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Differential Proteins Expressed in Rice Leaves and Grains in Response to Salinity and Exogenous Spermidine Treatments

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Abstract: Exogenous application of spermidine (Spd) has been reported to modulate physiological processes and alleviate salt-induced damage to growth and productivity of several plants including rice. Employing a proteomic approach, we aimed at identifying rice leaf and grain proteins differentially expressing under salt stress, and in response to Spd prior to NaCl treatment. A total of 9 and 20 differentially expressed protein spots were identified in the leaves of salt-tolerant (Pokkali) and salt-sensitive (KDML105) rice cultivars, respectively. Differential proteins common to both cultivars included a photosynthetic light reaction protein (oxygen-evolving complex protein 1), enzymes of Calvin cycle and glycolysis (fructose-bisphosphate aldolase and triose-phosphate isomerase), malate dehydrogenase, superoxide dismutase and a hypothetical protein (OsI_18213). Most proteins were present at higher intensities in Pokkali leaves. The photosynthetic oxygen-evolving enhancer protein 2 was detected only in Pokkali and was up-regulated by salt-stress and further enhanced by Spd treatment. All three spots identified as superoxide dismutase in KDML105 were up-regulated by NaCl but down-regulated when treated with Spd prior to NaCl, indicating that Spd acted directly as antioxidants. Important differential stress proteins detected in mature grains of both rice cultivars were late embryogenesis abundant proteins with protective roles and an antioxidant protein, 1-Cys-peroxiredoxin. Higher salt tolerance of Pokkali partly resulted from higher intensities and more responsiveness of the proteins relating to photosynthesis light reactions, energy metabolism, antioxidant enzymes in the leaves, and stress proteins with protective roles in the grains.

Key words: *Oryza sativa* L.; proteomics; salt stress; spermidine

Salt stress is one of the major abiotic stresses prevalent in agricultural areas worldwide. Excess salts in soils inhibit plant growth and productivity for two main reasons. The first one is an osmotic effect that reduces the capacity of plant water uptake and causes a slow plant growth, and the second is the toxic effect due to the entry of salts that cause tissue damage of the leaves (Zhu, 2002; Khan et al, 2007). Rice (*Oryza sativa* L.) is more sensitive to salt stress compared with other cereals (Shannon et al, 1998) and the threshold above which rice yields reduced ranges from 1.9 to 3.0 dS/m (Grattan et al, 2002). Salinity affects all stages of growth and development of rice, particularly early seedling, pollination and fertilization stages (Lutts et al, 1995; Gregorio et al, 1997). Different varieties of rice express widely different levels of salt tolerance (Gregorio et al, 2002; Zeng et al, 2003; Kanawapee et al, 2012). Mechanisms for increasing tolerance to salt stress involve the production of stress proteins, the accumulation of compatible solutes (sugars, amino and organic acids, betaines and polyamines), and the expression of different sets of genes (Jimenez-Bremont et al, 2007). Plant growth regulators including polyamines (PAs) and other groups of chemicals have been used to treat rice plants exogenously at various growth stages to increase salt tolerance by alleviating

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salt-induced damages and lead to improved growth and productivity (Roychoudhury et al, 2011; Plaut et al, 2013).

In plants, PAs are known to play regulatory roles in various physiological events, such as cell division, root initiation, flower development, and fruit ripening and senescence, and also have beneficial effects on abiotic stress responses (Alcazar et al, 2006). For salinity, exogenous PAs have been documented to reduce salinity stress-induced damages including the accumulation of Na^+, loss of K^+, chlorophyll degradation, inhibition of photochemical reaction of photosynthesis, reactive oxygen species (ROS) accumulation, lipid peroxidation and membrane damage, and the enhancement of activity of antioxidative enzymes and non-enzymatic antioxidants and compatible osmolytes (Chattopadhyay et al, 2002; Roy et al, 2005; Roychoudhury et al, 2011). Exogenous spermidine (Spd) reverses the inhibitory effects of salt stress in both salt-tolerant (Pokkali) and salt-sensitive (KDML105) rice cultivars (Saleethong et al, 2011). Salt-induced damage including chlorophyll loss, membrane leakage, ROS (H_2O_2) accumulation, lipid peroxidation, Na^+ accumulation and K^+ reduction were all alleviated by Spd pretreatment, resulting in improved growth performance (Zhu et al, 2006; Duan et al, 2008; Saleethong et al, 2011). In reproductive stage, salinity treatment imposed during late booting stage to maturity stage resulted in significant reductions in yield and yield-related parameters of both Pokkali and KDML105, and pretreatment by foliar spraying of Spd to rice plants during the early booting stage significantly improved yields particularly of KDML105. Spd also increased K^+ and reduced Na^+ concentration in the mature grains, leading to higher K^+/Na^+ ratio (Saleethong et al, 2013).

Over the past several years, proteomics studies in rice have provided a more critical understanding towards functions of rice proteins under favourable as well as various stress conditions. A proteomic approach was adopted to identify proteins that increase in abundance in leaf sheaths, roots, and leaf blades of 2-week-old rice seedlings subjected to salt stress (Abbasi and Komatsu, 2004). More than 1 100 proteins were reproducibly detected, including 34 up-regulated and 20 down-regulated ones when rice seedlings were exposed to NaCl stress (Yan et al, 2005). Kim et al (2005) identified 47 salt-induced proteins which were involved in major metabolic processes, such as photosynthetic assimilation of carbon dioxide, photorespiration and oxidative damage. In rice leaf lamina, Parker et al (2006) found that 32 out of 2 500 proteins were significantly regulated by salinity. Zhang et al (2009) identified an apoplastic protein in rice roots and found that 10 protein spots which were significantly increased or decreased in abundance during the initial phase of salt stress. These proteins included some well-known biotic and abiotic stress related proteins such as *O. sativa* root meander curling which drastically increased in abundance. Lee et al (2011) investigated salt-induced leaf proteins in two rice cultivars with different salt sensitivity and found 23 up-regulated protein spots, of which six were newly reported (class III peroxidase 29 precursor, beta-1,3-glucanase precursor, a putative transcription factor, putative chaperonin 21 precursor, Rubisco activase small isoform precursor, and drought-induced S-like ribonuclease). Comparing leaf proteomes of two rice cultivars differing in salt tolerance, ten different up-regulated proteins and elevated levels of corresponding mRNA transcripts were detected in the salt-tolerant rice cultivar Pokkali, while only three proteins were detected in the salt-sensitive rice cultivar KDML105 (Jankangram et al, 2011). Recently, a proteomic study in early salt stressed roots of SnRK2 transgenic rice revealed 52 early salt-responsive protein spots (Nam et al, 2012). The major up-regulated proteins were enzymes related to energy regulation, amino acid metabolism, methyl glyoxal detoxification, redox regulation and protein turnover.

