Proteomic analysis of halotolerant proteins under high and low salt stress in *Dunaliella salina* using two-dimensional differential in-gel electrophoresis

Yan-Long Jia¹², Hui Chen¹, Chong Zhang¹, Li-Jie Gao³, Xi-Cheng Wang¹, Le-Le Qiu³ and Jun-Fang Wu³

¹Pharmacy College, Xinxiang Medical University, Xinxiang 453003, Henan, China.
²Henan Collaborative Innovation Center of Molecular Diagnosis and Laboratory Medicine, Xinxiang Medical University, Xinxiang 453003, Henan, China.
³School of Basic Medicine, Xinxiang Medical University, Xinxiang 453003, Henan, China.

Abstract

*Dunaliella salina*, a single-celled marine alga with extreme salt tolerance, is an important model organism for studying fundamental extremophile survival mechanisms and their potential practical applications. In this study, two-dimensional differential in-gel electrophoresis (2D-DIGE) was used to investigate the expression of halotolerant proteins under high (3 M NaCl) and low (0.75 M NaCl) salt concentrations. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) and bioinformatics were used to identify and characterize the differences among proteins. 2D-DIGE analysis revealed 141 protein spots that were significantly differentially expressed between the two salinities. Twenty-four differentially expressed protein spots were successfully identified by MALDI-TOF/TOF MS, including proteins in the following important categories: molecular chaperones, proteins involved in photosynthesis, proteins involved in respiration and proteins involved in amino acid synthesis. Expression levels of these proteins changed in response to the stress conditions, which suggests that they may be involved in the maintenance of intracellular osmotic pressure, cellular stress responses, physiological changes in metabolism, continuation of photosynthetic activity and other aspects of salt stress. The findings of this study enhance our understanding of the function and mechanisms of various proteins in salt stress.

Keywords: *Dunaliella salina*, halotolerant protein, proteomics, two-dimensional differential in-gel electrophoresis.

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Introduction

Salt stress is a major natural abiotic stress and plants have evolved sophisticated mechanisms to adapt to saline environments (Zhang *et al*., 2012). *Dunaliella salina*, a unicellular eukaryotic alga, can survive in environments containing 0.5-5 M NaCl (Mishra *et al*., 2008). Various studies have used salt-tolerant algae as model organisms to investigate the mechanisms of salt tolerance (Liska *et al*., 2004; Ören, 2014). In addition to the study of glycerin synthesis in several salt-tolerant algal species (Goyal, 2007), some proteins involved in adaptation to salt have been isolated, *e.g.*, membrane structures associated with heat shock proteins 70 and 90 (HSP70 and HSP90, respectively), glucose-6-phosphate dehydrogenase and nitrate reductase (Liska *et al*., 2004; Katz *et al*., 2007; Lao *et al*., 2014). However, there is little genetic bioinformation about these proteins, which limits further research. High-throughput proteomics is a powerful tool for in-depth exploration of the mechanism of salt tolerance in algae (Liska *et al*., 2004). Gel electrophoresis, particularly two-dimensional differential in-gel electrophoresis (2D-DIGE) (Tonge *et al*., 2001), has been used to simultaneously analyze multiple samples that are imaged separately in order to detect protein differences of < 10% at the 95% confidence level. When combined with mass spectrometry (Katz *et al*., 2007), this technique can precisely determine molecular mass and analyze the molecular structure (Hu *et al*., 2005; Jin *et al*., 2007; Brechlin *et al*., 2008).

In the present study, proteomic analysis by 2D-DIGE was used to investigate the total protein content of *D. salina* cultured under two levels of salinity. A differential protein expression map, mass spectrometry and bioinformatics analysis were used to analyze and identify the differentially expressed proteins in order to improve our understanding of their function in salt tolerance.

