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Proteome and Phosphoproteome Differential Expression under Salinity Stress in Rice (*Oryza sativa*) Roots

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Salinity stress is a major abiotic stress that limits agriculture productivity worldwide. Rice is a model plant of monocotyledons, including cereal crops. Studies have suggested a critical role of protein phosphorylation in salt stress response in plants. However, the phosphoproteome in rice, particularly under salinity stress, has not been well studied. Here, we use Pro-Q Diamond Phosphoprotein Stain to study rice phosphoproteome differential expression under salt stress. Seventeen differentially upregulated and 11 differentially downregulated putative phosphoproteins have been identified. Further analyses indicate that 10 of the 17 upregulated proteins are probably upregulated at post-translational level instead of the protein concentration. Meanwhile, we have identified 31 salt stress differentially regulated proteins using SYPRO Ruby stain. While eight of them are known salt stress response proteins, the majority has not been reported in the literature. Our studies have provided valuable new insight into plant response to salinity stress.

Keywords: phosphoproteome · rice · salt stress · differential regulation

1. Introduction

Salinity, drought, and cold are among the most common abiotic stresses encountered by plants owing to their sessile life style. It is estimated that salinity stress may affect half of all arable lands by 2050 and will be a major factor of arable land loss for the coming decades.[1] Salinity stress imposes both osmotic stress and ion toxicity to plants. To survive, a sophisticated signal perception, transduction, and cellular and morphological response network have been evolved to combat the stress. Both gene expression profile changes and post-translational modifications play a key role in mediating the response to salt stress.[2] The role of transcriptional regulation in salt stress has been clearly illustrated in genetic manipulations of several groups of transcriptional factors. Overexpression of transcriptional factors DREB1A,[3] CBF4,[4] Tsi,[5] OSISAP1,[6] and SNAC1[7] has increased the tolerance to salinity, drought, and low temperature in *Arabidopsis*, rice, or other plant species.

The completion of genome sequencing in both *Arabidopsis* and rice and the advances in genomic and proteomic technologies have significantly facilitated the studies of plant response to salinity stress. As a model plant of monocot and a staple crop, rice responses to salinity stress have been investigated using proteome approaches for the identification of salt stress differentially regulated proteins.[8−12] Overall, proteins corresponding to about 50 genes have been shown to be differentially regulated and identified using mass spectrometry analyses in these proteome studies. Meanwhile, DNA microarray analyses have revealed that 5−30% of the genes in rice are differentially regulated under salinity stress, suggesting that either the cellular mRNA and protein levels are not correlated for most of the genes or many differentially regulated proteins have not yet been identified in the proteome studies.[13−15] The salt stress differentially regulated proteins identified thus far have the following features. (1) Most of the salt-induced proteins are involved in photosynthesis, photorespiration, or oxidative stress pathways.[12] Proteins involved in protein synthesis, heat shock responses, amino acid synthesis, iron homeostasis, and calcium binding have also been shown to be induced by salt.[8−12] (2) The salt-induced proteins identified by different groups are very different although some proteins have been identified by more than one group. For example, none of the 33 protein spots identified by Kim et al. are among the 12 protein spots identified by Yan et al.[11,12] This discrepancy may be because different tissues have been used or each study has identified only a subset of a large differentially regulated protein pool. (3) A large number of the salt-inducible genes reported in the literature have not been identified using proteome approaches thus far. (4) Most of these studies have mainly focused on rice tissues with 24 h and longer time of salt treatments.

Protein phosphorylation is a major type of post-translational modification observed during salinity stress in plants. Protein kinase activity assays, yeast two-hybrid analyses, and studies with mutants have identified a MAPK signaling pathway, consisting of AtMEKK1, AtMEK1/AtMKK2, and AtMPK4/AtMPK6, that transduces salt and other abiotic stress signals in *Arabidopsis*. Overexpression of AtMKK2 in *Arabidopsis* resulted in constitutive activation of AtMPK4 and AtMPK6 and enhanced salt and freezing tolerance. Meanwhile, *mkk2* mutants exhibited

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hypersensitivity to salt and cold stress.[2,16−18] In rice, OsMAPK5 kinase activity and gene expression are regulated by salt, drought, wounding, cold, and ABA. Overexpression of OsMAPK5 resulted in an increase in tolerance to several abiotic stresses, including salt stress.[19] Protein phosphorylation also plays a critical role in ion homeostasis under salinity stress.[20,21] The Na^+/H^+ antiporter SOS1 mediates Na^+ efflux under salt stress. SOS1 is regulated by the SOS3−SOS2 kinase complex. Salt stress-induced Ca^{2+} signatures are sensed by SOS3, which transduces the salt stress signal by activating SOS2, a Ser/Thr protein kinase with a N-terminal kinase catalytic domain. SOS2 regulates the activity of SOS1. Although evidence of protein phosphoregulation in salt stress response is overwhelming, rice phosphoproteome differential regulation under salt stress has not been well studied using proteome approaches thus far. One reported study used an in vitro isotope labeling approach to investigate salt stress-induced protein phosphorylation.[9] Four protein spots have displayed salt-induced phosphorylation and two of them have been identified after mass spectrometry analysis. The two identified proteins are cytoplasmic malate dehydrogenase and calreticulin.

The Pro-Q Diamond Phosphoprotein Stain (Molecular Probes) is a phosphoprotein specific fluorescence stain that has been used widely in detecting phosphoproteins following separation on 2-DE gels.[22−26] Using this dye, one can follow quantitative changes of particular protein spots on 2-DE gels in a time course under a treatment or at different developmental stages. It is a useful tool in revealing differential regulation of phosphoproteins.

In this paper, we investigate protein differential regulation in the time course of salinity stress in rice roots using both SYPRO Ruby stain, whose stain intensity has a linear correlation with protein levels, and the Pro-Q Diamond Phosphoprotein Stain. We focused our studies on protein differential regulation within the first 24 h of salinity stress. Thirty-one differentially regulated proteins revealed by SYPRO Ruby stain and 28 differentially regulated putative phosphoproteins revealed by Pro-Q Diamond stain have been identified using mass spectrometry analyses.

2. Materials and Methods

2.1. Rice Culture and Salt Treatment. Rice seeds (*Oryza sativa*, cv. Nipponbare) were germinated at 25 °C in Hoagland solution.[27] After 20 days, seedlings were treated with 150 mM NaCl in Hoagland solution and the roots were harvested at 0, 10, and 24 h of treatment.

2.2. Protein Extraction. Rice root tissues were ground in liquid nitrogen with mortar and pestle into fine powders. Proteins were extracted using a modified phenol extraction protocol[28] as follows: Ground tissues were suspended in an extraction buffer (0.9 M sucrose, 0.5 M Tris-HCl, 0.05 M EDTA, 0.1 M KCl, and 2% β-mercaptoethanol added freshly, the final pH was 8.7), were mixed with an equal volume of saturated phenol (pH 8.0), and then were homogenized for 10 min. The homogenate was centrifuged at 2500*g* for 10 min, the phenol phase was recovered, and the phenol extraction was repeated three times. The final collection of phenol was mixed with five volumes of precipitation buffer (methanol with 0.1 M ammonium acetate and 1% β-mercaptoethanol). Precipitation was carried out at −70 °C overnight. The precipitant was recovered by centrifugation at 13 400*g* for 10 min, and the pellet was washed three times with cold precipitation buffer and another three times with ice cold 70% ethanol. The protein pellet was lyophilized to powder in a speed vacuum (LABCONCO, model LYPH-LOCK 6) and was stored at −70 °C. At least three biological replicas were extracted for each treatment.

