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ORIGINAL RESEARCH

Proteomic Analysis of Salt Stress Responses in Rice Shoot

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Abstract To gain a better understanding of the mechanism of rice (*Oryza sativa* L.) in response to salt stress, we performed a proteomics analysis of rice in response to 250 mM NaCl treatment using shoots of 3-day-old nascent seedlings. The changes of protein patterns were monitored with two-dimensional gel electrophoresis. Of 57 protein spots showing changes in abundance in response to salt stress, 52 were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The identified proteins were classified into eight functional categories. Several novel salt stress-responsive proteins, including protein synthesis inhibitor I, photosystem II stability/assembly factor HCF136, trigger factor-like protein and cycloartenol-C24-methyltransferase are upregulated upon salt stress. In order to figure out the different and similar molecular mechanism among salt and other stresses, regulation of some salt responsive proteins under other abiotic stress (cold and dehydration) and abscisic acid application was also analyzed. The possible molecular mechanism of rice seedlings in response to salinity and other stresses were discussed.

Keywords Mass spectrometry · *Oryza sativa* · Proteomics · Salt stress · Two-dimensional gel electrophoresis

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Introduction

Rice (*Oryza sativa* L.), as one of the major cereal crops, serves the staple food for more than one third of the world's population. However, rice is grown under different agro-ecological conditions and easily subjected to salt stress throughout its life cycle. Salt stress is one of the serious environmental stresses in agricultural production all over the world. Understanding the mechanisms through which plant cells cope with salt stress is essential for the improvement of crop salinity tolerance by genetic engineering (Xiong et al. 2002). Plant responses to salt stress have been extensively studied involved in structural, physiological, and biochemical levels of most life processes in plants, such as photosynthesis, protein synthesis, energy, and lipid metabolism (Flowers et al. 1977; Zhu 2002; Pandit et al. 2010). Plant response to salinity is a very complicate biological process, including regulation and compartmentalization of different ions, biosynthesis of compatible solutes, induction of antioxidative enzymes, and plant hormones, etc. Physiological or metabolic regulations at the cellular level are the main responses amenable to the alteration in gene expression, thus an effective way to study the cellular responses is to examine gene expression at the mRNA level in a large scale using techniques such as cDNA microarrays, serial analysis of gene expression, cDNA-amplified fragment length polymorphism and massively parallel signature

sequencing (Breyne and Zabeau 2001). A large numbers of stress-inducible genes had been identified (e.g., Kawasaki et al. 2001; Rabbani et al. 2003; Sun et al. 2010). Undoubtedly, studying the rice gene expression under salt stress has deepened our understanding on the mechanism of salt response.

However, the techniques applied above can only provide us limited insights on the regulation of final gene products, i.e., proteins, which are the true executor of physiological function in cell. A high level of the mRNA of one gene is not necessarily correlated with a high level of the protein (Yang and Komatsu 2004; Yan et al. 2006; Lee et al. 2009). Furthermore, in order to localize and function properly in cell, some proteins have been reported to undergo post-translational modifications, which could not be detected at mRNA level. Proteomics is an arising powerful tool for the investigation of complex cellular process (Jorrín et al. 2007). Unlike the genome, which is essentially the same in all somatic cells of an organism, the proteome is a dynamic entity that is affected not only by the physiological status but also by the cell type and development stage. The proteomics technique was also applied to investigate the rice response to salt stress (Salekdeh et al. 2002; Yan et al. 2005; Cheng et al. 2009; Li et al. 2010; Ruan et al. 2011).

The early stage of rice development after germination is crucial for rice to cultivate strong seedlings. The nascent green rice shoots grow rapidly and are sensitive to subtle alternations of growth environments. To our knowledge, there are no reports on the response of rice shoots in this stage to salt stress by application of proteomics strategy. On the other hand, the signaling pathways of salt, dehydration, cold and abscisic acid (ABA) are often mutually influenced (Wang et al. 2003). Given that there was only very few reports to compare the response of plants to salt stress and other abiotic stress at protein level, we have also made the comparison in this study among salt and other treatments (dehydration, cold and ABA). Our findings may provide helpful insights into understanding the tolerance mechanism of plants to salt stress.

Materials and Methods

Plant Material and Treatments

The seeds of rice (*Oryza sativa* L. ssp. *Indica*) cultivar 93–11 were soaked in water for 24 h and then sprouted on wetted filter paper for 24 h. The germinated seeds were grown in plastic containers containing Kimura B nutrient solution. Seedlings were grown under white light (150 μmol photons/m^2s; 14 h light/10 h dark period) at 26°C in a growth chamber. Three-day-old seedlings were subjected to Kimura B nutrient solution containing 250 mM NaCl (salt stress) for 48 h. Seedlings grown in initial conditions for 48 h were used as control.

Plant Growth

The growth rates of rice shoots were investigated after 48 h of salt treatment. The heights of shoots in container were measured before and after treatment. The height increase during salt treatment was calculated.

Determination of Lipid Peroxidation

The level of lipid peroxidation in rice shoots was determined in terms of the peroxidation byproduct malondialdehyde (MDA) in the samples. Rice shoot segments of approximately 250 mg were shock frozen in liquid nitrogen and ground in 2.5 mL of 10% trichloroacetic acid. After centrifugation at 12,000×*g* for 10 min at 4°C, the supernatant was collected as a trichloroacetic acid extract for the determination of MDA content. The levels of lipid peroxidation were determined from the MDA contents resulting from the thiobarbituric acid (TBA) reaction as described by Health and Packer (1968). Of the supernatant fraction, 2 mL was mixed with 2 mL of 25% TBA solution and the mixtures were heated at 95°C for 30 min. To stop reaction, 0.1 mL HCl was added and followed by a centrifugation at 10,000×*g* for 5 min. The absorbance of the supernatant was calculated based on A532–A600 with the extinction coefficient of 155 mM^{-1} cm^{-1}.