Although several reports on rice proteomes in response to salinity stress are available, few have included a comparison between salt-tolerant and salt-sensitive cultivars, and none have addressed the effects of exogenous growth regulators on modulation of expression of salt-induced proteins. This study aimed to identify proteins differentially expressed in leaves and grains of two rice cultivars differing in salt tolerance, in response to salt stress and also examine their modulated expression due to pretreatment with Spd. This study is expected to contribute to continuing efforts to determine key processes involved in salt stress tolerance and elucidate the beneficial mechanisms elicited by exogenous Spd.

MATERIALS AND METHODS

Plant materials and salinity treatments at the vegetative stage

Two rice cultivars, Pokkali (salt-tolerant) and KDML105 (salt-sensitive), were used. Seeds were surface-

sterilized, soaked in sterile distilled water for 48 h and germinated in Petri plates lined with two layers of paper towel moistened with sterile distilled water. Seven-day-old seedlings of uniform size with three leaves were transplanted to plastic containers holding 20 L full-strength Yoshida nutrient solution (Yoshida et al, 1976) with pH 5.0 (50 seedlings per container). The solution was renewed weekly and the pH was daily adjusted to 5.0. Two treatment groups of 30-day-old seedlings were pretreated by adding 1.0 mmol/L Spd into nutrient solution. After 24 h of Spd pre-treatment, plants of one treatment group (Spd) were transferred to normal nutrient solution containing 150.0 mmol/L NaCl (NaCl+Spd), and plants of the other group were transferred from normal nutrient solution to the one only containing 150.0 mmol/L NaCl (NaCl). One set of plants cultured in normal nutrient solution throughout the experiment served as the control. After 7 d of salt treatment, the leaves were collected, immediately frozen in liquid nitrogen and stored at -80 °C. The experiment was carried out in a greenhouse under natural photoperiod at the Faculty of Agriculture, Khon Kaen University, Thailand.

Salinity treatments at the reproductive stage

The experiment was conducted in a wire house (with walls of nylon net) during the months of June to November in 2010. The potting mix was composed of sandy clay loam soil and farm yard manure in the volume ratio of 3:1. Ten kilograms of the potting mix was filled in each plastic pot (height: 0.30 m; diameter: 0.25 m). Five seeds were sown in each pot, and the plants were thinned to three per pot at 14 d after emergence. The water level in each pot was maintained at 5 cm above the soil surface using tap water. The plants were fertilized at the tillering stage with chemical fertilizer at 0.45 g per pot. At the early booting stage, the plants were divided into four groups, one control and three treatments including Spd, NaCl and NaCl+Spd. Ten pots were set up per treatment. For the Spd and NaCl+Spd treatments, the plants were treated with 1.0 mmol/L Spd by foliar spraying at 18:00 pm for 7 d. The control plants were sprayed with distilled water. The volume of Spd solution or distilled water was 100 mL per pot. After the Spd treatment was completed, salinity treatment was imposed to NaCl and NaCl+Spd treatments by irrigating with 25.0 mmol/L NaCl instead of tap water, while the control and the Spd treatments continued to be fed with tap water until harvest. The hulls were manually removed from the mature grains and the brown grains were used for protein extraction.

Protein extraction

The leaves and grains were ground on ice and proteins were extracted using extraction buffer [for leaves: 30.0 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L EDTA, 6.0 mmol/L ascorbic acid, 5.0 mmol/L $MgCl_2$, 1% polyvinyl pyrrolidone, 0.02% β-mercaptoethanol, 1% glycerol; for grains: 20.0 mmol/L Tris-base (pH 8.0), 5.0 mmol/L EDTA, 2.0 mmol/L phenylmethane sulfonyl fluoride]. The homogenate was vortexed and incubated at -20 °C for 40 min, centrifuged at 13 000 r/min for 1 h. Supernatants were collected and contaminating substances were removed by 2D-clean up kit (Amersham Biosciences, Sweden). The protein sample was transferred to a microcentrifuge and centrifuged at 13 000 r/min for 5 min. The protein mixture was mixed by vortexing and incubated on ice (4–5 °C) for 15 min. The 300 μL co-precipitant was added to the reaction mixture and centrifuged at 13 000 r/min for 5 min. The supernatant was removed and discarded using a pipette tip. The distilled water was added on the top of the layer pellet. The tube was vortexed for several seconds. The pellet was washed with 1 mL wash buffer and 5 μL wash additive buffer at -20 °C for at least 1 h. The supernatant was discarded, the pellet was dried and solubilized with rehydration buffer [8.0 mol/L urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane slfonate (CHAPS), 0.002% bromophenol blue, 2.0 mmol/L dithiothreitol, 0.8% immobiline pH gradient gels (IPG) buffer]. Then the protein concentration was determined by Bradford assay (Bradford, 1976).Three biological replications from each treatment group were used to perform protein electrophoresis.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

First dimensional isoelectric focusing (IEF)

Protein samples were resuspended in the rehydration buffer. Samples were allowed to rehydrate for 1 h on ice. In this study, Ettan IPGphor II Isoelectric Focusing (Amersham Biosciences, USA) and Immobiline Drystrip pH 3–10 were used in the first dimensional isoelectric focusing. IEF was performed on 7 cm IPG strips. Then, strips were rehydrated with sample and rehydration buffer on Strip Holder. The adequate amount of protein was loaded on the Strip Holder (150 μg for 7 cm IPG strip). Then, the cover fluid was pipetted into the Strip Holder until the IPG strip was covered (approximately 500 μL) and the lid was

placed onto the Strip Holder and the IPG strip was allowed to rehydrate at 20 °C in Ettan IPGphor II Isoelectric Focusing. A minimum of 12 h is required for rehydration. Finally, IPG strip was run in 5 steps according to the manufacturer's instruction manual i.e. step 1 at 150 V/2 kVh, step 2 at 300 V/0.2 kVh, step 3 at 1 000 V/0.3 kVh, step 4 at 5 000 V/4.0 kVh and step 5 at 5 000 V/2.0 kVh. After isoelectric focusing, IPG strip was equilibrated in equilibration solution [75.0 mmol/L Tris-HCl (pH 8.8), 6.0 mol/L urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 0.002% bromophenol blue] by soaking for 15 min in DTT containing 5 mL equilibration solution. The strip was placed on a shaker. Then, equilibration was performed in iodoacetamide (IAA) containing equilibration solution for 15 min.

