Protein Profiles in Response to Salt Stress in Seedling of Salt Tolerant Rice Mutants

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Abstract - Proteomic analysis was performed in order to identify proteomic changes by salt stress between the Japonica cv. Donganbyeo (WT) and two salt-tolerant (ST) mutant lines by using the SDS-PAGE and 2-DE. Two salt tolerant rice mutant lines, ST-87 and ST-301, were selected by *in vitro* mutagenesis with gamma-ray. Three-week-old seedlings were treated with 171 mM NaCl for 7 days. In the SDS-PAGE, three proteins with molecular weights of 27, 46 and 58 kDa were highly increased under salt treatment. Total proteins from shoots of both WT and ST-lines were separated by two-dimensional gel electrophoresis. In 2-DE, 201, 226, 217 and 213 protein spots were detected in the untreated-or treated-WT and untreated- or treated-ST-87, respectively. Of theses, 17 and 10 protein spots were up- and down-regulated under salt stress in the WT, respectively. While, 16 and 8 protein spots were up- and down-regulated under salt stress in the ST-87, respectively, compared with the untreated plants. High intensity or de novo synthesized proteins were analyzed by MALDI-TOF/MS analysis.

Key words : Gamma-ray, Mutant, Protein, Rice, Salt tolerance

INTRODUCTION

Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50%. Salinity also causes adverse effects on the growth of plants and the productivity of crops. One third of the irrigated land in the world is affected by high salinity (Mass and Hoffmann 1977). Salinity affects at least 20% of world's cultivated land and more than 45% of irrigated land (Rhoades and Loveday 1990). This slat stress is limiting agricultural expansion in many areas of the world and is the most serious problem in the aspects of crop productivity (Epstein *et al.* 1980; Yancey *et al.* 1982). Therefore, understanding the mechanisms of plant tolerance to salt stress and identification of stress responses will provide the breeding of effective strategies leading to greater salt stress tolerance (Bartels *et al.* 2005).

The analysis of osmotic stress-responsive in plants is an important way to the discovery of genes conferring stress tolerance and their use in breeding programs. A proteomic approach, the level of proteins, is not only a powerful molecular tool used at the organelle, cell, organ, and tissue levels (Lee *et al.* 2007), but it can also compare proteomes affected by different physiological conditions, such as those resulting from the exposure to abiotic stress including salinity. In

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widely, a high salt concentration causes cellular dehydration with concomitant osmotic changes including ion imbalance, deficiencies of mineral nutrition and water, oxidative damage and reduction of photosynthetic efficiency (Tsai *et al.* 2004). To defend against abiotic stress, plants use several strategies, one of which is regulation of gene expression at the mRNA level. The transcriptome analyses of gene expression at the mRNA level have contributed greatly to our understanding of the stress responses (Seki *et al.* 2002; Rabbani *et al.* 2003). These approaches allowed identification of many salt stress responsive genes in plants. However, the amount of translation, proteins, does not always correlated with the level of transcription, mRNA (Yan *et al.* 2005; Yan *et al.* 2006). Therefore, it is insufficient to predict protein expression level from quantitative mRNA and is necessary to study the salt stress response at the protein level. During the past several years, the rice proteomic analysis has increased power as a result of improvements in two-dimensional polyacrylamide gel electrophoresis (2-DE) coupled with mass spectrometry (MS).

In our previous study, we isolated 2 ST-lines, ST-87 and ST-301, with salt tolerance by gamma ray *in vitro* mutagenesis (Song *et al.* 2012). These ST-lines showed lower levels than the activity of the enzymatic catalase in the contents of EL and MDA and the higher peroxidase under the salt condition compared with the wild type. The total amino acid contents in the seedling leaves of the ST-lines were 2.2 and 1.4-times higher than in the WT. The ratio of K^+/Na^+ of the ST-87 (2.7 fold) was significantly higher than that of the WT (1.4 fold). In this study, we analyzed the proteomic comparisons of the protein changes between the 2 ST-lines and the original variety (WT) through the sodium dodecyl sulphate (SDS-PAGE) and two-dimensional polyacrylamide gel electrophoresis (2-DE) after salt stress treatment.

MATERIALS AND METHODS

1. Plant materials and stress conditions

The 2 ST-lines, ST-87 and ST-301, and WT (*Oryza sativa* cv. Dongan) plants were imbibed in 171 mM NaCl solution at 28±1˚C for 7 days at the 3-week-old seedling stage. After salt treatment, the shoots harvested at the indicated time, frozen in liquid nitrogen and stored at −80˚C until protein extraction for sodium dodecyl sulphate (SDS) and two-dimensional polyacrylamide gel electrophoresis (2-DE).

2. SDS-PAGE analysis in the salt tolerant mutant lines

The leaf tissues of each lines were ground under liquid nitrogen and the total crude protein was then extracted in 5 ml of 100 mM Tris-HCl (pH 8.2) containing 20 mM calcium chloride from 100 mg of fresh leaves. The suspension was centrifuged at 13,000 rpm for 30 minutes. Protein content of the clear supernatants was determined by the Bradford method (Bradford 1976).

