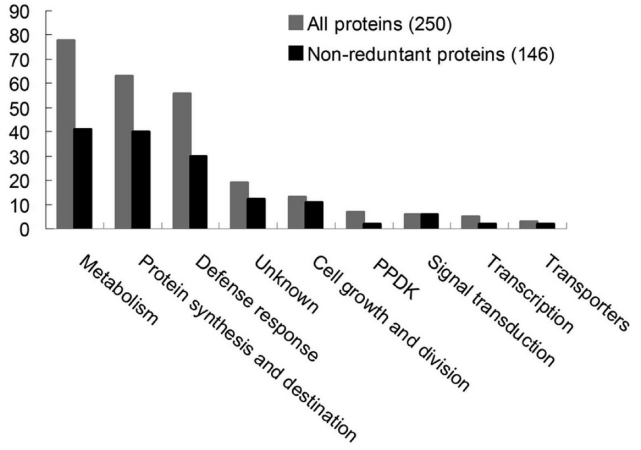

Figure 3. Functional classification of proteins with differential expression. A total of 250 proteins (gray columns) representing 146 unique proteins (black columns) were distributed in different groups. The raw data are in Supplemental Table S2.

According to annotations and a Blast search, we classified these proteins into 8 functional categories: metabolism, protein synthesis and destination, defense response, cell growth and division, pyruvate orthophosphate dikinases (PPDKs), signal transduction, transcription, and transporters (Figure 3). Proteins without defined annotations were grouped into "unknown". Most of the DEPs (78.8%) were involved in three functional categories: metabolism (31.2%), protein synthesis and destination (25.2%), and defense response (22.4%). In particular, 64.1% of the metabolism-related proteins were involved in central carbon metabolism (TCA cycle and glycolysis) and starch synthesis, and 74.6% of the protein synthesis and destination-associated proteins were involved in protein folding and modification (Supplemental Table S2). Thus, proteins implicated in central carbon metabolism, starch synthesis, protein folding and modification, and defense response showed altered expression in response to natural genetic variation, conventional genetic breeding, and transgene modification.

Hierarchical Clustering Analysis and PCA Analysis of Proteins with Differential Expression

To examine the expression changes of the identified DEPs in more detail, we analyzed the expression patterns of 218 DEPs using GeneCluster 2.0 with exclusion of spots containing 2 proteins. These DEPs were grouped into 6 clusters (c0, c1, c2, c3, c4, and c5). Interestingly, the 6 clusters were further grouped into three antagonistic pairs (clusters pairs: c0 vs c3, c1 vs c4, c2 vs c5) (Figure 4). Proteins in cluster c0 (31 identities) showed high expression in D68 and MH63, and low in MH86 and ZH10, with cluster c3 (40 identities) showing the reverse pattern. Therefore, proteins in c0c3 sets were assumed to contribute to the variability between D68/MH63 and MH86/ZH10. Proteins with lower (42 identities) or higher (36 identities) expression in the 2 mingHui varieties than in D68 and ZH10 were grouped into c1c4 sets, which possibly separated MingHui from D68/ZH10. The expression of proteins in c2 (34 identities) was higher in *japonica* (ZH10) than in *indica* rice (D68, MH63, and MH86). In contrast, the expression of 35 proteins in c5 was high in *indica* and low in *japonica* rice. The c2c5 sets may be the main contributors to the

