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RESEARCH ARTICLE

Comparative analysis of panicle proteomes of two upland rice varieties upon hyper-osmotic stress

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Abstract Drought is a major environmental factor that limits the yield of rice dramatically. Upland rice is now regarded as a promising rice cultivar in water saving agriculture. Two varieties of upland rice Zhonghan 3 and IR29 were used to compare the physiological and proteomic responses to hyper-osmotic stress induced by 15% polyethyleneglycol (PEG) at the reproductive stage. Osmotic stress affected the growth development and caused the loss of production especially the grain yield. IR29 was more tolerant to PEG than Zhonghan 3 as shown by less yield loss under osmotic stress conditions. Comparative proteomic analysis of the panicle suggested that the up-regulation of glycolysis related proteins and defense proteins may contribute to the better osmotic tolerance in IR29.

Keywords upland rice, panicle proteome, grain yield, glycolysis, cytosolic ascorbate peroxidase

1 Introduction

Drought is a major production constraint factor of rice in rain-fed ecosystems. More than a half of the world's rice (*Oryza sativa* L.) is on non-irrigated lands while they produce only a quarter of total rice output (Venuprasad et al., 2007). Upland rice varieties are regarded as promising rice cultivars for their drought-tolerant characteristics (Bernier et al., 2008). The Philippines' Department of Agriculture is guiding farmers to shift from the traditional lowland rice farming toward an upland rice based production to fight the drought threat due to climate change.

Many efforts in breeding have been made to improve drought resistance in upland rice (Bernier et al., 2008). It is proven to be an effective strategy by directly selecting the yield performance under drought stress at the reproduction stage for drought tolerant rice (Venuprasad et al., 2007). Using the strategy, a large-effect quantitative trait locus (QTL) for grain yield under drought stress was isolated which can increase the harvest index, biomass yield and plant height under drought stress conditions at the reproductive stage (Bernier et al., 2007).

The complete sequencing of the genome of rice makes the functional genomic studies convenient. Different approaches such as macro and microarray, oligoarray, real-time quantitative PCR (RT-qPCR), serial analysis of gene expression (SAGE), and massive parallel signature sequencing (MPSS) were used for transcriptomic research of rice to identify and evaluate stress-responsive genes. As a complement to the genomic studies, proteomic studies give new insights into the stress response of specific cellular compartments and organelles (Salekdeh et al., 2002). It was reported that by using suppression subtractive hybridization (SSH) of cDNA libraries from a drought susceptible variety and a drought tolerant upland rice variety, several genes expressed exclusively in susceptible or tolerant variety were identified. Additionally, two-dimensional electrophoresis (2-DE) analysis of the root proteomes allowed the identification of 22 proteins associated with drought tolerance (Rabello et al., 2008). The result suggested that the drought susceptibility was related to less expression of genes involved in the protection of cell damage. Rice is particularly susceptible to water stress during reproductive growth. Drastic reduction of grain yield is caused when rice encounters water stress at its irreversible reproductive stage (Yue et al., 2006). Therefore, improvement of drought resistance is especially important and also challenging. However, most

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of the studies on drought responsive gene/protein identification were focused on lowland rice genotypes at the seedling stage. Only a few studies used upland rice and investigated the drought stress responses at the reproductive stage. Here, we used two upland rice varieties, Zhonghan 3 and IR29 that are different in hyper-osmotic tolerance, to compare the proteomic responses to polyethyleneglycol (PEG) induced water deficit at the reproductive stage, aiming at identification of the functional proteins that may be associated with stress tolerance abilities.

2 Materials and methods

2.1 Plant materials and stress treatment

Zhonghan 3 and IR29 were germinated in water for 10 days, and then cultivated with Kimura B solution in a phytotron (photo flux density of 300–350 $mol \cdot m^{-2} \cdot s^{-1}$, 60%–80% relative humidity, 16/8 h day night cycle, (28±2)°C). When the plants were grown to the sixth stage of panicle primordial differentiation (Sun et al. 1993), the seedlings were treated with 15% PEG 6000 for 10 days followed by cultivation in Kimura B solution till harvest. Plant height (PlH) was measured before harvest. Overground parts were dried. Biomass (BiM), fertility (Fer) and grain yield (GrY) per plant were examined. For proteomic analysis, panicles at the same developmental stage were collected on the 3rd day of treatment with untreated panicles as controls.