In the SDS-PAGE, protein (10 μg) was added to an equivalent amount of 5x SDS-sample buffer [10% w/v sodium dodecyl sulphate (SDS), 50% v/v glycerol, 1.96% v/v b-mercaptoethanol, and 1 M Tris- HCl, pH 6.8]. The samples were held in boiling water for 5 min and then on ice for 5 min, immediately and centrifuged at 13,000 rpm for 5 min. The separation was performed with a 10% separating gel and 5% stacking gel using Bio-Rad Mini-PROTEAN 3 Cell (Bio-Rad, Richmond, CA). Electrophoresis was started at 100 volt until dye reached the end of the gel. The gel was stained with silver nitrate, according to the method of Ceils *et al.* (2006).

3. 2-DE analysis in the salt tolerant mutant lines

Shoots were ground in liquid nitrogen and suspended in ice cold 10% trichloroacetic acid (TCA) in acetone containing 0.07% 2-mercaptoethanol, incubated at −20˚C for 1 h and followed by centrifugation at 13,000 rpm for 20 min at 4˚C. The pellets were resuspended two times with 0.07% 2-mercaptoethanol in acetone, incubated at −20˚C for 1 h and centrifuged at 13,000 rpm for 20 min at 4˚C. The pellets were dried under vacuum for 30 min. The resulting powder was resuspended in lysis buffer [9 M urea, 1% CHAPS, 1% ampolytes (pH 4∼7), and 1% DTT] followed by sonication on ice for 30 min and centrifuged at 13,000 rpm for 20 min at 4˚C. The supernatant was used in the 2-DE analysis.

For the 2-DE analysis, 150 mg of proteins were loaded onto analytical gels. The IPG strips were rehydrated for 16 h in 340 ml rehydration buffer containing protein samples. The first-dimension isoelectric focusing gel electrophoresis (IEF) was performed using pH 4∼7 IPG strips (17 cm, linear) in a IPGphor apparatus (GE Healthcare, Piscataway, NJ), according to the manufacturer's recommendations. For the

second dimension, the gel strips were equilibrated for 15 min in 10 ml equilibration buffer [75 mM Tris-HCl buffer, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT, and 0.002% bromophenol blue] and subsequently placed on to 12% polyacrylamide gels prepared. Electrophoresis was also performed according to the manufacturer's recommendation. The protein spots in analytical gels were visualized by silver staining (Ceils *et al.* 2006). At least three replicates were performed for each sample.

The gels were scanned by i-MAXTM Gel Image Analysis System (CoreBio, Seoul, KO). Image analysis was accomplished using PDQuest 7.3 software (Bio-Rad). After automated detection and matching, manual editing was carried out.

4. In-gel digestion and MALDI-TOF/TOF MS analysis

Protein spots were excised from the preparative gels, washed three times with ultrapure water, destained twice with 50 mM NH_4HCO_3 in 50% acetonitrile, reduced with 10 mM DTT in 50 mM NH_4HCO_3, alkylated with 40 mM iodoacetamide in 50 mM NH_4HCO_3, dried twice with 100% acetonitrile, and digested overnight at 377°C with sequencing grade modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3. The peptides were extracted twice with 0.1% TFA in 50% acetonitrile. Extracts were pooled and lyophilized. The resulting lyophilized tryptic peptides were dissolved in 5 mg Ml^{-1} CHCA containing 0.1% TFA and 50% acetonitrile. MS analysis was conducted with a MALDI-TOF/TOF mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Data were analyzed using GPS Explorer software (Applied Biosystem) and MASCOT software (Matrix Science, London, UK). NCBInr and rice was selected as the database and taxonomy, respectively.

RESULTS

1. SDS-PAGE analysis of 2 ST-lines after salt treatment

In a previous report, we selected 2 ST-lines tolerant to high slat stress *in vitro* and on reclaimed saline land (Song *et al.* 2012). For the investigation of the protein changes in response to the salt stress, the WT and 2 ST-lines were subjected in the 171 mM NaCl nutrient solution at the 3-week-old seedling stage. SDS-PAGE analysis was performed to confirm changes in total proteins and to determine the profiles of proteins separated on the basis on their solubility properties in salt tolerant lines. SDS-PAGE of total proteins revealed few protein profile differences between the WT and 2 ST-lines, ST-87 and ST-301 (Fig. 1). Salt stress cause reductions in the contents of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) as well as enhancement of varied proteins marked by arrowheads, as compared with the WT. The RuBisCO in both ST-lines and WT plant showed almost no difference under normal conditions. However, the RuBisCO content in WT plant was decreased after salt treatment, while ST-lines significantly increased at 7 days after the salt treatment. Three proteins with molecular weights of 27, 46 and 58 kDa were highly increased under salt treatment.