Materials and methods

Algal culture

*Dunaliella salina* (UTEX-LB-1644, Culture Collection of Algae, University of Texas, USA) was cultured in modified medium at low (0.75 M NaCl) or high (3 M NaCl) salinity at 26 °C and an illumination of 4500 Lux for 12 h/day (Ben-Amotz and Avron, 1990). Before being used,
cultured algae were examined microscopically to ensure that the cells were axenic, motile and flagellated, and that cell debris was minimal. Viability curves for *D. salina* cells in different NaCl concentrations over time were obtained to ensure that a high salt stress (3 M NaCl) did not affect cell growth. *Dunaliella salina* cells in the logarithmic phase of growth (density: ~2 x 10^6 cells/mL) were collected for further analysis.

**Protein extraction, desalting, freeze-drying and quantification**

*Dunaliella salina* proteins were isolated using the methods of Hirano *et al.* (2006) and Natarajan *et al.* (2005), with minor modifications (Jia *et al.*, 2010). Initially, 2 mL of ice-cold freezing solution (10 mM Tris-MPOS, 2 mM MgCl2 and 10 mM KCl at pH 7.5) was added to a tube containing *D. salina* (2 x 10^6 cells), mixed and the suspension placed in liquid nitrogen for 2 min. Three freeze-thaw cycles were applied to thoroughly lyse the cells. Next, 6 mL of ice-cold TCA/acetone buffer [acetone with 10% (w/v) TCA and 0.07% (w/v) β-mercaptoethanol] was added, the proteins were precipitated at -20 °C overnight, and the tubes were then centrifuged (20,000 g, 15 min, 4 °C). The supernatant was decanted, the pellet was washed with chilled wash buffer [acetone with 0.07% (w/v) β-mercaptoethanol and 2 mM EDTA] plus 0.5 mL of a protease inhibitor cocktail (Sigma) to a final volume of 50 mL and the acetone mixture was then removed by centrifugation. The pellet was re-suspended in buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% Bio-Lyte 3/10 Ampholyte (BIORAD) and 65 mM dithiothreitol (DTT)] and 1% (w/v) P9599 protease inhibitor cocktail (Sigma) to a final volume of 50 mL, incubated at 4 °C for 30 min with occasional vortex mixing, and then centrifuged (20,000 g, 20 min, 10 °C). The total protein extracted from *D. salina* by this procedure was either immediately subjected to further analysis or stored in aliquots at -80 °C.

The extracted proteins from *D. salina* were desalted, freeze-dried and concentrated. A disposable PD-10 desalting column (GE Healthcare, Munich, Germany) was used to recover a desalted sample according to the manufacturer’s instructions. Since the total volume of each sample increased to about 3.5 mL during desalting, the samples were again concentrated by freeze-drying. The protein lysate was reconstituted and stored at -20 °C. The protein concentration was determined by the Bradford protein assay, using bovine serum albumin (BSA) as the standard.

**Protein labeling and 2D-DIGE analysis**

The *D. salina* protein samples were labeled with fluorescent CyDyes for DIGE (Cy2, Cy3 and Cy5; GE Healthcare), according to the manufacturer’s instructions. The order of staining with the dyes was altered among the protein samples in order to avoid artefacts caused by preferential labelling. Briefly, 50 µg of protein sample (pH 8.5) was labelled with 400 pmol of Cy3 or Cy5 minimal dye according to the experimental design, while a pool consisting of the same amount of each sample was labeled with Cy2 as an internal standard to control for quantitative comparisons. All of the individual samples were biological replicates. Protein sample labeling was done on ice in the dark for 30 min and then quenched by incubation with 1 µL of 10 mM L-lysine (GE Amersham Biosciences) on ice in the dark for 10 min. The labeled samples were then analyzed by 2D-DIGE.

