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DIFFERENTIAL PROTEOMIC ANALYSIS OF SALT STRESS RESPONSE IN JUTE (*CORCHORUS CAPSULARIS & OLITORIUS* L.) SEEDLING ROOTS

HONGYU MA[1,2], RUIFANG YANG[3], LIRU SONG[1], YAN YANG[1], QIUXIA WANG[2], ZHANKUI WANG[1], CAI REN[1] AND HAO MA[1*]

[1]*State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China*
[2]*College of Plant Protection, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China*
[3]*Ramie Research Institute, Hunan Agricultural University, Changsha, Hunan 410128, China*
[*]*Corresponding author's e-mail: Lq-ncsi@njau.edu.cn; Tel.: +86-25+84395324*

Abstract

Jute (*Corchorus capsularis & olitorius* L.) is mostly grown in Southeast Asian countries and has been recently suggested as a promising candidate for planting in wetland and saline soils in China. To effectively breed more salt-tolerant jute cultivars, it is necessary to understand its salt stress-responsive mechanism at molecular level. Morphological, physiological and proteomic analyses were performed on seedlings of two jute genotypes exposed to 50, 100 and 150 mM NaCl, respectively, for four days. Our results indicated that genotype 9511, with lower degree of average index of salt harm (AISH) in leaf, less fallen leaf number/ten plants and higher root proline (Pro) content, was more salt tolerant than genotype Mengyuan. Two-dimensional gel electrophoresis (2-DE) showed that expressions of 44 protein spots were significantly changed in the seedling roots of the two genotypes in response to salt stress. Thirty-nine (39) differentially expressed proteins were identified by MALDI-TOF-TOF MS, and classified into nine groups. Based on most of the 39 identified salt-responsive proteins, a salt stress-responsive protein network in jute seedling roots was proposed. After the persistent (for 4 d) salt stress, jute seedling would adapt to salt stress through altering signal transduction, accelerating ROS scavenging, impairing energy metabolism, enhancing nucleotide metabolism, lipid metabolism and cell wall metabolism, as well as altering cytoskeleton in roots. NaCl-responsive protein data will provide insights into salt stress responses and for further dissection of salt tolerance mechanisms in jute.

Key words: Jute, Root, 2-DE, Salt stress, Proteomic.

Introduction

Salinity is one of the major environmental constraints limiting yield of crop plants in many semi-arid and arid regions around the world. It is estimated that about 20% of the earth's land mass and nearly half of all irrigated land are affected by salinity. Increased salinization of arable land is expected to have devastating global effects, with predictions of 30% land loss within the next 25 years, and up to 50% by the year 2050 (Xu *et al*., 2008). Under this situation, studies of crop salt tolerant mechanisms are both necessary and impending (Swami *et al*., 2011). Root as a site of perception and injury for salt stress, it is its salt stress sensitivity that limits the productivity of the entire plant in many circumstances (Jiang *et al*., 2007). Using salt-stressed roots of *Oryza sativa*, *Arabidopsis*, and *Elsholtzia splendens* as research materials in recent years, many new insights into salinity stress responses have been obtained by comparative proteomics studies (Jiang *et al*., 2007; Li *et al*., 2009; Yan *et al*., 2005; Sobhanian *et al*., 2010). The increased understanding of molecular responses of roots to salt stress has facilitated the development of crops with increased tolerance to salt stress.

Jute (*Corchorus capsularis & olitorius* L.) is an herbaceous annual plant (family Tiliaceae), mostly grown in Southeast Asian countries (Carlosdel *et al*., 2009). Jute fibre can be separated from the bast or outer region of the stem after retting of the whole plant and is mainly used for the manufacture of cordage, carpets, bagging and wrapping materials, with an annual production of 2.65 million tons in the world (Carlosdel *et al*., 2009). In addition, jute fibre is a good source of different grades of paper pulp (Jahan 2001). In recent years, demand for jute fibre has enormously increased in China. Due to lack of redundant fertile land for its planting in China, jute has been recently suggested as a candidate for planting in wetland and saline soils (Ma *et al*., 2009; Javed *et al.*, 2014). Therefore, jute cultivars with increased salt tolerance are required for these aims. To effectively breed more salt-tolerant jute cultivars, it is necessary to understand its salt stress-responsive mechanism at molecular level. However, reports on molecular study of salt tolerance in jute have not yet been found to date.

Jute genotypes Mengyuan and 9511 have been reported to be salt sensitive and salt tolerant, respectively (Ma *et al*., 2011). In the present study, moderate NaCl stresses (50, 100, and 150 mM NaCl, respectively, for four days) were applied to the seedling roots of these two hydroponic-cultured jute genotypes. Through morphological, physiological and proteomic analyses, the main objectives of this study were: to gain insight into metabolic changes induced by salt toxicity in jute roots; to explore possible salt tolerance/accumulation mechanisms existing in jute roots; to identify candidate proteins for enhancing salinity tolerance in jute roots.

Materials and Methods

Plant materials: Seeds of two jute (*Corchorus capsularis & olitorius* L.) genotypes (salt-sensitive genotype Mengyuan and salt-tolerant genotype 9511), which were supplied by the Institute of Bast Fibre Crops, Chinese Academy of Agricultural Sciences, were sterilized with 0.1% $HgCl_2$ for 15 min. After three rinses with sterilized distilled water, the seeds were germinated on wet filter papers in the dark for 72 h at 28 ^{o}C. Uniformly germinated seeds were transplanted onto half-strength Hoagland nutrient solution (Ma *et al*., 2009), which was replaced with fresh one every third day. The seedlings were grown in a growth chamber with 25/20^{o}C temperature (day/night), photon flux density of 700~800 µmol m^{-2} s^{-1}, 14 h photoperiod, and relative humidity of 60~80%. Thereafter, fifty uniform seedlings, 3-weeks-old (about six leaves each plant), were selected to grow in each tank (30 cm×15 cm×10 cm) with 4 L half-strength Hoagland nutrient solution including 50, 100, and 150 mM NaCl for 4 d, respectively, in three independent experiments. Untreated plants (0 mM NaCl for 4 d) were set as control. Roots from plants after 4 d stress and non-treated corresponding plants grown for 4 d were separately harvested, washed, frozen in liquid nitrogen and kept at -70^{o}C for proline quantification and protein extraction.

Morphological measurement: Average index of salt harm (AISH) in leaf is one of the most important morphological parameters for evaluating salt tolerance of jute (Ma *et al*., 2009). Therefore, in the present study, AISH in leaf of jute seedlings under salt stress was determined. Four days after treatments (0, 50, 100, and 150 mM NaCl), 50 seedlings from each treatment were investigated for their appearance. Each treatment was repeated three times. At the same time, fallen leaf number/ten plants were investigated. According to their appearance, all seedlings were classified into five grades (0, 1, 2, 3, and 4) as follows:

0. Seedling grows normally without any injury.
1. The edge of one or two leaves of seedling turns yellow and presents some black spots or withers.
2. One whole leaf of seedling yellowly withers, bestrewing with black spots, or falls off.
3. Seedling growth is restrained with two or three leaves severely withering, turning yellow or falling off.
4. Seedling growth is severely restrained with many leaves severely withering and falling off or the whole seedling on the verge of death.

Then, the AISH in leaf of each genotype was calculated by the following formula:

$$\text{AISH}\ (\%) = \frac{\sum[(number\ \ of\ '0'\times 0)+(number\ \ of\ '1'\times 1)+(number\ \ of\ '2'\times 2)+(number\ \ of\ '3'\times 3)+(number\ \ of\ '4'\times 4)]}{4\times 50}\times 100$$

where “number of ′0′ ” represents the seedling number of Grade 0, and so forth.

The grade of salt tolerance of each jute genotype was determined according to Table 1.