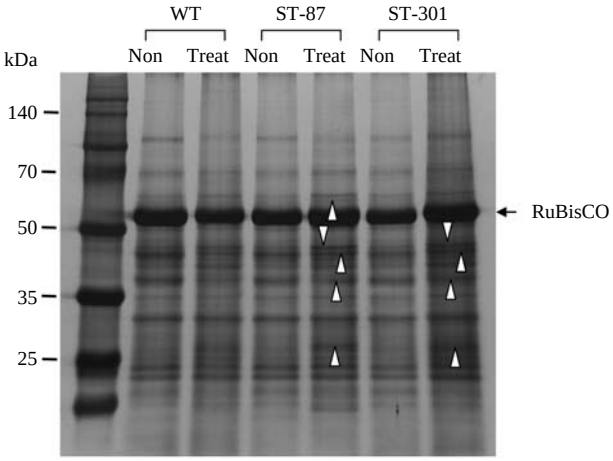

Fig. 1. SDS-PAGE profiles of total protein in WT, ST-87, and ST-301 before and after 171 mM salt treatment for 7 days. The proteins were resolved in a 10% SDS polyacrylamide gel and silver stained. Molecular weights are described on the left of the panel. The white arrowheads indicate the enhanced proteins in 2 ST-lines compared with those of the WT after salt treatment.

2. Two-dimensional gel electrophoresis analysis of total proteins in 2 ST-lines

In order to investigate the changes of protein profile in response to salt stress, we carried out 2-DE analysis of total proteins of the WT and 2 ST-lines after slat treatment for 7 days. For each sample, at least triplicate gels were performed, and they showed a high level of reproducibility. The representative gels are shown in Fig. 2. We have marked only

(A)

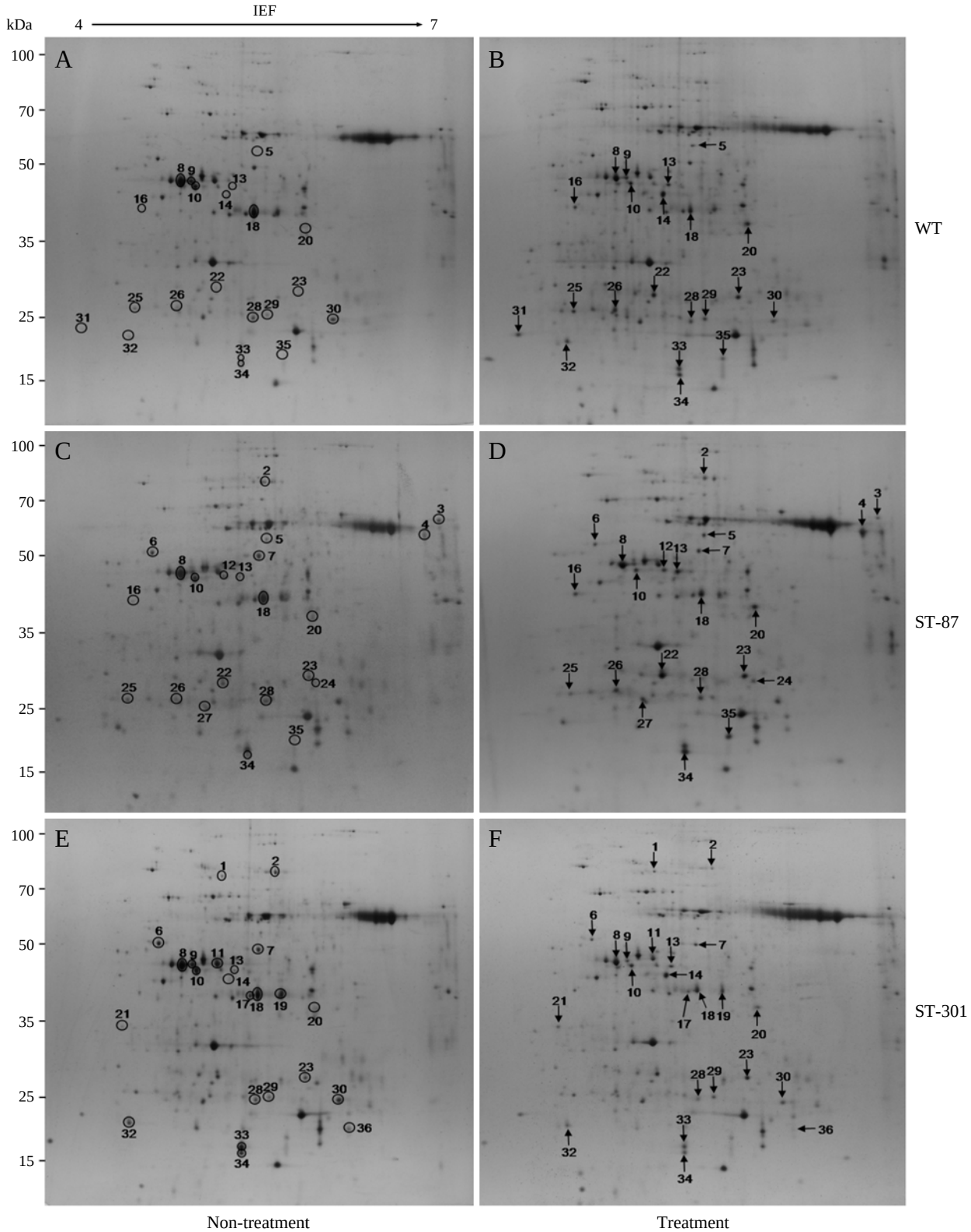

Fig. 2. Protein expression patterns in the shoots of the WT and 2 ST-lines exposed to salt stress. Proteins were separated by 2D-PAGE and stained by silver nitrate. Arrows indicate the positions of proteins changed by salt treatment, and circles mark the position of the same proteins in the non-treatment (a). The relative levels of protein expression were analyzed in the WT and 2 ST-lines by using the PDQest software. The white and black bars show the salt non-treated and treated plants, respectively (b).