Electrophoresis was done as described by Alban *et al.* (2003) and Tonge *et al.* (2001). Reagents and equipment used for DIGE were purchased from GE Healthcare. For each gel in DIGE, the protein samples labeled with Cy2, Cy3 or Cy5 (50 µg each) were pooled and an equal volume of rehydration buffer (8 M urea, 4% CHAPS, 2% DTT and 2% IPG buffer pH3-10) was added (the final concentration of DTT and IPG buffer was 1%). Isoelectric focusing of the pooled protein samples was done on non-linear IPG strips (24 cm long, pH 3-10) using an Etan II IPG-phor apparatus (GE Healthcare). The strips were rehydrated at 30 V for 12 h at room temperature and isoelectric focusing was done at 500 V for 0.5 h, followed by 1000 V for 0.5 h, 4000 V for 2 h, 10,000 V for 3 h and then 10,000 V 70 h to reach a total of 70 Kvh. After isoelectric focusing, the strips were incubated for 15 min in equilibration buffer [50 mM Tris-HCl, 6 M urea, 20% (v/v) glycerol and 2% (w/v) SDS supplemented with 1% (w/v) dithiothreitol] and then for 15 min in 2.5% (w/v) iodoacetamide. The proteins were separated on 12.5% SDS-PAGE gels at 10 mA/gel for 15 min and then at 20 mA/gel at 20 °C until they reached the end of the plate. The analysis of cell lysates was done using at least three independent replicates and the protein spots used for comparisons were detected on all of the gels.

**Scanning of electrophoretic patterns and image analysis**

The maps labeled with Cy2, Cy3 or Cy5 fluorescent dye were scanned with a Typhoon 9410 scanner (GE Healthcare) at wavelengths of 488/520 nm, 532/580 nm and 633/670 nm, respectively. Scan values ranged from 60,000 to 90,000 units, with differences of ~5,000 units among the three replicate gels for each sample. DeCyder v.5.02 DIGE image analysis software was used to analyze the images (DIA and BVA) and to identify the spots that differed between the high and low salinity treatments. When the presence of protein spots differed between the salinity treatments in at least two of the three analyzed gels (*i.e.*, in six of nine analyzed images), this was designated a significant change. In addition, when the ratio between the standardized average spot volumes exceeded 1.5, this was statistically significant using Student’s *t*-test at *p* < 0.05.
**Protein identification by mass spectrometry (MS)**

The differentially expressed protein spots of interest were further identified by MS. Briefly, unlabeled pooled protein samples (800 μg) of each salinity group were run in parallel on separate preparative polyacrylamide gels and stained with Coomassie brilliant blue (Colloidal Blue stain kit; Invitrogen, Carlsbad, CA, USA) to facilitate MS analysis. The spots of interest were selected and manually cut out from the preparative gels. Tryptic digests were prepared according to the manufacturer’s instruction. Briefly, the excised gel pieces containing the proteins of interest were destained by ultrasound with 25 mM NH₄HCO₃ (Fluka, USA) in 50% acetonitrile (ACN) (Merck, Germany) for 10 min and then lyophilized. Fifteen microliters of digestion buffer [10 ng of trypsin/μL (Promega, Madison, WI, USA) in 25 mM NH₄HCO₃] was added and the samples were digested overnight at room temperature. Peptides were extracted twice with 5% trifluoroacetic acid (TFA; ACROS, Belgium) for 1 h each and with 2.5% TFA/50% acetonitrile (ACN) for 1 h. The extracted peptides were pooled, dried completely by centrifugal lyophilization and re-suspended in 0.1% TFA. Equal volumes of the sample solution and CHCC matrix (5 mg/mL, dissolved in 50% ACN/0.1% TFA; Sigma-Aldrich, USA) were mixed and spotted onto the matrix-assisted laser desorption/ionization (MALDI) target plate.