Ion Leakage Measurement

Membrane damage was assayed by measuring ion leakage from rice shoots. For each measurement, 15 rice shoots (cut into 0.5 cm long segments) in beaker were floated on 15 mL of double-distilled water and vacuumized by air pump until all the segments subsided, and then the conductivity of the bathing solution was measured with a conductivity meter (as value *A*). After that, all the inclusion in beaker was transferred into sealed tubes and was boiled in the bathing solution for 15 min. After cooling to room temperature, the conductivity of the bathing solution was measured again (as value *B*). For each measurement, ion leakage was expressed as percentage leakage, i.e., (value *A*/value *B*)×100.

Statistical Analyses

The data was statistically analyzed by one-way ANOVA procedure using SPSS software (SPSS, Inc., Chicago, IL,

USA). The differences between least square means for fixed effects were established using the standard error of a difference based on a t test when $p<0.05$.

Protein Extraction and Two-Dimensional Gel Electrophoresis

Of the rice shoots, 250 mg were cut into small pieces in a pre-cooled mortar containing 1 mL lysis buffer (8 M urea, 2% NP-40, 2% Triton X-100, 2% ampholine pH 3.5–10, 5% 2-mercaptoethanol, 5% polyvinyl pyrrolidone-40) and ground into homogenate using a pestle. The extract was centrifuged twice at 15,000×g at 4°C for 15 and 10 min, respectively. The supernatant was saved and stored at −80°C for use. Three replicates were performed for each sample.

Two-dimensional gel electrophoresis (2-DE) was carried out according to the method described previously (Li et al. 2010). The first dimensional isoelectric focusing (IEF) was performed in a 13-cm long glass tube with a 3 mm diameter. The gel solution consisted of 8 M urea, 3.6% acrylamide, 2% NP-40, and 5% ampholine (one part pH 3.5–10, one part pH 5–8). IEF was performed successively at 200 V for 0.5 h, 400 V for 16 h, and 600 V for 1 h. Electrode solutions were 50 mM phosphoric acid at the anode and 50 mM sodium hydroxide at the cathode. About 300 μg of protein was loaded on the top of the tube gel (cathode). After the first dimensional electrophoresis, IEF gels were equilibrated for 15 min twice in equilibration solution containing 62.5 mM Tris-HCl, pH 6.8, 2.5% sodium docecyl sulfate (SDS), 10% (v/v) glycerol, and 5% 2-mercaptoethanol. The second-dimensional SDS electrophoresis was performed on vertical slab gels (175×200×1 mm), Laemmli buffer system was used to cast 5% stacking gel and 15% resolving gel. The tube gels were sealed on the top of the slab gels with 1% agarose and electrophoresis was carried out at 40 mA per gel for 3–4 h. The gels were visualized by Coomassie brilliant blue (CBB) R-250 staining after electrophoresis.

Image and Data Analysis

The stained gels were scanned using a UMAX Power Look 2100XL scanner (UMAX Inc., Taipei, China) in transmission mode with a resolution of 600 dots per inch. The data was analyzed using ImageMaster™ 2D Platinumsoftware 5.0 (GE Healthcare Bio-Science). In order to minimize the variability due to CBB staining and to reflect the quantitative variations in intensity of protein spots, all spot volumes were normalized as a percentage of the total volume in all of the spots present in the gel. Protein spots with changes in volume more than 1.5-fold under salt stresses (Student's t test, $p<0.05$) were considered as differently expressed proteins.

MALDI-TOF MS Analysis

Protein spots were excised from the gels manually and cut into small pieces. Protein digestion and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis were performed according to a method described previously (Shen et al. 2003a) with slight modifications. Briefly, each small gel piece was destained with 50 mM NH_4HCO_3 in 50% ethanol for 1 h at 40°C. The protein in the gel piece was reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 60°C and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature in the dark. The gel pieces were minced and lyophilized, then rehydrated in 25 mM NH_4HCO_3 with 10 ng sequencing grade modified trypsin (Promega, Madison, WI, USA) at 37°C overnight. After digestion, protein peptides were collected and the gels were washed with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile three times to collect the remaining peptides. The peptides were desalted by ZipTipC 18™ pipette tips (Millipore, Bedford, MA, USA) and cocrystallized with an equivalent volume of saturated α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile containing 1% TFA. Tryptic peptide masses were measured with an AXIMA-CFR plus MALDI-TOF MS (Shimadzu Biotech, Kyoto, Japan).

A search was performed in National Center for Biotechnology Information database for the acquired peptide mass fingerprinting data using the Mascot software available at http://www.matrixscience.com. *Oryza sativa* was chosen for the taxonomic category. Database queries were carried out for monoisotopic peptide masses using the following parameters: enzyme of trypsin, mass error tolerance set to 0.3 Da, maximum of one missing cleavage site, and modifications allowed for carboxyamidomethylation of cysteine. To determine the confidence of the identification results, the following criteria were used: in addition to a minimum of 61 in MOWSE score, sequence coverage of the protein should be no less than 11% by the matching peptides. Only the best matches with high confidence levels were selected.

Results

Plant Growth and Physiological Changes of Rice Seedlings Under Salt Stress

Stresses have negative effects on plant growth, which could be reflected at molecular, physiological, and morphological levels. After 48 h treatment with salt, phenotypic analysis showed that rice seedlings grew slower than under normal conditions (Fig. 1a). The growth rate of rice shoots was decreased significantly ($p<0.01$). The height of control shoots was 3.5±0.25 cm, which was 4.53-fold that of salt

treated shoots (Fig. 1b). At the physiological level, oxidative stress and ion leakage are usually regarded as two immediate results of different abiotic stresses. Malondialdehyde (MDA) occurs as a soluble byproduct during lipid peroxidation and has been proven to be a reliable marker for oxidative stress. In the present work, the content of MDA in the shoot of rice seedlings under salt stress was 2.74-fold as much as in control ($p<0.01$; Fig. 1c), while the relative ion leakage of salt-treated rice shoots was as 3.21-fold as that in control tissues ($p<0.01$; Fig. 1d).

