Chinese Science Bulletin

Proteomic analysis of long-term salinity stress-responsive proteins in *Thellungiella halophila* leaves

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Salinity is one of the most severe environmental factors that may impair crop productivity. A proteomic study based on two-dimensional gel electrophoresis is performed in order to analyze the long-term salinity stress response of *Thellungiella halophila*, an *Arabidopsis*-related halophyte. Four-week-old seedlings are exposed to long-term salinity treatment. The total crude proteins are extracted from leaf blades, separated by 2-DE, stained with Coomassie Brilliant Blue, and differentially displayed spots are identified by MALDI-TOF MS or QTOF MS/MS. Among 900 protein spots reproducibly detected on each gel, 30 spots exhibit significant change and some of them are identified. The identified proteins include not only some previously characterized stress-responsive proteins such as TIR-NBS-LRR class disease resistance protein, ferritin-1, and pathogenesis-related protein 5, but also some proteins related to energy pathway, metabolism, RNA processing and protein degradation, as well as proteins with unknown functions. The possible functions of these proteins in salinity tolerance of *T. halophila* are discussed and it is suggested that the long-term salinity tolerance of *T. halophila* is achieved, at least partly, by enhancing defense system, adjusting energy and metabolic pathway and maintaining RNA structure.

Thellungiella halophila, proteomics, salinity stress, mass spectrometry, two-dimensional gel electrophoresis

Salinity is one of most severe factors that may impair global crop productivity. It is estimated that about 20% of the agricultural land in the world is affected by salinity[1] and salinity is a crucial constraint to realizing sustainable development of agriculture in future. Therefore it is of great significance to investigate the molecular mechanism of salinity tolerance in plants, and improve stress tolerance of crops by genetic engineering.

The salinity tolerance mechanism has been intensively studied over the past years and most studies have been preformed by using the model plant, such as *Arabidopsis thaliana* and rice, while few were done on halophytes. In recent years, *Thellungiella halophila*, a close relative of *A. thaliana*, called also *Thellungiella salsuginea*, was increasingly used as a model system in plant abiotic stress researches[2]. *T. halophila* is able to tolerate salinity shock up to 500 mmol/L NaCl and grows in salt with significantly higher capability than *Arabidopsis*[3]. At the same time, it is a close relative of *A. thaliana* and *Brassica napus*, and expressed sequence tags (EST) analysis reveals that it shares 94% homology in cDNA sequence with *A. thaliana* and 98% homology with Brassicacea family. With a short life cycle, small plant size, copious seed production and small genome size, it is regarded as an ideal model for the researches on plant abiotic tolerances.

In the past few years, several research teams performed physiological, biochemical and transcriptional analysis to investigate the molecular mechanism of

Received May 4, 2008; accepted July 3, 2008
doi: 10.1007/s11434-008-0455-6
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Supported by the National Basic Research and Development Program of China (Grant Nos. 2006CB100100, 2004CB117303), National Natural Science Foundation of China (Grants Nos. 30670203, 30570434), Beijing Municipal National Natural Science Foundation (Grant No. 5062021), CUN 985-3-3 and Open Fund of Key Laboratory of Cell Proliferation and Regulation Biology, Ministry of Education of China.

salinity tolerance in *T. halophila*. A NaCl-treated EST library of Shandong ecotype, EST and SSH libraries of cold-, drought-, and salinity-stressed plants from the Yukon ecotype were constructed[4,5]. GeneChip array and full-length cDNA microarray of *Arabidopsis* were also used to probe the gene expression profiles of *T. halophila* under salinity stress, due to lack of its own microarray[6—8]. Recently, a cDNA microarray consisting of 3628 unique genes of *T. halophila* was used to analyze the transcript profiles in response to cold, salinity, drought, and rewatering after drought[9].