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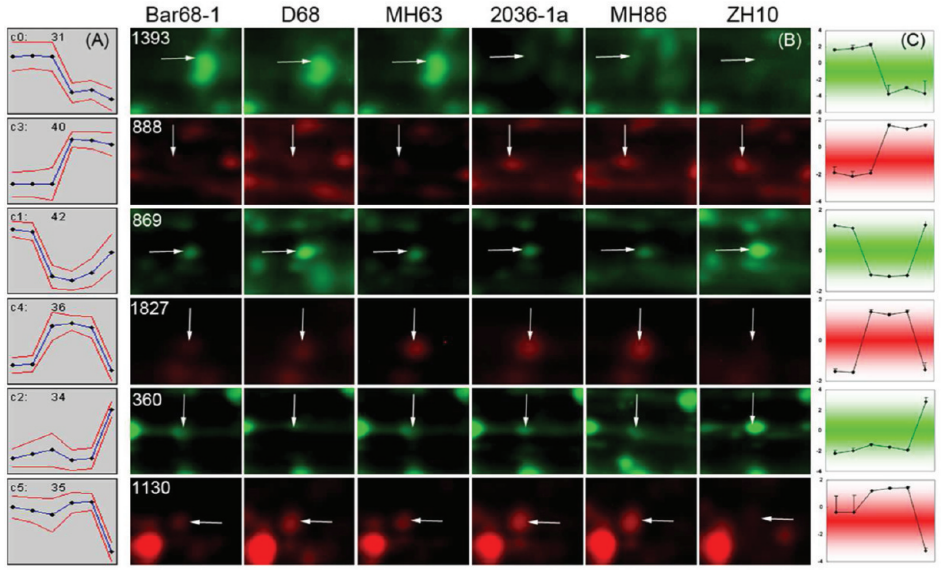

Figure 4. Hierarchical clusters of 218 differentially expressed proteins in rice seed proteomes. Spots containing 2 proteins were not included. The clusters were produced by GeneCluster 2.0. (A) The numbers in each cluster represent the numbers of proteins included in the cluster. The red lines represent the centroid data of proteins in each cluster, and the blue line represents the distances from the centroid data. Expression profiles of representative protein spots in each cluster (Spot 1393 in c0, 888 in c3, 869 in c1, 1827 in c4, 360 in c2, and 1130 in c5) in Bar68-1, D68, MH63, 2036-1a, MH86, and ZH10 are shown (B and C). Six representative proteins are indicated by arrows. Mean standardized abundances from three biological repeats were used. The raw data are in Supplemental Table S3. The sample distributions are Bar68-1, D68, MH63, 2036-1a, MH86, and ZH10 from left to right in A and C.