Proteome Profile of Rice Shoots and its Changes in Response to Salt Stress

In order to uncover the correlation of the reduced growth of rice seedlings and molecular changes under salt stress, proteins in rice shoots were extracted and separated through 2-DE. Three 2-DE gel replicates were performed for each sample under salt stress and control conditions. More than 700 reproducible protein spots were detected on the gels of the control and treated plants. These protein spots were mainly resolved in the range of p*I* 3.5–8 and molecular mass of 20–110 kDa (Fig. 2).

The gels were digitized with ImageMaster-2D software (GE health, version 5.0). Comparative analysis results showed that 57 individual protein spots changed more than 1.5-fold (Student's *t* test, $p<0.05$) in abundance. Among them, 24, 28, and five individual protein spots were up-, downregulated and induced by salt stress, respectively (Fig. 2). These changed levels of proteins can be regarded as salt stress-responsive proteins.

Protein Identification

All the 57 differently expressed protein spots under salt stress were analyzed by MALDI-TOF MS. According to the criteria described in the "Materials and Methods" section, 52 were identified. Among them, two proteins (U7 and I3) were assigned as *S*-adenosylmethionine

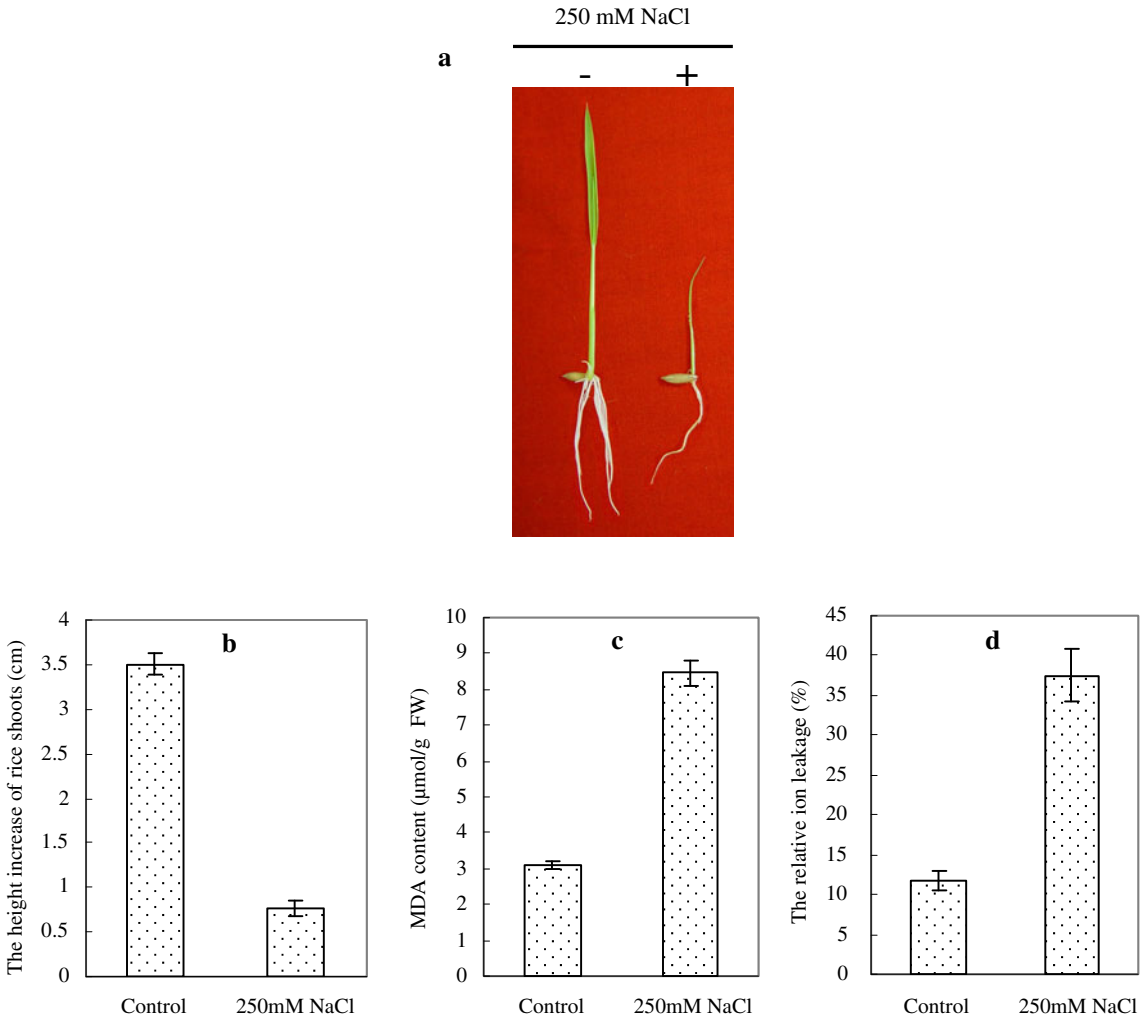

Fig. 1 Changes in phenotype (**a**), height increase (**b**), MDA content (**c**), and relative ion leakage (**d**) of rice shoots under 250 mM NaCl stress. Data are presented as means±SD ($n=3$)