2.3. Two-Dimensional Polyacrylamide Gel Electrophoresis (2D PAGE). Proteins were dissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPSO, 1% dithiotreitol (DTT), and 0.2% ampholines) and were centrifuged at 12 000*g* for 10 min to remove undissolved content. The supernatant was quantified using a Bio-Rad Rc Dc protein assay kit according to the instructions of the manufacturer. The quantified proteins were then used for 2D PAGE. Isoelectric focusing (IEF) was carried out using a Bio-Rad PROTEAN IEF cell on 24-cm 3−10 pH nonlinear IPG strips (Bio-Rad). One milligram of protein in 400 μL of rehydration buffer was loaded into the IEF tray and active rehydration was carried out at 23 °C for 12 h, followed by 250 V for 2 h, and a linear increase of voltage to 10 000 V for 4 h, and the isoelectric focusing was performed at 23 °C for a total of 90 000 VH. After the completion of IEF, the strips were equilibrated in an equilibrating buffer containing 6 M urea, 0.375 M Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, and 2% dithiothreitol for 15 min followed by equilibration for another 15 min in a buffer containing 6 M urea, 0.375 M Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.1% bromophenol blue, and 2.5% iodoacetamide.

The equilibrated IPG strips were loaded on horizontal slab gels (25 × 20.5 × 1.5 mm) containing 12% (w/v) separating gel and 4% stacking gel (w/v). Electrophoresis was carried out in a Bio-Rad PROTEAN PLUS horizontal Dodeca cell at 20 mA/gel.

The gels were stained with SYPRO Ruby fluorescence stain (Bio-Rad) according to the protocols provided by the manufacturer and were scanned with a VersaDoc4000 image system (Bio-Rad). The images were analyzed with PDQUEST 7.4.0. software (Bio-Rad, Hercules, CA). For each treatment, at least three 2-DE gels representing the three biological replicas were used for data analyses. The cutoff for differentially regulated proteins was 2-fold of change in all biological replicas.

2.4. Identification of Phosphoproteins Using Pro-Q Diamond Phosphoprotein Stain. Detection of phosphoproteins after separation on 2-DE gels followed the instructions from the manufacturer except that only half of the recommended concentration was used (Molecular Probes). In brief, 2-DE gels were fixed in solution containing 50% methanol and 10% acetic acid, were washed with several changes of water to remove SDS, and were stained with the Pro-Q Diamond dye. After destaining, the gel images were recorded using VersaDoc4000 (Bio-RAD). The spots consistently stained with Pro-Q Diamond dye in all three biological replicas were considered as putative phosphoproteins. The proteins with 2-fold differential regulations in all three biological replicas were considered as differentially regulated putative phosphoproteins. Protein gels of the same protein samples were also stained with SYPRO Ruby to correlate the protein spots revealed by these two different dyes.

2.5. In-Gel Digestion and Mass Spectrometry. After PDQUEST analysis, the spots of interest were robotically excised from 2-DE gels by Proteome Works Spot Cutter (Bio-Rad). In-gel trypsin digestion was performed using the ProPrep (Genomic Solutions) robotic digester/spotter. The samples were subjected to disulfide bond reduction and alkylation with DTT (dithiotreitol) and iodoacetamide, respectively. The resulting peptide mix was desalted with C18 ZipTips (Millipore) and was spotted on a MALDI plate in a solution containing 70%

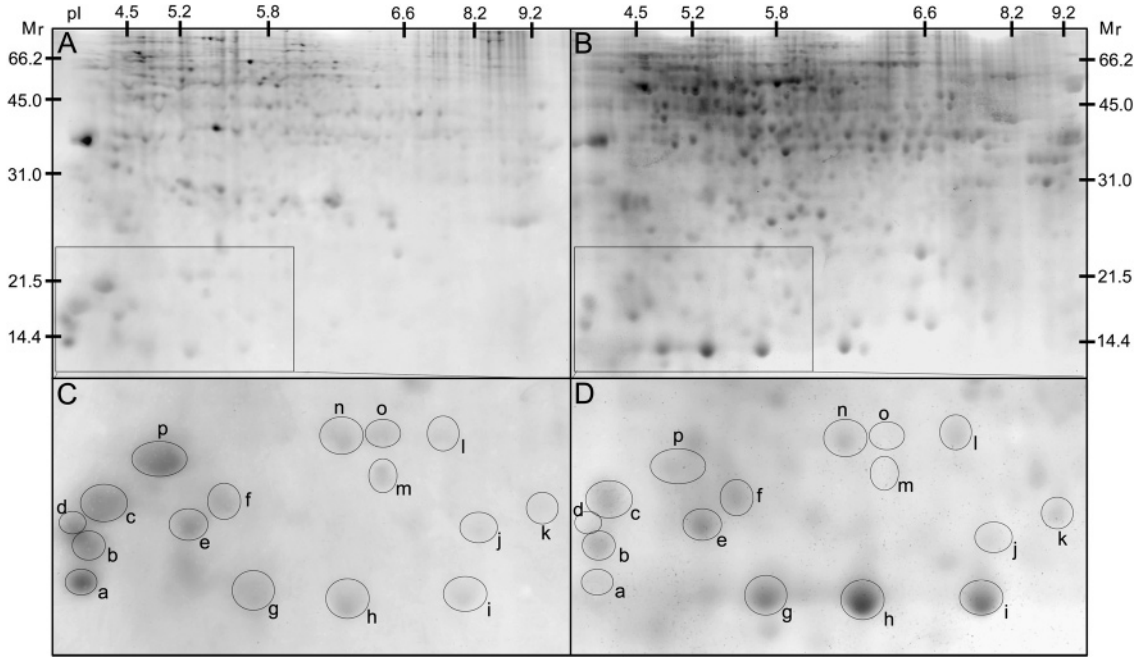

Figure 1. 2-DE gel images of rice root proteome revealed by Pro-Q Diamond in Gel Stain and SYPRO Ruby fluorescence stain. Proteins were extracted from 20-day-old rice roots, were separated on 2-DE gels, and were stained with Pro-Q Diamond dye and SYPRO Ruby. (A) 2-DE gel image revealed by Pro-Q Diamond in Gel stain. (B) 2-DE gel image revealed by SYPRO Ruby stain. (C) An enlarged section of the image shown in A. The spot positions of several major proteins are marked and labeled for comparison. (D) An enlarged section of the image shown in B. The spot positions of several major proteins are marked and labeled for comparison.

acetonitrile, 0.1% trifluoroacetic acid, and 5 mg/mL matrix (alfa-cyano-4-hydroxycinnamic acid).

Mass spectra (MS) were collected on an ABI 4700 Proteomics Analyzer (Applied Biosystems) MALDI TOF/TOF mass spectrometer, and protein identification (ID) was performed using the result dependent analysis (RDA) of ABI GPS Explorer software, version 3.5 (Applied Biosystems). Some of the crucial parameters were set as follows: Digestion enzyme: trypsin with one missed cleavage; MS (precursor-ion) peak filtering: 800−4000 m/z interval, monoisotopic, minimum signal-to-noise ratio (S/N) = 10, mass tolerance = 150 ppm. MSMS (fragment-ion) peak filtering: monoisotopic, M + H^+, minimum S/N = 3, MSMS fragment tolerance = 0.2 Da; database used: *Oryza* taxonomic sub-database of "nr" (nonredundant) database of the National Center for Biotechnology Information (NCBI).

During the initial MS scan, data were analyzed as peptide mass fingerprinting (PMF), and preliminary protein ID was done by searching against the database using the MASCOT (Matrix Science) algorithm. Proteins with high-confidence ID (cross confidence interval C.I. % > 95%) were automatically selected for in silico digestion, and their three most prevalent corresponding peptides-precursor ions present in the MS spectra were selected for MSMS analysis: RDA_1 (top protein confirmation). The sample spots not yielding high-confidence ID after preliminary PMF ID or after RDA_1 ID were subjected to RDA_2 by selecting the first 20 most intense precursor ions in the MS spectra for MSMS analysis. The spectral data from the PMF (initial MS scan), RDA_1, and RDA_2 MSMS were together subjected to combined MASCOT search. Only proteins with total protein score C.I. % >95% were considered as a positive ID.