Determination of proline (Pro): Determination of free Pro content in jute seedling roots was conducted according to Bates *et al*. (1973). Fresh root samples (about 0.5 g) from each treatment were homogenized in 5 ml sulphosalicylic acid [3% (w/v)] and the homogenate was filtered through filter paper. After addition of 2 ml acid ninhydrin [2.5% (w/v)] and 2 ml glacial acetic acid, the homogenate was heated at 100^{o}C for 1 h in water bath. Reaction was then stopped by ice bath. The mixture was extracted with 4 ml toluene, and the absorbance of the fraction with toluene aspirated from liquid phase was read at 520 nm. Free Pro content was determined using calibration curve and expressed as µmol proline/g FW.

Protein extraction and assay: Ten roots of jute seedling, which composed one sample, were ground in liquid nitrogen and suspended in ice-cold 10% (w/v) trichloroacetic acid (TCA) in acetone containing 0.1% (w/v) dithiothreitol (DTT) and 0.02% (w/v) phenylmethanesulfonyl fluoride (PMSF), incubated at -20^{o}C for 2 h and centrifuged for 20 min at 4^{o}C, 35,000 × *g*. The pellets were resuspended in 0.07% (w/v) DTT and 0.02% (w/v) PMSF in acetone, incubated at -20^{o}C for 1 h and centrifuged for 15 min at 4^{o}C, 30,000 × g. This step was repeated three times and the pellets were lyophilized. The resulting powder was resuspended in solubilization buffer [42% (w/v) urea, 15.2% (w/v) thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylamonio]-1-propanesulphonate (CHAPS), 1% (w/v) DTT, 0.2% (v/v) carrier ampholytes (Bio-Rad, pH 4-7), 0.02% (w/v) PMSF]. The different samples obtained from each treatment were pooled together and used to perform three replicates for each two-dimensional electrophoresis (2-DE) map. Protein quantification was carried out by Bradford method (Bio-Rad, Labs, Hercules, CA, USA; Bradford, 1976), with 0.2 mg mL^{-1} of bovine serum albumin (BSA) as a standard.

Two-dimensional electrophoresis (2-DE): Isoelectric focusing (IEF) was carried out with 300 µg of protein sample in 350 µl of 2-DE rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 0.2% (v/v) carrier ampholytes (pH 4-7), 0.02% (w/v) PMSF] for 17 cm immobilized pH gradient (IPG) strips. Protein was loaded by the active rehydration at 50 V for 13 h onto IEF strips (pH range 4-7). The IPG strips were passively rehydrated for 13 h. Focusing was performed using an IPGphor system (Bio-Rad, Labs, Hercules, CA, USA) at 18^{o}C in five steps: 250 V for 1 h (slow), 500 V for 1 h (slow), 2,000 V for 1 h (linear), 8,000 V for 3 h (linear) and then a voltage rapid up to 60,000 Vh. The focused strips were subjected to equilibration buffer [6 M urea, 2% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 375 mM Tris-HCl (pH 8.8)] with 2% (w/v) DTT in 10 ml of for 20 min and followed by alkylation with 2.5% (w/v) iodoacetamide in the same buffer. The IPG strips were then loaded on top of 12% polyacrylamide gels for SDS-PAGE using the PROTEAN II xi cell system (Bio-Rad, Hercules, CA, USA) and a running buffer containing 192 mM glycine, 0.1% SDS and 25 mM Tris (pH 8.3). The voltage for running SDS-PAGE was 80 V for 1.5 h and 200 V for 4 h. The protein spots were visualized by silver staining (Yan *et al*., 2005).

Table 1. Grading standard of salt tolerance of jute based on average index of salt harm.

Grades of salt tolerance	Average index of salt harm (AISH, %)	Degree of salt tolerance
1	0-20.0	High salt tolerance
2	20.1-40.0	Salt tolerance
3	40.1-60.0	Medium salt tolerance
4	60.1-80.0	Sensitivity
5	80.1-100.0	High salt sensitivity

Gel scanning and image analysis: Gel images were digitized with a gel scanner system (Powerlook 2100XL, UMAX) equipped with a 12-bit camera, then analyzed with the PDQuest^{TM} software package (Version 7.2.0; BioRad). After automated detection and matching, manual editing was carried out. In the statistic sets, the Student's t test and significance level of 95% were chosen. In the quantitative sets, the upper limit and lower limit were set to 1.5 and 0.66 fold, respectively. Then the Boolean analysis sets were created between the statistic sets and the quantitative or qualitative sets. The spots from the Boolean sets were compared among three biological replicates. Only spots displaying reproducible change patterns were considered to be differentially expressed proteins.

In-gel digestion and protein identification: Protein spots with differential expression patterns on gels were manually excised from gels, washed with Millipore pure water for three times, destained twice with 30 mM $K_3Fe(CN)_6$ for silver staining spots, 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 37 ^oC with sequencing grade modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3. The trypsin digested peptides were extracted from the gel pieces with 0.1% trifluoroacetic acid in 50% acetonitrile three times with sonication (Li *et al*., 2009). The peptide solution was desalted with ZipTip C-18 pipette tips (Millipore). The desalted peptide solution was analyzed by nanoLC-MS/MS (UltiMate 3000 system, Dionex, Sunnyvale, CA, USA; LTQ Orbitrap MS, Thermo Fisher Scientific, Waltham, MA, USA). The peptides were eluted from the trap column using 0.1% formic acid in acetonitrile on a 75 μm ID×15 cm C18 nanocolumn at a flow rate of 200 nl/min. Full scan mass spectra were obtained by the Orbitrap at 300-2,000 m/z with a resolution of 30,000. The three most intense ions were determined at a threshold above the 1,000 ion trap at a normalized collision energy of 35%.

Acquired MS/MS spectra were searched against the database of the Viridiplantae taxonomy of the NCBInr protein database using MASCOT search engine (http://www.matrixscience.com). Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionines was set as a variable modification, peptide mass tolerance of ± 100 ppm, fragment mass tolerance of ± 0.5 Da, and mass values monoisotopic. Peptides from MS/MS were searched in NCBInr protein database. Only significant hits, as defined by the MASCOT probability analysis (p<0.05), were accepted.

Statistical analysis: All analyses were done on a completely randomized design. All data obtained was subjected to two-way analyses of variance (ANOVA) and mean differences were compared by lowest standard deviations (LSD.) test. Experiment for determination of physiological indexes was conducted twice for each genotype with 3 repeated measurements (n = 6) and comparisons with p<0.05 were considered significantly different.

Results

Morphological changes and physiological responses induced by salt stress in the two jute genotypes: In our pilot study, seedlings of jute genotypes Mengyuan and 9511 were treated in 200 mM NaCl solution, respectively, and were found to display very severe symptoms after 30 min treatment (Ma *et al*., 2009). Therefore, in the present experiments, jute seedlings were treated in 0, 50, 100, and 150 mM NaCl solution, respectively. Images of the plants under 150 mM NaCl salt stress for four days are shown in Fig. 1. Genotype Mengyuan displayed more severe symptoms than genotype 9511 under 150 mM NaCl salt stress for four days. It is known that salt tolerant genotypes under salt stress normally have lower AISH in leaf, lower fallen leaf number, and higher Pro content than salt sensitive genotypes (Bates *et al*., 1973; Gzik *et al*., 1996; Koca *et al*., 2007; Ma *et al*., 2009). Therefore, to understand the morphological changes and physiological responses of the seedlings of both the genotypes under the salt stress, AISH in leaf, fallen leaf number/ten plants and root Pro content were investigated. The grade of salt tolerance of each jute genotype was determined according to Table 1. As presented in Fig. 2A, both the genotypes had the increased (p<0.05) AISH in leaf with the increase of salt concentrations, but genotype 9511 showed lower (p<0.05) increase than genotype Mengyuan under 100 and 150 mM NaCl stresses. Based on AISH in leaf, genotype 9511 fell into grade 2 of salt tolerance, but genotype Mengyuan belonged to grade 4 of salt tolerance. Similar change pattern was also observed in the fallen leaf number/ten plants (Fig. 2B). Fallen leaf number/ten plants in both the genotypes were increased (p<0.05) with the increase of salt concentrations, but it was less (p<0.05) increased in genotype 9511 than in genotype Mengyuan under 100 and 150 mM NaCl stresses. The effect of NaCl stress on root Pro content is shown in Fig. 2C. Both the genotypes increased root Pro content gradually in response to the increase of salt concentrations, but genotype 9511 had higher (p<0.05) root Pro content than genotype Mengyuan under 150 mM NaCl stress. These results further testified that genotype 9511, with lower degree of AISH in leaf, lower fallen leaf number/ten plants and higher root Pro content, was more salt tolerant than genotype Mengyuan (Ma *et al*., 2011).