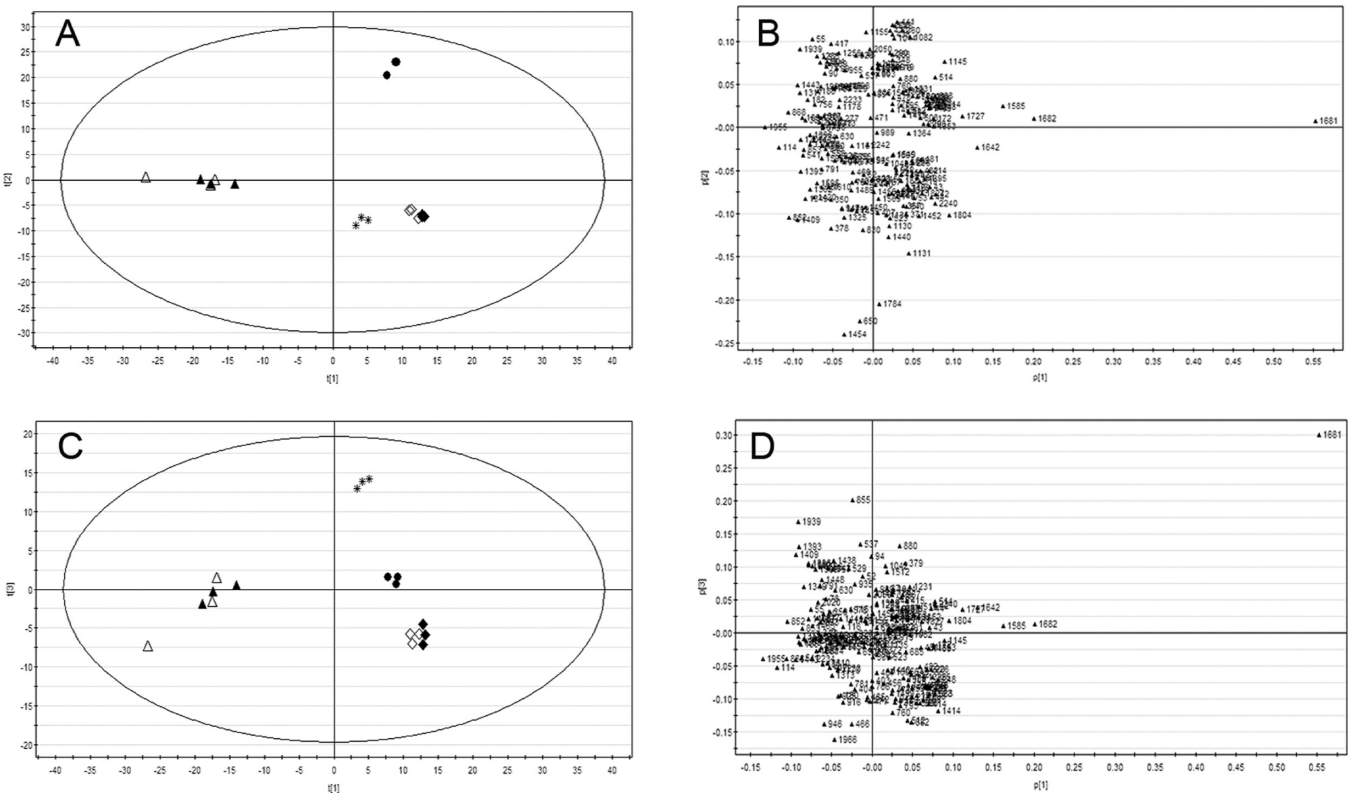

Figure 5. Principal component analysis (PCA) score plots (A and C) and loading plots (B and D) for Bar68-1 (▲), D68 (Δ), MH63 (∗), 2036-1a (◆), MH86 (◊), and ZH10 (●). The score plots show distinct separation of the 4 nontrangenic rice. The loading plots show the impact of proteins on the separation. Standardized abundances of 218 spots containing only 1 protein were used for PCA. The raw data are in Supplemental Table S3.

separation between *japonica* and *indica* rice. Despite the large changes in expression of these proteins among nontransgenic varieties, their expression was similar between transgenic lines and their respective controls in all clusters.

We further performed PCA for the 218 DEPs to estimate the contribution of DEPs to the total variability observed within rice lines and identify proteins responsible for the variability (Figure 5). The results were consistent with that of the first

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Table 2. Principal Component Analysis of Proteins with Large Loadings (Absolute Value >3) in the First Three Principal Components