Samples were analyzed using MALDI-time-of-flight (TOF)/TOF MS with a proteomics analyzer (4800 plus, Applied Biosystems SCIEX, USA). Mono-isotopic peak masses were acquired in a mass range of 700 to 4,000 Da. Ten of the most intense ion signals (signal/noise ratio or S/N > 20), excluding common trypsin autolysis peaks and matrix ion signals, were selected as precursors for MS/MS acquisition. Protein identification of the peptide mass fingerprint combined MS/MS data was done using Global Proteome Server (GPS) Explorer software (version 3.6, Applied Biosystems SCIEX, Framingham, MA, USA) with the NCBI non-redundant protein database (ncbi2009). The search parameters were set as follows: Taxonomy - all or plant, Enzyme - trypsin, peptide mass tolerance - ± 100 ppm, Fragment ion mass tolerance - ± 0.2 Da, Max missed cleavages - 2, Static modification - Carbamidomethyl (C) (57.021 Da), Dynamic modification - M oxidation (15.995 Da). The criterion for successful identification of proteins was a 95% confidence interval (95%CI) for protein scores and peptide mass fingerprint and MS/MS data. The results were further confirmed in the SwissProt protein database (SwissP.sprot_1105).

**Validation of selected proteins by western blotting**

To further validate the alterations of selected proteins identified in the proteomic analysis, we examined the expression of glutamine synthetase (GS) by western blotting of protein samples from low and high salt conditions. Briefly, after determining protein concentrations by the Bradford method, protein samples were boiled in loading buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol) for 5 min. Equal amounts of protein (20 μg/well) of each sample were then separated by electrophoresis in a 12% SDS-polyacrylamide gel and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with 5% (w/v) non-fat milk in TBST (20 mM Tris-HCl, pH7.6, 136 mM NaCl and 0.1% Tween-20) for 1 h at room temperature and rinsing, the blot was incubated overnight at 4 °C with goat polyclonal anti-GS (1:500; sc-6640, Santa Cruz, CA, USA) as primary antibody. The membranes were then washed four times with TBST (5 min each) and incubated at room temperature for 1.5 h with anti-goat secondary horseradish peroxidase-conjugated antibody (1:2000, SC-2768, Santa Cruz, CA, USA). After incubation with BeyoECL Plus (Beyotime Biotechnology, Nantong, China), the bands were visualized by using a ChemiDoc-It®2 810 Imager (UVP) and quantified by densitometric analysis. As an internal control for protein loading, the blots were stripped and probed with a mouse monoclonal anti-β-actin antibody (1:2000; SC-2048, Santa Cruz); the resulting immunoreactive bands were used to normalize the densities of the GS bands.

**Statistical analysis**

The results were expressed as the mean ± standard deviation (SD), where appropriate. Statistical comparisons of the protein levels between the two groups were done using Student’s unpaired t-test and one-way analysis of variance (ANOVA), with a value of p < 0.05 indicating significance. All of these statistical analyses were done using SPSS 13.0 software (SPSS, Chicago, IL, USA).

**Results and Discussion**

Sample preparation is a very important step in proteomics. We therefore initially examined the cell morphology of *D. salina* microscopically and determined the viability curves in different salt concentrations over time. Figure 1 shows that *D. salina* grew well and showed similar morphology and growth curves over time in both salinities. These findings indicated that *D. salina* cultured in low and high salinities was suitable for further study.

In order to extend our understanding of the molecular mechanisms of halotolerance in *D. salina*, we undertook a comparative proteomic analysis of *D. salina* grown in high (3 M) and low (0.75 M) salinity. As shown in Figure 2, the 2D gels of protein samples from *D. salina* grown in 3 M and 0.75 M NaCl were labeled with the fluorescent dyes Cy2, Cy3 or Cy5. Based on detailed image analysis, 141 protein spots that differed between the 3 M and 0.75 M salinity treatments were observed (Figure 3, Table 1). Further analysis of these 141 spots identified 33 spots containing 20
proteins that differed between the salinity treatments (Table 2). Among these proteins, heat shock protein (HSP), the αβ subunit of mitochondrial ATP synthase, GS, the light-harvesting protein of photosystem II, major light-harvesting complex II protein m7, sedoheptulose-1,7-bisphosphatase (SBPase), chlorophyll a-b binding protein of LHCII, and aspartate aminotransferase were up-regulated in high salinity (3 M), whereas α-tubulin, β-tubulin 2, major light-harvesting chlorophyll a/b protein 3 and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit were down-regulated.