2-DE analysis of soluble proteins in the roots of both jute genotypes: To counteract salt stress, plants can change their gene expression and protein accumulation. Some salt stress-responsive genes were found to be mainly, or more strongly, induced in plant roots than in other organs (Yan *et al*., 2005). To investigate the short-term changes of protein profiles under salt stress, an in-depth 2-DE analysis of the soluble proteins in seedling roots of genotypes Mengyuan and 9511 in response to salt stress was carried out. For each salt concentration point (0, 50, 100, and 150 mM NaCl for 4 d, respectively), three biological replicate 2-DE gels were run, and then computationally combined into a representative standard gel (Fig. 3). For both genotypes, the representative gels are shown in Fig. 4A. Quantitative image analysis on silver-stained gels using PDQuest 7.2.0 software revealed a total of 44 protein spots that changed their intensities significantly (p<0.05) by more than 1.5-fold or less than 0.66 at least at one salt concentration point.

Identification and functional classification of differentially expressed proteins: Due to its poor protein and DNA sequence database coverage, identification of proteins of jute by MALDI-TOF-MS was rather difficult. Therefore, in this study, MS/MS was used to identify the differentially expressed proteins. Peptides from MS/MS were searched in NCBInr protein database**.** Only significant hits, as defined by the MASCOT probability analysis (p<0.05), were accepted. Consequently, of 44 differentially expressed proteins, 39 proteins were successfully identified by MS/MS (Table 2). Among the identified proteins, five were annotated either as unknown and hypothetical proteins. To gain the functional information about these proteins, we searched their homologues with BLASTP (www.ncbi.nlm.nih.gov/BLAST/) using their protein sequences as queries. Five corresponding homologues with the highest homology are shown in Table 3. All spots shared more than 50% positives with homologues at the amino acid level, indicating that they might have similar functions.

Based on the metabolic and functional features of jute seedling root, the 39 identified proteins were classified into nine groups according to KEGG (http://www.kegg.jp/kegg/pathway.html) and literatures, including signal transduction (25%), ROS scavenging (23%), energy metabolism (13%), nucleotide metabolism (5%), lipid metabolism (3%), cell wall metabolism (3%), cytoskeletal (5%), and unclassified proteins (23%) (Fig. 5 and Table 2). The unclassified proteins included function-unknown proteins and unknown and hypothetical proteins.

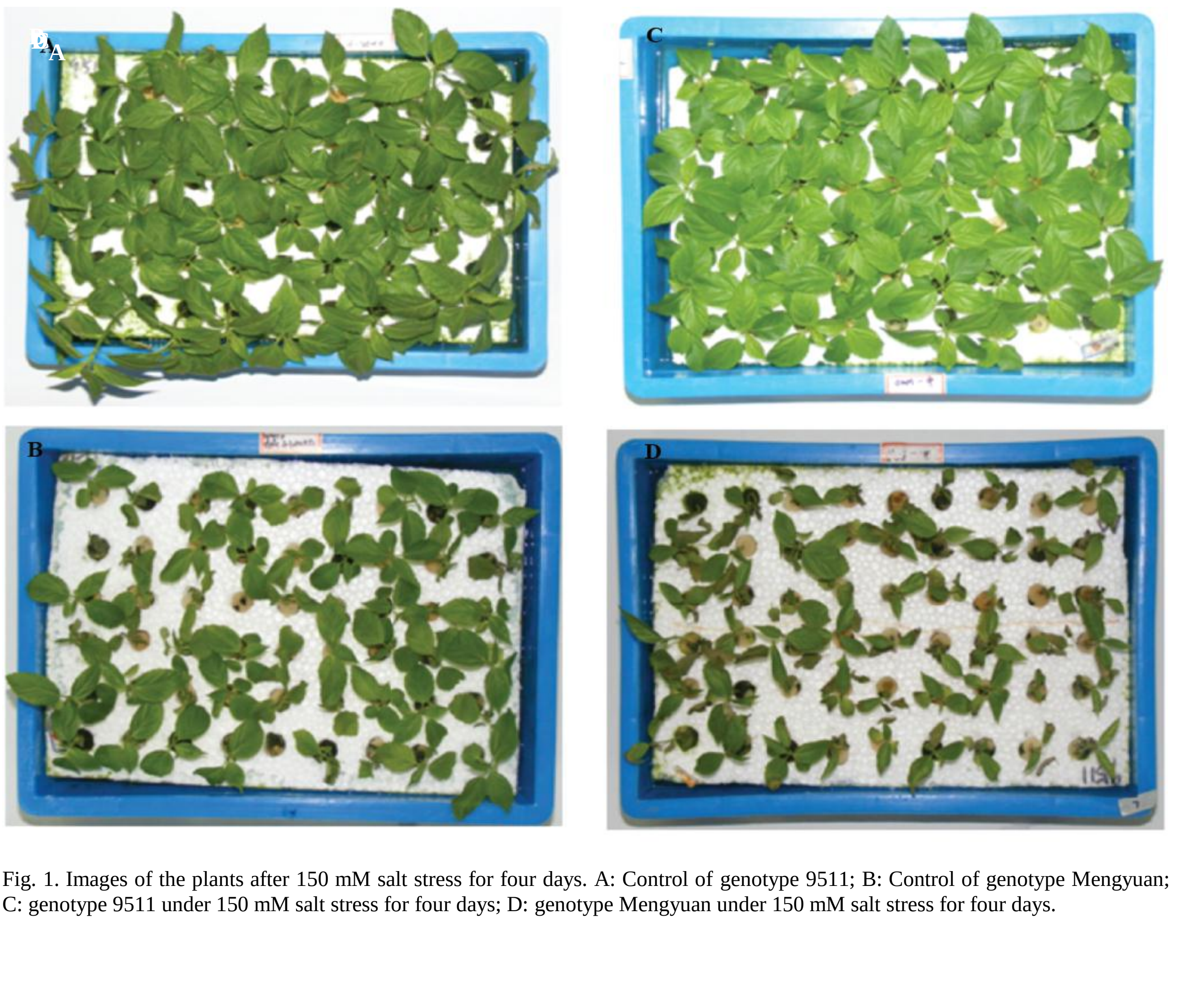

Fig. 1. Images of the plants after 150 mM salt stress for four days. A: Control of genotype 9511; B: Control of genotype Mengyuan; C: genotype 9511 under 150 mM salt stress for four days; D: genotype Mengyuan under 150 mM salt stress for four days.