Second dimensional polyacrylamide gel electrophoresis (SDS-PAGE)

After equilibration, the second dimension was performed using SDS-PAGE with 10% separating gel. The relative molecular weight of each protein was determined using the broad range molecular weight standard proteins (Fermentas, France). The equilibrated IPG gel strip was placed on top of the vertical SDS gel and overlaid with agarose solution. Then the agarose was solidified for at least 5 min. The gel was inserted in the electrophoresis apparatus and the SDS-gel was run at 30 V per gel for 10 min, subsequently increased to 75 V per gel until finished.

Comparative analysis of 2D-gels

Gel was stained with 0.2% Coomassie Brilliant Blue G-250 and scanned using a high-resolution scanner (GS-800 Calibrated Imaging Densitometer, Bio-Rad, California, USA). Spot detection was carried out using the Dimension software (SineGene, USA) and Image Master 2D platinum (Amersham Biosciences, USA). Comparative analysis of the protein expression on 2D-gels between control and treatment groups were responded (unaffected, up-regulated and down-regulated) by the percentage of total volume in spot proteins on gel.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

The protein spots were digested with trypsin and analyzed at Proteomics Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand. The protein digest was analyzed in an Ultimate 3000 LC System (Dionex, USA) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker, Germany) with electrospray at a flow rate of 0.3 μL/min to a nanocolumn (Onyx monolithic HDC18, 0.2 mm ×150 mm). The raw MS/MS data was submitted for a database search using the Mascot software (Matrix Science, London, UK; http://www.matrixscience.com). The data was searched against the NCBI database for protein identification. Database interrogation was taxonomy (*Oryza sativa*: rice), enzyme (trypsin), variable modifications (carbamidomethyl, oxidation of methionine residues), mass values (monoisotopic); protein mass (unrestricted), peptide mass tolerance (1.2 Da), fragment mass tolerance (± 0.6 Da), peptide charge state (1+, 2+ and 3+) and maximum missed cleavages (3). Identified proteins had at least one peptide with an individual Mascot score corresponding to $P < 0.05$.

RESULTS

2D-gel analysis of proteins in leaf extract

Comparisons of protein patterns in the leaves of Pokkali between four pairs of 2D-gels (Fig. 1-A and Table 1) showed nine differential protein spots. The intensity of protein spots 2A, 7A and 9A remained unaffected by salt stress when the control gel was compared with the NaCl gel, whereas the protein spots 3A and 4A were down-regulated and protein spots 1A, 5A, 6A and 8A were up-regulated. When the control gel was compared with the Spd gel, all protein spots were down-regulated except the protein spot 6A, which remained unaffected by Spd. When the Spd gel was compared with Spd+NaCl gel, the protein spots 2A, 3A and 5A–7A were unaffected, while the protein spots 1A, 4A, 8A and 9A were up-regulated. When the plants were pretreated with Spd, all protein spots (except the protein spot 8A) were down-regulated when compared with the plants treated with NaCl without Spd pretreatment.

Comparisons of protein patterns in the leaves of KDML105 between four pairs of 2D-gels (Fig. 1-B and Table 2) showed 12 differential protein spots. Protein spots 2B and 8B were unaffected, however, protein spots 1B, 3B, 4B, 6B, 7B and 10B were down-regulated, and protein spots 5B, 9B, 11B and 12B were up-regulated when the NaCl-stressed plants were compared with the control. When the control gel was compared with the Spd treatment, the protein spots 1B–6B and 8B were down-regulated, and the protein spots 7B and 9B–12B were up-regulated. Pretreatment with Spd followed by salt stress resulted in the

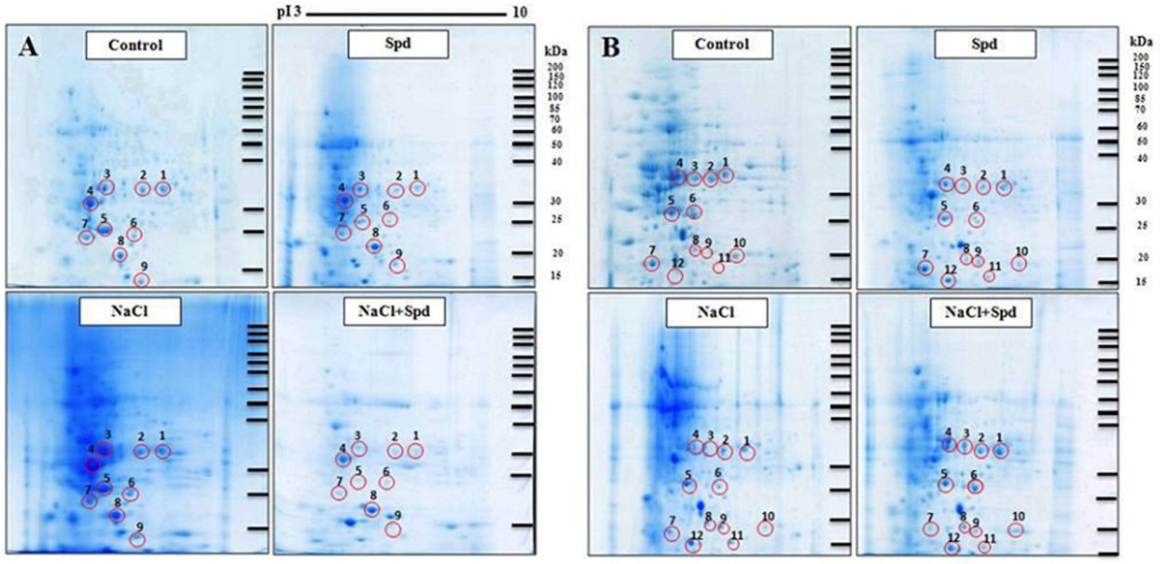

Fig. 1. Two-dimensional electrophoretic patterns of soluble proteins in rice leaf.