spot no.	identification	function category	cluster
	First Principal Component		
114	putative seed maturation protein	cell growth and division	c1
868	methylisocitrate lyase 2	TCA pathway	c1
1585	glutathione *S*-transferase, N-terminal domain containing protein	defense response	c3
1642	expressed protein	unknown	c4
1681	late embryogenesis abundant protein	defense response	c3
1682	19 kDa globulin	defense response	c3
1955	retrotransposon protein, putative, unclassified	defense response	c1
	Second Principal Component		
286	heat shock cognate 70 kDa protein, putative, expressed	protein folding/ modification	c2
290	heat shock cognate 70 kDa protein, putative, expressed	protein folding/ modification	c2
360	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	glycolysis	c2
378	pyruvate decarboxylase isozyme 2	glycolysis	c5
438	OsPDIL1-1 protein disulfide isomerase PDIL1-1	protein folding/ modification	c2
441	OsPDIL1-1 protein disulfide isomerase PDIL1-1	protein folding/ modification	c2
470	OsPDIL1-1 protein disulfide isomerase PDIL1-1	protein folding/ modification	c2
636	UTP−glucose-1-phosphate uridylyltransferase	starch synthesis	c2
648	UTP−glucose-1-phosphate uridylyltransferase	starch synthesis	c2
650	UTP−glucose-1-phosphate uridylyltransferase	starch synthesis	c5
792	*S*-adenosylmethionine synthetase	defense response	c5
830	adenylosuccinate synthetase	nucleotides metabolism	c5
852	DJ-1 family protein	proteolysis	c0
1044	kinase, pfkB family	unknown	c2
1082	60S acidic ribosomal protein P0	protein synthesis	c2
1130	malonyl CoA-acyl carrier protein transacylase	lipid and sterol metabolism	c5
1131	glucose and ribitol dehydrogenase homologue	defense response	c4
1155	malonyl CoA-acyl carrier protein transacylase	lipid and sterol metabolism	c2
	Second Principal Component		
1325	late embryogenesis abundant protein D-34	defense response	c5
1440	triosephosphate isomerase	glycolysis	c5
1450	soluble inorganic pyrophosphatase	sugars conversion	c5
1454	triosephosphate isomerase	glycolysis	c5
1459	stress responsive A/B Barrel domain containing protein	defense response	c5
1784	MYB family transcription factor	transcription	c5
	Third Principal Component		
94	pyruvate, phosphate dikinase, chloroplast precursor	PPDK	c0
209	DnaK family protein	protein folding/ modification	c3
414	retrotransposon protein, putative, unclassified	transcription	c3
466	retrotransposon protein, putative, unclassified	transcription	c5
506	granule-bound starch synthase I	starch synthesis	c3
508	granule-bound starch synthase I	starch synthesis	c3
512	granule-bound starch synthase I	starch synthesis	c3
537	UDP-glucose 6-dehydrogenase	starch synthesis	c2
760	pro-resilin precursor	transporters	c3
795	aspartate aminotransferase	amino acid metabolism	c3
855	DJ-1 family protein	proteolysis	c0
880	DJ-1 family protein	proteolysis	c2
882	monodehydroascorbate reductase	defense response	c3
946	late embryogenesis abundant protein 1	defense response	c1
1393	desiccation-related protein PCC13-62 precursor	defense response	c0
1396	desiccation-related protein PCC13-62 precursor	defense response	c0
1414	desiccation-related protein PCC13-62 precursor	defense response	c3
1438	desiccation-related protein PCC13-62 precursor	defense response	c0
1499	chaperonin	protein folding/ modification	c3
1939	universal stress protein domain containing protein	defense response	c1
1966	ribosomal protein L7Ae	protein synthesis	c1

PCA performed on proteome profiles (Supplemental Figure S2). Score plots were used to describe the grouping of samples (Figure 5A,C), and corresponding loading plots were used to represent the impact of each protein on this grouping (Figure 5B,D). PCA confirmed our findings from cluster analysis that proteins in 3 clusters pairs (c1c4, c2c5, and c0c3) may have a distinct contribution to the entire variability of the data set. The first 3 principal components (PC1, PC2, and PC3) showed high correlation with the 3 pairs (c1c4, c2c5, and c0c3), respectively (Figures 4A and 5; Supplemental Figure S3 and Table S3). We found 52 proteins located far from the origin in the loading plots and having absolute value >3 in contribution plots in PC1 to PC3 (Table 2, Supplemental Figure S3). Among proteins contributing to PC1 separating D68/Bar68-1 from the other varieties (MH63, MH86/2036-1a, and ZH10), several proteins are involved in defense response, including late embryogenesis abundant protein (spot 1681), 19 kDa globulin (spot 1682), and glutathione *S*-transferase (spot 1585). Proteins with large loadings in PC2 separating *japonica* from *indica* varieties were mainly related to glycolysis, starch synthesis, protein folding and modification, and defense response, while starch synthesis and defense response-related proteins could distinguish between the 2 mingHui varieties in PC3 (Table 2).