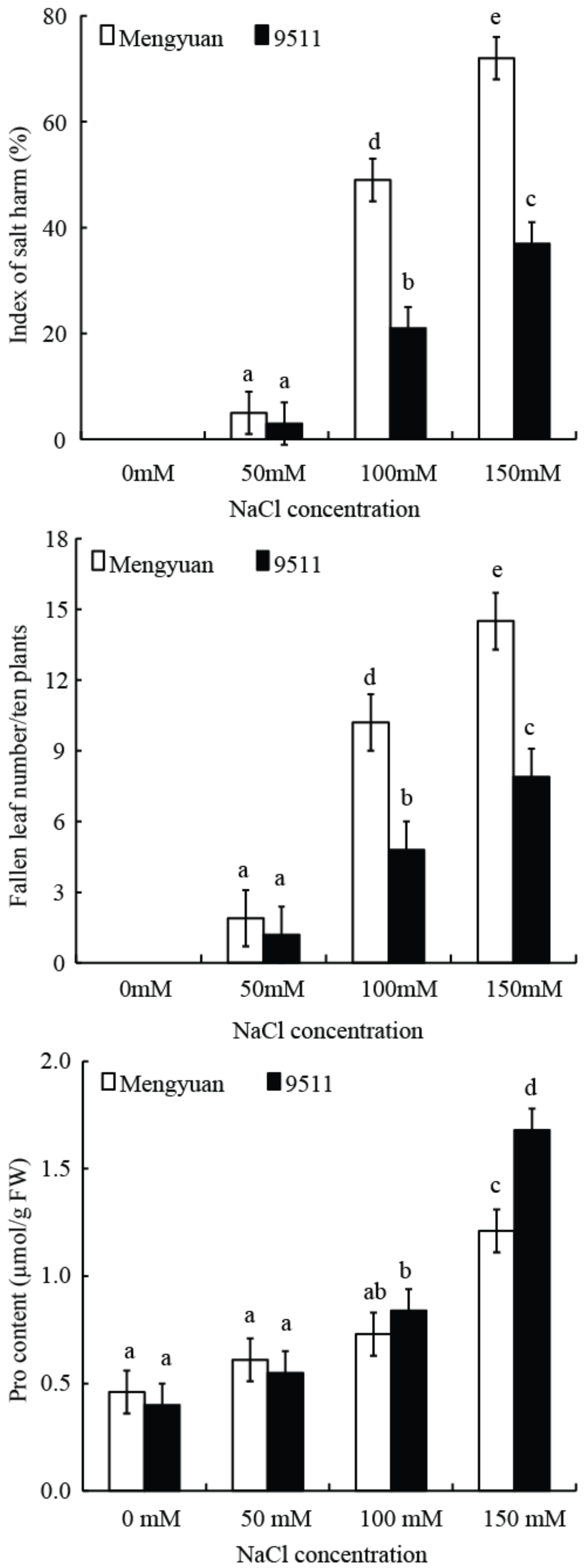

Fig. 2. AISH in leaf, fallen leaf number/ten plants, and root Pro content of the two jute genotype seedlings under salt stress. The changes of AISH, fallen leaf number/ten plants, and Pro content are shown in A, B, and C, respectively. Vertical bars represent standard errors (n=6). Bars with different letters are significantly different at $p<0.05$.

Discussion

Identified proteins related to signal transduction: Signal transduction pathways, which transmit information within the individual cell and throughout the plant, are activated when on recognition of biotic and abiotic stresses at the cellular level, leading to changes of many metabolism pathways, such as ROS scavenging, energy metabolism and nucleotide metabolism, etc (Bhushan *et al*., 2007; Mehede *et al.*, 2014). In this study, ten identified proteins were found to be implicated in signal transduction pathways.

Of the ten identified proteins involved in signal transduction, eight (spots # 4818, 0821, 6714, 8713, 3328, 8222, 7818 and 8021) were found to be up-regulated under NaCl stress in the seedling roots of both jute genotypes. Spot # 4818, which was identified as allene oxide synthase (AOS), is the first enzyme in the lipoxygenase pathway that leads to the formation of jasmonic acid, and also has been suggested as a control point in jasmonic acid biosynthesis (Wu *et al*., 2004). Jasmonic acid functions as signaling molecules to activate the genes involved in plant defense responses (Alvarez *et al*., 2009). Protein spots #0821, 6714 and 8713 were identified as F-box family protein, kelch repeat-containing F-box family protein, and F-box domain containing protein, respectively. F-box proteins are an expanding family of eukaryotic proteins, which have shown in some cases to be critical for the controlled degradation of cellular regulatory proteins via the ubiquitin pathway and regulate many phytohormone signalling pathways, including the jasmonate, gibberellin and ethylene pathways (Staswick 2008). Spot #3328 was identified as Wiscott-Aldrich syndrome, C-terminal (WASP), which is involved in cellular signals through various protein-protein interactions leading to kinase action (Ow 1996). Spot #8222 was identified as NPR1-like protein, which is encoded by nonexpressor of pathogenesis-related gene 1(*NPR1*). *NPR1* is a key gene involved in regulation of plant disease resistance and plays a pivotal role not only in systemic acquired resistance (SAR) and inducing systemic resistance, but also in basic resistance and resistance gene-dependent resistance (Steven *et al*., 2009). Furthermore, *NPR1* has been found to regulate *PR* (pathogenesis-related) gene expression through interaction with TGA transcription factors whose binding motif has been shown to be essential for salicylic acid-responsiveness of PR-1 gene (Steven *et al*., 2009). Spot #7818 was identified as Calreticulin. Calreticulin is known as a Ca^{2+} signal transduction related protein that functions during various stresses, especially under salt conditions (Hashimoto & Komatsu 2007). Spot #8021 was identified as Polcalcin Jun o 2, which is a calcium-binding allergen and related with Ca^{2+} signal transduction (Raffaella *et al*., 1998). The above up-regulated proteins implied that many signal (jasmonate, gibberellin, ethylene, and Ca^{2+}, etc.) transduction pathways might be enhanced by NaCl stress for counteracting salt attack in jute seedling roots.

Table 2. Identification of differentially expressed proteins in the seedling roots of genotypes Mengyuan and 9511 under control and salt stresses by MS/MS.