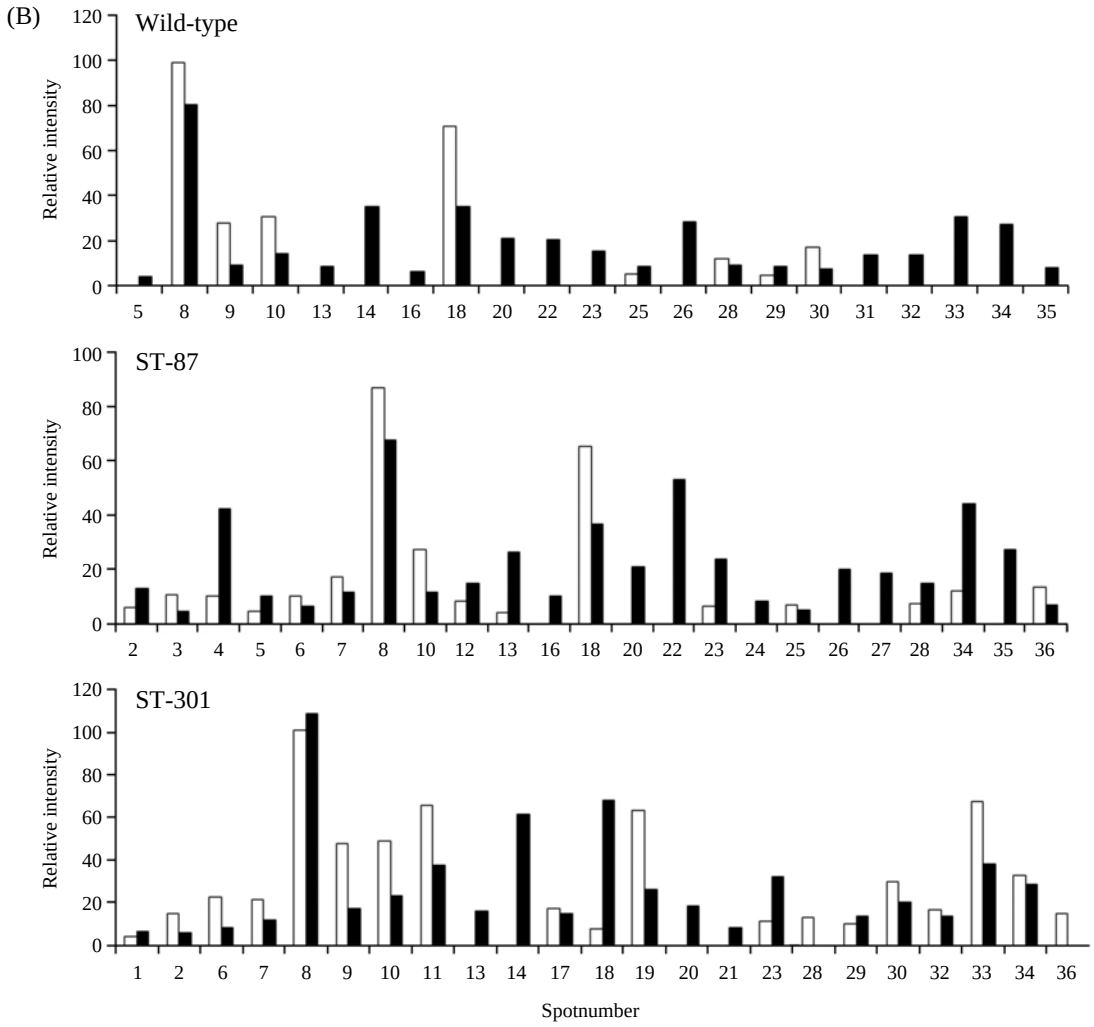

Fig. 2. Continued

those major proteins that were most affected by salt treatment and which showed reproducible and obvious differences after salt treatment. In the 2-DE, more than 200 protein spots were detected in the untreated-or treated-WT, ST-87, and ST-301, respectively. Of theses, 36 proteins were differentially expressed under salt stress in one or other lines. Among 36 proteins, 21 protein spots were up- and down-regulated under salt stress in the WT. In the ST-87 and ST-301 lines, 22 and 23 protein spots were up- and down-regulated under salt stress, respectively, compared with the non-treated plants (Fig. 2a). Relative intensity of protein spots on the 2D-PAGE gels were analyzed by using the PDQuest software. Shoots of the WT and 2 ST-lines showed no reproducible differences in the expression of proteins between non-treated and salt-treated plants. In the WT, the relative intensity of 15 proteins increased from 1.8 fold (spot 29) to 35.2 fold (spot 14) and 6 proteins were decreased from 1.2 fold (spot 8) to 3 fold (spot 9) under salt stress condition. In the ST-87, the relative