2.2 Protein extraction and 2-DE

Protein extraction was performed as described with some modifications (Yan et al. 2005). Samples were ground in liquid nitrogen and incubated in ice-cold 10% w/v trichloroacetic acid (TCA)/acetone with 0.07% w/v dithiothreitol (DTT) at −20°C for 1 h, and then centrifuged at 35 000 × *g* for 25 min. The pellets were suspended in ice-cold acetone with 0.07% w/v DTT at −20°C for 1 h and centrifuged at 12 000 × *g* for 25 min. This step was repeated three times and the pellets were lyophilized. The resultant powder was solubilized in lysis buffer consisting of 9 mol/L urea, 4% w/v CHAPS, 1% w/v DTT, 1% v/v pH 3–10 immobilized pH gradient (IPG) buffer. Protein concentration was quantified by the Bradford assay with bovine serum albumin (BSA) as the standard. For isoelectric focusing (IEF), the Ettan IPGphor system (Amersham Biosciences, Uppsala, Sweden) and pH 4–7 IPG strips (18 cm, linear) were used according to the manufacturer's recommendations. The IPG strips were rehydrated at 50 V for 12 h in 350 μL rehydration buffer (8 mol/L urea, 20 mmol/L DTT, 2% w/v CHAPS, 0.5% v/v pH 4–7 IPG buffer) with 100 μg protein. The IEF conditions were set as follows: 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 1 h and 8000 V for 6 h. Each gel strip was equilibrated for 15 min in 5 mL equilibration buffer (50 mmol/L Tris-HCl buffer, pH 8.8, 6 mol/L urea, 30% v/v glycerol, 2% w/v sodium dodecyl sulfate (SDS), 1% w/v DTT and trace bromophenol blue grains). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% gels using the PROTEAN II XL Cell system (Bio-Rad, Hercules, CA, USA). The gels were run at 20 mA per gel for the first 30 min and followed by 30 mA per gel. The protein spots were visualized by silver staining.

2.3 Image acquisition and data analysis

The silver-stained gels were scanned using Fluorescent Image Analyzer FLA-9000 (Fujifilm, Japan). 500 V was applied for the photo-multipliers (PMT) as an integral value within the predetermined range. The pixel size was set at 100 μm for reading. Spot detection and quantification were done using Progenesis SameSpots (Nonlinear, USA). Only the spots with reproducible 2-fold changes in normalized percentage volume (%vol) and shown statistical significance ($P < 0.01$) were considered to be differentially expressed protein spots.

2.4 Liquid chromatography coupled with tandem mass spectrometry (LC-MS)/MS analyses and database search

Protein spots were excised from silver-stained gels. The gel was first washed twice with water, destained in solution (15 mmol/L $K_3Fe(CN)_6$, 50 mmol/L NaS_2O_3) and again washed with water. The gel was dehydrated in 50% (v/v) acetonitrile (ACN) and 100% (v/v) ACN and dried by vacuum centrifugation. The sample was in-gel digested with 0.02 μg/μL trypsin and 25 mmol/L NH_4HCO_3 in 10 μL of digesting solution overnight at 37°C and the peptides were lyophilized. The lyophilized peptide was dissolved in 0.1% trifluoroacetic acid (TFA) and mass spectrometry (MS) analysis was conducted with a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF mass spectrometer 4800-plus Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA).

Database search was performed by MASCOT (V2.1, Matrix Science, London, UK) and the local database is NCBInr containing 2 367 365 protein sequences and 802 797 248 amino acid sequences (updated in July, 2008). The search parameters were set as follows: using trypsin as the digestion enzyme, allowing up to 1 missed cleavage, carbamidomethyl (Cys) and oxidation (Met) as variable modification, no fixed modification, peptide tolerance is 60 ppm and MS/MS tolerance is 0.25 Da. The keratin contamination was removed and the MASCOT score which was above 65 ($P < 0.05$) or the secondary monoisotopic mass score which was above 30 ($P < 0.05$) was considered as positive hit.

3 Results

3.1 Physiological responses of Zhonghan 3 and IR29 to the hyper-osmotic stress

We first analyzed the change in productivity after the PEG treatment. The growth and development of both varieties were affected by the stress treatment as shown in decreased plant height, biomass, fertility and grain yield (Fig. 1). The plant heights were decreased for both varieties as compared to their controls and no difference was observed between the two varieties (Fig. 1A and B). The biomass decreased for Zhonghan 3 but not for IR29 (Fig. 1C), whereas the fertility and grain yield decreased for both varieties, but the reduction was more significant for Zhonghan 3 (Fig. 1D and E). In IR29, the grain yield of stressed plant was approximately 79.5% of the untreated control, while it was decreased to 53.5% in Zhonghan 3 (Fig. 1F). These data suggested that IR29 is more tolerant to hyper-osmotic stress induced by PEG than Zhonghan 3 at the panicle primordial differentiation stage.