Spots	Description	Species	Accession no.	Score[a]	genotype Mengyuan				genotype 9511				SC[c]	Thr[d] MW /pI	Exp[e] MW/pI
					E[b]-0	E-1	E-2	E-3	E-0	E-1	E-2	E-3			
	Signal transduction														
4818	Allene oxide synthase	*Oryza sativa* L.	BAD17184	68/45	1.0	1.1	1.3	1.7	1.0	1.1	0.9	1.9	12%	55.3/6.91	56.3/5.75
0821	F-box family protein	*Arabidopsis thaliana* L.	NP_199308	68/45	1.0	1.1	1.6	2.0	1.0	1.5	1.6	1.5	7%	50.3/5.92	54.8/6.36
6714	Kelch repeat-containing F-box family protein	*Arabidopsis thaliana* L.	NP-566286	55/44	1.0	0.9	1.3	2.0	1.0	0.7	0.9	1.6	4%	45.0/6.00	47.5/5.37
8713	F-box domain containing protein, expressed	*Oryza sativa* L.	ABB46668	47/44	1.0	0.9	1.0	1.7	1.0	0.8	1.1	1.8	6%	41.0/5.10	46.9/4.85
3328	Wiscott-Aldrich syndrome, C-terminal	*Zea mays* L.	ACG30269	71/47	1.0	1.6	1.3	1.7	1.0	1.7	1.8	1.6	15%	20.0/6.38	27.4/5.88
8222	NPR1-like protein	*Prunus serrulata* L.	ABB59684	45/44	1.0	2.4	1.3	1.7	1.0	1.7	1.2	1.0	7%	29.0/5.44	28.3/4.55
7818	Calreticulin	*Zea mays* L.	CAA54975	46/44	1.0	1.5	0.8	1.5	1.0	1.6	0.7	1.6	4%	49.0/4.60	54.9/4.79
8021	Polcalcin Jun o 2	*Zea mays* L.	NP_001152603	48/43	1.0	1.0	0.9	3.4	1.0	1.2	1.4	3.9	7%	20.5/4.80	20.1/4.45
8812	Protein phosphatase 2c	*Ricinus communis* L.	XP-002524215	92/48	1.0	1.0	1.0	0.5	1.0	1.0	1.0	0.6	7%	45.4/5.35	53.4/4.84
2733	Ras-GTPase-activating protein-binding protein	*Ricinus communis* L.	XP-002528349	46/44	1.0	0.5	0.6	0.5	1.0	0.6	0.5	0.6	6%	52.3/5.41	53.2/6.03
	ROS scavenging														
8220	Glutathione S-transferase	*Pyrus communis* L.	ABI79308	48/44	1.0	1.6	0.9	1.2	1.0	7.8	5.7	4.4	4%	24.0/5.40	26.5/4.75
3428	Resveratrol synthase	*Arachis hypogaea* L.	CAA44186	101/49	1.0	1.4	1.7	1.8	1.0	1.2	1.2	1.1	10%	33.9/5.06	32.2/5.55
3822	Nucleic acid binding / zinc ion binding	*Arabidopsis thaliana* L.	NP-189933	58/46	1.0	1.2	1.5	1.3	1.0	1.3	1.3	1.7	5%	52.0/6.10	56.1/5.90
6018	Nucleic acid binding	*Arabidopsis thaliana* L.	NP-564325	49/45	1.0	3.8	1.7	14.9	1.0	3.4	4.8	13.2	11%	15.0/5.30	18.3/5.11
4426	NBS-containing resistance-like protein	*Platanus x acerifolia* L.	ABV30875	50/45	1.0	0.7	1.5	1.6	1.0	0.9	0.8	1.5	5%	31.0/6.78	34.3/5.71
6521	Disease resistance protein-related	*Glycine max* L.	NP-001235526	49/46	1.0	1.6	1.3	1.6	1.0	2.3	1.9	2.0	7%	39.0/5.20	39.6/5.11
5128	HSP20/alpha crystallin family molecular Chaperone	*Methanobrevibacter smithii DSM* L.	ZP-05975685	49/44	1.0	1.2	1.4	11.4	1.0	10.0	15.9	25.9	7%	23.0/5.20	25.0/5.25
3325	Tasselseed2-like short-chain dehydrogenase/ reductase	*Pharus lappulaceus* L.	ABD39550	73/50	1.0	1.1	1.0	0.3	1.0	1.2	0.9	0.6	15%	25.7/6.08	28.8/5.90
7322	Short-chain dehydrogenase/reductase SDR	*Arabidopsis thaliana* L.	YP-002131654	46/43	1.0	0.1	0.2	0	1.0	0.2	0.2	0	6%	27.2/5.70	29.2/4.96
	Energy metabolism														
7721	UDP-glycosyltransferase 76G1	*Stevia rebaudiana* L.	ACT33422	49/44	1.0	0.8	0.9	0.4	1.0	0.8	0.9	0.8	6%	52.0/5.50	50.2/4.91
9117	Gluconate operon transcriptional repressor	*Staphylococcus haemolyticus* L.	YP-252489	48/44	1.0	0.6	0.6	0.6	1.0	0.6	0.6	0.6	4%	26.0/5.30	24.6/4.40
7820	Glycoprotein	*Zea mays* L.	NP-001151220	48/44	1.0	0.6	0.8	0.6	1.0	0.7	0.6	0.5	7%	54.0/6.40	57.8/5.03
5520	Alcohol dehydrogenase	*Hordeum vulgare subsp. spontaneum* L.	AAG42507	84/46	1.0	0.6	0.6	0.2	1.0	0.9	0.7	0.5	8%	33.0/5.30	35.4/5.33
6717	ATP-citrate synthase	*Ricinus communis* L.	XP-002512567	73/44	1.0	1.5	1.6	1.1	1.0	1.3	1.5	1.2	7%	47.0/5.36	50.3/5.09
	Nucleotide metabolism														
7821	Ectonucleotide pyrophosphatase/phosphodiesterase	*Ricinus communis* L.	XP-002514102	45/45	1.0	3.4	3.1	2.6	1.0	2.1	1.8	1.8	6%	54.0/5.10	58.9/4.89
7020	Cytidine deaminase	*Cicer arietinum* L.	ACG27051	50/44	1.0	1.8	1.5	1.9	1.0	1.1	1.3	1.6	7%	20.0/5.38	19.5/4.86
	Lipid metabolism														
1915	Lipase class 3 family protein	*Arabidopsis thaliana* L.	NP-567482	48/44	1.0	0.8	1.9	2.4	1.0	1.7	1.4	1.6	2%	69.0/5.50	65.0/6.15
	Cell wall metabolism														
2519	Polysaccharide biosynthesis protein Capd	*Heliobacterium modesticaldum* L.	YP-001679368	46/43	1.0	1.0	1.0	1.3	1.0	1.2	1.5	2.2	6%	37.4/5.75	36.1/5.92
	Cytoskeletal														
7523	Tubulin folding cofactor C	*Arabidopsis thaliana* L.	AAM22959	44/44	1.0	0.8	0.6	0.6	1.0	1.0	0.7	0.6	3%	37.7/5.89	37.2/5.02
7819	Actin related protein Arp3 subunit	*Physcomitrella patens subsp. patens* L.	XP-001766600	45/42	1.0	0.3	0.4	0.4	1.0	0.6	0.5	0.6	3%	47.0/5.40	55.6/4.98
	Unclassified proteins														
3222	Transposon protein, putative, CACTA	*Oryza sativa* L.	ABA93472	98/45	1.0	1.5	1.8	2.3	1.0	1.6	1.6	2.1	12%	39.0/5.70	29.9/5.82
1520	Retrotransposon protein	*Oryza sativa* L.	ABB47461	48/43	1.0	0.3	0.4	0.4	1.0	0.4	0.3	0.2	4%	45.0/5.22	38.8/6.05
2111	DREPP2 protein	*Nicotiana tabacum* L.	CAB91552	68/49	1.0	0.9	0.7	1.6	1.0	0.9	1.0	2.1	15%	23.0/4.98	25.5/5.95
1223	Tropinone reductase I	*Anisodus acutangulus* L.	ACB71202	49/45	1.0	1.7	1.2	1.1	1.0	3.2	2.0	2.3	8%	29.0/6.45	26.9/6.27
3326	Hypothetical protein	*Vitis vinifera* L.	CAN76209	67/45	1.0	1.4	0.6	2.2	1.0	1.2	1.0	1.8	15%	22.1/6.06	28.5/5.83
8613	Os03g0774800	*Oryza sativa* L.	NP_001051425	44/42	1.0	4.3	5.8	1.6	1.0	2.5	3.6	1.1	5%	48.0/5.20	45.4/4.73
7826	Hypothetical protein	*Vitis vinifera* L.	CAN79663	52/47	1.0	2.3	2.3	2.0	1.0	2.0	2.1	2.0	2%	60.0/6.40	58.9/4.90
1322	Hypothetical protein OsI_26905	*Oryza sativa* L.	EEC82464	56/47	1.0	4.6	3.4	5.8	1.0	4.2	4.1	5.2	6%	32.0/6.78	29.2/6.38
9611	Predicted protein	*Ricinus communis* L.	XP-002303948	112/49	1.0	1.0	1.3	1.6	1.0	0.8	1.3	1.7	7%	37.0/5.30	40.9/4.46

Note: [a]Score of each protein and minimum score for significant hit of mascot search; Score was MOWSE score probability for the entire protein. [b]Protein spot abundance is given by the mean normalized spot volume of three biologically independent experiments as determined by the image analysis software. E-0, E-1, E-2, and E-3 represent 0, 50, 100, and 150 mM NaCl concentration treatments, respectively. Significant differences within genotype treatment-specific (more than 1.5-folds or less than 0.66-fold) are underlined. [c]The sequence coverage of identified proteins. [d]The theoretical values of molecular weight (MW/kDa) and isoelectric point (pI). [e]The experimental values of molecular weight (MW/kDa) and isoelectric point (pI).