intensities of 15 proteins increased from 2.0 fold (spot 28) to 53.2 fold (spot 22) and decreased from 1.3 fold (spot 25) to 2.3 fold (spot 10) and in the ST-301, increased from 1.1 fold (spot 8) to 61.7 (spot 14) and decreased from 1.1 fold (spot 34) to 2.3 fold (spot 36), compared with the non-treated plants (Fig. 2b). Although most proteins showed quantitative changes, some proteins showed *de novo* expression. For example, 5 spots [spots 5 (U1), 13 (U2), 16 (U3), 20 (U4), and 23 (U5)] absent in the salt non-treatment were induced after salt treatment (Figs. 2 and 3). They were visible only in the treated samples, suggesting that they were newly synthesized after salt stress treatment or in very low abundance without salt stress. The other differentially increased or decreased proteins showed quantitative changes in after

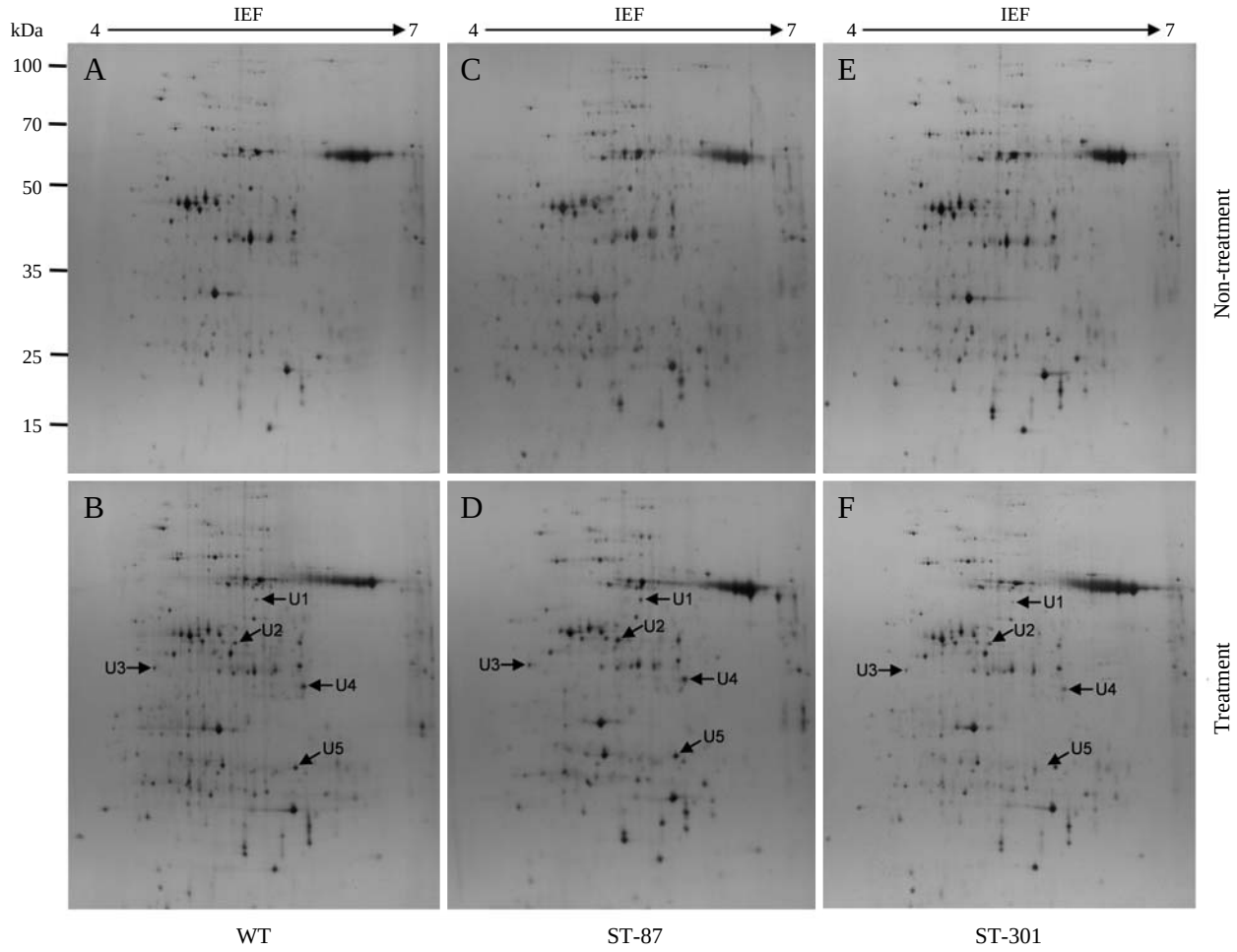

Fig. 3. Representative *de novo* proteins on the 2-DE gels of the WT and 2 ST-lines proteins. Newly accumulated protein spots are indicated by arrows. Five *de novo* proteins (U1-U5) are shown in response to salt stress as compared with the salt non-treated condition.