These studies provided valuable information for elucidation of salinity tolerance mechanism in *T. halophila.* First1y, the rosette leaves of *T. halophila* show more efficient tolerance for Na^+ metabolic toxicity than *A. thaliana* and the capability is associated with higher expression of CDSP32 thioredoxin. Secondly, the root of *T. halophila* is able to withstand high level Na^+ via expression of specific Na/K antiporter[10]. Thirdly, stress tolerance of *T. halophila* may be due to constitutive overexpression of many known abiotic- and biotic-stress inducible genes in *Arabidopsis*[8]. Finally, the stress-tolerance of *T. halophila* is achieved not only by well-documented stress genes, but also by some novel stress genes[5].

In general, the level of mRNA does not always correlate well with the level of protein mainly owing to post-transcriptional and post-translational regulation, and protein subcellular transport as well[11]. Focusing on complete set of proteins encoded by the genome, proteomics is regarded as an efficient approach to addressing plant response to different environmental factors[12]. Proteomic analysis of salinity stress response was performed in leaves of rice and *Suaeda aegyptiaca*[13,14], roots of rice and *Arabidopsis*[15,16], and pea mitochondrial[17]. These studies have provided new insights into plant salinity stress tolerance investigation.

There is no report on proteomic analysis of abiotic stress tolerance mechanism in *T. halophila* as yet. It is of great importance to investigate the salinity response at the protein level. In this study, the molecular responses to long-term salinity stress were investigated by using proteomic approach in leaves of *T. halophila.* Thirty differentially expressed proteins were detected and some of them were further identified by MS analysis. These proteins were involved in various cellular processes, such as defense, energy pathway, RNA processing and protein degradation. The possible functions of the identified proteins in long-term salinity adaptation were discussed.

1 Materials and methods

1.1 Plant materials and salinity treatment

T. halophila (ecotype Shandong) seedlings are germinated and grown in soil under fluorescent light (330 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 16 h light/8 h dark) at 25℃ and 35% relative humidity in a growth chamber.

Two week old seedlings are transferred onto plastic pots filled with a 1∶1 (perlite∶vermiculite) and watered with half strength Hoagland solution every 3—4 d. Two weeks after transfer, the salinity treatment was applied. The high salinity treatments were applied by incremental increases of NaCl in half strength Hoagland solution until the 300 mmol/L were reached. The seedlings in control group were watered only by half strength Hoagland solution. Twenty five days later, the leaves were collected and frozen immediately in liquid nitrogen, and then stored at −70℃ for protein extraction.

1.2 Protein extraction and 2-DE

Leaves were ground in liquid nitrogen, suspended in ice-cold 10% (w/v) trichloroacetic acid in acetone containing 0.07% (w/v) DTT and incubated at −20℃ for 1 h, and then centrifuged for 15 min at 35000×*g*. The pellets were resuspended in 0.07% (w/v) DTT in acetone, incubated at −20℃ for 1 h and centrifuged for 15 min at 12000×*g*. After repeating 3 times the above steps, the pellets were lyophilized. Then the dry powder was solubilized in lysis buffer (9 mol/L urea, 35 mmol/L Tris, 4% (w/v) CHAPS, 1% (v/v) pH 4—7 IPG buffer, 1% (w/v) DTT) and centrifuged for 15 min at 12000×*g*. The proteins in liquid were precipitated by adding 4 volumes of ice-cold acetone, incubated at −20℃ for at least 2 h and centrifuged again for 15 min at 12000×*g*. The pellets were finally dissolved in rehydration buffer (8 mol/L urea, 20 mmol/L DTT, 2% (w/v) CHAPS, 0.5% (v/v) pH 4—7 IPG buffer). Protein concentration was determined using the Bradford assay (Baitech, Beijing, China) and then stored at −70℃.