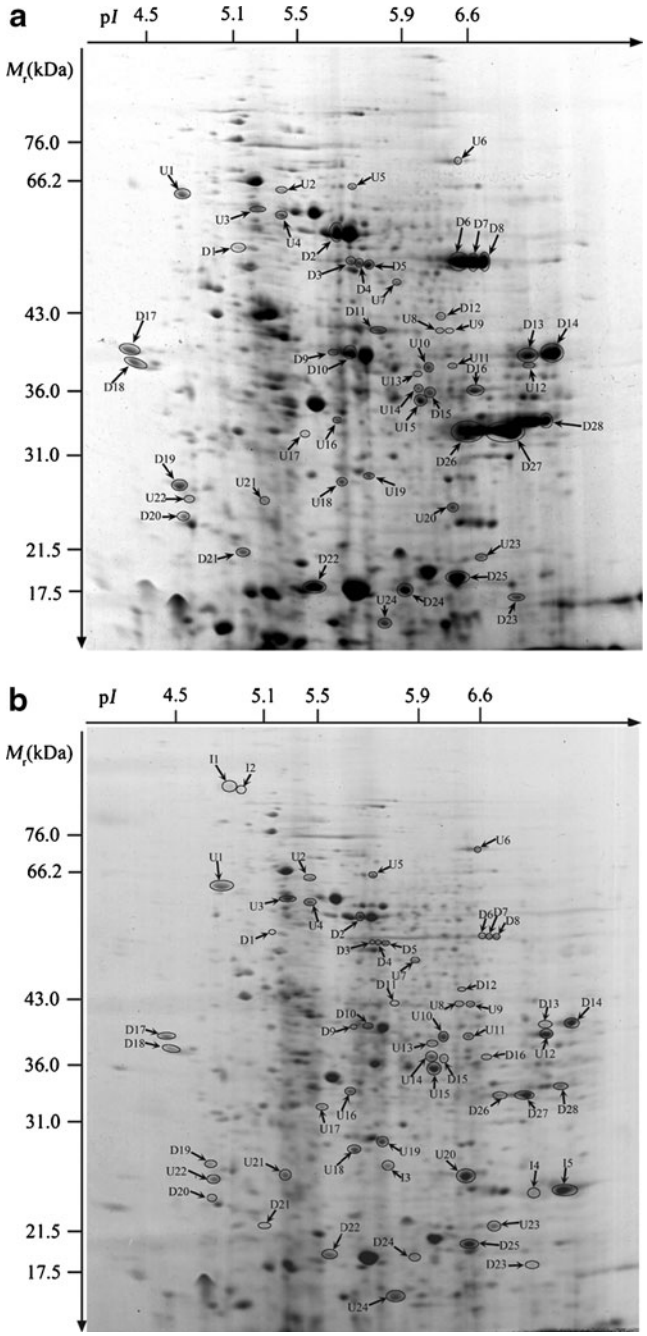

Fig. 2 Comparison of the proteome patterns of 3-day-old rice seedlings after 48 h salt stress. Differently expressed protein spots are circled and indicated in the images of control sample (**a**) and NaCl-treated sample (**b**) by arrows. U, D, and I represent the upregulated, downregulated, and induced protein spots, respectively

synthetase (EC 2.5.1.6) and late embryogenesis abundant (LEA) protein in group 3, respectively, based on a Basic Local Alignment Search Tool (BLAST) search (Table 1). Five protein spots (I1, I2, I4, I5, and D26) could not get any reliable matches in the database currently. The identified proteins could be categorized into eight predominant classes according to their functions (Table 1). These functional classes included photosynthesis and carbon assimilation, protein synthesis, transport, fold and assembly, antioxidation and detoxification, metabolism (miscellaneous), transporters, signal transduction, cell structure, and unknown function.

Proteins involved in photosynthesis and carbon assimilation include eight ribulose bisphosphate carboxylase large chains, RuBisCO activase small isoform precursor, ribulose-1, 5-bisphosphate carboxylase activase (EC 6.3.4.-), two isoforms of ATP B gene product which code the β subunits of extrinsic CF1 complex of ATP synthetase (Groth and Strotmann 1999) and photosystem II stability/assembly factor HCF136 of chloroplast. Except for the last one which was described to be essential for the stability of PSII in higher plant chloroplast (Meurer et al. 1998), all the proteins in this functional group were decreased under the salt stress (Fig. 3a). These results indicated the inhibitory effect of salt stress on the photosynthesis.

In contrast to the decrease of photosynthetic proteins, the metabolic proteins and antioxidation and detoxification related proteins were mainly upregulated upon the salt treatment (Fig. 3b and c). Proteins belonging to the former group were mainly involved in carbohydrate metabolism, such as the proteins in glycolysis (U5, U18, and U19) and in the tricarboxylic acid cycle (U14 and U15) processes; while the latter included the universal antioxidation proteins such as protein disulfide isomerase (U3, EC 5.3.4.1), putative heat shock protein (U17), ascorbate peroxidase (APX; U21, EC 1.11.1.11) and superoxide dismutase (SOD; U24, EC 1.15.1.1), along with some other proteins like phenylalanine ammonia-lyase (U6, EC 4.3.1.24), Osr40c1 (U12), and LEA (I3). For those proteins belonging to other functional group, some of them were upregulated and some of them were downregulated by the salinity (Fig. 3d and e). The change patterns of different proteins might be determined by their functions.

Among all these identified proteins, most of them have previously been identified as salt responsive proteins. Noticeably, we identified several novel salt responsive proteins such as protein synthesis inhibitor I (U8 and U9), photosystem II stability/assembly factor HCF136 (U13), trigger factor-like protein (D1), and cycloartenol-C24-methyltransferase (SMT1, U11, EC 2.1.1.41). Based on a BLAST search, protein synthesis inhibitor I was assigned as the functional homolog of ribosome-inactivating proteins (RIP). RIP can inhibit the translation process by removing single adenine residues of the large rRNA from the universally conserved sarcin/ricin loop (Endo and Tsurugi 1988). HCF136 was demonstrated as an assembly factor of PSII reaction center (Plucken et al. 2002). Trigger factor is a molecular chaperone that is present in all species of eubacteria. It binds to the ribosomal 50S subunit near the translation exit tunnel and is thought to be the first protein