2.6. Gene Ontology (GO) Annotation. Functional categorization of proteins was carried out according to the GO rules using gene ontology tools at http://www.agbase.msstate.edu.[29] There were three independent sets of ontologies that were used to describe a gene product: (1) the biological process (BP) in which the gene product participates; (2) molecular function (MF) that describes the gene product activities, such as catalytic or binding activities, at the molecular level; and (3) the cellular component (CC) where the gene product can be found. The GO annotations and numbers were retrieved using the GORetriever tool and were grouped into different levels, and pie charts were generated using the analysis results of the GOSlimViewer tool at AgBase.

3. Results and Discussion

3.1. Rice Root Proteome Map Revealed by Pro-Q Diamond Phosphoprotein in Gel Stain and SYPRO Ruby Stain. Pro-Q Diamond Phosphoprotein in Gel Stain is a fluorescence dye specific for phosphoproteins. To examine the phosphoproteome and proteome in rice roots, we stained proteins from 20-day-old rice roots with Pro-Q Diamond dye and SYPRO Ruby dye, respectively, following protein separation on 2-DE gels. As shown in Figure 1A, the majority of the Pro-Q Diamond dye-stained protein spots had a relatively low pI, and it was very obvious for those heavily stained protein spots. In contrast, the SYPRO Ruby stained proteins were more evenly distributed on the 2-DE gel (Figure 1B). Many protein spots that were heavily stained with SYPRO Ruby were not stained or were weakly stained by Pro-Q Diamond dye. Conversely, many spots that were weakly stained by SYPRO Ruby were heavily stained by Pro-Q Diamond dye as revealed by comparing the spots labeled in Figure 1C and 1D. Since SYPRO Ruby stain intensity has a linear correlation to protein concentration in a broad range,[30] the results suggest that Pro-Q Diamond dye stain intensity is not proportional to protein concentration. One possibility is that the stain intensity of the Pro-Q Diamond dye is proportional to the level of phosphorylation although further

tests are required to confirm this. About 214 valid protein spots were consistently detected when stained with Pro-Q Diamond dye and about 733 protein spots were consistently detected when stained with SYPRO Ruby in all three biological replicas (data not shown). We found that too much Pro-Q Diamond dye resulted in a high background just as applying too much antibodies in Western blots causes nonspecific staining. Using half of the manufacturer recommended concentration of the dye avoided the problem. This observation was consistent with reported results.[24]

3.2. Protein Differential Regulation under NaCl Stress Revealed by Pro-Q Diamond Dye. To study how the putative phosphoproteins stained by Pro-Q Diamond dye respond to NaCl stress, we examined the rice root proteome with the Pro-Q Diamond dye after 10 and 24 h of 150 mM NaCl treatments. Twenty Pro-Q Diamond dye-stained protein spots displayed over 2-fold upregulation and 18 protein spots displayed over 2-fold downregulation in all three biological replicas (Figure 2 and data not shown). These differentially regulated protein spots were excised, digested with trypsin in-gel, and analyzed using an ABI 4700 Proteomics Analyzer (Applied Biosystems). As shown in Table 1, molecular identities of 17 of the 20 upregulated and 11 of the 18 downregulated putative phosphoproteins were obtained with high confidence. Among the upregulated proteins, the salt-induced protein (SalT, P24120) and mannose-binding rice lectin (O64441) are typical salt stress response proteins.[31] Another interesting protein is glutathione S-transferase, whose overexpression has resulted in salt stress tolerance in tobacco.[32] The small GTP binding protein and transposase may also be involved in salt stress response.[33,34] Other proteins included dnaK-type Hsp70, putative disease resistant protein, putative dimethylaniline monooxygenase, and six hypothetical and function unknown proteins. Since different stress response pathways often cross talk to each other,[1,2] it is not surprising that dnaK-type Hsp70, putative disease resistant protein, and putative dimethylaniline monooxygenase were also induced under salt stress. Interestingly, none of the above 17 identified upregulated proteins were identified as salt-induced protein in previously published rice salt stress proteome studies, suggesting that the Pro-Q Diamond in gel stain is a very useful tool in identifying salt-responsive proteins. The 11 differentially downregulated proteins included putative glucan endo-1,3-beta-D-glucosidase, putative protein kinase, putative retroelement, glyceraldehyde 3-phosphate dehydrogenase, and function unknown proteins.

3.3. Protein Differential Regulation under NaCl Stress Revealed by SYPRO Ruby Stain. Protein differential expression in rice roots, after 10 and 24 h of salt treatment, was also examined using SYPRO Ruby stain following 2-DE separation. Thirty-one protein spots displayed over 2-fold upregulation and 13 protein spots displayed over 2-fold downregulation in all three biological replicas (Figure 3 and data not shown). After MALDI-TOF/TOF analyses, 23 of the 31 upregulated proteins and 8 of the 11 downregulated proteins had been identified with high confidence as shown in Table 2. Among the 23 upregulated proteins, six have been reported to be differentially upregulated under salinity stress in prior rice proteome studies, including triosephosphate isomerase, peroxidase, malate dehydrogenase, dihydrolipoamide dehydrogenase, aldolase, and phosphoglycerate kinase.[8−12] In addition to these six proteins, the roles of cysteine synthase and glyoxalase in salt stress are well documented.[35−37] Other upregulated proteins include putative CRK1, actin 7, small GTP-binding protein OsRac2,