Most plants can adapt to low or moderate salinity (Hasegawa et al., 2000). However, D. salina can adapt to a wide range of salt concentrations. In recent years, several studies have used proteomic or genomic methods to identify proteins of D. salina that are affected by salinity (Liska et al., 2004; Liu et al., 2014). Previous work concentrated on subcellular structures such as chloroplasts and the plasma membrane as demonstrated by Katz et al. (2007). Although knowledge of the genomics and protein sequences of D. salina is very limited, the analytical approach described here (i.e., extraction of total protein of D. salina, 2D-DIGE analysis, comprehensive analysis of differences in protein expression under high- and low-salt conditions and the identification of 20 proteins) may contribute to our understanding of the physiological processes of salt adaptation, as suggested by Pick (1992). Salt stress leads to multi-

Figure 1 - The appearance (A,B) and growth rate (C) of D. salina cells in high salinity (3 M) (A) and low salinity (0.75 M) (B) were very similar.

Figure 2 - 2-D DIGE gel images of proteins after exposure to salt stress in high (3 M) and low (0.75 M) salinity. The proteins were labeled with Cy2 [column 1, a pool (0.75M + 3M) consisting of the same amount of each sample as an internal standard to control for quantitative comparisons, scanning at wavelength of 488/520 nm], Cy3 (column 2, scanning at wavelength of 532/580 nm) or Cy5 (column 3, scanning at wavelength of 633/670 nm) fluorescent dyes; column 4 is an overlay of the first three columns. Gel1, 2, 3: triplicate gels for protein samples to reduce the gel-to-gel variations. Gel1: Cy2(0.75M+3M) + Cy3(0.75M) + Cy5(3M); Gel2: Cy2(0.75M+3M) + Cy3(3M) + Cy5(0.75M); Gel3, Cy2(0.75M+3M) + Cy3(0.75M) + Cy5(3M).
ple changes in basic biological functions such as photosynthesis, photorespiration and the synthesis of amino acids and carbohydrates (Kawasaki et al., 2001; Ozturk et al., 2002; Seki et al., 2002). The proteins identified in the present work also revealed that exposure to high salt in the environment altered the expression of many D. salina proteins involved in physiological and biochemical processes such as photosynthesis, stress defense, metabolism, molecular chaperones and cell structure. The functional significance and potential roles of the differentially expressed proteins associated with halotolerance in D. salina are discussed below.

HSPs are a family of proteins that are produced by cells in response to different environmental stress conditions, including exposure to heat shock, cold, UV light, nitrogen deficiency or water deprivation (Li and Srivastava, 2004). Therefore, up-regulation of HSP in D. salina can also be described more generally as part of the stress response (Santoro, 2000). In contrast, α-tubulin and β-tubulin 2 are down-regulated in D. salina under high salinity (3 M). We suspect that down-regulation of tubulin in response to high salinity may result in reduced cell motility, but further experiments are required to verify the hypothesis.

As shown in Figure 4, western blotting, used to validate the enhanced expression of GS, confirmed that the ex-

Table 1 - Protein spots with significant changes between the treatment with 3 M and 0.75 M NaCl. The changes are expressed as the ratio 3 M/0.75 M.