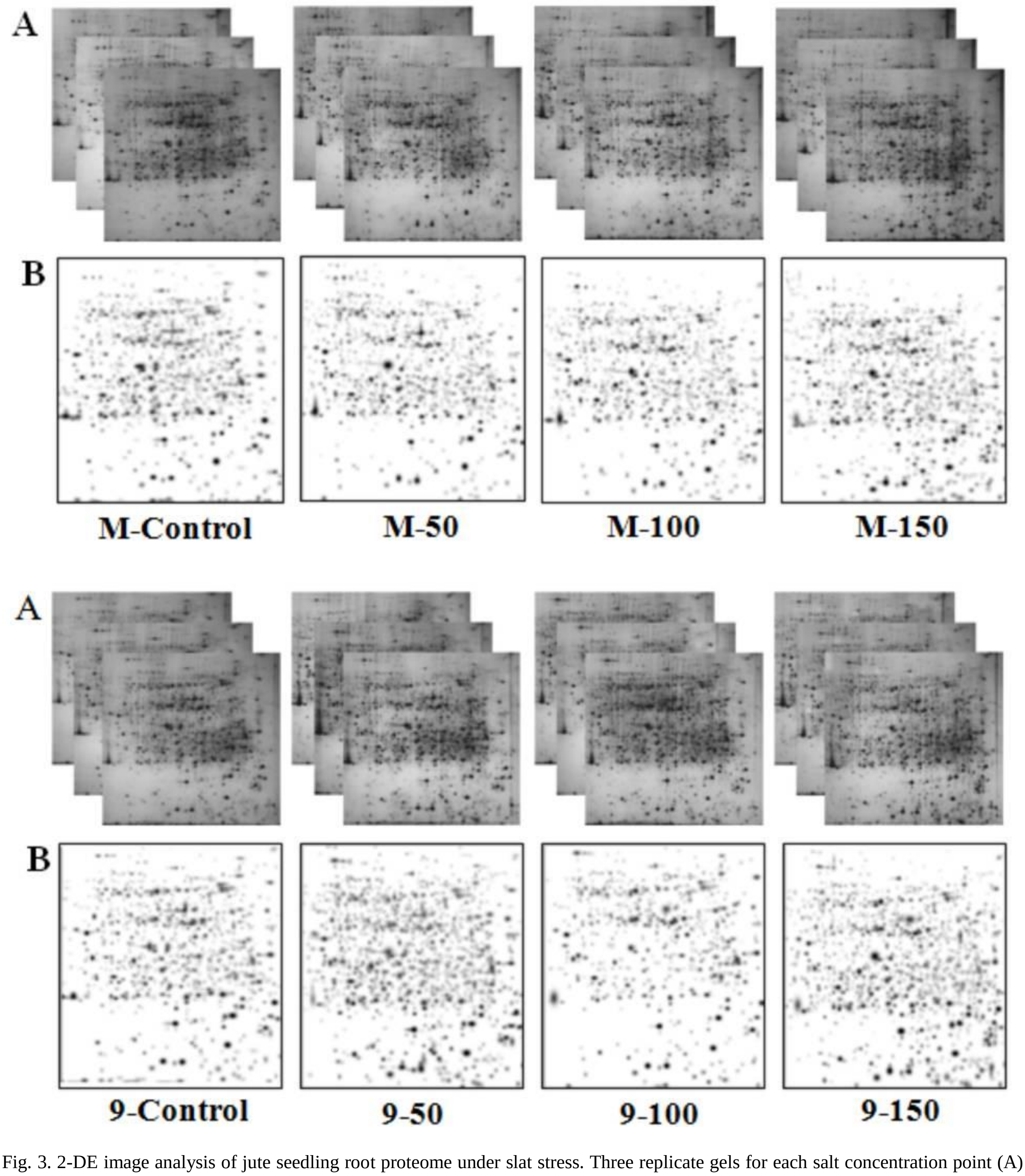

Fig. 3. 2-DE image analysis of jute seedling root proteome under slat stress. Three replicate gels for each salt concentration point (A) were computationally combined using PDQuest 7.2.0 software to generate the standard gels (B).

Table 3. The homologues of five unknown and hypothetical proteins.

Spots[a]	NCBI accession No.	Homologue				
		NCBI accession No.[b]	Description	Organism	Ident.[c] %	Pos.[d] %
3326	CAN76209	XP_002515412	RNA binding protein, putative	*Ricinus communis*	62	74
8613	NP_001051425	ABA97953	Transposon, putative mutator sub-class	*Oryza sativa*	69	78
7826	CAN79663	BAG68656	Jasmonate ZIM-domain protein 2	*Nicotiana tabacum*	45	56
1322	EEC82464	ACG24884	FIP1	*Zea mays*	64	70
9611	XP_002303948	XP_002538120	Flavonol 4'-sulfotransferase, putative	*Ricinus communis*	58	77

Note: [a]The accession number of the unknown and hypothetical proteins in Table 2. [b]The accession number of the homologues. [c]Identities. [d]Positives means similarities