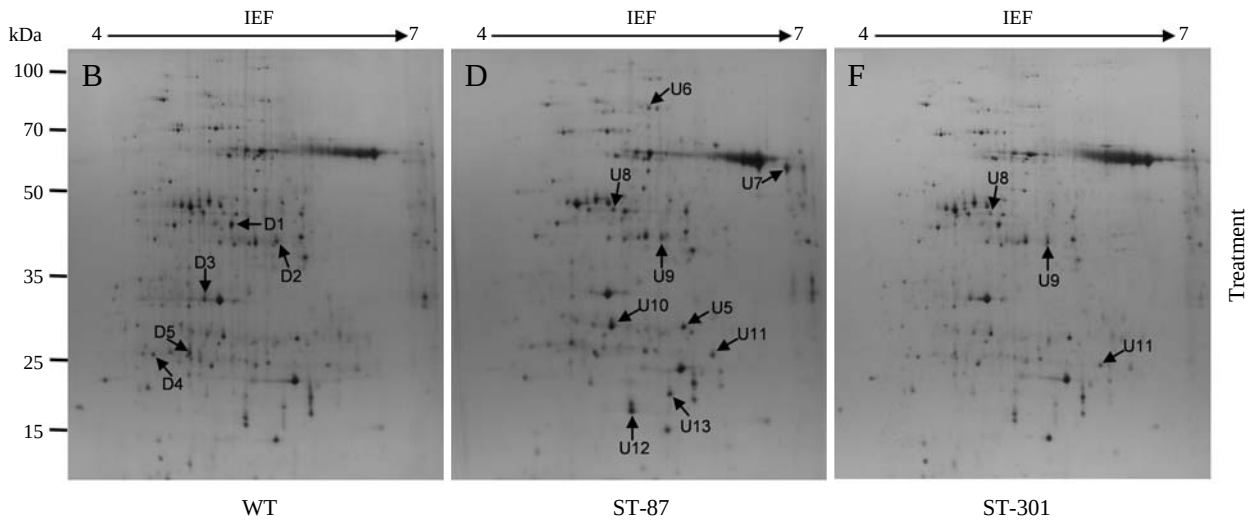

Fig. 4. Differentially increased or decreased proteins on the 2-DE gels of the WT and 2 ST-lines proteins. Differentially accumulated protein spots are indicated by arrows. All marked spots indicated changes between WT and ST-lines in response to salt stress as compared to WT.

Table 1. Differentially expressed proteins in the WT (D1) and ST-87 (U1-U13) seedlings under salt stress

Spot no.	NCBI accession no.	Protein name	M_r/pI[a]	Score[b]	C (%)[c]	Sequence[d]
U1	AAA84588	atpB gene product	53.9/5.3	100	2	AHGGVSVFGGVGER
U2	AAA84588	atpB gene product	53.9/5.3	301	11	AHGGVSVFGGVGER IVGNEHYETAQR FVQAGSEVSALLGR VALVYGQMNEPPGAR
U3	AAT07576	Putative chloroplast phosphor-glycerate kinase	32.3/9.9	164	11	LVSELPNGSVLLLENVR LASLADLYVNDAFGTAHR
U4	AAC28134	rubisco activase	47.8/5.85	197	7	VYDDEVR FYWAPTR GQSIDFFGALR
U5	NP_001058042	Os06g0608700 (hypothetical protein)	37.7/7.57	255	9	VGAEVIGEYTVAALR YYEAGAR LASINVENVEPNR
U7	NP_039391	rubisco activase	52.8/6.22	291	12	TFQGPPHGIQVER DTDILAAFR DNGLLLHIHR VALEACVQAR GGLDFTKDDENVNSQPFMR
U10	ABA91631	Fructose-bisphosphate aldolase, chloroplast precursor, putative, expressed	41.5/6.07	457	14	GLVPLAGSNNESWCQGLDGLASR LASIGLENTEANR EAAYYQQGAR EAAWGLAR
U11	NP_001060047	Os07g0570700 (hypothetical protein)	29.6/9.35	49	4	AIETVQNNFNTVR
U12	NP_001044494	Os01g0791600 (hypothetical protein)	22.7/8.24	274	20	TFQGPPHGIQVER DTDILAAFR LTYYTPEYETK ALRLEDLR
U13	ABA96140	Ribulose bisphosphate carboxylase large chain precursor, putative	56.0/9.04	178	4	TFQGPPHGIQVER DTDILAAFR
D1	AAC28134	rubisco activase	47.8/5.85	192	12	MCCLFINDLDAGAGR VYDDEVR SFQCELVFAK FYWAPTR IVDSFPGQSIDFFGALR

[a]Molecular weight and isoelectric point.
[b]Probability-based molecular weight search (Mowse) score.
[c]Sequence coverage of matched peptides.
[d]The amino acid sequence of the peptide with the highest score identified by MS/MS

salt stress treatment. For example, 13 spots [spots 2 (U6), 4 (U7), 12 (U8), 14 (D1), U9, D2, D3, 25 (D4), 26 (D5), 22 (U10), 30 (U11), 34 (U12), and 35 (U13)] showed quantitative changes between the WT and 2 ST-lines under salt stress treatment (Figs. 2 and 4).