Expression Patterns of Functional Categories and Subcategories

We evaluated the distribution of 218 DEPs involved in different function categories and subcategories in the cluster pairs c0c3, c1c4, and c2c5. This analysis clearly demonstrated different functional categories and subcategories-related proteins distributed heterogeneously in these clusters pairs (Table 3). For example, metabolism-related proteins were mainly in c1c4 (29/67) and c2c5 (25/67), but less so in c0c3 (13/67). Protein synthesis and destination-related proteins were more frequently in c0c3 (23/58) and c2c5 (21/58) than in c1c4 (14/58). Most of the defense response-related proteins were in c0c3 (21/48) and c1c4 (18/48), with only 9 out of 48 in c2c5. The different distribution of

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Table 3. Distribution of 218 Differentially Expressed Proteins Involved in Each Category or Subcategory in Different Clusters[a]

category or subcategy	c0	c3	**c0c3**	c1	c4	**c1c4**	c2	c5	**c2c5**	**total**
01 metabolism	5	8	**13**	12	17	**29**	11	14	**25**	**67**
01.01 sugars conversion	0	1	**1**	0	1	**1**	2	1	**3**	**5**
01.02 glycolysis	2	2	**4**	2	4	**6**	4	4	**8**	**18**
01.03 alcoholic fermentation	0	0	**0**	0	2	**2**	0	1	**1**	**3**
01.04 TCA pathway	0	0	**0**	7	2	**9**	0	0	**0**	**9**
01.05 starch synthesis	0	3	**3**	1	5	**6**	4	3	7	**16**
01.06 amino acid metabolism	0	1	**1**	2	1	**3**	0	2	**2**	**6**
01.07 nitrogen and sulfur metabolism	1	0	**1**	0	1	**1**	0	0	**0**	**2**
01.08 nucleotides metabolism	0	0	**0**	0	0	**0**	0	1	**1**	**1**
01.09 lipid and sterol metabolism	0	0	**0**	0	0	**0**	1	1	**2**	**2**
01.10 secondary metabolism	2	1	**3**	0	1	**1**	0	1	**1**	**5**
02 protein synthesis and destination	5	18	**23**	9	5	**14**	12	9	**21**	**58**
02.01 protein synthesis	0	1	**1**	3	0	**3**	2	1	**3**	**7**
02.02 protein folding and modification	1	17	**18**	6	4	**10**	9	7	**16**	**44**
02.03 proteolysis	4	0	**4**	0	1	**1**	1	1	**2**	**7**
03 cell growth and division	3	2	**5**	2	0	**2**	3	0	**3**	**10**
04 signal transduction	3	0	**3**	1	1	**2**	0	0	**0**	**5**
05 transporters	0	1	**1**	0	0	**0**	1	0	**1**	**2**
06 transcription	0	2	**2**	1	0	**1**	0	2	**2**	**5**
07 PPDK	2	0	**2**	0	3	**3**	1	0	**1**	**6**
08 defense response	13	8	**21**	11	7	**18**	3	6	**9**	**48**
09 unknown	0	1	**1**	6	3	**9**	3	4	7	**17**

[a]Numbers of proteins in cluster 0-cluster 3 (c0c3), c1c4, and c2c5 are shown in bold.