For two-dimensional gel electrophoresis, 400 μg of extracted proteins in a certain volume were loaded onto semipreparative gels. For IEF, the Ettan IPGphor3 system (Amersham Biosciences, Uppsala, Sweden) and pH

4—7 IPG strips (13 cm, linear) were used according to the manufacturer's recommendations. The IPG strips were rehydrated for 13 h in 250 μL rehydration buffer containing protein samples. Focusing was performed in 3 steps: 500 V for 1 h, 1000 V for 1 h and 8000 V for 7 h. The gel strips were equilibrated for 15 min in 10 mL equilibration buffer (50 mmol/L Tris-HCl buffer, pH 8.8, 6 mol/L urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT and 0.002% (w/v) bromophenol blue). SDS-PAGE was performed with 12.5% gels using the PROTEAN II xi Cell system (Bio-Rad, Hercules, CA, USA). The gels were run at 10 mA per gel for the first 45 min and followed by 30 mA per gel. The gels were visualized with Coomassie Brilliant Blue R-250. The gels were scanned using an optical scanner in transmittive mode (Umax) at 400 dpi and analyzed with ImageMaster 2-D Elite software (Amersham Biosciences, Uppsala, Sweden). Each sample was performed by 2-DE at least three repetitions for further analysis. The abundance of each protein spot was estimated from the relative volumes values (%Vol). Only those protein spots with significant and reproducible changes were determined to be differentially expressed proteins.

1.3 In-gel digestion and MS analysis

Spots of differentially expressed proteins were excised from the semipreparative gels, washed 3 times with ultrapure water, destained twice with 50 mmol/L NH_4HCO_3 in 50% acetonitrile, dried with 100% acetonitrile and digested overnight at 37℃ with sequencing grade modified trypsin (Promega, Madison, WI, USA) in 25 mmol/L 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 CHCA containing 0.1% TFA and 50% acetonitrile. MALDI-TOF MS analysis was conducted with a MALDI-TOF/TOF mass spectrometer, a Bruker Autoflex mass spectrometer (Bruker-Franzen Bremen, Germany). Database searches, including PMF and MS/MS, were performed using MASCOT program (http://www.matrix science.com). The database was set to NCBInr (updated on January 14, 2008). The taxonomic category selected was green plants. The other parameters for searching were enzyme of trypsin, one missed cleavage, fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M), peptide tolerance of 100 ppm, MS/MS tolerance of 0.4 Da, peptide charge of 1^+, and monoisotopic. According to the MASCOT probability analysis (P<0.05), only significant hits were accepted for identification of the protein samples.

For proteins that could not be identified by MALDI-TOF MS, ESI-MS/MS was performed on a Qstar Pulser I Quadrupole TOF-MS (Applied Biosystems/MDS Sciex, Toronto, Canada). The protein identification was determined by MS/MS fragment ion using MASCOT software through searching the Swiss-Prot database. The parameters used were: monoisotopic peptide masses, ±0.2 Da peptide mass tolerance, one missed cleavage, modifications allowed for oxidation of methionine and carboxyamidomethylation of cysteine.

2 Results

2.1 Two-dimensional electrophoresis analysis of total proteins of *T. halophila* leaves

To investigate the long-term changes of protein profiles after salinity stress, we carried out 2-DE analysis of the total proteins in *T. halophila* rosette leaves from 3 biological replicates and each protein sample comprised 4—5 pooled plants. For each sample, triplicate gels were performed, and they showed a high level of reproducibility. The representative gels are shown in Figure 1. There are 1240 ± 191 and 1281 ± 198 protein spots detected by ImageMaster 2-D Elite software respectively in CBB-stained gels for treated group and control group. 939 and 903 protein spots detected reproducibly on gels for treated group and control group respectively. Quantitative image analysis revealed that a total of 30 protein spots changed their intensities significantly (P < 0.05) by more than 1.5-fold compared with the control. Four typical regions are enlarged in Figure 2. Among the 30 differentially accumulated protein spots, 27 spots showed quantitative changes, with 16 more abundant and 11 less abundant, and 3 spots appeared absent in the control but present after salinity treatment.