Table 1 Proteins identified through MALDI-TOF MS

Spot no.	Accession no.	Description	Peptides matched	Mascot score	Coverage (%)	Theoretical M_r (kDa)/pI
Photosynthesis and carbon assimilation						
D2	AAA84588	ATP B gene product	14	153	42	53,977/5.3
D6	CAG34174	Ribulose bisphosphate carboxylase large chain	11	98	21	53,331/6.23
D7	CAG34174	Ribulose bisphosphate carboxylase large chain	9	83	17	53,331/6.23
D8	CAG34174	Ribulose bisphosphate carboxylase large chain	7	64	14	53,331/6.23
D10	AAX95072	Fructose-bisphosphate aldolase chloroplast precursor (ALDP)	11	118	25	42,122/7.6
D12	AAA84588	ATP B gene product	18	137	54	53,977/5.3
D13	CAG34174	Ribulose bisphosphate carboxylase large chain	6	70	12	52,760/6.23
D14	CAG34174	Ribulose bisphosphate carboxylase large chain	10	75	19	53,331/6.23
D16	AAX95285	RuBisCO activase small isoform precursor	10	129	38	39,858/6.66
D21	AAK31173	Ribulose-1,5-bisphosphate carboxylase activase	8	97	39	21,681/4.78
D24	CAG34174	Ribulose bisphosphate carboxylase large chain	14	117	30	53,331/6.23
D27	AAP53258	Putative rbcL; RuBisCO large subunit from chromosome 10 chloroplast insertion	12	91	20	53,433/6.45
D28	CAG34174	Ribulose bisphosphate carboxylase large chain	11	68	17	52,760/6.23
U13	BAD62115	Putative photosystem II stability/assembly factor HCF136, chloroplast	11	87	34	45,441/9.02
Protein synthesis, transport, fold and assembly						
D1	BAD62306	Trigger factor-like	14	142	32	59,894/5.21
D3	AAL37431	Translational elongation factor Tu	7	95	22	50,614/6.19
D4	AAL37431	Translational elongation factor Tu	11	118	35	50,614/6.19
D5	XP_466527	Translational elongation factor Tu	15	141	41	50,610/6.19
D17	XP_483744	Putative nucleic acid-binding protein	8	87	34	33,237/4.36
D18	BAD46651	Putative nucleic acid-binding protein	10	127	32	35,403/4.41
U2	AAP44754	Putative 60 kDa chaperonin alpha subunit	6	62	11	61,363/5.36
U4	NP_910308	Putative chaperonin 60 beta precursor	10	98	17	64,046/5.6
U8	NP_909370	Putative protein synthesis inhibitor I	7	81	16	30,458/9.3
U9	NP_909370	Putative protein synthesis inhibitor I	7	63	23	30,458/9.3
U10	XP_477140	Putative mRNA binding protein precursor	8	102	31	41,268/7.68
Antioxidation and detoxification						
U3	AAX85991	Protein disulfide isomerase	6	63	17	56,820/4.95
U6	S06475	Phenylalanine ammonia-lyase	10	70	22	76,305/8.53
U12	CAA64683	Osr40c1	9	118	37	35,545/6.6
U16	BAB71741	Glyoxalase I	7	76	34	32,861/5.51
U17	AAP53794	Putative heat shock protein	7	62	40	22,063/5.07
U20	AAV44199	Dehydroascorbate reductase	8	104	54	23,726/5.81
U21	XP_479627	Ascorbate peroxidase	6	95	40	27,215/5.2
U23	AAP55038	Putative nucleoside diphosphate kinase	6	66	41	16,769/6.85
U24	XP_483791	Putative superoxide dismutase [Cu-Zn], chloroplast precursor	6	93	43	20,633/5.79
I3	XP_473850	LEA protein in group 3	10	101	37	28,848/9.09
Metabolism (miscellaneous)						
D9	BAD87047	Putative plastidic cysteine synthase 1	10	99	36	42,104/6.28
U5	BAD82294	Putative phosphoglycerate mutase	8	110	25	60,980/5.42
U7	NP_908684	S-adenosylmethionine synthetase	11	121	39	43,330/5.68
U11	XP_477078	Cycloartenol-C24-methyltransferase	9	69	34	38,986/5.98
U14	AAP54283	Cytoplasmic malate dehydrogenase	11	87	40	35,888/5.75
U15	AAP54283	Cytoplasmic malate dehydrogenase	11	108	43	35,888/5.75
U18	XP_462797	Putative triosephosphate isomerase	5	64	34	27,274/5.38
U19	XP_462797	Putative triosephosphate isomerase	9	123	52	27,274/5.38

Table 1 (continued)

Spot no.	Accession no.	Description	Peptides matched	Mascot score	Coverage (%)	Theoretical M_r (kDa)/p*I*
Transporters						
D19	XP_467812	Putative H (+)-transporting ATP synthase	6	87	34	26,202/4.98
D20	XP_467812	Putative H (+)-transporting ATP synthase	7	84	28	26,202/4.98
U22	XP_467812	Putative H^+-transporting ATP synthase	10	103	32	26,202/4.98
Signal transduction						
D15	NP_916988	Guanine nucleotide-binding protein beta subunit-like protein (GPB-LR) (RWD)	7	70	30	36,665/5.97
D25	XP_475372	Putative GTP-binding protein	7	75	40	24,073/8.35
U1	XP_477251	Putative calreticulin precursor	11	90	26	48,279/4.47
Cell structure						
D11	BAC82429	Beta-tubulin	10	78	28	50,538/4.82
Unknown function						
D22	NP_909667	Hypothetical protein	6	64	39	19,082/9.58
D23	AAR00599	Hypothetical protein	5	73	38	15,495/8.71

The spot numbers correspond to those given in Fig. 1. Theoretical Mr/p*I* indicates theoretical values for molecular weight and isoelectric point. Protein identities were from searching in NCBInr database. U, D, and I represent upregulated, downregulated, and induced spots, respectively

to interact with nascent polypeptides emerging from the ribosome (Ludlam et al. 2004). One gene encoding trigger factor-like protein was found in the *Arabidopsis* genome and named as *AtTIG* (He et al. 2004). It is noteworthy that trigger factor is only found in bacteria and in the chloroplast so far. The function of trigger factor in the chloroplast is unknown but it may also function in the chloroplast like its bacterial homolog in the protein synthesis and targeting processes (He et al. 2004). SMT1 catalyzed cycloartenol to 24-methylene cycloartenol (Sitbon and Jonsson 2001) to produce the 24-alkylations, which are typical of sterols from higher plants.