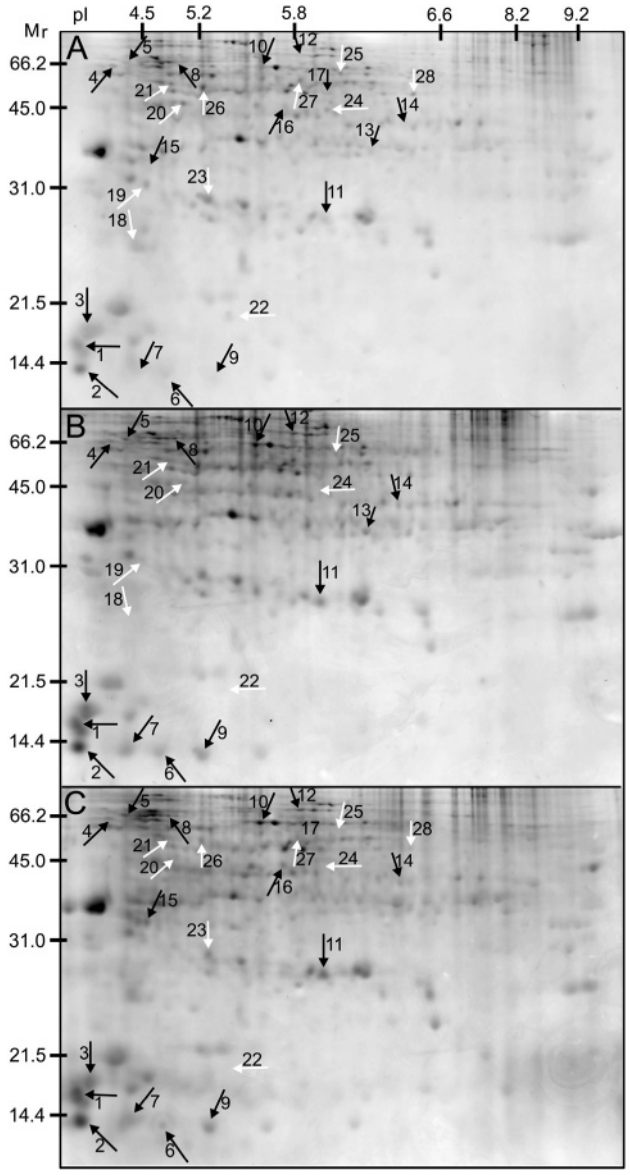

Figure 2. Protein differential regulation under salinity stress revealed by Pro-Q Diamond Phosphoprotein Gel Stain. Proteins were extracted from 20-day-old control and salt-treated rice roots, were separated on 2-DE gels, and were stained with Pro-Q Diamond dye. The cutoff of differential regulation was 2-fold of change in three biological replicas. (A) Putative phosphoproteome image of rice roots without salt stress. All protein spots subjected to upregulation under salinity stress are marked with black arrows and all protein spots subjected to downregulation are marked with white arrows. (B) Putative phosphoproteome image after 10 h of NaCl (150 mM) treatment. Proteins upregulated after 10 h of salt treatment are marked with black arrows and the proteins downregulated after 10 h of salt treatment are marked with white arrows. (C) Putative phosphoproteome image after 24 h of NaCl (150 mM) treatment. Proteins upregulated after 24 h of salt treatment are marked with black arrows and the proteins downregulated after 24 h of salt treatment are marked with white arrows.

putative disease resistant protein, putative transposase, sugar metabolic pathway proteins, hypothetical proteins, and function unknown proteins. Seven of the 23 proteins are common to the differentially upregulated proteins identified using Pro-Q Diamond dye as indicated in Figure 4. The Pro-Q Diamond

Table 1. Differentially Expressed Putative Phosphoproteins in the Time Course of Salt Stress[a]

Proteins with at least Two fold up regulation after 10 hour induction

Spot #	Protein Identified	MW / pI	No. of Peptides	C.I.%	Express. Graph	Ratio	Protein ID
1	OSJNBa0060N03.24 protein	17.8 / 3.5	11	95.1		2.0	Q7XPD8
2	OSJNBa0059H15.10 protein	15.1 / 3.6	19	95.2		2.2	Q7XXH9
3	Putative dimethylaniline monooxygenase	18.4 / 3.7	12	97.3		2.5	NP_911087
4	Putative ribosomal protein S29	65.1 / 3.9	08	94.9		2.4	NP_919056
5	OSJNBb0034I13.10 protein	67.1 / 4.2	23	99.9		2.3	Q7XTU6
6	Salt-stress induced protein (Salt protein)	14.5 / 4.9	08	100		2.6	P24120
7	Hypothetical protein	15.1 / 4.5	11	95.8		3.3	AAO37494
8	dnaK-type molecular chaperone hsp70 - rice (fragment)	66.1 / 5.1	17	100		2.0	S53126
9	MRL (Mannose-binding RICE lectin) (Salt-induced protein)	13.8 / 5.3	06	100		2.2	O64441
10	Pyruvate decarboxylase isozyme 2 (EC 4.1.1.1)	66.1 / 5.6	09	99.9		3.0	P51848
11	Putative glutathione S-transferase	27.6 / 5.9	05	99.9		2.0	Q9AS53
12	OSJNBa0063G07.8 protein	68.9 / 5.8	12	99.6		3.3	Q7XP43
13	Putative disease resistant protein	36.6 / 6.2	26	98.1		2.7	Q8W5N5
14	Hypothetical protein	43.1 / 6.4	14	95.7		2.9	Q8S6Y5

Proteins with at least Two fold up regulation after 24 hour induction

Spot #	Protein Identified	MW / pI	No. of Peptides	C.I.%	Express. Graph	Ratio	Protein ID
1	OSJNBa0060N03.24 protein	17.8 / 3.5	09	95.1		2.1	Q7XPD8
2	OSJNBa0059H15.10 protein	15.1 / 3.6	19	95.2		2.3	Q7XXH9
3	Putative dimethylaniline monooxygenase	18.4 / 3.7	12	97.3		2.3	NP_911087
4	Putative ribosomal protein S29	65.1 / 3.9	08	94.9		3.0	NP_919056
5	OSJNBb0034I13.10 protein	67.1 / 4.2	23	99.9		2.6	Q7XTU6
6	Salt-stress induced protein (Salt protein)	14.5 / 4.9	08	100		4.0	P24120
7	Hypothetical protein	15.1 / 4.5	11	95.8		3.9	AAO37494
8	dnaK-type molecular chaperone hsp70 - rice (fragment)	66.1 / 5.1	17	100		2.4	S53126
9	MRL (Mannose-binding RICE lectin) (Salt-induced protein)	13.8 / 5.3	06	100		2.5	O64441
10	Pyruvate decarboxylase isozyme 2 (EC 4.1.1.1)	66.1 / 5.6	09	99.9		3.0	P51848
11	Putative glutathione S-transferase	27.6 / 5.9	05	99.9		2.6	Q9AS53
12	OSJNBa0063G07.8 protein	68.9 / 5.8	12	99.6		2.4	Q7XP43
14	Hypothetical protein	43.1 / 6.4	14	95.7		2.5	Q8S6Y5
15	Putative transposase	34.3 / 4.6	13	95.2		3.7	Q75I14
16	OSJNBa0032I19.18 protein	47.6 / 5.7	18	100		2.1	Q7XQ36
17	Small GTP-binding protein OsRac2	51.9 / 5.9	08	96.8		2.3	Q9SSW9

Proteins with at least Two fold down regulation after 10 hour induction

Spot #	Protein Identified	MW / pI	No. of Peptides	C.I.%	Express. Graph	Ratio	Protein ID
18	OSJNBa0017P10.22 protein	24.7 / 4.4	19	98.7		3.8	Q7XLU5
19	Putative glucan endo-1,3-beta-D-glucosidase	31.3 / 4.6	06	100		2.5	NP 914652
20	Putative protein kinase	49.4 / 5.1	11	98.8		2.5	NP 921616
21	OSJNBb0016D16.17 protein	56.7 / 5.0	19	94.4		3.2	Q7XN16
22	P0496H05.16	20.1 / 5.4	07	99.8		3.3	NP 918414
24	Hypothetical protein	46.4 / 5.9	09	91.2		2.8	NP 915705
25	Hypothetical protein~similar to chromosome 4 OSJNBb0080H08.22	60.2 / 6.1	20	97		2.6	NP 914725

Proteins with at least Two fold down regulation after 24 hour induction

Spot #	Protein Identified	MW / pI	No. of Peptides	C.I.%	Express. Graph	Ratio	Protein ID
20	Putative protein kinase	49.4 / 5.1	11	98.8		3.8	NP 921616
21	OSJNBb0016D16.17 protein	56.7 / 5.0	19	94.4		3.5	Q7XN16
22	P0496H05.16	20.1 / 5.4	07	99.8		3.6	NP 918414
23	Putative retroelement	30.1 / 5.2	26	99.8		2.5	Q8S782
24	Hypothetical protein	46.4 / 5.9	09	91.2		3.1	NP 915705
25	Hypothetical protein~similar to chromosome 4 OSJNBb0080H08.22	60.2 / 6.1	20	97		3.0	NP_914725
26	ATP synthase beta chain, mitochondrial precursor (EC3.6.3.14)	54.8 / 5.2	17	100		3.6	Q01859
27	OSJNBa0089K21.1 protein	57.3 / 5.8	11	99.8		2.5	Q7XQN5
28	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	52.4 / 6.4	12	100		2.3	Q42977