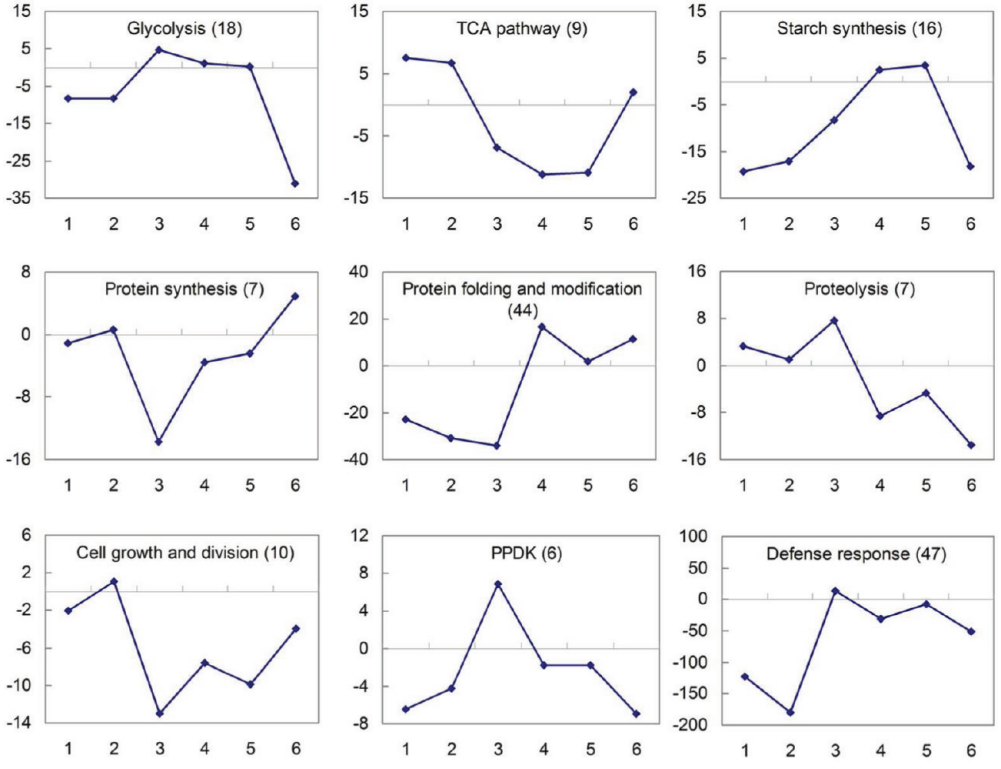

Figure 6. Composite expression profiles of protein groups associated with 9 functional categories and subcategories. The profiles were established by the sum of standardized abundances for protein components in a given functional category/subcategory in each sample. The total numbers of proteins (identities) used for the expression profiles are in parentheses. The raw data are in Supplemental Table S3. The sample distributions are Bar68-1, D68, MH63, 2036-1a, MH86, and ZH10 from left to right.

functional categories and subcategories in these clusters confirmed differences in biological processes in all analyzed rice lines.

To investigate the particular changes in biological processes as a result of natural genetic variation, conventional genetic breeding, and transgene modification, we performed expression profile analysis of protein groups associated with 9 functional categories and subcategories showing significant contribution to the differences (Figure 6). The expression patterns of proteins

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involved in glycolysis and starch synthesis were similar, with relatively high levels in 2 nontransgenic MingHui varieties and low levels in D68 and ZH10. TCA pathway-related proteins showed high expression in D68 and ZH10 and low in the other 2 nontransgenic MingHui varieties. In addition, the expression profiles of proteins involved in protein synthesis and cell division and growth were similar, which was opposite for those of the proteolysis subcategory. The 3 other major categories, protein folding and modification, defense response, and PPDKs-related proteins, showed specific expression patterns. In all analyzed groups, the expression of proteins in transgenic lines and their respective controls was changed, but the changes fell in the change ranges observed between certain nontransgenic varieties.

Expression Features of Isoforms in Functional Families

Protein isoforms are generally derived from alternative splicing or post-translational modification of the same gene product.[41−43] Among 146 unique proteins, 121 had multiple isoforms and were grouped into 37 function families consisted of 2 to 9 members (Supplemental Table S4). The molecular mass, p*I*, and expression level of isoforms varied in analyzed varieties, indicating the common presence of post-translational modification in different rice varieties having undergone distinct environments, including transformation, conventional genetic breeding, and natural evolution and selection.

DISCUSSION

To clearly understand whether transgenes in GM crops cause the unintended effects, we systematically compared seed proteomes of rice resulted from transgene, natural variation, and conventional genetic breeding by DIGE-based comparative proteomic analysis. This experimental design involved (1) GM rice and their control lines, (2) *japonica* and *indica* cultivars generated by natural variation, and (3) varieties generated by conventional genetic breeding. Thus, we could perform an objective comparison of the proteome differences of GM rice and their control line and lines produced by natural genetic variation and conventional genetic breeding.