| Position | Master number | t-test | Average ratio (3 M/0.75 M) |
|----------|---------------|--------|---------------------------|
| 1        | 575           | 1.80E-07 | 6.34                      |
| 2        | 553           | 5.60E-07 | 11.06                     |
| 3        | 1878          | 8.10E-07 | -1.69                     |
| 4        | 573           | 1.30E-06 | 9.87                      |
| 5        | 1201          | 1.70E-06 | 5.77                      |
| 6        | 1634          | 1.80E-06 | 5.26                      |
| 7        | 576           | 2.20E-06 | 9.18                      |
| 8        | 333           | 2.30E-06 | 1.97                      |
| 9        | 529           | 2.40E-06 | 2.04                      |
| 10       | 571           | 3.40E-06 | 9.13                      |
| 11       | 475           | 5.60E-06 | 2.15                      |
| 12       | 1205          | 6.30E-06 | 1.82                      |
| 13       | 1635          | 7.00E-06 | 4.03                      |
| 14       | 1617          | 7.20E-06 | 4.65                      |
| 15       | 551           | 7.50E-06 | 1.92                      |
| 16       | 567           | 7.80E-06 | 5.23                      |
| 17       | 1744          | 7.80E-06 | -5.72                     |
| 18       | 593           | 9.70E-06 | -1.8                      |
| 19       | 746           | 1.20E-05 | 2.25                      |
| 20       | 443           | 1.40E-05 | 1.55                      |
| 21       | 1377          | 1.40E-05 | 1.59                      |
| 22       | 1290          | 1.70E-05 | 1.73                      |
| 23       | 1612          | 1.80E-05 | 6.8                       |
| 24       | 336           | 2.00E-05 | 1.94                      |