3.2 2-DE analysis of the differentially expressed proteins under hyper-osmotic stress

To compare the differentially expressed proteins in response to the PEG treatment which might be related to the different stress tolerance in the two varieties, a 2-DE based proteomic approach was used. More than 800 spots were reproducibly detected on silver-stained gels. Among them, 38 spots showed significant changes ($P < 0.01$) after stress treatment (Fig. 2). Quantification of the expressional changes of the 38 spots in IR29 and Zhonghan 3 varieties was provided as supplemental information. Twenty-six differently expressed protein spots were found in Zhonghan 3, including 10 up-regulated and 16 down-regulated ones, respectively (Fig. 2A and B). In IR29, there were 20 differentially expressed spots, including 14 up-regulated and 6 down-regulated ones (Fig. 2C and D). According to their expression patterns, these spots could be divided into 6 groups as shown in Fig. 3. Groups A and B were only up- or down-regulated in Zhonghan 3 after PEG treatment (Fig. 3A and B). Groups C and D were only up- or down-regulated in IR29 (Fig. 3C and D). There were 5 common up-regulated spots (31–35) (Fig. 3E) and 3 common down-regulated spots (36–38) (Fig. 3F) in both varieties, suggesting similar cellular responses induced by the hyper-osmotic stress.

3.3 Identification of differentially expressed proteins

Mass spectrometry analysis allowed the identification of 22 out of the 38 differentially expressed proteins. They were classified into 4 functional categories (Table 1). Seven proteins were involved in energy production and metabolism, respectively. Seven proteins were involved in defense. Interestingly, 5 defense proteins were cytosolic ascorbate peroxidases (APX), among which 3 were APX1 (spots 22, 24 and 25) and 2 were APX2 (spots 11 and 35). One protein was found to be related to protein destination.

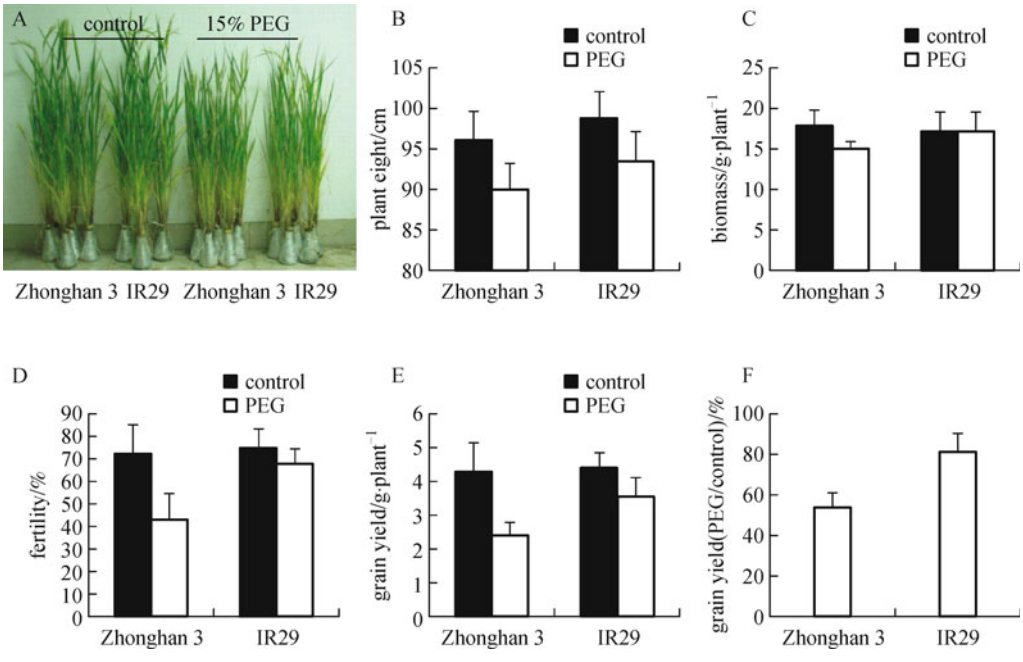

Fig. 1 The effects of osmotic stress on Zhonghan 3 and IR29. Growth of the rice was inhibited by polyethyleneglycol (PEG). The photo was taken 10 days after treatment with 15% PEG (A). The plant height (B), biomass (C), fertility (D), grain yield (E) and drop index of grain yield (F) were plotted. The experiment was performed with 6 plants and repeated for 3 times. The values were mean±SD of 18 plants from 3 replicates.