A, Pokkali obtained from control, Spermidine (Spd), NaCl and NaCl+Spd treatments; B, KDML105 obtained from control, Spd, NaCl and NaCl+Spd treatments.

Differential protein spots are indicated with a circle and a number.

reduction of protein spots 7B, 9B, 10B and 12B, and induction of protein spots 1B, 2B, 5B and 6B. Comparison of leaf proteins in plants pretreated with Spd before NaCl with those treated with NaCl without Spd found the up-regulation of protein spots 1B, 4B and 6B and down-regulation of spots 2B, 3B, 5B, 7B–9B, 11B and 12B.

2D-gel analysis of proteins in grains extracts

Comparisons of protein patterns in mature grains of Pokkali between four pairs of 2D-gels (Fig. 2-A and Table 3) showed seven differential protein spots. The protein spots 1A–3A and 6A were down-regulated and protein spots 5A and 7A were up-regulated when the plants were salt-stressed compared with the control. While the intensity of protein spot 4A remained constant. When the control gel was compared with Spd treatment, the protein spots 2A and 3A remained unaffected. However, protein spot 1A was down-regulated, and protein spots 4A–7A were up-regulated.

Table 1. Identification of differential proteins and changes in the intensity of each protein spot in the leaves of Pokkali.

Protein No.	Increasing or decreasing component (Fold)				pI	Mw (kDa)	Accession no.	Homologous protein	Score	Function
	C/NaCl	C/S	S/S+NaCl	NaCl/S+NaCl						
1A	↑(4.03)	↓(4.58)	↑(4.67)	↓(3.96)	7.4	32	NP_001056389	Os05g0574400	377	Similar to malate dehydrogenase
2A	-	↓(8.48)	-	↓(2.96)	6.7	32	NP_001043717	Os01g0649100	369	Similar to malate dehydrogenase
3A	↓(2.68)	↓(3.92)	-	↓(2.20)	5.4	32	ABA91631	Fructose bisphosphate aldolase	451	Fructose-bisphosphatealdolase activity
4A	↓(1.05)	↓(3.54)	↑(1.57)	↓(2.15)	4.9	29	NP_001043134	Os01g0501800	1 633	Similar to photosystem II oxygen-evolving complex protein 1
5A	↑(2.00)	↓(11.78)	-	↓(17.03)	5.3	24	AAB63603	Triosephosphate isomerase	251	Triose-phosphate isomerase activity
6A	↑(2.28)	-	-	↓(3.34)	6.4	24	EEC78412	Hypothetical protein OsI_18213	251	Putative uncharacterized protein
7A	-	↓(3.71)	-	↓(6.71)	4.8	23	BAD35228	Putative chaperonin 21 precursor	606	Chaperonin ATPase activity
8A	↑(1.04)	↓(1.60)	↑(2.36)	↑(1.12)	5.9	21	NP_001058863	Os07g0141400	976	Similar to photosystem II oxygen-evolving enhancer protein 2
9A	-	↓(6.06)	↑(2.80)	↓ (2.07)	6.6	18	AAA33917	Superoxide dismutase	60	Superoxide dismutase copper chaperone activity

C/NaCl, Pokkali collected from 30-day-old plants treated with 150 mmol/LNaCl for 7 d compared with those grown in the control solution; C/S, Pokkali grown in the control solutions with spermidine (Spd) pretreatment compared with those without Spd pretreatment; S/S+NaCl, Pokkali pretreated with Spd then transferred to the solution with NaCl compared with Spd treatment; NaCl/S+NaCl, Pokkali pretreated with Spd then transferred to solution with NaCl compared to those grown in NaCl; pI, Isoelectric point; Mw, Molecular weight.

Table 2. Identification of differential proteins and changes in the intensity of each protein spot in the leaves of KDML105.

Protein No.	Increasing or decreasing component (Fold)				pI	Mw (kDa)	Accession No.	Homologous protein	Score	Function
	C/NaCl	C/S	S/S+NaCl	NaCl/S+NaCl						
1B	↓(2.02)	↓(1.66)	↑(2.39)	↑(2.91)	7.8	34	NP_001056389	Os05g0574400	386	Similar to malate dehydrogenase
2B	-	↓(2.33)	↑(1.53)	↓(1.65)	7.0	34	NP_001043717	Os01g0649100	311	Malate dehydrogenase
3B	↓(1.34)	↓(3.43)	-	↓(2.73)	6.4	34	ABG66141	Malate dehydrogenase	193	Malate metabolic process
4B	↓(4.11)	↓(2.01)	-	↑(1.97)	5.7	35	ABA91631	Fructose-bisphosphate aldolase	806	Fructose-bisphosphatealdolase activity
5B	↑(1.58)	↓(2.65)	↑(2.73)	↓(1.53)	5.6	27	AAB63603	Triosephosphate isomerase	727	Triose-phosphate isomerase activity
6B	↓(3.41)	↓(4.62)	↑(3.81)	↑(2.81)	6.9	27	EEC78412	Hypothetical protein OsI_18213	248	Putative uncharacterized protein
7B	↓(1.18)	↑(1.43)	↓(6.01)	↓(3.58)	4.7	19	NP_001065834	Os11g0165700	89	Jacalin-related lectin domain containing protein
8B	-	↓(1.49)	-	↓(1.28)	6.5	20	2002393A	Oxygen-evolving complex protein 1	97	Oxygen sensor activity
9B	↑(9.85)	↑(7.57)	↓(1.36)	↓(1.77)	6.8	20	AAA33917	Superoxide dismutase	55	Superoxide dismutase copper chaperone activity
10B	↓(1.59)	↑(1.48)	↓(2.73)	-	8.6	20	NP_001067074	Os12g0569500	100	Thaumatin, pathogenesis-related family protein
11B	↑(11.94)	↑(5.92)	-	↓(1.54)	7.2	17	BAC10110	Copper/zinc-superoxide dismutase	71	Antioxidant activity
12B	↑(41.74)	↑(31.19)	↓(1.18)	↓(1.58)	5.8	16	BAD09607	Putative superoxide dismutase [Cu-Zn]	389	Antioxidant activity