Response to Dehydration, Cold Stresses, and ABA Treatment

In order to figure out the different and similar molecular mechanism among abiotic stresses, 3-day-old seedlings were also subjected to different treatments with Kimura B nutrient solution containing 25% polyethylene glycol (dehydration stress) and 50 μM ABA for 48 h, respectively. For cold stress, seedlings were transferred to a growth chamber and grown at 10°C for 48 h. Like salt stress, three replications were also performed in each treatment. The 2-DE images of the shoot under different treatments were obtained and analyzed as mentioned above and provided as supplementary material (Figure S1). Regulation of salt responsive proteins under dehydration, cold, salinity, and ABA treatments are available as supplementary material (Table S1). It was found that nine salt-responsive proteins, including ribulose bisphosphate carboxylase large chain (D13), RuBisCO activase small isoform precursor (D16), ribulose-1, 5-bisphosphate carboxylase activase (D21), ATP B gene product (D12), guanine nucleotide-binding protein beta subunit-like protein (D15), GTP-binding protein (D25), beta-tubulin (D11), glyoxalase I (Gly I, U16, EC 4.4.1.5), and dehydroascorbate reductase (DHAR, U20, EC 1.8.5.1) were regulated by all treatments (Fig. 4). Among the other salt-responsive proteins, 11 were common regulated by three treatments (salt, dehydration, and ABA), such as trigger factor (D1), two protein synthesis inhibitor (U8 and U9), Osr40c1 (U12) and LEA (I3) (Fig. 4); ten were changed under two treatments (salt and cold stress), such as calreticulin (U1) (Fig. 4); and ten were regulated by salt stress, such as SMT1 (U11) and HCF136 (U13) (Fig. 4).

Discussion

Salt stress undoubtedly results in the changes of many physiological processes in plant, therefore decreasing plant growth. Proteomics and its related techniques are very efficient approach in monitoring the changes at the proteins level (Jorrín et al. 2007). In this work, we have performed a proteomic analysis of rice shoots subjected to salt stress. We found that 57 responsive proteins were regulated under salt stress (Table 1), including several novel salt stress-responsive proteins that were reported for the first time in rice.

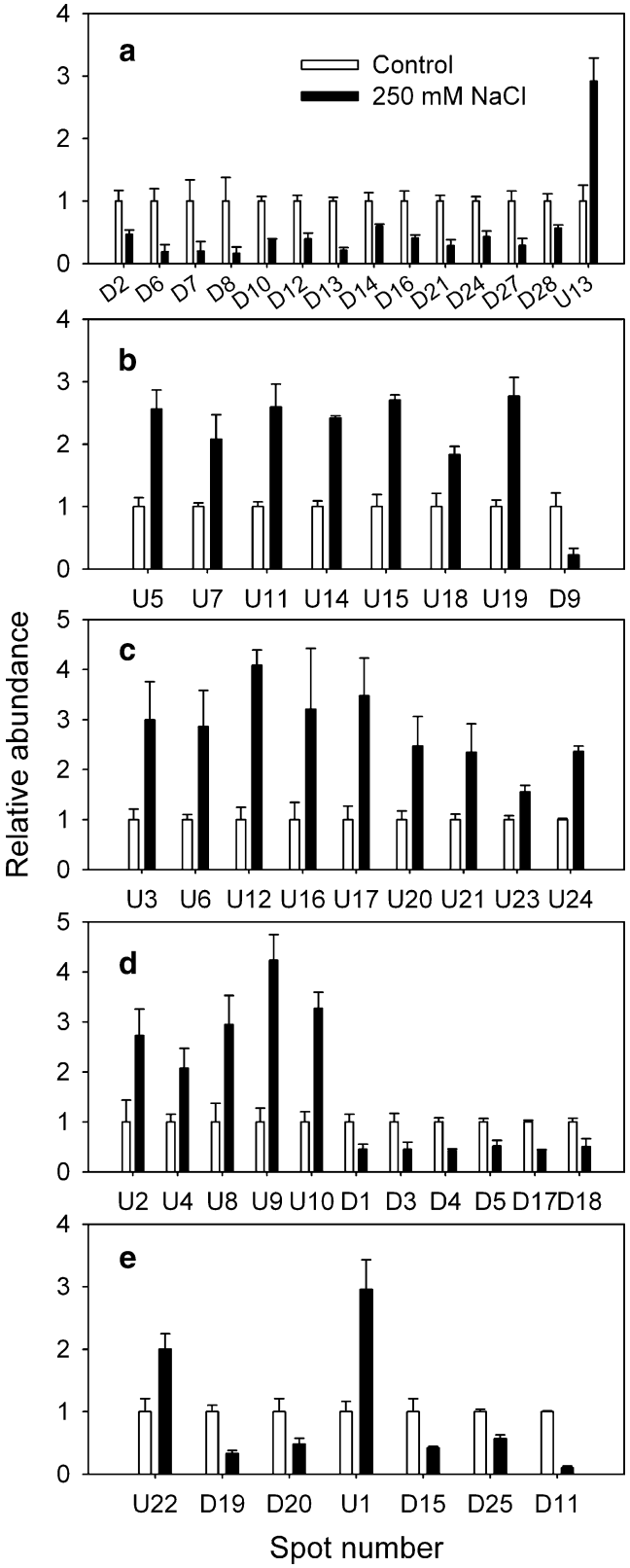

Fig. 3 Quantitative changes of the identified proteins. **a** Photosynthesis and carbon assimilation related proteins, **b** metabolic proteins, **c** antioxidation and detoxification related proteins, **d** protein synthesis, transport, fold, and assembly related proteins, **e** other functional proteins. Data are representatives of three independent replicates and shown as mean±SD