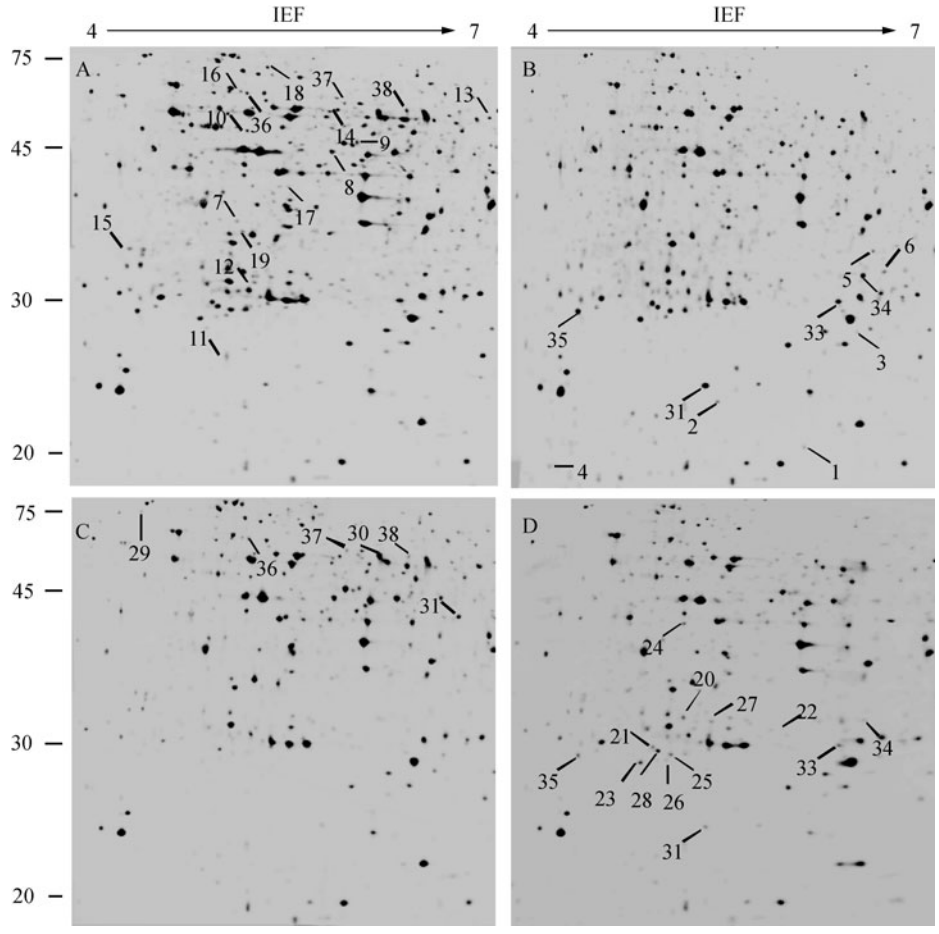

Fig. 2 Two-dimenosional electrophoresis (2-DE) analysis of proteins extracted from panicles of Zhonghan 3 and IR29. (A) and (B) are panicle proteins from Zhonghan 3 at normal growth conditions and treated with 15% PEG 6000 for 3 days, respectively. (C) and (D) are panicle proteins from IR29 at normal growth conditions and treated with 15% PEG 6000 for 3 days, respectively. The osmotic stress responsive proteins are numbered. IEF: isoelectric focusing.

4 Discussion

Drought is a major limiting factor for the crop productivity and therefore, investigation of the drought stress response aimed at identifying the functional genes and proteins in drought stress tolerance is a hot topic. For research purposes, drought stress can be triggered by withholding of irrigation or hyper-osmotic stress (PEG and mannitol, etc.), which imposes water deficit to plants. Because of the fact that PEG treatment is easier to manipulate and to control the strength of the stress in experimental repetitions, it is widely used in the physiological and molecular analysis of the responses in plants at water deficit conditions. It was reported that osmotic stress induced by PEG or mannitol caused a sustained inhibition of the growth in emerging first leaves of intact rice and changed the phenotypes of rice (Lu and Neumann, 1999; Zang and Komatsu, 2007). In the current study, we treated the rice with hyper-osmotic stress induced by 15% PEG 6000 to mimic the water deficit effect and study the proteomic response of two rice varieties. In agreement with the previous report that rice is especially sensitive to drought stress at the reproductive development stage (Liu et al., 2007), 10-day-PEG treatment at the reproductive stage significantly affected spikelet fertility, grain filling and final yield (Fig. 1). Although other researches suggested that Zhonghan 3 is drought tolerant and IR29 is salt sensitive (Xie et al., 2000; Liu et al., 2006), IR29 is more tolerant than Zhonghan 3 to hyper-osmotic stress in our study. Proteomic analysis revealed 38 differently expressed protein spots. The stress tolerant and susceptible varieties only shared 8 common responsible protein spots (Fig. 3E and F). Thirty spots showed unique expression pattern in response to PEG in two varieties (Fig. 3).