2.2 Identification of the differentially expressed proteins by MS

The differentially expressed proteins were excised from the gels, in-gel digested by trypsin, and analyzed by MALDI-TOF/TOF or q-TOF mass spectrometer. In total, 13 protein spots were successfully identified either by PMF or MS/MS analysis as shown in Tables 1 and 2. The proteins identified by MS are indeed the most homologous protein in green plant protein database since

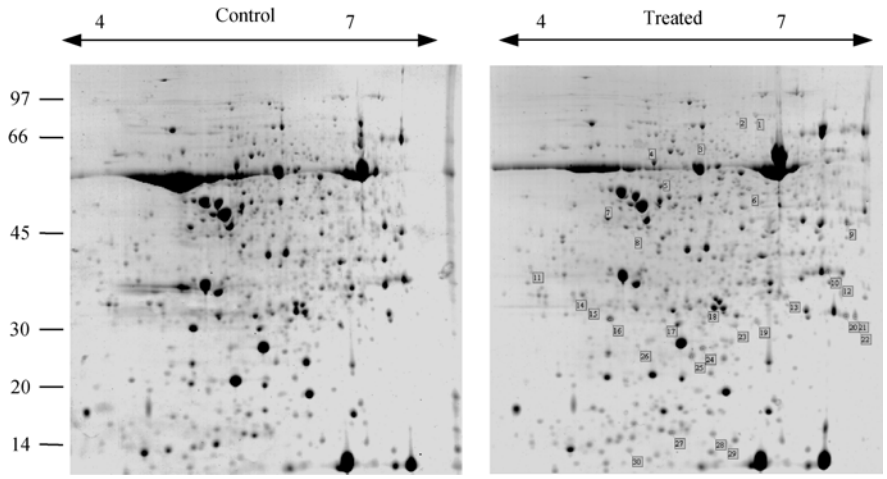

Figure 1 Representative 2-DE gels of *T. halophila* leaf proteins. Labeled spots indicate differentially expressed proteins showing at least a 1.5-fold change under NaCl treatments with $P < 0.05$. Positions and sizes of SDS-PAGE molecular weight markers (MW) are shown in kD.

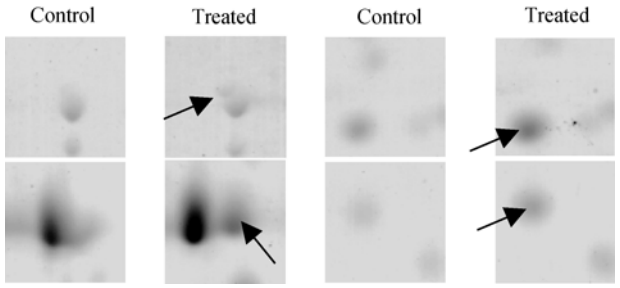

Figure 2 Examples of changed patterns of some protein spots after salinity treatment. Labeled spots were differentially expressed proteins.

the genome of *T. halophila* is not completely sequenced yet.

It is noteworthy that the spot 10 was identified as a mixture of two proteins. MS identification of spot 16 is illustrated in Figure 3. A total of 14 proteins were identified by MS analysis and these proteins participate in defense, energy pathway, RNA processing and protein degradation. It is suggested that the long-term salinity tolerance of *T. halophila* is achieved partly by enhancing defense system, adjusting energy and metabolic pathway and maintaining RNA structure.

3 Discussions

A comparative proteomics analysis was performed to screen and identify the proteins that respond to long-term salinity stress in *T. halophila* leaf. 14 proteins have been identified from 30 differentially expressed protein spots.