Changes of the Catabolism and Anabolism

One direct effect of salt stress on rice is the reduction of stomatal aperture of leaves for the lack of water (Fricke et al. 2004). The decrease of stomatal aperture will reduce the photosynthesis of the plant. In this study, downregulation of the photosynthetic related proteins, such as ATP synthetase F1F0 (D2 and D12), RuBisCO large chain (D8), ribulose-1, 5-bisphosphate carboxylase activase (D21), and RuBisCO activase small isoform precursor (D16) suggest that salinity affects photosynthesis through reducing the abundance of photosynthesis related proteins as well as the availability of CO_2. Among them, ATP synthetase F1F0 is a membrane integral enzyme, which couples the free energy of proton transfer down an electrochemical gradient to synthesize ATP. The reduction of photosynthesis resulted in shortage of energy and substrate for anabolism, and thereby the shoot growth was arrested. Furthermore, reduction of the photosynthesis seems to be the common result of different abiotic stresses, since D21 and D16 were also downregulated by other abiotic stresses.

Energy provision is essential for plants to survive under abiotic stresses (Riccardi et al. 1998). Therefore, as a strategy to fight against salt stress, upregulation of the catabolism (respiration) related proteins, such as phosphoglycerate mutase (U5, EC 5.4.2.1) and triosephosphate isomerase (U18 and U19, EC 5.3.1.1) which were important enzymes in the glycolytic pathway, to activate the energy generating processes was reasonable. Our results are in agreement with previous reports (Salekdeh et al. 2002; Yan et al. 2005).

Protein Synthesis, Transport, Fold, and Assembly

Protein synthesis inhibitor I (U8 and U9), as the functional homolog of RIP, was upregulated, while trigger factor (D1) and translational elongation factors (D3, D4 and D5) were downregulated by salt stress. These results indicated that the protein synthesis in rice was inhibited by salt stress. In this study, levels of protein synthesis inhibitor I and trigger factor which is involved in protein translation were changed in response to salt stress, which was not reported previously. Perhaps as for the rice seedlings in this stage, nutrition that mainly depends on the seed supply is limited, thus, they must employ more efficient strategy to decrease the consumption of energy in order to survive under salt

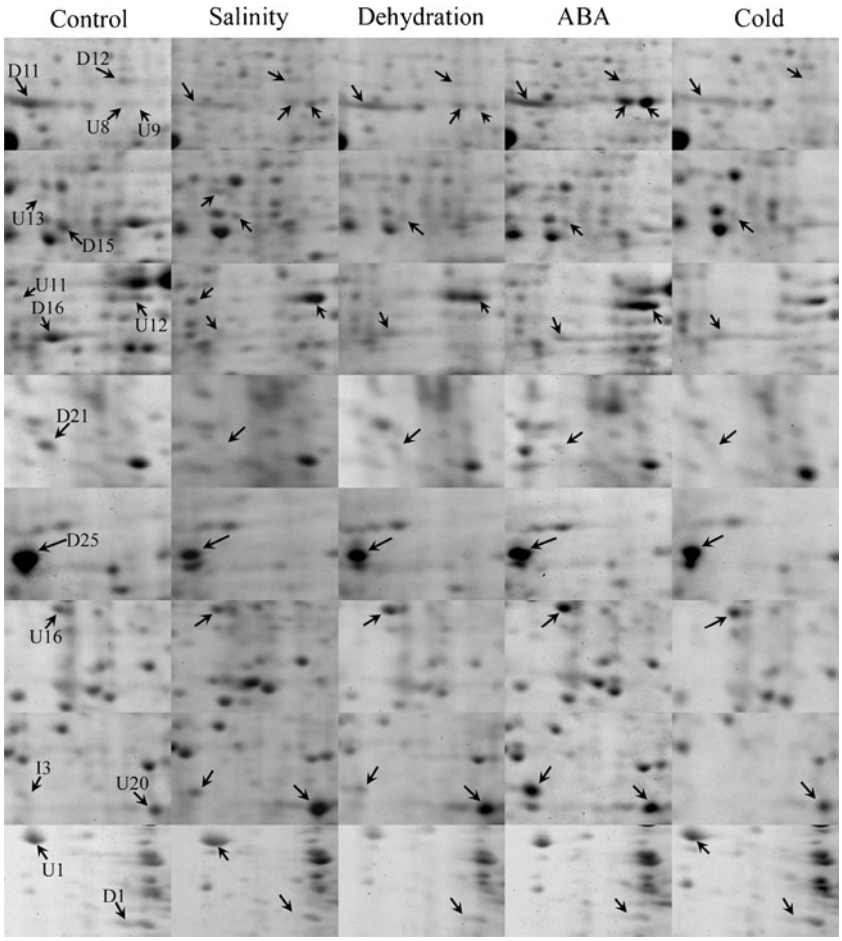

Fig. 4 Comparison of the regions that contain some salt stress-responsive proteins under salt, cold, dehydration, and ABA treatment. Only the differently expressed proteins compared with control was pointed by *arrow*

stress. The accumulation of RIP had been reported to be stimulated at transcription level by osmotic, heat stresses, and some signal compounds such as abscisic acid but not by cold (Stirpe et al. 1996). The inhibition of proteins synthesis through these two proteins might be a common mechanism in response to the abiotic stresses through ABA-dependent signaling pathway. The biological roles of the two proteins were poorly elucidated, so further work focused on them could be very interesting.