4.1 Energy production related proteins

Seven protein spots were identified as energy production related proteins including 5 different proteins (Table 1). Two proteins (spots 2 and 31) are photosynthesis related

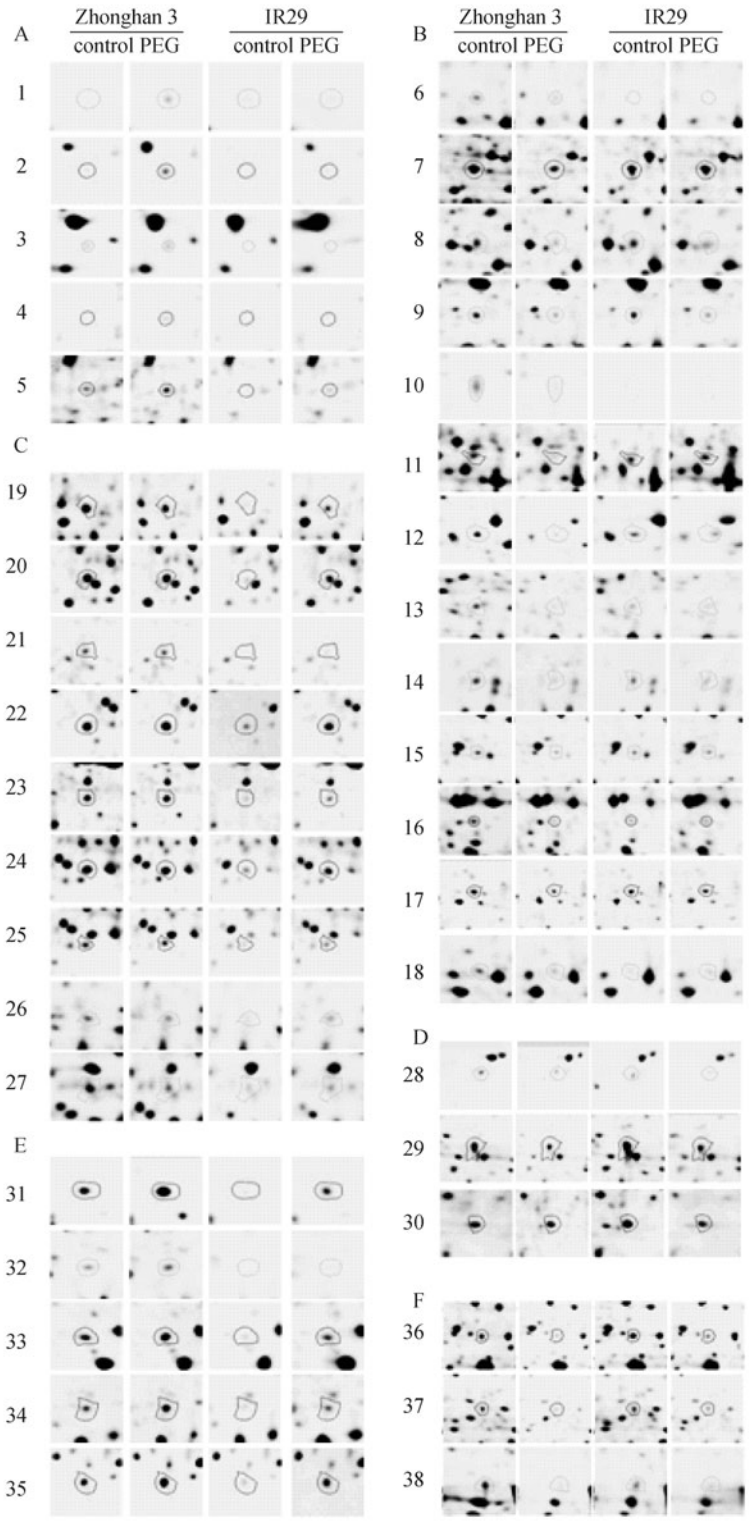

Fig. 3 Close-up view of 38 differentially expressed protein spots. A–F corresponds to 6 groups. A: Spots up-regulated only in Zhonghan 3; B: spots down-regulated only in Zhonghan 3; C: spots up-regulated only in IR29; D: spots down-regulated only in IR29; E: spots up-regulated in both varieties; F: spots down-regulated in both varieties.