Hydroponic culture methods are often used in investigation of salt tolerance mechanism of plant. In our study, plants grown at perlite:vermiculite (1∶1) with regular irrigation by half strength Hoagland solution was used as in previous study[8]. Anyway, hydroponic culture comprising solely nutrient solution was used in some studies[13]. According to our experience, the latter is more suitable for short-term stress response study, while the former is suitable for long-term study. 300 mmol/L NaCl was chosen for salinity treatment in this study, since the seedlings showed marked stress symptoms without death under such a stress[3]. This study design primarily focuses on long-term stress. A consideration for this choice is that a rapidly imposed stress can result in a large number of injury-related responses[18] and may not reveal adjustments required for long-term survival under salinity stress

Of the 30 differentially expressed proteins, 53% (16/30) was up-regulated, 37% (11/30) was down-regulated and 10% (3/30) was newly-induced. It is inferred that the salinity tolerance of *T. halophila* is achieved, at least partly, by adjusting some proteins abundance and *de novo* synthesis of a large number of proteins is not needed for long-term salinity adaptation.

In our study, a spot was identified as a mixture of two proteins with similar molecular weights. This phenomenon was reported in a previous study[15]. Although the 2-DE using IPG strips can realize higher resolution than classical 2-DE, the total number of expressed proteins in a cell is more than 10^4; therefore a spot in 2-DE gel composed of two or more proteins is inevitable.

Most of the identified proteins were well-documented stress related proteins, such as TIR-NBS-LRR class disease resistance protein, ferritin-1, and pathogenesis-related protein 5 (PR-5); however, some proteins with

Table 1 Data obtained from the MALDI-TOF analysis and Mascot PMF search

Spot No.	Homologous protein	Theor./exp. Mass (kD)	Theor./ exp. pI	Mascot Score	Peptides matched	Sequence coverage	Accession No.	Species	*Arabidopsis* locus	%Vol in Treatment /Control [a]	Fold change
1	disease resistance protein (TIR-NBS-LRR class)	138/92	6.0/6.0	70	20	22%	gi|15238808	*Arabidopsis thaliana*	AT5G18370	0.0052±0.00097/0.0015±0.00030	+3.56
10[b]	cytosolic malate dehydrogenase	36/36	7.0/6.7	67	11	33%	gi|21593565	*Arabidopsis thaliana*	AT5G43330	0.092±0.019/0.024±0.0040	+3.88
10[b]	glyceraldehyde-3-phosphate dehydrogenase	37/36	6.7/6.7	68	12	29%	gi|15222848	*Arabidopsis thaliana*	AT1G13440	–	–
13	cysteine proteinase inhibitor, putative 1	22/28	6.0/6.4	71	6	20%	gi|24899735	*Arabidopsis thaliana*	AT3G12490	0.031±0.0061/0.014±0.0025	+2.24
18	PAB1 (20S proteasome alpha subunit B1); peptidase	26/27	5.5/5.7	107	11	44%	gi|15219257	*Arabidopsis thaliana*	AT1G09780	0.052±0.0077/0.031±0.0052	+1.65
24	unknown protein	20/21	5.4/5.5	86	12	31%	gi|18391006	*Arabidopsis thaliana*	AT1G09310	0.069±0.011/0.036±0.0034	+1.92
3	putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	61/61	5.3/5.3	171	22	33%	gi|23397105	*Arabidopsis thaliana*	AT1G09780	0.016±0.0010/0.028±0.0015	–0.59
6	ATP sulfurylase	51/48	6.3/6.2	103	17	32%	gi|6606509	*Arabidopsis thaliana*	AT3G22890	0.047±0.0044/0.071±0.0017	–0.66

a) %Vol (the relative volumes values) was calculated with ImageMaster 2D Elite (Amersham Biosciences); b) This spot was identified as a mixture of two proteins.