C/NaCl, KDML105 collected from 30-day-old plants treated with 150 mmol/L NaCl for 7 d compared with those grown in the control solution; C/S, KDML105 grown in the control solutions with spermidine (Spd) pretreatment compared with those without Spd pretreatment; S/S+NaCl, KDML105 pretreated with Spd then transferred to the solution with NaCl compared with Spd treatment; NaCl/S+NaCl, KDML105 pretreated with Spd then transferred to solution with NaCl compared to those grown in NaCl; pI, Isoelectric point; Mw, Molecular weight.

When the plants were treated with Spd then NaCl, the intensities of protein spots 3A–5A decreased and 1A, 6A and 7A increased, whereas the spot 2A was unaffected. The down-regulation of protein spots 3A–5A and up-regulation of protein spots1A, 6A and 7A occurred when the plants were pretreated with Spd before exposed to NaCl compared with those treated with NaCl only (without Spd pretreatment). However, the spot 2A was unaffected.

Comparisons of protein patterns in rice grains of KDML105 between four pairs of 2D-gels (Fig. 2-B and Table 4) showed five differential protein spots.

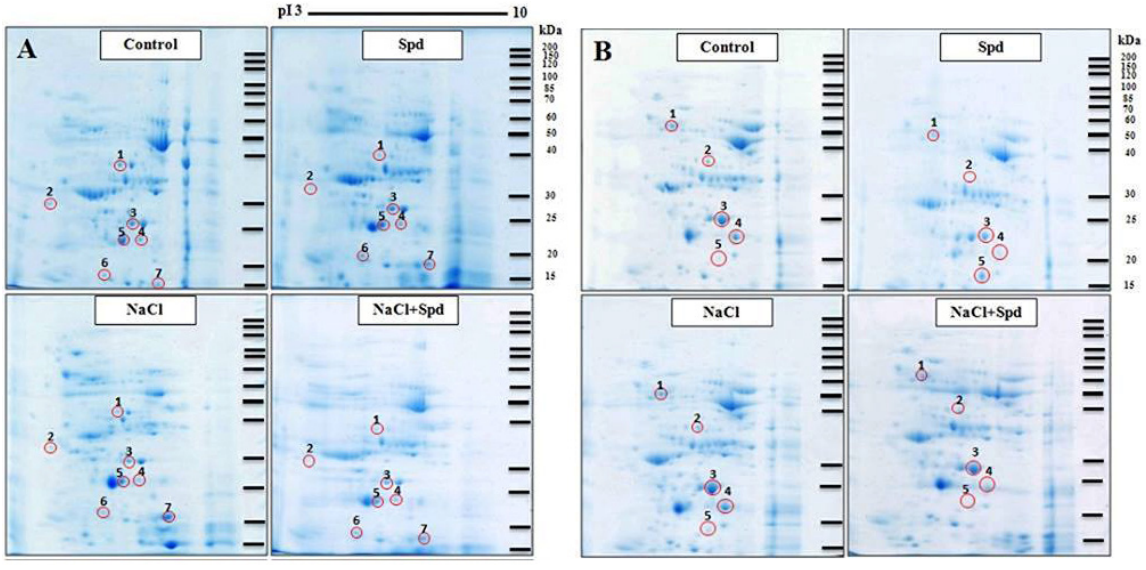

Fig. 2. Two-dimensional electrophoretic patterns of soluble grain proteins in rice grains.

A, Pokkali obtained from control, spermidine (Spd), NaCl and NaCl+Spd treatments; B, KDML105 obtained from control, Spd, NaCl and NaCl+Spd treatments.

Differential protein spots are indicated with a circle and a number.

Table 3. Identification of differential proteins and changes in the intensity of each protein in the mature grains of Pokkali.

Protein No.	Increasing or decreasing component (Fold)				pI	Mw (kDa)	Accession No.	Homologous protein	Score	Function
	C/NaCl	C/S	S/S+NaCl	NaCl/S+NaCl						
1A	↓(3.79)	↓(1.93)	↑(1.43)	↑(2.82)	6.5	38	A2XG55	Late embryogenesis abundant protein 1	295	Seed development
2A	↓(1.84)	-	-	-	3.4	31	NP_001049030	Os03g0159600	220	Late embryogenesis abundant protein D-34, putative, expressed
3A	↓(3.00)	-	↓(1.24)	↓(2.55)	6.8	27	P0C5C8	1-Cys peroxiredoxin A	412	Peroxiredoxin activity
4A	-	↑(1.45)	↓(1.32)	↓(1.09)	7.4	24	CAA92106	Group 3 LEA (type I) protein	342	3-beta-hydroxy-delta5-steroid dehydrogenase activity
5A	↑(1.71)	↑(1.10)	↓(1.24)	↓(1.93)	6.9	24	NP_001056195	Os05g0542500	190	Acyl-CoA dehydrogenase activity
6A	↓(1.68)	↑(1.22)	↑(1.26)	↑(2.59)	5.8	19	NP_001049657	Os03g0266300	282	Heat shock protein Hsp20 domain containing protein
7A	↑(1.64)	↑(1.47)	↑(1.45)	↑(1.30)	8.4	17	ACA50505	Seed allergenic protein RAG2	848	Serine-type endopeptidase inhibitor activity

C/NaCl, Pokkali collected from 30-day-old plants treated with 150 mmol/L NaCl for 7 d compared with those grown in the control solution; C/S, Pokkali grown in the control solutions with spermidine (Spd) pretreatment compared with those without Spd pretreatment; S/S+NaCl, Pokkali pretreated with Spd then transferred to the solution with NaCl compared with Spd treatment; NaCl/S+NaCl, Pokkali pretreated with Spd then transferred to solution with NaCl compared to those grown in NaCl; pI, Isoelectric point; Mw, Molecular weight.