Table 1 Differentially expressed proteins identified by liquid chromatography with tandem mass spectrometry (LC-MS)/MS

spot no.	accession no.	protein name	score	peptide	theoretical Mr/pI	observed Mr/pI	group[a]	expression change in ZH3/IR29[b]
				ENERGY				
2	NP_001058863	23 kDa polypeptide of photosystem II	284	EFPGQVLR QYYSVTVLTR TNTEFIAYSGEGFK	26.92/8.66	21.00/5.56	A	13/–
7	NP_001046020	phosphoglycerate kinase, cytosolic	427	YSLKPLVPR LAAALPEGGVLLLENVR LAAVADLYVNDAFGTAHR	42.08/5.64	44.80/5.87	B	3.3/5
20	NP_001042016	triosephosphate isomerase	99	WLAANVSAEVAESTR VATPDQAQEVHDGLRK	27.59/6.60	28.50/5.20	C	–/5.3
21	CAE02009	glyceraldehyde-3-phosphate dehydrogenase	93	GILGYVEEDLVSTDFQGDNR	42.03/6.41	31.00/5.90	C	–/2.6
23	NP_001046020	phosphoglycerate kinase, cytosolic	112	LAAALPEGGVLLLENVR	42.08/5.64	42.02/5.42	C	–/4.1
31	NP_039391	ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit	286	DTDILAAFR LTYYTPEYETK TFQGPPHGIQVER	52.85/6.22	22.03/5.50	E	6.1/9.9
34	AAA82047	glyceraldehyde-3-phosphate dehydrogenase	473	VPTVDVSVVDLTVR LVAWYDNEWGYSNR GIIGYVEEDLVSTDFVGDSR	36.47/6.61	32.50/6.32	E	2.4/3.1
				METABOLISM				
4	NP_001065060	glycine decarboxylase complex H-protein; hypothetical protein	228	YSSSHEWVK VKPSSPSELDALLDPAK KLSETPGLINSSPYEDGWMIK	17.36/4.92	17.51/4.61	A	13/–
5	NP_001053928	aminomethyl transferase, mitochondrial precursor	249	WHVHDER DGTGTLTVFTNDR TALYDFHVAHGGK TGYTGEDGFEISVPSENAVDLAK	43.94/8.53	34.34/6.41	A	2.5/–
16	NP_001052013	arginase	224	VIDASLTLIR VLTDVGDVPIQEIR DVLNILHNLQGDVVAGDV-VEFNPQR	36.92/5.90	41.08/5.63	B	0.36/–
29	NP_001051328	UDP-glucose 6-dehydrogenase	402	NLFFSTDVEK AADLTYWESAAR IYDNMQKPAFVFDGR AQISIYDPQVTEDQIQR	52.87/5.79	60.00/6.10	D	–/0.24
30	BAD22334	putative acetyl-CoA C-acyltransferase	92	NSGAFAWEIVPIEVPVGR	41.00/6.15	43.00/6.49	D	–/0.5
33	NP_001049723	UDP-glucuronic acid decarboxylase	378	QHGIEIR IFNTYGPR VVSNFIAQAVR GEPLTVQKPGTQTR	39.28/7.16	29.00/6.20	E	3/7.2
37	Q6K669	leucine aminopeptidase 2, chloroplastic	478	DVEFSEWK GLTFDSGGYNIK YANDLSSGVIFGK GDILAIAVTENDLVK QVDLIGFGSGPEVDQK	61.78/8.29	60.00/5.90	F	0.19/0.29
				DEFENSE				
11	NP_001060741	APX2	593	QDKPEPPPEGR LPDATQGSDHLR LAWHSAGTFDVSSR	27.10/5.21	28.0/5.4	B	0.24/–
17	NP_001048274	heat shock 70 kDa protein	667	HLNITLTR EVDEVLLVGGMTR AVITVPAYFNDAQR SQVFSTAADNQTQVGIR	72.85/5.49	73.00/5.51	B	0.42/–

(*Continued*)

spot no.	accession no.	protein name	score	peptide	theoretical Mr/pI	observed Mr/pI	group[a]	expression change in ZH3/IR29[b]
19	BAB71741	glyoxalase I	111	GNAYAQVAIGTEDVYK	32.53/5.51	30.00/5.37	C	–/7.8
22	XP_470658	putative ascorbate peroxidase (APX1)	417	SGFEGPWTR LAWHSAGTFDVSSK ALLSDPAFRPLVEK TPAELSHAANAGLDIAVR	27.14/5.42	26.00/5.15	C	–/4.5
24	NP_001049769	APX1	192	TPAELSHAANAGLDIAVR	27.14/5.42	27.00/5.30	C	–/3.6
25	NP_001049769	APX1	410	EDKPAPPPEGR AFFEDYKEAHLK TPAELSHAANAGLDIAVR	27.14/5.42	26.53/5.30	C	–/3.3
35	NP_001060741	APX2	298	LAWHSAGTFDVSSR YAADEDAFFADYAEAHLK	27.10/5.21	27.02/4.80	E	2.6/2.8
				PROTEIN DESTINATION				
36	BAA88950	importin alpha 1b	410	SPPIEEVINTGVVPR GKPQPNFEQVKPALSALQR	58.50/5.18	62.00/5.40	F	0.14/0.30

a: The spots were classified into six groups (A–F) according to the expression profiles shown in Fig. 3. b: The expression change was calculated by the spot %vol after PEG treatment/%vol of the control of the same variety. Mr: molecular weight; pI: isoelectric point.