Table 2 Data obtained from the ESI-MS/MS or MALDI-TOF-MS/MS analysis

Spot No.	Homologous protein	Theor./exp. Mass (kD)	Theor./ exp. pI	Score	Peptides matched	Accession No.	Species	*Arabidopsis* locus	%Vol in Treatment/Control	Fold change
14	Putative ribonucleoprotein At2g37220, chloroplast precursor	31/30	4.9/4.8	1565	VSEAEARPPREQSFSADLK	Q9ZUU4	*Arabidopsis thaliana*	AT2G37220	0.062±0.0098/ 0.027±0.0022	+2.29
17	Ferritin-1, chloroplast precursor	28/26	5.1/5.3	2090	ISDFITQLRGDALYAMELALSLEK	Q96540	*Brassica napus*	_	0.029±0.0028/ 0.018±0.0036	+1.66
27	Glycine-rich RNA-binding protein 7	16/16	6.0/5.5	2141	SITVNEAQSRCFVGGLAWATGDAELERTFSQFGEVIDSKGFGFVTFKDEK	Q03250	*Arabidopsis thaliana*	AT2G21660	0.13±0.0085/ 0.083±0.0087	+1.56
23	SOUL-like protein	26/25	4.5/4.8	162	EDGGVGSDSAYTVAQYNSPFEFSGR	gi|21592576	*Arabidopsis thaliana*	AT1G17100	0.027±0.0041/ 0.084±0.0090	–0.32
7	F-box protein PP2-A12 (Protein PHLOEM PROTEIN 2-LIKE A12) (AtPP2-A12)	34/41	7.9/4.8	400	TSGVCLSISAK	Q9LN77	*Arabidopsis thaliana*	AT1G12710	0.0086±0.0025/ 0.021±0.0033	–0.42
16	Pathogenesis-related protein 5 precursor (PR-5).	26 /26	4.5/4.8	2492	DFYDVSLVDGYNVK	P28493	*Arabidopsis thaliana*	AT1G75040	0.14±0.026/ 0.65±0.12	–0.22