Anti-Oxidation and Detoxification

The reduction of photosynthesis and enhancement of respiration will increase the formation of reactive oxygen species (ROS) and hence increase the accumulation and activity of enzymes that detoxify these species (Pellinen et al. 2002). In this study, besides APX and SOD, DHAR and Gly I were also upregulated by salt stress. Moreover, both of them were also upregulated by other stresses in our experiment. *Gly I* was found upregulated in response to heavy metal, salt, and dehydration stresses in *Brassica juncea* (Veena and Sopory 1999) and cold stress in *Arabidopsis* (Seki et al. 2001) at mRNA level. At protein level, it was also reported to be upregulated in response to dehydration, salt, cold, and ABA in leaf sheath of rice (Zang and Komatsu 2007). It seemed that Gly I did not have specific expression in different tissues and species. Furthermore, over-expressing *Gly I* showed significant tolerance to salt stress in transgenic tobacco (Veena and Sopory 1999). So our results affirmed that Gly I could be looked as a new promising candidate target for gene engineering to improve the tolerance of crops to abiotic stresses. Different *LEA* genes are induced by different abiotic stresses (Seki et al. 2002; Rabbani et al. 2003) and associated with a protective function for plants (Ingram and Bartels 1996). In the researches on *Arabidopsis*, Seki et al. (2002) found that most genes of LEA proteins were induced by drought, salinity and ABA. Osr40c1 is an ABA-responsive protein associated with salinity or osmotic stress tolerance (Moons et al. 1995). In this study, one LEA protein and Osr40c1 were induced or upregulated under salt and dehydration stresses and were also responsive to ABA, but not to cold. The results implied some similarities existed among responses to salinity, dehydration and ABA. Zhu (2001) indicated that in the cold conditions typically used in laboratories to induce cold-responsive genes in

plants, osmotic stress is minimal or does not exist. With this study, we found more proteins involved in antioxidation and detoxification in response to the abiotic stress treatment than previous reports (Salekdeh et al. 2002; Yan et al. 2005). This might be caused in young rice seedlings used in this study, due to their more sensitivity to the stresses than older ones used in previous studies.

Signaling in Response to Salinity

Under the salt treatment, stress is first perceived by the root and then the signal is transported upward to the shoot. This long-distance signaling is mediated by different kinds of second messengers, such as calcium and ROS (Chinnusamy et al. 2006). Some signaling components, such as Gβ (D15) and GTP-binding protein (D25) were downregulated in this study. At transcriptional level, GTP-binding protein had been reported to be repressed by abiotic stresses in *Arabidopsis* (Seki et al. 2002), while Gβ had been reported to express constitutively in rice (Iwasaki et al. 1995). Evidence revealed that G protein signaling is involved in stress-associated physiological processes (Joo et al. 2005). Our results showed that Gβ (D15) and GTP-binding protein (D25) were downregulated by all stress treatments, indicating that G protein might be a common component in the signaling network of abiotic stresses.

Calreticulin (CRT, U1), a major Ca^{2+}-sequestering protein, has been implicated in a variety of cellular functions such as Ca^{2+} storage, signaling, and chaperone activity within the cytoplasm and endoplasmic reticulum. Salinity can induce Ca^{2+} in cytosol and Ca^{2+} could play an essential role in abiotic stresses (Knight et al. 1997). Upregulation of CRT was correlated with the inhibition of seedling growth (Shen et al. 2003b), and it was regulated by cold and salt stress implied similarity exist in two stress signaling pathways.

Other Cellular Processes

In addition to the physiological processes mentioned above, the maintenance of ion homeostasis in cells was also important for plants in adaptation to abiotic stresses. It has been proved that H^+-transporting ATP synthase is one of the most important enzymes required for this process (Zhu 2003). Abiotic stress may lead to the membrane damage, and hence the increase of ion leakage (Fig. 1c and d), implying that the ion homeostasis was disturbed. H^+-transporting ATP synthase is suggested to be involved in the regulation of ion balance (Zhu 2003). In our study, three isoforms of this enzyme (U22, D19, and D20, EC 3.6.3.14) were up- or downregulated by the salt. Since these isoenzymes were encoded by one single gene, we reasoned that different modifications may exist for this enzyme, and each modification status might play a specific role in the regulation of ion homeostasis. The significance of the modification in ion homeostasis during abiotic stress adaptation remains to be elucidated. Our results will be favor to further understand the regulation of H^+-transporting ATP synthase under salt stresses, which cannot be determined just at mRNA level.

In addition, we also found that two novel salt stress-responsive proteins, STM1 (U11) and HCF136 (U13), were upregulated only by the salinity among all the stresses in this study. It has been suggested that increased SMT1 expression could activate the defense against ROS and/or lipid peroxides in the photo-oxidative stress under high light (Ledford et al. 2004). High level of HCF136 was reported to be in favor of forming a tighter structure of PSII core monomer (Chen et al. 2007). Compared with dehydration, cold, and ABA, salinity creates both ionic and osmotic double stresses, and high salt uptake under salt stress could compete with the uptake of other nutrient ions, especially K^+, leading to K^+ deficiency (Ball et al. 1987). Under such conditions of high salinity and K^+ deficiency, malfunction of photosystem II occurred (Ball et al. 1987). Base on the results in this study, it seems that upregulation of STM1 (U11) and HCF136 (U13) was to repair or avoid more serious damage to the photosystems. Photosystems was more sensitive to salt stress than to osmotic in the nascent rice seedlings. However, there was no report on the correlation of salinity stress with regulation of SMT1 and HCF136. It should be interesting to elucidate the roles of these two proteins in the rice seedling in response to salt stress.

Plant salt stress response is a very complicated process that is difficult to uncover its mechanisms only relying on the studies of a single gene or several genes. The "-omics" study has been proved to be a powerful method to study the complicated biological phenomenon. Future study combining the transcriptomics, proteomics, and metabonomics will be very helpful for elucidation of the mechanisms of salt stress response.

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