Table 4.Identification of differential proteins and changes in the intensity of each protein in the mature grains of KDML105.

Protein No.	Increasing or decreasing component (Fold)				pI	Mw (kDa)	Accession No.	Homologous protein	Score	Function
	C/NaCl	C/S	S/S+NaCl	NaCl/S+NaCl						
1B	↓(1.29)	↓(1.82)	↑(1.48)	-	5.0	54	ABB46862	Enolase, putative, expressed	442	Phosphopyruvatehydratase activity
2B	-	↓(7.8)	↑(7.77)	-	6.7	36	A2XG55	Late embryogenesis abundant protein 1	117	Sequence-specific DNA binding
3B	↓(15.83)	↓(1.92)	↑(1.98)	↓(16.36)	6.2	24	NP_001060407	Os07g0638300	247	Similar to 1-Cys peroxiredoxin
4B	↑(1.17)	↓(1.85)	↑(1.79)	↓(1.21)	7.9	22	CAA92106	Group 3 LEA (type I) protein	102	3-beta-hydroxy-delta5-steroid dehydrogenase activity
5B	↑(1.51)	↑(8.73)	↓(4.85)	-	7.2	18	AAR87267	Putative stress-related protein	122	Sequence-specific DNA binding transcription factor activity

C/NaCl, KDML105 collected from 30-day-old plants treated with 150 mmol/LNaCl for 7 d compared with those grown in the control solution; C/S, KDML105 grown in the control solutions with spermidine (Spd) pretreatment compared with those without Spd pretreatment; S/S+NaCl, KDML105 pretreated with Spd then transferred to the solution with NaCl compared with Spd treatment; NaCl/S+NaCl, KDML105 pretreated with Spd then transferred to solution with NaCl compared to those grown in NaCl; pI, Isoelectric point; Mw, Molecular weight.

The protein spots 1B and 3B were down-regulated and protein spots 4B and 5B were up-regulated when the plants were exposed to salt stress compared with the control. Only the protein spot 2B was unaffected by salt stress. When the control gel was compared with the Spd gel, the protein spots 1B–4B were down-regulated, whereas the protein spot 5B was up-regulated. The induction of protein spots 1B–4B and reduction in spot 5B was observed when the plants were treated with Spd then NaCl compared with those treated with Spd only. Protein spots 3B and 4B were down-regulated by the effect of Spd when the NaCl-stressed (NaCl) gel was compared with the Spd+NaCl gel. However, the protein spots 1B, 2B and 5B were unaffected.

Identification of differentially expressed proteins by mass spectrometry

Following the LC-MS/MS analysis of each differential protein spot, the empirical peptide mass values were matched with the theoretical digested mass and database sequence information by a Mascot search. The results showed that the homologous proteins in the leaves of Pokkali (spots 1A–9A in Fig. 1-A and Table1) were all identified with high Mascot scores. These proteins were Os05g0574400, Os01g0649100, fructosebisphosphate (FBP) aldolase, Os01g0501800, triosephosphate isomerase (TPI), hypothetical protein OsI_18213, putative chaperonin 21 precursor, Os07g0141400 and superoxide dismutase. The homologous proteins in rice leaves of KDML105 (spots 1B–12B in Fig. 1-B and Table 2) were Os05g0574400, Os01g0649100, malate dehydrogenase, FBP aldolase, TPI, hypothetical protein OsI_18213, Os11g0165700, oxygen-evolving complex protein 1, superoxide dismutase, Os12g0569500, copper/zinc-superoxide dismutase and putative superoxide dismutase [Cu-Zn]. The homologous proteins in rice grains of Pokkali (spots 1A–7A in Fig. 2-A and Table 3) were late embryogenesis abundant protein 1, Os03g0159600, 1-Cys peroxiredoxin A, group 3 LEA

(type I) protein, Os05g0542500, Os03g0266300 and seed allergenic protein RAG2. In addition, the homologous proteins in rice grains of KDML105 (spots 1B–5B and Fig. 2-B and Table 4) were enolase-putative-expressed, late embryogenesis abundant protein 1, Os07g0638300, group 3 LEA (type I) protein and putative stress-related protein.

DISCUSSION

Previous studies have reported that exogenous application of Spd was effective in raising salt tolerance of rice plants as indicated by modulation of specific physiological processes, leading to growth improvement (Roychoudhury et al, 2011; Saleethong et al, 2011, 2013). However, the mechanism of action of exogenous Spd was largely unknown. Identification of proteins up-or down-regulated by Spd treatment may contribute to a better understanding of the protective roles of Spd on salt-treated plants. Using a comparative proteomic approach, a number of differentially expressed proteins with diverse functions have been detected in the leaves and mature seeds of rice cultivars Pokkali and KDML105 subjected to salt stress and Spd pretreatment prior to salt stress.

The first group of differential proteins detected in the leaves of both rice cultivars included those involved in photosynthesis and energy metabolism. Two predicted proteins which function in photosynthetic oxygen evolution (OEC1 and OEE2) were detected in Pokkali (spots 4A and 8A in Fig. 1-A and Table 1) whereas only one was identified from KDML105 (spot 8B in Fig. 1-B and Table 2). In response to Spd and NaCl, these proteins were up-regulated in Pokkali but non-responsive or down-regulated in KDML105. The increased abundance of OEE2 protein was also detected in salt-treated barley (a relatively salt-tolerant cereal crop) leaves possibly to repair protein damage caused by NaCl-induced dissociation of the oxygen-evolving complex (Fatehi et al, 2012). The OEC proteins are assembled to form the site for light-induced water oxidation to provide electrons for the photosystem (PSII) reaction center producing molecular oxygen as the by-product (Ferreira et al, 2004; Cady et al, 2008). In previous reports, the OEC proteins are responsive to salt stress and cause changes in the activity of photosystem II (PSII) in coping with salt stress (Abbasi and Komatsu, 2004; Kim et al, 2005; Jankangramet al, 2011; Chang et al, 2012). The results from this study essentially confirmed our earlier report of the higher salt-induced expression of OEC proteins in the tolerant cultivar Pokkali as compared with the sensitive KDML105 (Jankangramet al, 2011), pointing to the higher adaptability to adjust the efficiency of its photosynthetic machinery in response to salt stress. The expression of the OEE2 protein (spot 8A in Table 1), in particular, was induced by NaCl and further enhanced by Spd in the tolerant cultivar Pokkali.