[a] Spot #: the spot number was given arbitrarily. MW/PI: experimental molecular weight and pI. No. of peptides: number of peptides that matched with the identified protein in mass analyses. C.I. %, cross confidence interval %. Over 95% represents high-confidence identification. Express graph: the three bars represent relative expression levels of the protein at 0, 10, and 24 h of salt treatment. The data was the average of the three biological replicas. The error bars are not presented here because the graphs are too small to reveal the differences of the error bars. Ratio: the up- or downregulation ratio of the protein at the time point of salt stress indicated in the table compared with the untreated sample. Protein ID: protein identification number provided by the NCBI data base and UniProt.

dye and the SYPRO Ruby each identified 10 and 16 unique proteins, respectively. Since SYPRO Ruby stain intensity is proportional to protein concentration in a wide concentration range,[30] the result suggests a possibility that the 10 putative

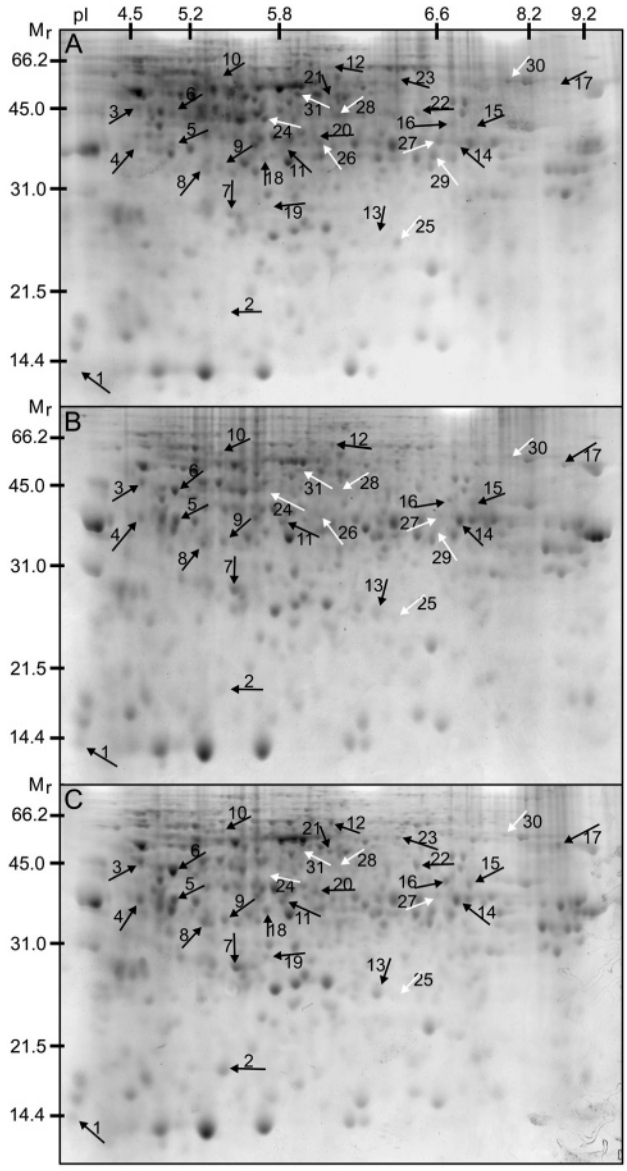

Figure 3. Protein differential expression under salinity stress revealed by SYPRO Ruby stain. Proteins were extracted from 20-day-old rice roots, were separated on 2-DE gels, and were stained with Pro-Q Diamond dye. The cutoff of differential regulation was 2-fold of change in three biological replicas. (A) Rice root proteome image without salt stress. All protein spots subjected to upregulation under salinity stress are marked with black arrows and all protein spots subjected to downregulation are marked with white arrows. (B) Rice root proteome image after 10 h of NaCl (150 mM) treatment. Proteins upregulated after 10 h of salt treatment are marked with black arrows and the proteins downregulated after 10 h of salt treatment are marked with white arrows. (C) Rice root proteome image after 24 h of NaCl (150 mM) treatment. Proteins upregulated after 24 h of salt treatment are marked with black arrows and the proteins downregulated after 24 h of salt treatment are marked with white arrows.

phosphoproteins identified by Pro-Q Diamond stain only were upregulated at the post-translational level instead of having their cellular protein concentration increased.

3.4. Gene Ontology (GO) Analysis. To understand the biological processes and cellular components involved in salt stress response in rice, gene ontology analyses were performed using the differentially regulated proteins, including both differentially up- and downregulated proteins, identified by SYPRO Ruby stain and Pro-Q Diamond stain. As shown in Figure 5, the differentially regulated proteins identified using these two dyes had very different distribution among the GO categories. The top two cellular components for the SYPRO Ruby stain identified proteins were cytoskeleton (33.3%) and cytoplasm (20%) and for the Pro-Q Diamond dye identified proteins were membrane (54.5%) and intracellular components (18.2%). The top two biological processes for the SYPRO Ruby stain-identified proteins were metabolism (33.3%) and response to biotic stimulus/amino acid and derivative metabolism/carbohydrate metabolism (9.5%) and for the Pro-Q Diamond identified proteins were transport (17.4%) and DNA metabolism/metabolism (13%). When analyzed on the basis of molecular functions, the top two categories for the SYPRO Ruby stain-identified proteins were catalytic activity (31.6%) and binding (21.1%) and for the Pro-Q Diamond stain identified proteins were nucleotide binding/catalytic activity (21.5%) and binding (15.7%). These results indicated that diversified cellular processes and cellular components are involved in salt stress response, and these two dyes covered a different spectrum of the salt stress response proteins. However, we should keep in mind that one protein could be grouped into more than one GO category sometimes. Therefore, the total percentage in each pie is over 100% when added together. In addition, the current GO system has not included all possible functions for proteins with multiple functions. For example, although the roles of aldolase, malate dehydrogenase, and glutathione S-transferase in salt stress have been reported in several publications, they were considered as metabolic enzymes with catalytic activities in GO analysis instead of stress response proteins. Therefore, the GO analysis results presented here should be taken with the above information in mind.

4. Conclusion Remarks

Studies in molecular biology, biochemistry, and genetics have indicated that protein phosphorylation plays a critical role in plant response to salinity stress. However, phosphoprotein differential regulation under salinity stress has not been well studied using proteome approaches in rice. Pro-Q Diamond in Gel Stain has been used extensively to identify phosphoproteins following 2-DE gel separation because of its specificity to phosphoproteins.[22−26] Here, we have investigated protein differential regulation under salt stress using the Pro-Q Diamond dye in rice. The majority of proteins stained heavily by Pro-Q Diamond dye have a relatively low pI. This observation is consistent with the fact that protein phosphorylation results in acidification of the protein. We have successfully identified 17 of the 20 upregulated and 11 of the 18 downregulated putative phosphoproteins. All these 17 proteins have not been reported in prior proteome studies on rice salinity stress although some of them are typical salt stress response proteins, suggesting that the Pro-Q Diamond in Gel Stain is a useful tool in identifying stress response proteins. We find that using an appropriate concentration of Pro-Q Diamond dye is critical for reducing nonspecific background. Direct visualization of the putative phosphoprotein spots using Pro-Q Diamond dye enables us to select the potentially interesting proteins for mass analyses. However, the limited protein quantity, from an excised protein spot of 2-DE gel, makes mapping the phosphorylated residues extremely challenging. Recently, several phosphoproteome analysis methods have been developed, including titanium dioxide microcolumn,[38] immobilized metal-

Table 2. Differentially Expressed Proteins in the Time Course of Salt Stress[a]