GM Does Not Alter Proteomes of Rice Seeds As Compared with Natural Genetic Variation and Conventional Genetic Breeding

A comparison of the proteome patterns of GM rice and their control lines, including Bar68-1 and D68, and 2036-1a and MH86, allowed for evaluating the effects caused only by exogenous genes insertion. *Japonica* cultivar ZH10 and *indica* cultivars D68, MH63, and MH86 were used to investigate the genotypic variation. Genotypic variation can result from conventional genetic breeding or interactions between genotype and growing environment,[44] the inherent mechanism of which is the joint action of gene flow and selection. This joint action occurs in natural populations, and is even stronger in hybrid populations. Therefore, a comparison between *japonica* and *indica* rice allowed for investigating the effects of natural gene flow and selection on seed proteome during rice evolutionary history. A comparison between MH86 and MH63 allowed for evaluating genotypic variation of seed proteomes caused by conventional genetic breeding.

PCA analysis of proteome patterns revealed GM lines and their controls clustered together, and *japonica* and *indica* cultivars or different *indica* varieties clearly separated (Supplemental Figure S2). This finding was consistent with results of pairwise comparison showing the largest set of protein spots with differential expression between *japonica* and *indica* cultivars (Table 1). *Japonica* and *indica* subspecies are genetically divergent, although their origins are a long-standing debate.[45,46] Several studies showed that *japonica* and *indica* rice varieties possess unique metabolite composition, and rice varieties classified closely by DNA polymorphisms showed similar metabolite composition.[47] Our proteomic data are compatible with this evidence.

Furthermore, some proteins with changed expression in Bar68-1 (spot 2242) and 2036-1a (spot 506) as compared with D68 and MH86 appeared to have the same identities as those with altered expression between nontransgenic varieties, but they presented as different spots in the gel possibly because of post-translational modification. For example, spot 2242 identified as activator of 90-kDa heat shock protein ATPase homologue had the same identity as spots 541 and 380.2. Spot 506 had the same identity as spots 407, 455.2, 508, 512, and 727, identified as granule-bound starch synthase I. In addition, as compared with MH86, 2036-1a showed 3 additional spots with differential expression (spots 622, 678, and 989); spot 622 was identified as a cupin domain containing protein (spot 622.1) and a tubulin/FtsZ domain containing protein (spot 622.2). Whether spot 622.1 or spot 622.2 or both changed, they all had isoforms that also changed between varieties (Supplemental Table S4). We identified no toxins- or allergens-related proteins in GM lines. However, the expression of Glb33 (spot 1364), a 33 kDa allergen showing glyoxalase I activity,[48] was high in MH86 and low in MH63 and D68 ($p < 0.01$), and another 2 glyoxalase family proteins (spots 1291 and 1727) (Supplemental Table S3) were expressed differentially among 4 nontransgenic varieties. That we did not find transgene-encoding proteins was possibly due to the low expression of the gene in rice seeds, which has been noted in other studies.[26,49]

Together, these lines of evidence demonstrated that the integration in rice genome and expression of *bar* or *cry1Ac/sck* do not change the proteome patterns as compared with natural genetic variation and conventional genetic breeding. Thus, the 2 sets of GM rice may be "substantially equivalent" to their controls, at least from a proteomic point of view. This conclusion appeared to be supported by other observations showing nutritional quality of Bar68-1 and transgenic rice carrying *cry1Ac/sck* genes substantially equivalent to their nontransgenic counterparts.[50,51] Metabolic profiling of transgenic rice carrying *cry1Ac* and *sck* genes and their wild type controls also showed only 3 affected metabolites by gene insertion.[20]

Metabolism, Protein Folding and Modification, and Defense Response Are Key Biological Processes Differentiating Rice Varieties