In relation to energy metabolism, two differential protein spots found in the leaves of both cultivars were FBP aldolase and TPI. FBP aldolase (spot 3A in Fig. 1-A and Table 1 for Pokkali and spot 4B in Fig. 1B and Table 2 for KDML105) is an enzyme catalyzing a reversible reaction that splits the aldol, fructose 1,6-bisphosphate, into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) in the Calvin cycle and also glycolysis (Patron et al, 2004; Qaisar et al, 2010).The expression level of FBP aldolase was found to increase in rice leaves under salt stress (Abbasi and Komatsu, 2004; Kim et al, 2005; Jankangram et al, 2011; Lee et al, 2011). In contrast, some previous reports found a decrease in its intensity under salt stress in rice (Kim et al, 2005), sorghum (Ngara et al, 2012) and a halophyte *Sueda salsa* (Li et al, 2011) and also under oxidative stress provoked by hydrogen peroxide (Wan and Liu, 2008). In this study, the intensity of FBP aldolase decreased in both rice cultivars under salt stress. When treated with Spd under salt stress, the expression levels decreased in Pokkali but increased in KDML105. The TPI protein (spot 5A in Fig. 1-A and Table 1 for Pokkali, and spot 5B in Fig. 1-B and Table 2 for KDML105) is an enzyme that catalyzes the reversible interconversion of DHAP and GAP in the Calvin cycle and glycolytic pathway (Henze et al, 1994; Sharma et al, 2012). This protein was reported to be responsive to salt stress (Yan et al, 2005). The expression levels of TPI increased under salt stress but decreased when treated with Spd in both Pokkali and KDML105. However, it is noted that the intensity of this protein was higher in Pokkali leaves under both control and salt-stress conditions. This also points to the better preparedness for stress in Pokkali. The glycolytic enzymes, such as FBP aldolase, apart from their significance in energy metabolism under salt stress, were recently reported to work in association with the V-ATPase subunit B to up-regulate the H^+-pump activity in the tonoplast of salt-treated *Mesembryanthemum crystallinum* (a halophyte) and resulted in increased efficiency of Na^+ compartmentation (Barkla et al, 2009).

The second group of differentially expressed proteins in rice leaves was predicted as superoxide

dismutase (SOD, AAA33917), copper/zinc-superoxide dismutase (BAC10110) and putative superoxide dismutase [Cu-Zn] (BAD09607). SOD catalyzes the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide. In higher plants, SODs act as antioxidants and protect cellular components from being oxidized by ROS (Smirnoff, 1993). The expression of SOD has been shown to be affected by miR398-directed mRNA cleavage regulated by various biotic and abiotic stress (Sunkar et al, 2006), and responsive to salt stress in rice in relation to protein intensities as well as enzymatic activities (Fadzilla et al, 1997; Abbasi and Komatsu, 2004; Kim et al, 2005; Yan et al, 2005). Nuanjan et al (2012) found that SOD activity in KDML105 seedlings increased by 2.5 folds after 6 d salt stress and returned to the same level as that of the control at 5 d after recovery. The intensities of SODs increased several folds in salt-sensitive rice KDML105 (spots 9B, 11B and 12B in Fig. 1-B and Table 2) under salt stress and in response to Spd, but were unaffected in Pokkali (spot 9A in Fig. 1-A and Table 1). When NaCl was treated in combination with Spd, the protein expression levels of SODs decreased in both cultivars. It should be noted that one isoform of SOD (AAA33917; spots 9A and 9B in Tables 1 and 2, Fig. 1), which was detected in both cultivars, was already present at higher intensity in Pokkali than KDML105 under the control condition pointing to the better preparedness for stress in Pokkali. Exogenous Spd-mediated reduction in ROS under salt-stress has been reported to be associated with increased activities of antioxidant enzymes including SODs (Kubis et al, 2005; Roychoudhury et al, 2008). Polyamines act directly as ROS scavengers (Bors et al, 1989; Ha et al, 1998; Du et al, 2010). Therefore, the observed reductions in intensities of the three SOD proteins in KDML105 leaves (spots 9B, 11B and 12B in Table 2) in response to Spd+NaCl treatment is presumed to be partly due to the direct action of exogenous Spd on ROS scavenging, hence reducing the need for the antioxidant enzymes.

Proteins forming the third functional group in rice leaves are those similar to malate dehydrogenase i.e. Os05g0574400 (NP_001056389), Os01g0649100 (NP_001043717) and ABG66141. Malate dehydrogenase (MDH) catalyzes the NAD/NADH-dependent inter-conversion of malate and oxaloacetate (Minarik et al, 2002). In *Arabidopsis thaliana*, the protein (At1g53240) similar to MDH was found to be responsive to various abiotic stress including high salinity (Seki et al, 2003; Ndimba et al, 2005). The roles of MDH in salinity are still unclear but it was proposed that increased concentrations of MDH in water-stressed leaves of *Fraxinus excelsior* inhibit stomatal opening (Patonnier et al, 1999). The intensity of MDH protein spot in Pokkali was up-regulated by salt stress (spot 1A in Fig. 1-A and Table 1), whereas the same protein (spot 1B in Fig. 1-B and Table 2) was down-regulated in KDML105. However, when treated with Spd followed by NaCl, it was down-regulated in Pokkali but up-regulated in KDML105 compared with plants treated with NaCl alone.

The remaining differential proteins were detected in only one cultivar. The protein predicted as putative chaperonin 21 precursor (BAD35228; spot 7A in Fig. 1-A) was detected only in Pokkali. This protein was also detected in rice leaves under salt stress and was supposed to play an important role in preventing inactivation of proteins by salt stress (Lee et al, 2011). Chaperonin 21 protein was previously reported to act as co-chaperonin, helping chaperonin 60, whose function is to facilitate the assembly of Rubisco in spinach chloroplast (Baneyx et al, 1995). The intensity of this protein was not affected by NaCl treatment but decreased when the plants were treated with Spd alone or in combination with NaCl.