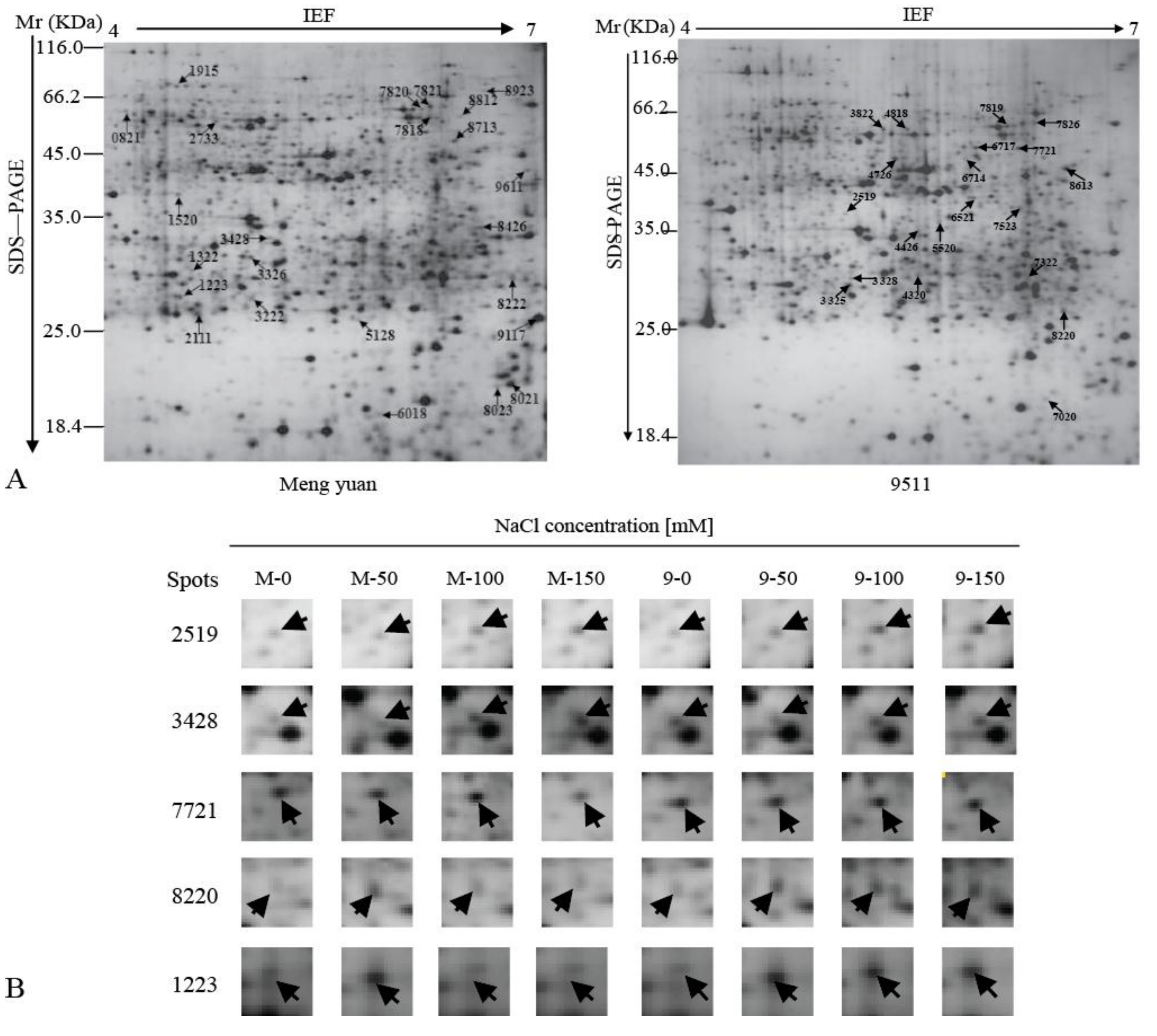

Fig. 4. A: Representative 2-DE gels from seedling root samples of both genotypes Mengyuan and 9511. The spot indicated in the gel of some genotype based on its higher protein expression in this genotype than the other. B: The section of 2-DE gel of 5 proteins for close-up view, M represents genotype 'Mengyuan', and 9 represents genotype '9511'.

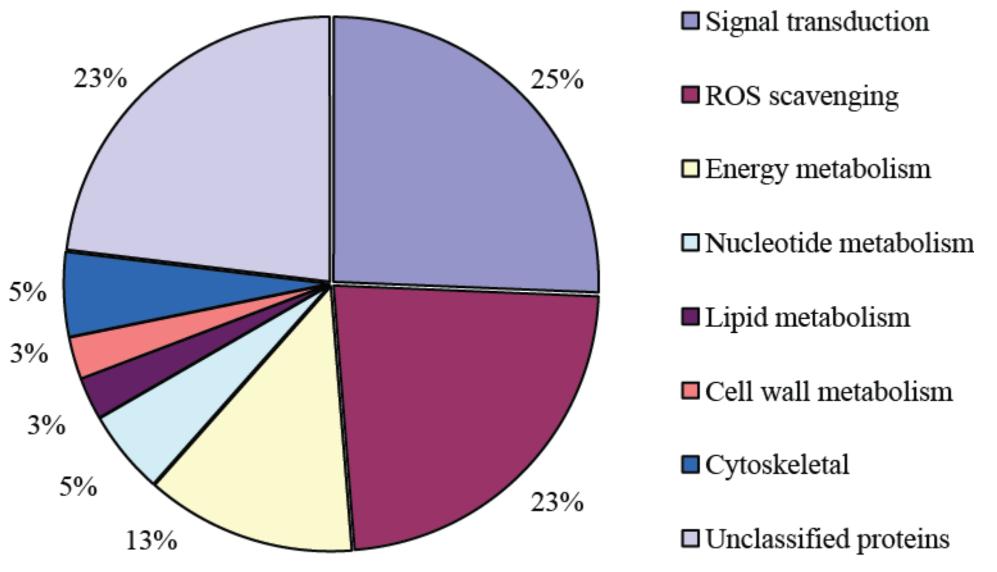

Fig. 5. Functional category distribution of the 39 differentially expressed proteins identified by MS/MS.

Of the ten identified proteins involved in signal transduction, two (spots #8812 and 2733) were found to be down-regulated under NaCl stress in the seedling roots of the two jute genotypes. One (spot #8812) was identified as protein phosphatase 2C, which acts as negative regulators of ABA signaling in *Arabidopsis*, but its target proteins are still unknown (He & Li, 2008). The down regulation of protein phosphatase 2C may increase ABA signal transduction pathway, which is supported by the fact that over-expression of protein phosphatase 2C in *Arabidopsis thaliana* lowers its tolerance to salt (Liu *et al*., 2009). The other (spot #2733) was identified as Ras-GTPase-activating protein-binding protein. Ras-GTPase-activating protein-binding protein is the essential negative regulator of the ras-signaling pathway (Pamonsinlapatham *et al*., 2009) and induced by salt stress in smooth cordgrass (Baisakh *et al*., 2008). The down-regulated Ras-GTPase-activating protein-binding protein indicated that ras-signaling pathway might be decreased under salt stress in jute seedling roots.

Identified proteins related to ROS scavenging: Growing evidences suggest that redox homeostasis is a metabolic interface between stress perception and physiological responses (Yan *et al*., 2006). But reactive oxygen species (ROS) produced readily in stress conditions act as signaling molecules for stress responses and also cause damage to cellular components (Li *et al*., 2009). Plants can scavenge the superfluous ROS by modulating related gene and protein expression to maintain cell redox homeostasis (Li *et al*., 2009). In this study, nine identified proteins were found to be involved in ROS scavenging.

From nine proteins involved in ROS scavenging, seven (spots #8220, 3428, 3822, 6018, 4426, 6521, and 5128) were found to be up-regulated by NaCl stress. Interestingly, two (spots #8220 and 3428) of them showed great difference in abundance between genotypes Mengyuan and 9511 after salt stress. Spot #8220, which had higher abundance in genotype 9511 ($\geq$ 4.4 fold) than in genotype Mengyuan ($\leq$ 1.6 fold) after salt stress, was identified as glutathione S-transferase (GST). GST is an important antioxidative enzyme involved in plant defense against both biotic and abiotic stresses by scavenging ROS produced during stress (Witzel *et al*., 2009). Spot #3428, which was up-regulated by NaCl stress in genotype Mengyuan but not regulated in genotype 9511, was identified as resveratrol synthase. Resveratrol synthase catalyzes one molecule of coumaroyl-CoA and three molecules of malonyl-CoA into resveratrol (Lim *et al*., 2005). Resveratrol has been reported for its possible antioxidant role and protective effects against certain forms of oxidant damage (Leonard *et al*., 2003). Up-regulated protein spots #3822 and 6018 were identified as nucleic acid binding/zinc ion binding protein and nucleic acid binding protein, respectively. These two nucleic acid binding proteins can protect cells by inhibition of ROS production (Chimienti *et al*., 2001). Spot #4426 was identified as NBS-containing resistance-like protein, which can also protect cells by inhibition of ROS production (Chimienti *et al*., 2001). Spot #6521 was identified as disease resistance protein-related. Disease resistance protein-related has been found to be involved in the scavenging of ROS, especially H_2O_2 (Li *et al*., 2009). Spot #5128 was identified as HSP20/alpha crystallin family molecular chaperone, which has been recognized to play protective roles against a variety of stresses (H_2O_2, salt and drought, etc) and promote resistance to environmental stress factors (Ouyang *et al*., 2009). In the present study, the up-regulation of these proteins indicated that jute genotype increased its salt tolerance through enhancing its ROS scavenging capacity.

The other two proteins (spots #3325 and 7322) involved in ROS scavenging were found to be down-regulated under NaCl stress in the seedling roots of the two jute genotypes. Both of them were identified as short-chain dehydrogenases/reductases (SDR), which are involved in regulating cell redox state (Li *et al*., 2009).