Proteins with at least Two fold up regulation after 10 hour induction

Spot #	Protein Identified	MW / pI	No. of Peptides	C.I.%	Express. Graph	Ratio	Protein ID
1	OSJNBa0059H15.10 protein	14.5 / 3.7	19	95.2		2.4	Q7XXH9
2	Hypothetical protein	19.9 / 5.4	14	95.7		3.8	Q8S6Y5
3	Alpha-amylase precursor (EC 3.2.1.1) (Isozyme 1B)	44.9 / 4.5	06	100.0		2.1	P17654
4	OSJNBa0006B20.3 protein	38.3 / 4.5	07	96.7		4.0	Q7XV63
5	Putative CRK1 protein	38.1 / 4.9	11	98. 7		4.3	Q8S1Y6
6	Actin 7	43.2 / 4.9	06	94.4		4.6	P17300
7	Triosephosphate isomerase, cytosolic (EC 5.3.1.1)	29.3 / 5.5	10	100.0		2.1	P48494
8	Cysteine synthase (EC 2.5.1.47) (CSase) (OAS-TL)	33.9 / 5.3	13	100.0		2.2	Q9XEA8
9	Glyoxalase I	35.1 / 5.4	12	100.0		3.0	Q948T6
10	Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	57.6 / 5.4	16	100.0		2.5	Q5KQH5
11	Cytoplasmic malate dehydrogenase (EC 1.1.1.37)	38.4 / 5.8	05	100.0		2.1	Q7XDC8
12	OSJNBb0059K02.15 protein	62.7 / 6.0	11	99.9		2.1	Q7XMP6
13	Putative disease resistant protein	27.1 / 6.3	26	98.1		2.2	Q8W5N5
14	CAA303717.1 protein	38.5 / 6.9	12	100.0		5.0	Q9ST80
15	Aldolase C-1	40.8 / 7.1	15	100.0		3.0	Q42476
16	Putative phosphoglycerate kinase	41.7 / 6.7	12	97.1		3.0	Q65X66
17	Putative transposase	57.1 / 8.9	10	95.2		3.2	Q75I14

Proteins with at least Two fold up regulation after 24 hour induction

Spot #	Protein Identified	MW / pI	No. of Peptides	C.I.%	Express. Graph	Ratio	Protein ID
1	OSJNBa0059H15.10 protein	14.5 / 3.7	19	95.2		2.3	Q7XXH9
2	Hypothetical protein	19.9 / 5.4	14	95.7		5.4	Q8S6Y5
3	Alpha-amylase precursor (EC 3.2.1.1) (Isozyme 1B)	44.9 / 4.5	06	100.0		3.2	P17654
4	OSJNBa0006B20.3 protein	38.3 / 4.5	07	96.7		8.3	Q7XV63
5	Putative CRK1 protein	38.1 / 4.9	11	98. 7		5.2	Q8S1Y6
6	Actin 7	43.2 / 4.9	06	94.4		5.5	P17300
7	Triosephosphate isomerase, cytosolic (EC 5.3.1.1)	29.3 / 5.5	10	100.0		2.6	P48494
8	Cysteine synthase (EC 2.5.1.47) (CSase) (OAS-TL)	33.9 / 5.3	13	100.0		2.5	Q9XEA8
9	Glyoxalase I	35.1 / 5.4	12	100.0		3.8	Q948T6
10	Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	57.6 / 5.4	16	100.0		2.8	Q5KQH5
11	Cytoplasmic malate dehydrogenase (EC 1.1.1.37)	38.4 / 5.8	05	100.0		2.2	Q7XDC8
12	OSJNBb0059K02.15 protein	62.7 / 6.0	11	99.9		2.1	Q7XMP6
13	Putative disease resistant protein	27.1 / 6.3	26	98.1		3.4	Q8W5N5
14	CAA303717.1 protein	38.5 / 6.9	12	100.0		4.3	Q9ST80
15	Aldolase C-1	40.8 / 7.1	15	100.0		4.0	Q42476
16	Putative phosphoglycerate kinase	41.7 / 6.7	12	97.1		4.3	Q65X66
17	Putative transposase	57.1 / 8.9	10	95.2		3.9	Q75I14
18	Peroxidase precursor (EC 1.11.1.7)	36.8 / 5.7	04	99.7		2.7	Q43006
19	OSJNBa0032I19.18 protein	30.3 / 5.7	18	100.0		3.9	Q7XQ36
20	Small GTP-binding protein OsRac2	39.7 / 5.9	08	96.8		2.7	Q68Y52
21	OSJNBa0063G07.8 protein	49.2 / 6.0	12	99.6		4.0	Q7XP43
22	Carboxypeptidase precursor-like protein	43.5 / 6.4	11	100.0		2.9	Q9FP87
23	Putative dihydrolipoamide dehydrogenase	56.9 / 6.3	12	100.0		2.4	Q9ASP4

Proteins with at least Two fold down regulation after 10 hour induction

Spot #	Protein Identified	MW / pI	No. of Peptides	C.I.%	Express. Graph	Ratio	Protein ID
24	Hypothetical protein	43.4 / 5.7	17	95.6		3.7	Q8S6K4
25	OSJNBa0020I02.19 protein	24.3 / 6.3	08	94.9		4.9	Q7XVZ7
26	Hypothetical protein	39.1 / 5.9	06	97.8		2.8	Q94LT8
27	OSJNBa0087O24.20 protein	39.4 / 6.5	20	96.3		2.2	Q7XPJ0
28	Contains similarity to retrotransposable elements TNP2	43.4 / 6.1	19	99.3		3.1	Q8H898
29	Hypothetical protein	36.8 / 6.5	09	98.2		4.2	Q9LWI0
30	Guanine nucleotide-exchange protein GEP2	55.4 / 7.9	20	98.3		3.0	Q8S565
31	P0690B02.19 protein	50.1 / 5.9	14	96.4		3.2	Q8W0D2

Proteins with at least Two fold down regulation after 24 hour induction

Spot #	Protein Identified	MW / pI	No. of Peptides	C.I.%	Express. Graph	Ratio	Protein ID
24	Hypothetical protein	43.4 / 5.7	17	95.6		6.5	Q8S6K4
25	OSJNBa0020I02.19 protein	24.3 / 6.3	08	94.9		6.4	Q7XVZ7
27	OSJNBa0087O24.20 protein	39.4 / 6.5	20	96.3		2.5	Q7XPJ0
28	Contains similarity to retrotransposable elements TNP2	43.4 / 6.1	19	99.3		3.7	Q8H898
30	Guanine nucleotide-exchange protein GEP2	55.4 / 7.9	20	98.3		2.5	Q8S565
31	P0690B02.19 protein	50.1 / 5.9	14	96.4		3.2	Q8W0D2

[a] Spot #: the spot number was given arbitrarily. MW/PI: experimental molecular weight and pI. No. of peptides: number of peptides that matched with the identified protein in mass analyses. C.I. %: cross confidence interval %. Over 95% represents high-confidence identification. Express graph: the three bars represent relative expression levels of the protein at 0, 10, and 24 h of salt treatment. The data was the average of the three biological replicas. The error bars are not presented here because the graphs are too small to reveal the differences of the error bars. Ratio: the up- or downregulation ratio of the protein at the time point of salt stress indicated in the table compared with the untreated sample. Protein ID: protein identification number provided by the NCBI data base and UniProt.

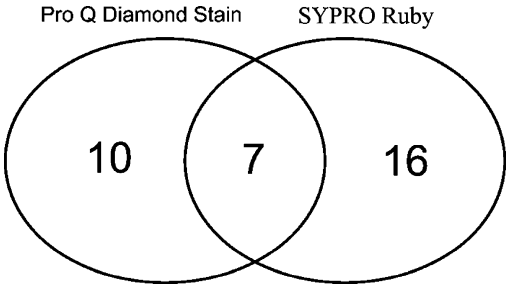

Figure 4. Venn diagram analyses of the differentially upregulated proteins under salinity stress. Differentially upregulated proteins revealed by SYPRO Ruby and Pro-Q Diamond Phosphoprotein Gel Stain were analyzed.

affinity chromatography method,[39] phosphopeptide enrichment by IEF,[40] and the phosphoprotein extraction kit of QIAGEN.[41] The combination of the 2-DE gel based Pro-Q Diamond stain and the phosphopeptide-enriching microcolumn may help mapping the phosphorylated residues of these putative phosphoproteins in the future.