3. Salt stress-responsive proteins identified by MALDI-TOF/TOF MS analysis

The differentially expressed proteins were excised from the preparative gels, in-gel digested by trypsin, and analyzed by a MALDI-TOF/TOF mass spectrometer. Among 36 differentially accumulated proteins, 18 spots were analyzed by the MALDI-TOF- TOF MS/MS. Three (U9, D2, and D3) of them had no MS and MS/MS data. Fifteen protein spots representing 8 different proteins were identified with high probability (Table 1). The MS/MS result of spot U13 is shown in Fig. 5 as an example. The proteins were identified as atpB gene product (spots U1 and U2), rubisco activase (spots U4, U7, and D1) as the same protein, putative chloroplast phosphor-glycerate kinase (spot U3), fructose-bisphosphate aldolase, chloroplast precursor, putative, expressed (spot U10), and ribulose bisphosphate carboxylase large chain precursor, putative (spot U13). Spots U5 and 11 were identified as unknown proteins, Os06g0608700 and Os07g0570700, respectively (Fig. 4).

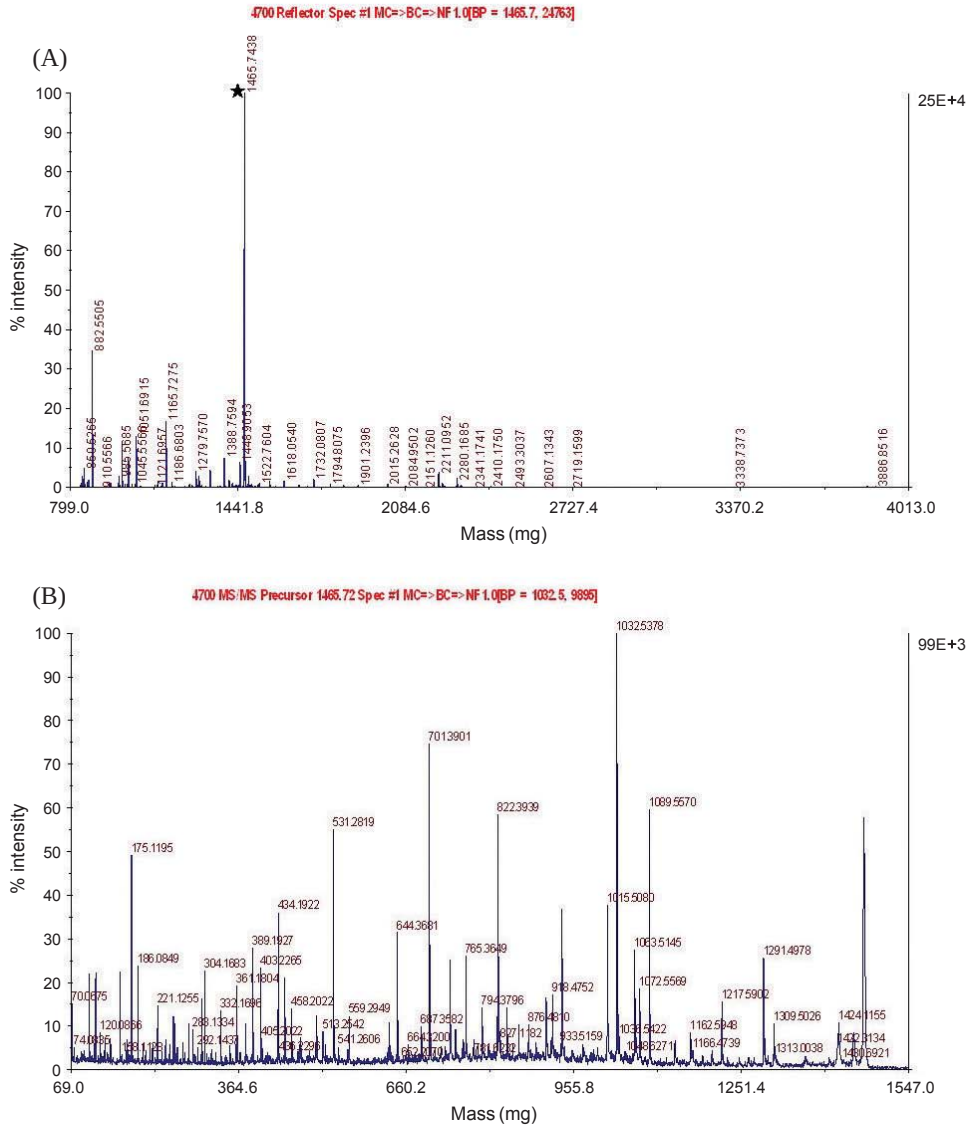

Fig. 5. MS analysis of spot U13. The protein excised from gels was digested with trypsin and the resulting peptides were analyzed using a 4700 Proteomics Analyzer. A. MALDI-TOF MS analysis. The spectral peaks show the intensities of different peptides. The 1465.74 m/z ion (marked with an asterisk) was further analyzed by MS/MS. B, MALDI-TOF MS/MS analysis of the 1465.74 m/z ion. The protein was identified as the Ribulose bisphosphate carboxylase large chain precursor (NCBI accession number ABA96140) after database searching.