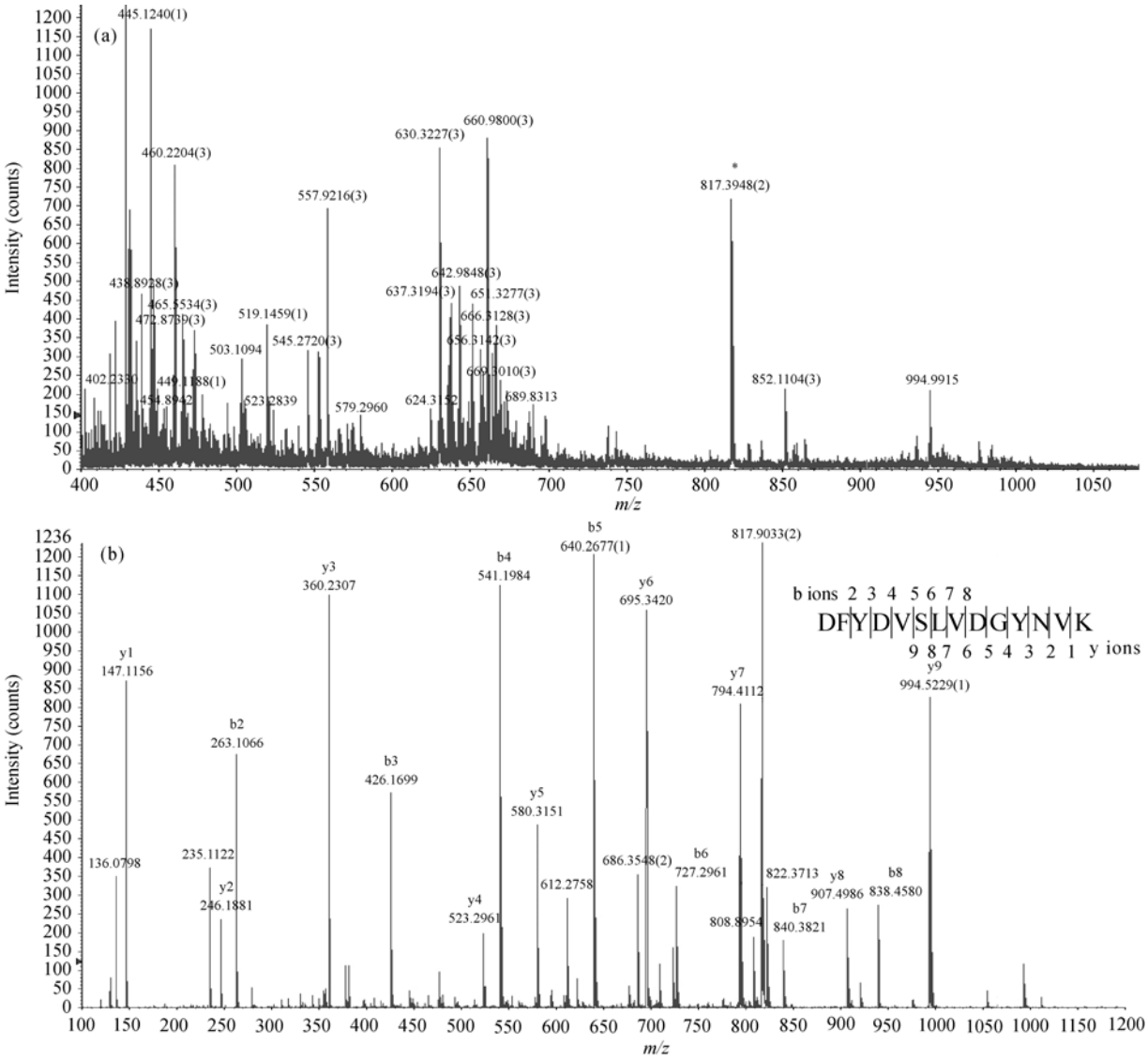

Figure 3 MS analysis of protein spot 16. The protein excised from gels was digested with trypsin and the resulting peptides were analyzed using a Qstar Pulser I Quadrupole TOF-MS spectrometry. (a) MS analysis. The spectral peaks show the intensities of different peptides. The 817.3946 *m/z* ion (marked with an asterisk) was further analyzed by MS/MS. (b) MS/MS analysis of the 817.3946 *m/z* ion. The b ions, y ions and the resulting peptide sequence were shown. The b1 ions were not included in this region. Database searching identified the protein as pathogenesis-related protein 5 precursor (PR-5).

unknown functions were also detected, such as cysteine proteinase inhibitor, and some proteins that had not been reported to relate with salinity response were also identified, such as SOUL-like protein and F-box protein PP2-A12. The presence of these novel salinity-responsive proteins implied that there might be some novel mechanisms for salinity tolerance of *T. halophila*.

Three defense-related proteins, ferritin-1 chloroplast precursor, disease resistance protein (TIR-NBS-LRR class) and PR-5, were found to respond to long-term salinity stress in our study. Fe is an important element in photosynthesis apparatus and free Fe participates in the production of excess hydroxyl radicals through Haber-Weiss reaction[19]. Ferritin-1, an iron-binding protein located in chloroplast, can store iron in a complex form and release them if necessary. Ferritin-1 is supposed to protect plants from oxidative damage in chloroplast induced by manifold stresses[20]. mRNA and protein abundance of ferritin was reported to increase in response to photoinhibition in *A. thaliana*[21]. In this study, the increase of ferritin-1 chloroplast precursor in protein abundance is thought to be associated with the excess Reactive Oxygen Species (ROS) in chloroplast caused by salinity stress.

TIR-NBS-LRR class disease resistance protein (At5g41550) increased in abundance after long-term

salinity stress. However, cDNA microarray analysis revealed that the protein's mRNA was down-regulated in response to drought and short-term salinity stress[9]. It appears that the same stress related gene responds differentially to short-term and long-term salinity stress. Pathogenesis-related protein 5 precursor (PR-5) showed a constitutive expression pattern with a markedly high abundance (spot 16 in Figure 1) and the protein abundance decreased after long-term salinity. This result is consistent with the cDNA microarray analysis, in which the mRNA of PR-5 was down-regulated by short-term salinity stress[9]. However, a recent study demonstrated that the expression of PR-5 is not affected by salinity stress, but induced by drought and cold stress in *A. thaliana*[22]. Therefore it seems that PR-5 is regulated differently in *T. halophila* from *A. thaliana* under salinity stress and it is suggested that the decrease of PR-5 abundance is a strategy of *T. halophila* to better acclimate to high salinity stress.