proteins. Both of the proteins were up-regulated but showed lower Mr than their theoretical ones (Table 1), suggesting that these two spots were the products of protein partial degradation. In consistence with previous reports, the components of photosynthesis apparatus are sensitive to environmental stresses. Degradation of the photosynthesis related proteins may directly affect the photosynthesis rate (Yan et al., 2006).

The other 3 proteins are involved in glycolysis (Table 1). Glycolysis plays an important role in life cycle in resisting all kinds of stresses (Abbasi and Komatsu, 2004; Suzuki et al., 2005; Agrawal and Rakwal, 2006; Xiao et al., 2009). Triosephosphate isomerase (TPI), which catalyzes the inter-conversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP), is on the fifth step of glycolysis. Several reports showed that a variety of stresses induced TPIs which can produce more energy and photosynthetic products to cope with these dangerous situations and permit the plant's survival (Riccardi et al., 1998; Del Buono et al., 2009).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the next step of TPI to break down glucose for energy and carbon molecules. Phosphoglycerate kinase is a transferase enzyme that transfers a phosphate group from 1,3-biphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate. *OsGAPDH* was shown to be induced by drought (Pillai et al., 2000). These data suggested that enhanced rate of the glycolysis was one of the immediate cellular responses to water deficit in plants, which could fulfill the energy demand in stress conditions and during recovery (Velasco et al., 1994). Interestingly the three enzymes which function in the consecutive steps in glycolysis pathway were induced by hyper-osmotic stress mainly in IR29 variety, indicating the difference in efficiency of energy supply in the two varieties. This may explain why IR29 suffered less from PEG stress than Zhonghan 3.

4.2 Proteins involved in metabolism

Seven spots were identified to be 6 different proteins involved in metabolism (Table 1). These proteins are involved in the metabolism of amino acid and protein (spots 4, 5, 16 and 37), sugar (spots 29 and 33) and fatty acid (30), etc.

The mitochondrial glycin decarboxylase complex (GDC) including P-, H-, T-, and L-proteins is a multi-enzyme system that catalyzes glycine to serine during the course of photorespiration. Among them H-protein acting as a mobile substrate and commuting between the other three proteins plays a pivotal role while the T-protein as an aminomethyltransferase transfers methylamine in the GDC catalytic cycle (Vauclare et al., 1996; Douce et al., 2001). Evidence has shown that a cytosolic product of lipid peroxidation, 4-hydroxy-2-nonenal (HNE), rapidly inhibits the activity of GDC and therefore GDC is a major target for the oxidative damage in the mitochondria in plants (Taylor et al., 2002). However, it is not clear for the reason that two components of the GDC, namely the H-protein (spot 4) and the T-protein (spot 5), were up-regulated only in the Zhonghan 3 variety.

Arginase is one of the key enzymes in the ornithine pathway of proline biosynthesis. Drought and hyper-osmotic stress as well as oxidative stress could induce the proline biosynthesis and lead to a significant proline accumulation in plant cells, which is served as osmolite to protect cellular damage from dehydration (Jubault et al., 2008). Different from the previous report that arginase

activity was increased after stress treatment (Yang et al., 2009), the arginase (spot 16) was found to be down-regulated in Zhonghan 3. Decreased amount of arginase is not favored for the biosynthesis of proline and therefore might be a reason why Zhonghan 3 is less tolerant to PEG treatment than IR29.

Both UDP-glucose-6-dehydrogenase (UGDH) and UDP-D-glucuronic acid decarboxylase (UXS) are important for cell wall synthesis. UGDH converts UDP-glucose to UDP-glucuronate and thereby participates in the biosynthesis of glycosaminoglycans. In plants, UGDH is an important enzyme in the synthesis of hemicellulose and pectin (Johansson et al., 2002), which are the components of newly formed cell walls. UXS catalyzes the conversion of UDP-D-glucuronate to UDP-D-xylose. Arabinoxylans in crop plants are the major sugar components of the cell walls, and UDP-xylose is a key substrate in the biosynthesis of xylans. Seven OsUXS were found in rice genome including the cytosolic and membrane bound forms. The *OsUXS* genes were changed in the developing rice seeds and the enzymatic activities were correlated well with the protein abundance (Suzuki et al., 2004; Zhang et al., 2005). Here we found that the OsUXS protein was up-regulated by the PEG treatment in both of the two varieties, suggesting that OsUXS activity is important for the developing seeds upon hyper-osmotic stress.