We detect 31 upregulated and 13 downregulated protein spots when the 2-DE gels are stained by SYPRO Ruby. Among these 44 differentially regulated protein spots, molecular identities of 23 upregulated and 8 downregulated proteins have been obtained. While eight are well-known salt stress response proteins, salt stress differential regulation of the majority of the other proteins, including the seven function unknown proteins, has been not reported in literature. Gene ontology analyses revealed that the differentially regulated proteins participate in diversified biological processes.

As shown in Figure 4, seven proteins displayed differential expression whether the gel was stained by Pro-Q Diamond dye or SYPRO Ruby. The other differentially regulated proteins were specific either to Pro-Q Diamond stain or SYPRO Ruby stain. These results suggest that most proteins with quantity change during salinity stress are not phosphoproteins or have no change in phosphorylation level. On the other hand, most differentially regulated phosphoproteins have modification level change instead of quantity change during salinity stress. The functions of the differentially regulated proteins in salinity stress can be further tested using reverse genetic approaches. In addition, it will be very interesting to identify the kinases that phosphorylate these phosphoproteins in response to salinity stress. Our studies have provided valuable new insight into plant response to salinity stress.

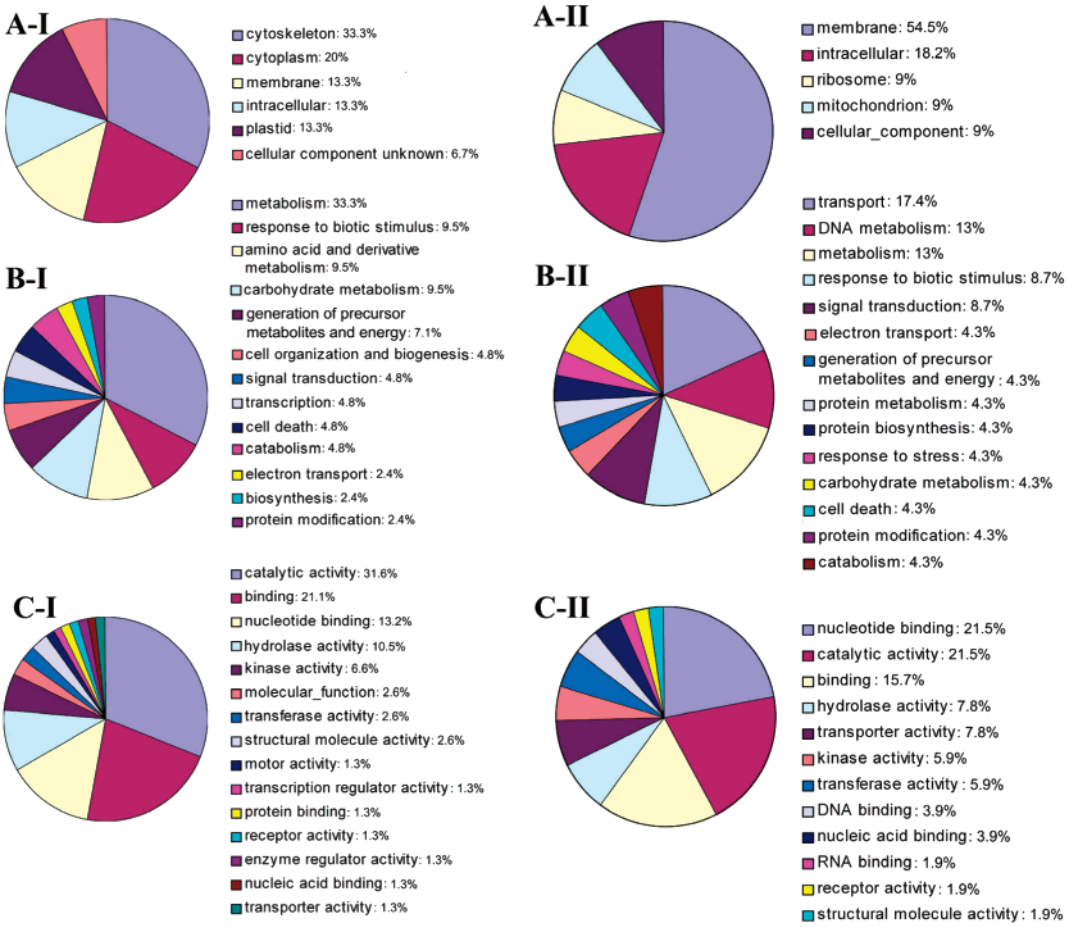

Figure 5. Distribution of the differentially regulated proteins among different GO categories. All differentially up- and downregulated proteins within the 24 h salt stress period were included. The differentially regulated proteins identified using SYPRO Ruby stain were presented in A-I, B-I, and C-I. The differentially regulated proteins identified using Pro-Q Diamond stain were presented in A-II, B-II, and C-II. Percentage distribution of the differentially regulated proteins was used. The pie charts were generated using the analysis results of the "GOSlimViewer" tool at AgBase. (A-I and A-II) Protein distribution based on cellular localizations. (B-I and B−II) Protein distribution based on biological processes. (C-I and C-II) Protein distribution based on molecular functions.