Plant will decrease energy metabolism to respond to salinity stress, thus reducing the excess production of ROS. As expected, 3 proteins involved in glycolysis were observed to change their abundance under long-term salinity treatment. 2, 3-bisphosphoglycerate-independent phosphoglycerate mutase was previously reported to respond to cold stress in *Arabidopsis* leaf[23], rice leaf[24] and anther[25]. It was found to be down-regulated under salinity stress in our study. Malate dehydrogenase (MDH) catalyzes the reversible reaction of oxaloacetate (OAA) to malate and is widely distributed in mammals and plants. Malate dehydrogenase was reported to be responsive to salinity stress in root and suspension cell of *Arabidopsis*[16,26] and pea root as well[27]. In the present study, a cytosolic malate dehydrogenase was up-regulated by long-term salinity stress. It is suggested that MDH has a crucial biological function in plant metabolism in various tissues. Plant GAPDH has been considered to be primarily a housekeeping enzyme involved in the glycolytic pathway. However, several studies are indicating that GAPDH is a multifunctional protein involved in numerous physiological processes, such as heat shock[28] and oxidative stress response[29]. GAPDH was shown to be translocated from the cytosol to the nucleus following oxidative stress where it is thought to act as a DNA repair enzyme[30]. In our study, GAPDH was up-regulated by long-term salinity stress and it is suggested to be resulted from the oxidative stress caused by long-term salinity.

ATP sulfurylase, a metabolism related protein was down-regulated in this study. ATP sulfurylase is the first enzyme in the sulfate assimilation pathway and it is the key enzyme for cysteine biosynthesis[31], suggesting that the abundance of ATP sulfurylase is related with the GSH metabolism under stress condition.

Two RNA processing related RNA binding proteins, Glycine-rich RNA-binding protein 7 (GRP7) and putative ribonucleoprotein At2g37220 (cp29), were up-regulated after long-term salinity stress. Plant RNA binding proteins, a family of proteins with RNA Recognition Motif (RRM), participate in mRNA synthesis, splicing of hnRNA and post-translational regulation of chloroplast genes[32]. Glycine-rich proteins (GRPs) play certain roles in post-transcriptional regulation of gene expression in plants under various stress conditions and their synthesis is part of the plant's defense mechanism[33]. Although the function of plant GRPs is not clear, they may play important roles in stress responses, as their mRNA levels increased after exposure to cold, wounding, water stress, plant hormones or viral infection[34]. A recent study showed that AtGRP7 exhibited RNA chaperone activity and was able to promote the cold adaptation process in *Escherichia coli*[35]. The GRP7 serve to maintain mRNA structure in saliity-stressed *T. halophila.* RNA-binding protein cp29 is a subunit of the photosystem II and it belongs to a family of chloroplast RNA-binding proteins[15]. The phosphorylation of cp29 was related with the cold tolerance in maize[36]. In a recent proteomics study, cp29 was verified to be induced by cold in *A. thaliana*[23]. The increase in cp29 protein abundance is presumed to maintain the translation efficiency of chloroplast proteins.

26S proteasome is presumed to play an essential role in the removal of short lived regulatory proteins and abnormal polypeptides in plants during stress. 20S proteasome alpha subunit B1 (PAB1) was reported to be involved in ubiquitin-dependent protein catabolic process[37]. In this study, a PAB1 was increased after long-term salinity treatment. This may indicate that a high protein turn-over rate was needed to eliminate the abnormal polypeptides for plant adaptation to long-term salinity stress.

In conclusion, these results demonstrate that the tolerance of *T. halophila* for long-term salinity stress mainly rely on regulation of the basic metabolism, meanwhile, some unknown mechanism may exist, which need further investigation.

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