Taken together, our above results indicated that ROS scavenging capacity might be increased in jute seedling roots in response to salt response. Moreover, most of the identified proteins involved in ROS scavenging in genotype 9511 showed more abundance than these in genotype Mengyuan, indicating that genotype 9511 might have stronger ROS scavenging capacity than genotype Mengyuan. This result was in accordance with our previous one, which indicated that genotype 9511 has stronger ROS scavenging capacity and lower MDA content than genotype Mengyuan (Ma *et al*., 2011).

Identified proteins related to primary metabolisms: The primary metabolisms, such as metabolisms of energy, nucleotide, lipid, and cell wall, need to be modulated to establish a new homeostasis under salt stress (Yan *et al*., 2006). As expected, energy metabolism was altered under the salt stress as revealed by the altered expression of five identified proteins (spots #7721, 9117, 7820, 5520 and 6717) in this study. Of them, protein spot #7721, which was down-regulated after salt stress in genotype Mengyuan but not regulated in genotype 9511, was identified as UDP-glycosyltransferase 76G1 (UG-76G1). In plants, UG-76G1 catalyzes the products of photosynthesis into disaccharide, oligosaccharide, and polysaccharide (Ross *et al*., 2001). Three identified proteins (spots #9117, 7820 and 5520) were down-regulated by salt stress in the seedling roots of the two jute genotypes. Spot #9117 was identified as gluconate operon transcriptional repressor, which is carbon and energy source (Letek *et al*., 2006). Spot #7820 was identified as glycoprotein. Glycoprotein is involved in glycan biosynthesis and metabolism and also provides energy source (Berger *et al*., 1982). Spot #5520 was alcohol dehydrogenase, which is involved in glycolysis (Alam *et al*., 2010). Only one identified protein (6717) involved in energy metabolism was up-regulated by salt stress in the seedling roots of the two jute genotypes. This protein was ATP-citrate synthase, which is a key synthase for citrate synthesizing in the TCA cycle (Fuente *et al*., 1997). Taken together, the above results implied that energy metabolisms might be impaired by salt stress in jute seedling roots.

Two identified proteins (spots #7821 and 7020) were found to be involved in nucleotide metabolism. And both of them were up-regulated by the salt stress in the seedling roots of the two jute genotypes. One of them, spot #7821, was identified as ectonucleotide pyrophosphatase/

phosphodiesterase (E-NPP), which belongs to a family of membrane proteins and is related with various physiological processes, including nucleotide recycling, phospholipids signaling, proliferation and motility of cells (Stefan *et al*., 2005). The other (spot #7020) was identified as putative cytidine deaminase (CDM), which exerts partially known functions in the intracellular regulation of nucleotide metabolism (Donadelli *et al*., 2007). Our results indicated that nucleotide metabolism might be enhanced in jute seedling roots by salt stress.

Lipases are ubiquitous enzymes and play important roles in lipid metabolism, including catalyzing hydrolysis and synthesis of triglycerides and other water insoluble esters (Fischer *et al*., 2003). One up-regulated identified protein (spot #1915) involved in lipid metabolism was identified as lipase class 3 family protein in this study. This protein has been found in response to salt stress (Nguyen *et al*., 2007). The up-regulated lipase class 3 family protein in this study implied that the lipid metabolism might be enhanced under salt stress.

In response to salt stress, the metabolism of cell wall can be modulated and its composition might be changed (Li *et al*., 2009). Spot #2519, which was up-regulated under salt stress in genotype 9511 but not changed in genotype Mengyuan, was identified as polysaccharide biosynthesis protein Capd, putative (Capd). This protein has been predicted to be involved in cell wall biosynthesis (Li *et al*., 2009). The up-regulation of this protein in genotype 9511 implied that cell wall biosynthesis might be enhanced in genotype 9511 seedling roots in response to salt stress.

Two proteins (#7523 and 7819) were identified as components of plant cytoskeleton. Both of them were down-regulated by salt stress in the seedling roots of the two jute genotypes. Spot #7523, which was identified as tubulin folding cofactor C, is an important component of plant cytoskeleton (Li *et al*., 2009). The other spot (#7819) was identified as actin related protein Arp3 subunit (Arp3 subunit). Arp3 subunit is also an important component of plant cytoskeleton and contributes to cell elongation and mixed disulphides formation (Li *et al*., 2009). Our results suggested that plant cytoskeleton in jute seedling roots were severely affected by salt stress.

The difference of differentially expressed proteins between the two jute genotypes: A number of salt stress-responsive proteins in jute seedling roots have been identified in the present study. Of them, five could be used as potential candidates for in-depth salt tolerance study, and are listed in Table 4. The standards for choosing them were as follows: 1) proteins had higher abundance (more than 2.0 fold) in genotype 9511 than in genotype Mengyuan after salt stress; 2) proteins had lower abundance (lower than 0.5 fold) in genotype 9511 than in genotype Mengyuan after salt stress. In the first group, four proteins were presented, including glutathione S-transferase involving in ROS scavenging, UDP-glycosyltransferase 76G1 involving in energy metabolism, polysaccharide biosynthesis protein Capd involving in cell wall metabolism, and tropinone reductase I which was not classified. In the second group, only one protein (resveratrol synthase) involving in ROS scavenging was presented. The sections of 2-DE gel of these proteins for close-up view are shown in Fig. 4B. These proteins maybe have important roles in jute seedling roots in response to salt stress.

Table 4. Differentially expressed proteins between the seedling roots of the two genotypes Mengyuan and 9511.

Higher abundance in genotype 9511 than in genotype Mengyuan after salt stress	**Functions**
Glutathione S-transferase	ROS scavenging
UDP-glycosyltransferase 76G1	Energy metabolism
Polysaccharide biosynthesis protein Capd	Cell wall metabolism
Tropinone reductase I	Unclassified protein
Lower abundance in genotype 9511 than in genotype Mengyuan after salt stress	
Resveratrol synthase	ROS scavenging

A possible salt stress-responsive protein network in jute seedling roots: In the present study, a salt stress-responsive protein network was proposed with most of the 39 salt-responsive proteins identified in jute seedling roots. This network consists of several functional components, including ROS scavenging, signal transduction, energy metabolism, nucleotide metabolism, lipid metabolism, cell wall metabolism, and cytoskeleton etc.

Under salt stress, jute seedling roots can perceive salt stress signals through putative sensors and transmit them to the cellular machinery by many signal transduction pathways, including jasmonate, gibberellin, ethylene, ABA, ROS and Ca^{2+} signal transduction pathways, leading to changes of many metabolism pathways and cellular processes. After the persistent (for 4 d) salt stress, jute seedling would adapt to salt stress through altering signal transduction, accelerating ROS scavenging, impairing energy metabolism, enhancing nucleotide metabolism, lipid metabolism and cell wall metabolism, as well as altering cytoskeleton in roots. Such a protein network allows us to further understand and describe the possible management strategy of cellular activities occurring in salt-treated jute seedling.

Furthermore, genotype 9511 possessed the ability of higher ROS scavenging, stronger cell wall biosynthesis in the seedling roots than genotype Mengyuan, which may be the major reasons why genotype 9511 is more salt tolerant than genotype Mengyuan.

Conclusions

To investigate changes of proteome under salt stress, we performed a comparative proteome analysis of seedling roots of two jute genotypes (salt sensitive genotype

Mengyuan and salt tolerant genotype 9511) using NaCl as a model for salt stress. About 44 protein spots on the 2-DE gel image were found to be differentially expressed in the salt-treated jute seedling roots. Of them, 39 were successfully identified by MS/MS. These identified proteins were involved in nine metabolic pathways and cellular processes. Five of the identified proteins could be used as potential candidates for in-depth salt tolerance study. For example, the study of their function may be important for plant in response to salt stress. Based on most of the 39 identified salt-responsive proteins, a salt stress-responsive protein network in jute seedling roots was proposed. Such a molecular mechanism will provide insights into salt stress responses and for further dissection of salt tolerance mechanisms in jute.

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