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References

(1) Wang, W.; Vinocur, B.; Altman, A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* **2003**, *218* (1), 1−14.
(2) Chinnusamy, V.; Zhu, J.; Zhu, J. K. Salt stress signaling and mechanisms of plant salt tolerance. *Genet. Eng. (N. Y.)* **2006**, *27*, 141−77.
(3) Kasuga, M.; Liu, Q.; Miura, S.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.* **1999**, *17* (3), 287−91.
(4) Haake, V.; Cook, D.; Riechmann, J. L.; Pineda, O.; Thomashow, M. F.; Zhang, J. Z. Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. *Plant Physiol.* **2002**, *130* (2), 639−48.
(5) Park, J. M.; Park, C. J.; Lee, S. B.; Ham, B. K.; Shin, R.; Paek, K. H. Overexpression of the tobacco Tsi1 gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *Plant Cell* **2001**, *13* (5), 1035−46.
(6) Mukhopadhyay, A.; Vij, S.; Tyagi, A. K. Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proc. Natl. Acad. Sci. U.S.A.* **2004,** *101* (16), 6309−14.
(7) Hu, H.; Dai, M.; Yao, J.; Xiao, B.; Li, X.; Zhang, Q.; Xiong, L. Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (35), 12987−92.
(8) Abbasi, F. M.; Komatsu, S. A proteomic approach to analyze salt-responsive proteins in rice leaf sheath. *Proteomics* **2004**, *4* (7), 2072−81.
(9) Khan, M.; Takasaki, H.; Komatsu, S. Comprehensive phosphoproteome analysis in rice and identification of phosphoproteins responsive to different hormones/stresses. *J. Proteome Res.* **2005**, *4* (5), 1592−9.
(10) Parker, R.; Flowers, T. J.; Moore, A. L.; Harpham, N. V. An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. *J. Exp. Bot.* **2006**, *57* (5), 1109−18.
(11) Yan, S.; Tang, Z.; Su, W.; Sun, W. Proteomic analysis of salt stress-responsive proteins in rice root. *Proteomics* **2005**, *5* (1), 235−44.
(12) Kim, D. W.; Rakwal, R.; Agrawal, G. K.; Jung, Y. H.; Shibato, J.; Jwa, N. S.; Iwahashi, Y.; Iwahashi, H.; Kim, D. H.; Shim Ie, S.; Usui, K. A hydroponic rice seedling culture model system for investigating proteome of salt stress in rice leaf. *Electrophoresis* **2005**, *26* (23), 4521−39.
(13) Chao, D. Y.; Luo, Y. H.; Shi, M.; Luo, D.; Lin, H. X. Salt-responsive genes in rice revealed by cDNA microarray analysis. *Cell Res.* **2005**, *15* (10), 796−810.
(14) Rabbani, M. A.; Maruyama, K.; Abe, H.; Khan, M. A.; Katsura, K.; Ito, Y.; Yoshiwara, K.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant Physiol.* **2003**, *133* (4), 1755−67.
(15) Kawasaki, S.; Borchert, C.; Deyholos, M.; Wang, H.; Brazille, S.; Kawai, K.; Galbraith, D.; Bohnert, H. J. Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell* **2001**, *13* (4), 889−905.
(16) Teige, M.; Scheikl, E.; Eulgem, T.; Doczi, R.; Ichimura, K.; Shinozaki, K.; Dangl, J. L.; Hirt, H. The MKK2 pathway mediates cold and salt stress signaling in *Arabidopsis*. *Mol. Cell* **2004**, *15* (1), 141−52.
(17) Ichimura, K.; Mizoguchi, T.; Irie, K.; Morris, P.; Giraudat, J.; Matsumoto, K.; Shinozaki, K. Isolation of ATMEKK1 (a MAP kinase kinase kinase)-interacting proteins and analysis of a MAP kinase cascade in *Arabidopsis*. *Biochem. Biophys. Res. Commun.* **1998**, *253* (2), 532−43.
(18) Mizoguchi, T.; Irie, K.; Hirayama, T.; Hayashida, N.; Yamaguchi-Shinozaki, K.; Matsumoto, K.; Shinozaki, K. A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis* thaliana. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93* (2), 765−9.
(19) Xiong, L.; Yang, Y. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell* **2003**, *15* (3), 745−59.
(20) Liu, J.; Zhu, J. K. A calcium sensor homolog required for plant salt tolerance. *Science* **1998**, *280* (5371), 1943−5.
(21) Zhu, J. K. Regulation of ion homeostasis under salt stress. *Curr. Opin. Plant Biol.* **2003**, *6* (5), 441−5.
(22) Schulenberg, B.; Goodman, T. N.; Aggeler, R.; Capaldi, R. A.; Patton, W. F. Characterization of dynamic and steady-state protein phosphorylation using a fluorescent phosphoprotein gel stain and mass spectrometry. *Electrophoresis* **2004**, *25* (15), 2526−32.
(23) Steinberg, T. H.; Agnew, B. J.; Gee, K. R.; Leung, W. Y.; Goodman, T.; Schulenberg, B.; Hendrickson, J.; Beechem, J. M.; Haugland, R. P.; Patton, W. F. Global quantitative phosphoprotein analysis using Multiplexed Proteomics technology. *Proteomics* **2003**, *3* (7), 1128−44.
(24) Agrawal, G. K.; Thelen, J. J. Development of a simplified, economical polyacrylamide gel staining protocol for phosphoproteins. *Proteomics* **2005**, *5* (18), 4684−8.
(25) Stasyk, T.; Morandell, S.; Bakry, R.; Feuerstein, I.; Huck, C. W.; Stecher, G.; Bonn, G. K.; Huber, L. A. Quantitative detection of phosphoproteins by combination of two-dimensional difference gel electrophoresis and phosphospecific fluorescent staining. *Electrophoresis* **2005**, *26* (14), 2850−4.
(26) Agrawal, G. K.; Thelen, J. J. Large Scale Identification and Quantitative Profiling of Phosphoproteins Expressed during Seed Filling in Oilseed Rape. *Mol. Cell. Proteomics* **2006**, *5* (11), 2044−59.
(27) Hoagland, D. R.; Arnon, D. R. The Water Culture Method for Growing Plants without Soil. *Calif. Agric. Exp. Stn. Circ.* **1938**.
(28) Hurkman, W. J.; Tanaka, C. K. Solubilization of Plant Membrane Proteins for Analysis by Two-Dimensional Gel Electrophoresis. *Plant Physiol.* **1986**, *81* (3), 802−6.
(29) McCarthy, F. M.; Bridges, S. M.; Wang, N.; Magee, G. B.; Williams, W. P.; Luthe, D. S.; Burgess, S. C. AgBase: A Unified Resource for Functional Analysis in Agriculture. *Nucleic Acids Res.* **2007**, in press.
(30) Berggren, K.; Steinberg, T. H.; Lauber, W. M.; Carroll, J. A.; Lopez, M. F.; Chernokalskaya, E.; Zieske, L.; Diwu, Z.; Haugland, R. P.; Patton, W. F. A Luminescent Ruthenium Complex for Ultrasensitive Detection of Proteins Immobilized on Membrane Supports. *Anal. Biochem.* **1999**, *276* (2), 129−43.
(31) Hirano, K.; Teraoka, T.; Yamanaka, H.; Harashima, A.; Kunisaki, A.; Takahashi, H.; Hosokawa, D. Novel mannose-binding rice lectin composed of some isolectins and its relation to a stress-inducible salt gene. *Plant Cell Physiol.* **2000**, *41* (3), 258−67.
(32) Roxas, V. P.; Smith, R. K., Jr.; Allen, E. R.; Allen, R. D. Overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nat. Biotechnol.* **1997**, *15* (10), 988−91.
(33) Lewis, A.; Di Ciano, C.; Rotstein, O. D.; Kapus, A. Osmotic stress activates Rac and Cdc42 in neutrophils: role in hypertonicity-induced actin polymerization. *Am. J. Physiol. Cell Physiol.* **2002**, *282* (2), C271−9.
(34) Liu, X.; Baird, W. V. Differential expression of genes regulated in response to drought or salinity stress in sunflower. *Crop Sci.* **2003**, *43*, 678−87.
(35) Veena Reddy, V. S.; Sopory, S. K. Glyoxalase I from Brassica juncea: molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. *Plant J.* **1999**, *17* (4), 385−95.
(36) Barroso, C.; Romero, L. C.; Cejudo, F. J.; Vega, J. M.; Gotor, C. Salt-specific regulation of the cytosolic O-acetylserine(thiol)lyase gene from *Arabidopsis* thaliana is dependent on abscisic acid. *Plant Mol. Biol.* **1999**, *40* (4), 729−36.
(37) Singla-Pareek, S. L.; Reddy, M. K.; Sopory, S. K. Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (25), 14672−7.

(38) Larsen, M. R.; Thingholm, T. E.; Jensen, O. N.; Roepstorff, P.; Jorgensen, T. J. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell Proteomics* **2005**, *4* (7), 873−86.
(39) Ficarro, S. B.; McCleland, M. L.; Stukenberg, P. T.; Burke, D. J.; Ross, M. M.; Shabanowitz, J.; Hunt, D. F.; White, F. M. Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. *Nat. Biotechnol.* **2002**, *20* (3), 301−5.
(40) Maccarrone, G.; Kolb, N.; Teplytska, L.; Birg, I.; Zollinger, R.; Holsboer, F.; Turck, C. W. Phosphopeptide enrichment by IEF. *Electrophoresis* **2006**, *27* (22), 4585−95.
(41) Jones, A. M.; Bennett, M. H.; Mansfield, J. W.; Grant, M. Analysis of the defence phosphoproteome of *Arabidopsis* thaliana using differential mass tagging. *Proteomics* **2006**, *6* (14), 4155−65.

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