4.3 Stress defense proteins

In our experiment, 7 spots were identified as stress defense proteins. They are an HSP70, a glyoxalase I and 5 ascorbate peroxidases (APX) including 3 APX1s and 2 APX2s (Table 1).

The glyoxalase system, which consists of glyoxalase I (Gly I) and glyoxalase II (Gly II), catalyzes the conversion of methylglyoxal (MG) to D-lactate through a glutathione (GSH)-glutathione disulfide (GSSG) cycle (Thornalley, 1990). MG is a cytotoxic by-product produced mainly from triose phosphates and the level increases significantly in plants in response to different abiotic stresses (Yadav et al., 2005a). Transgenic plants overexpressing Gly I or Gly II or both can resist an increase in MG and improve stress tolerance (Yadav et al., 2005b; Singla-Pareek et al., 2006). We found that both TPI (spot 20) and Gly I (spot 19) were up-regulated in IR29 after stress treatment, but not in Zhonghan 3 (Table 1 and Fig. 3). The coordination of the two enzymes in expression upon hyper-osmotic stress may contribute to the release of the toxicity of MG and therefore enhance the tolerance of IR29 variety.

All kinds of stresses including biotic and abiotic stimulate the generation of reactive oxygen species (ROS), which cause oxidation damage (Király, 1998; Smirnoff, 1998). APX is thought to play the most essential role in scavenging ROS and protecting cells against these toxic effects in higher plants, algae, euglena and other organisms (Lu et al., 2007). There are at least 8 APX isoenzymes in plants (Shigeoka et al., 2002). We found 5 APX isoforms (spots 11, 22, 24, 25 and 35) with similar Mr and different pIs (Fig. 2). Except for spot 11, which was down-regulated by stress treatment in Zhonghan 3, 4 APX isoforms were up-regulated in IR29 and only one APX2 (spot 35) was up-regulated in Zhonghan 3. The different APX expression patterns in the two varieties correlated well with the difference in osmotic stress sensitivity. Intriguingly, the APXs were expressed at a higher level in Zhonghan 3 (spots 22, 24, 25 and 35) at normal and even stressed conditions; however, the induction was only significant in IR29 (Fig. 3). It is possible that the induction of APX upon stress conditions rather than the basal expression level is more important for plant stress tolerance. Further biological experiments are worth carrying out to test the hypothesis.

4.4 Proteins for protein destination

Importin is involved in nucleocytoplasmic protein transport. Proteins containing classic nuclear localization signals (NLS) are initially recognized and bound in the cytoplasm by the NLS receptor which consists of importin alpha and beta heterodimer, and subsequently translocated into the nucleus mediated by GTPase. Multiple isoforms of importin alpha were identified in many vertebrates and plants with different substrate preference. In rice, constitutive photomorphogenesis 1 (COP1) protein bears a bipartite-type NLS and its nuclear transport is mediated preferentially by importin alpha 1b (Jiang et al., 2001). A proteomic analysis of wheat response to salt and drought stress also showed the up-regulation of importin alpha 1b in stressed roots (Peng et al., 2009). Here we found that the importin alpha 1b (spot 36) was down-regulated in stressed panicles in both Zhonghan 3 and IR29 varieties, suggesting a down-regulation of protein nucleocytoplasmic transportation.

In summary, comparative proteomic analysis of two upland rice varieties, Zhonghan 3 and IR29, in response to hyper-osmotic stress at the reproductive stage allowed us to identify stress responsive proteins. The differentially expressed proteins especially in groups B and C showed a good correlation in expression and stress tolerance abilities. These proteins are mainly involved in stress defense (APXs) and energy generation (glycolysis enzymes), suggesting the importance for maintaining the energy supply and removing the ROS at the stress conditions. Improving the stress tolerance of crops is a big project for plant biologists in the changing environment. Our study provides some starting materials for the future in-depth investigation of stress tolerance mechanisms and engineering of stress tolerance crops. For example, it will be worthy to express the APX in rice under the control of stress inducible promoter rather than the constitutive promoter and test the stress tolerance. In addition, some of differentially expressed proteins have the

potential to be served as protein markers in the stress tolerant rice breeding.

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