Two differential protein spots were detected only in KDML105 (spots 7B and 10B in Fig. 1-B and Table 2). The protein spot 7B (Fig. 1-B) was identified as a jacalin-related lectin domain containing protein. Zhang et al (2000) firstly reported the detection and characterization of a jacalin-related lectin in salt-stressed rice plants and suggested that protein-carbohydrate interactions may play an important role in plant defense against stress. Recently, the carbohydrate-binding lectin superfamily including the jacalin families in rice, soybean and *Arabidopsis* has been implicated as being involved in abiotic stress regulations such as cold, drought and high salinity (Jiang et al, 2010). The protein spot 10B (Os12g0569500) identified as thaumatin-like pathogenesis-related protein. The thaumatin-like protein family which has long been reported to confer resistance to a variety of fangal pathogens (Mahdavi et al, 2012) and recently found to alleviate stress caused by high NaCl (Singh et al, 2013).

The closest homologues of proteins differentially expressed in rice grains were predicted as shown in Table 2. Comparative proteomics led to the detection

of function and biological processes of proteins, such as late embryogenesis abundant (LEA) proteins, antioxidant proteins, stress-related proteins and hypothetical proteins.

The first group of differentially expressed proteins detected in rice grains is the rice LEA proteins. Two LEA proteins which were detected in both rice cultivars are LEA protein 1 (A2XG55; spots 1A and 2B in Fig. 2 and Tables 3 and 4), and Group 3 LEA (type I) protein (CAA92106; spots 4A and 4B in Fig. 2 and Tables 3 and 4). Both proteins showed similar pattern of expression in KDML105 grains, being down-regulated when treated with Spd but up-regulated in the presence of both Spd and NaCl. While the LEA protein 1 was up-regulated, Group 3 LEA (type 1) protein was down-regulated in the presence of Spd+NaCl. The LEA proteins generally accumulate to high levels at the onset of seed maturation to provide desiccation tolerance to the embryos and also in vegetative tissues subjected to water stress to protect tissues from damage caused by water deficit (Dure et al, 1981; Battaglia et al, 2008). Although abundant in seeds and pollen, LEA proteins have been found to protect against desiccation, cold and high salinity in a variety of organisms (Duan and Cai, 2012). The LEA protein 1 (accession No. A2XG55) firstly described by Chourey et al (2003) to accumulate to high levels during salinity-triggered growth arrest of an indica rice and then to be mobilized during the recovery of seedlings from salinity stress. The gene encoding Group 3 LEA protein (accession No. CAA92106), first described in rice roots in response to abscisic acid and salt stress, was more highly expressed in the salt-tolerant cultivar Pokkali than the salt-sensitive Taichung N1 (Moons et al, 1997). The physicochemical properties and *in vitro* assays of LEA proteins suggested that they provide the hydrogen bonds to other molecules such as enzymes or sugars, or reorganize the available water molecules to maintain the functional structure of the associated molecules (Olvera-Carrillo et al, 2011). In dry seeds, LEA proteins contribute to the formation of tight hydrogen-bonding network with sugars to promote a long-term stability of sugar glasses during dehydration (Wolkers et al, 2001).

The proteins forming the second functional group in rice grains possess the antioxidant activity which included 1-Cys peroxiredoxin A or 1-Cys Prx A (P0C5C8), enolase putative expressed (ABB46862) and Os07g0638300 (NP_0010407). The protein 1-Cys Prx A (spots 3A in Fig. 2-A and Table 3 for Pokkali) and Os07g0638300 (it is similar to 1-Cys peroxiredoxin; spot 3B in Fig. 2-B and Table 4 KDML105), members of one of the four subclasses of Prx, a family of thiol dependent peroxidases having important functions in catalyzing the decomposition of toxic peroxides (Tripathy et al, 2009). It was specifically expressed in the embryo and aleurone layer of developing seeds (Stacy et al, 1999). The function of this 1-Cys Prx A is to protect the embryo and the aleurone cells from oxidative damage during development and desiccation of seeds (Dietz, 2003) as well as during water uptake and resumption of respiration during seed imbibition (Bhatt and Tripathi, 2011).

The remaining differential stress-related proteins detected in Pokkali grains are Os05g0542500, Os03g0266300 (heat shock protein Hsp20 domain containing protein) and seed allergenic protein RAG2 (spots 5A–7A in Fig. 2-A and Table 3). Heat shock proteins and putative allergenic protein with molecular weight were also identified during the desiccation phase of grain filling in rice cultivar Nipponbare and presumed to be involved in acquisition of desiccation tolerance (Sano et al, 2013). Of particular interest is the seed allergenic protein RAG2 having serine-type endopeptidase inhibitor activity, which was up-regulated in response to both NaCl and Spd separately and in combination. This protein may play important roles in preventing protein degradation during seed development and drying.

CONCLUSIONS

Four groups of differentially expressed proteins in response to salt stress which were modulated by exogenous Spd were detected in the leaves of both salt-tolerant (Pokkali) and salt-sensitive (KDML105) cultivars. These included light reaction PSII OEC1, two enzymes in Calvin cycle and glycolysis (FBP aldolase and triosephosphate isomerase), malate dehydrogenase, antioxidant enzymes, SODs, and hypothetical protein OsI_18213. In most cases, the intensities of proteins were higher in Pokkali than KDML105. Differential proteins detected only in Pokkali included PSII oxygen-evolving enhancing protein 2 (OEE2) and putative chaperonin 21 precursor. The OEE2 protein was up-regulated by both salt and exogenous Spd. Stress-related proteins detected only in KDML105 included jacalin-related lectin domain containing protein and thaumatin pathogenesis-related protein. An interesting and consistent pattern of

expression was noted for three SOD isoforms in KDML105, which were up-regulated in response to NaCl but down-regulated with Spd pretreatment prior to NaCl stress. Differentially expressed proteins which were detected in mature seeds of both Pokkali and KDML105 but with different patterns of expression were LEA proteins and 1-Cys Prx. Three proteins were detected in only Pokkali grains. Of interest is the seed allergenic RAG2 protein, which was up-regulated by both NaCl and Spd treatments. Future in-depth analyses of proteins which are more highly expressed in the salt-tolerant cultivars may provide a better understanding of the specific mechanisms which endow these cultivars with higher level of salt tolerance.

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