PCA analysis revealed DEPs in seeds involved in metabolism, protein folding and modification, and defense response are the major contributors discriminating *japonica* and *indica* varieties, as well as different *indica* varieties (Table 2). Composite expression profiles of proteins implicated in the 3 biological processes differed significantly among varieties as well (Figure 6). Most metabolism-related proteins were associated with central carbon metabolism and starch synthesis (Table 3; Supplemental Table S2). Central metabolism is important to provide energy, cofactors and carbon skeletons for interconversion and synthesis of metabolites, and the generated metabolite concentration gradients usually act as signals to regulate diverse

processes.[52] Thus, central metabolism plays an important role in seed development and reserve accumulation.[39] Starch is the major reserve material accounting for more than 85% of the reserve material in cereal seeds.[53] Its quality and yield are determined by coordinated action of a group of enzymes.[54] Our study revealed 5 pullulanases and 6 granule-bound starch synthases (GBSS), two important enzymes for starch synthesis, with differential expression in nontransgenic varieties (Supplemental Table S3). Pullulanase is involved in yield and structure modification of starch.[54] GBSS, also called Wx protein, is encoded by rice *waxy* gene and is required for the synthesis of amylose in rice endosperms, where a proportion of amylose and amylopectin in starch granules affects the physicochemical characteristics and texture properties of rice grain.[55] Cultivars with high amylose content in grains had high levels of Wx proteins.[56] Furthermore, the expression levels of high *Mr* Wx isoforms were correlated with the amylose content of rice grains.[57] Here, the abundance of 3 pullulanases (spots 42, 43, and 44) was higher in two MingHui varieties than in D68 and ZH10, and the other two pullulanases (spots 48 and 55) showed a reverse pattern (Supplemental Table S3). The expression of Wx proteins (spots 407, 506, 508, 512 and 727, with exclusion of spot 455 containing two proteins) was largely low in D68 and MH63, and high in MH86 and ZH10, except for spots 407 and 727 with low levels in ZH10 (Supplemental Table S3). These data suggested a difference in expression of starch synthesis and modification-related proteins in varieties, thus resulting in distinct starch yields and qualities.

Immovability of plants requires the plant to have a comprehensive ability to deal with environmental stress, and the responses to stress usually cause alteration in the plant genome, transcriptome, proteome, and metabolome, which continues throughout the plant's life.[58] The rice varieties we used had distinct breeding histories and usually had optimal growing conditions, such as temperature and humidity. Therefore, they have developed diversified strategies and protective systems compatible with their own lifestyle. Consistent with these features, these varieties were distinct in the accumulation of proteins associated with defense response and protein folding and modification (Table 3). These defense response proteins were involved in multiple processes such as redox homeostasis regulation, desiccation stress response, and GSH cycles (Supplemental Table S2). The protein folding and modification category contained a large set of chaperone proteins and proteins involved in disulfide bond regulation (Supplemental Table S2), which is crucial for regulation of protein structure and function. The combination of the 2 function categories should be important to guarantee optimal adaptation of these varieties to environmental stress, although detailed mechanisms remain to be further investigated.

In conclusion, we used the sensitive 2D-DIGE approach to evaluate seed proteome variation caused by transgene, conventional genetic breeding, and natural genetic variation among diverse rice varieties, including GM rice lines and their controls and *indica* and *japonica* cultivars. Our data show GM does not significantly alter the rice seed proteomes as compared with natural genetic variation and conventional genetic breeding. Furthermore, the proteomics data of seeds indicated most of the proteins differentially expressed in nontransgenic rice varieties were implicated in central carbon metabolism, starch synthesis, protein folding and modification, and defense response, thus suggesting these processes could be important in differentiating rice varieties.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1, Experimental designs for CyDye label and DIGE images. **Figure S2**, PCA t1/t2 (A) and t1/t3 (B) score plots. **Figure S3**, Score-contribution plots of 218 identified DEP spots containing single protein showing how analyzed samples separated from each other. **Figure S4**, PMF-based protein identification. **Table S1**, Expression profile data for 1233 protein spots present in at least 21 images of 2D-DIGE analysis. **Table S2**, Identities of differentially expressed proteins determined by MALDI-TOF-TOF MS. **Table S3**, Identities, standardized abundances, and the clusters of differentially expressed proteins. **Table S4**, Isoforms of differentially expressed proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tai Wang Key Laboratory for Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, 20 Nanxincun, Xiangshan, Haidianqu, Beijing 100093, China. Tel: 0086-10-62836210. Fax: +86-10-62594170. E-mail: twang@ibcas.ac.cn.

Author Contributions

#These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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