DISCUSSION

In an attempt to understand the molecular basis of salt tolerance, proteomics using a combination of SDS-PAGE and 2-DE was used to identify proteins involved in salt stress response for the selection of the salt tolerant mutant lines. The protein profiles in salt non-treated and treated shoots in the WT and 2 ST-lines using the SDS-PAGE revealed significant changes in the expression pattern of proteins. It was found that three protein bands with molecular weights of 27, 46 and 58 kDa were highly increased and clearly observed in the ST-87 and ST-301 than that of the WT after salt treatment (Fig. 1). These results revealed that these proteins were expressed in specific regions of rice plants adapted to salt stress and suggested that the decrease of the protein content was attributed to the decrease rate of protein synthesis, the decreased availability of amino acids, or the denaturation of the enzymes involved in amino acid and protein synthesis (Dubey 1994). Salinity is a major factor in reducing the

growth and productivity of plants. The reduction in plant dry matter might have been due to inhibition of photosynthesis as a result of direct effects of salinity on the photosynthetic apparatus (Dubey 1994).

The synthesis, accumulation, and degradation of protein in plants are highly regulated in response to environmental changes. Proteomic analysis of this study revealed that proteins associated with photosynthesis were differentially expressed upon salt stress. Rubiscos are the most prevalent enzymes in the plant. They form about 30～50% of the total soluble protein content of the chloroplast. Oxidative stress may lead to small-subunit degradation, subsequently leading to translational arrest of the large subunit. In the present study, the RuBisCO large subunit in both ST-lines and WT plant showed almost no difference under normal growth conditions. However, after salt treatment, the WT plant was significantly decreased in the amount of the RuBisCO, while, the ST-87 and ST-301 plants enhanced greater than that of the tolerant lines under non-treatment (Fig. 1). Also, in the 2-DE analysis, RuBisCO activase (spots U4 and U7) showed a significant increase after 7 days salt-treatment. Increased activity of RuBisCO subunits in maize and rice under salt stress has also been demonstrated (Kim *et al.* 2005). Spots U1 and U2 were highly abundant proteins on 2-DE gels in the saline condition compare to the WT (Fig. 4). These proteins were identified as a rice atpB gene product of chloroplast ATP synthase (Table 1). The atpB gene encodes β subunit of chloroplast ATP synthase and is co-transcribed in plant species (Westhoff *et al.* 1981). Chloroplast ATP synthase plays an important role in biological energy metabolism in plant cells (Botha *et al.* 2006). In addition, the atpB gene encodes the β-subunit of ATP synthase complex, which has a highly conserved structure that couples proton translocation across membranes with the synthesis of ATP (Gatenbey *et al.* 1989). The decrease in the level of atpB protein indicates that the ATP-synthesis system in the photosynthesis process in the WT plant is seriously affected by salt stress. Spot U3 was identified as putative chloroplast phosphorglycerate kinase. Phosphoglycerate kinase (PGK) is a monomeric enzyme, which catalyzes the transfer of the high-energy phosphate group of 1,3-bisphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate. In this study, the molecular responses to salt stress were investigated at the protein level in rice. Thirty-six differentially expressed proteins were revealed, and 15 of them were further identified by the MS/MS analysis. These proteins were involved in several processes that might work under salt stress. These results provides not only understands into salt stress responses but also a good starting point for further dissection of their functions using genetic and other approaches.

CONCLUSION

In the SDS-PAGE, three proteins with molecular weights of 27, 46 and 58 kDa were highly increased under salt treatment. Total proteins from shoots of both WT and ST-lines were separated by two-dimensional gel electrophoresis. In 2-DE analysis, more than 200 protein spots were detected in the untreated-or treated-WT, ST-87 and ST-301, respectively. Of theses, 36 proteins were differentially expressed under salt stress in one or other lines. Of 36 proteins, 21 protein spots were up- and down-regulated under salt stress in the WT. In the ST-87 and ST-301 lines, 23 and 23 protein spots were up- and down-regulated under salt stress, respectively, compared with the untreated plants. Among 36 differentially accumulated proteins, 18 spots were analyzed by MALDI-TOF/TOF MS/MS. The proteins were identified as atpB gene product (spots U1 and U2), rubisco activase (spots U4, U7, and D1) as the same protein, putative chloroplast phosphor-glycerate kinase (spot U3), fructose-bisphosphate aldolase, chloroplast precursor, putative, expressed (spot U10), and ribulose bisphosphate carboxylase large chain precursor, putative (spot U13). Spots U5 and U11 were identified as un-known proteins, Os06g0608700 and Os07g0570700, respectively. High intensity or *de novo* synthesized proteins were analyzed by MALDI-TOF MS/MS analysis.

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