| Position | Master number | t-test | Average ratio (3 M/0.75 M) |
|----------|---------------|--------|---------------------------|
| 25       | 524           | 2.10E-05 | 2.17                      |
| 26       | 546           | 2.40E-05 | 3.09                      |
| 27       | 474           | 3.00E-05 | 1.9                       |
| 28       | 577           | 3.40E-05 | 4.61                      |
| 29       | 578           | 3.40E-05 | 4.78                      |
| 30       | 905           | 3.40E-05 | 1.71                      |
| 31       | 970           | 3.60E-05 | 2.12                      |
| 32       | 486           | 3.80E-05 | 1.61                      |
| 33       | 1169          | 3.80E-05 | 1.6                       |
| 34       | 1378          | 3.90E-05 | 1.66                      |
| 35       | 1575          | 4.40E-05 | 1.88                      |
| 36       | 572           | 4.60E-05 | 6.21                      |
| 37       | 1590          | 4.90E-05 | 1.92                      |
| 38       | 975           | 5.10E-05 | 2.17                      |
| 39       | 1584          | 5.50E-05 | -1.59                     |
| 40       | 1197          | 5.80E-05 | 1.66                      |
| 41       | 754           | 6.20E-05 | 2.07                      |
| 42       | 1206          | 6.50E-05 | 1.76                      |
| 43       | 170           | 6.60E-05 | 1.86                      |
| 44       | 538           | 7.20E-05 | 1.9                       |
| 45       | 1731          | 7.50E-05 | -5.31                     |
| 46       | 438           | 7.70E-05 | 1.7                       |
| 47       | 521           | 8.10E-05 | 7.1                       |
| 48       | 943           | 8.70E-05 | -1.78                     |
| Position | Master number | t-test | Average ratio (3 M/0.75 M) |
|----------|---------------|--------|-----------------------------|
| 49       | 528           | 9.70E-05 | 2.3                        |
| 50       | 979           | 0.0001  | 1.65                       |
| 51       | 1291          | 0.00011 | 1.84                       |
| 52       | 527           | 0.00013 | 2.1                        |
| 53       | 1346          | 0.00013 | 1.51                       |
| 54       | 1542          | 0.00013 | 2.04                       |
| 55       | 580           | 0.00014 | -1.93                      |
| 56       | 902           | 0.00014 | 1.56                       |
| 57       | 1538          | 0.00014 | 1.86                       |
| 58       | 1037          | 0.00016 | 2.02                       |
| 59       | 1192          | 0.00016 | 1.58                       |
| 60       | 1379          | 0.00016 | 1.74                       |
| 61       | 958           | 0.00017 | 1.66                       |
| 62       | 563           | 0.00018 | 5.63                       |
| 63       | 1574          | 0.00018 | 1.73                       |
| 64       | 582           | 0.00019 | -1.63                      |
| 65       | 649           | 0.00019 | 2.03                       |
| 66       | 700           | 0.00021 | -1.87                      |
| 67       | 1143          | 0.00021 | 1.58                       |
| 68       | 568           | 0.00022 | 6.68                       |
| 69       | 789           | 0.00024 | -1.53                      |
| 70       | 1691          | 0.00025 | -1.51                      |
| 71       | 914           | 0.00026 | 1.68                       |
| 72       | 175           | 0.00029 | 1.6                        |
| 73       | 337           | 0.00029 | 2.05                       |
| 74       | 549           | 0.00031 | 2.04                       |
| 75       | 788           | 0.00031 | -1.54                      |
| 76       | 1632          | 0.00031 | 1.8                        |
| 77       | 1637          | 0.00033 | 3.47                       |
| 78       | 607           | 0.00034 | -1.84                      |
| 79       | 911           | 0.00035 | -1.64                      |
| 80       | 1119          | 0.00035 | 1.59                       |
| 81       | 735           | 0.00036 | 1.53                       |
| 82       | 1912          | 0.00038 | 2.63                       |
| 83       | 476           | 0.00039 | 1.65                       |
| 84       | 1901          | 0.00039 | -2.36                      |
| 85       | 368           | 0.00044 | -1.88                      |
| 86       | 1295          | 0.00044 | 1.62                       |
| 87       | 1365          | 0.00044 | 1.69                       |
| 88       | 588           | 0.00046 | -1.77                      |
| 89       | 676           | 0.00046 | 3.17                       |
| 90       | 1502          | 0.00047 | -1.58                      |
| 91       | 1761          | 0.00047 | 2.47                       |
| 92       | 1330          | 0.00049 | 1.53                       |
| 93       | 782           | 0.0005 | -1.72                      |
| 94       | 530           | 0.00055 | 1.86                       |
| 95       | 825           | 0.00055 | 1.56                       |
| 96       | 1401          | 0.00055 | 1.97                       |
| 97       | 400           | 0.00059 | -2.13                      |
| 98       | 1375          | 0.00059 | -1.66                      |
| 99       | 603           | 0.00061 | -2.05                      |
| 100      | 1512          | 0.00062 | -1.57                      |
| 101      | 759           | 0.00066 | 1.82                       |
| 102      | 404           | 0.0007 | -1.6                       |
| 103      | 1552          | 0.00072 | 1.94                       |
| 104      | 543           | 0.00074 | 1.73                       |
| 105      | 1422          | 0.00076 | 1.51                       |
| 106      | 1387          | 0.00078 | 1.52                       |
| 107      | 1976          | 0.00079 | -1.76                      |
| 108      | 1031          | 0.001  | 2.03                       |
| 109      | 745           | 0.0012  | 2.49                       |
| 110      | 1204          | 0.0012  | 1.55                       |
| 111      | 558           | 0.0013  | 1.52                       |
| 112      | 1747          | 0.0013  | -1.55                      |
| 113      | 721           | 0.0014  | 1.57                       |
| 114      | 1210          | 0.0014  | 1.73                       |
| 115      | 1198          | 0.0016  | 1.78                       |
| 116      | 1212          | 0.0017  | 1.99                       |
| 117      | 335           | 0.0021  | 1.58                       |
| 118      | 1570          | 0.0021  | 1.77                       |
| 119      | 1776          | 0.0022  | -2.26                      |
| 120      | 548           | 0.0023  | 1.56                       |
| 121      | 1399          | 0.0023  | 1.51                       |
| 122      | 660           | 0.0028  | 1.5                        |
| 123      | 1739          | 0.0029  | -2.27                      |
| 124      | 1926          | 0.0035  | -2.25                      |
| 125      | 1891          | 0.0038  | -2.46                      |
| 126      | 1886          | 0.0045  | -1.62                      |
| 127      | 171           | 0.0047  | 2                          |
| 128      | 1372          | 0.0061  | -1.71                      |
| 129      | 1506          | 0.0064  | 1.63                       |
| 130      | 910           | 0.0067  | -1.59                      |
| 131      | 1436          | 0.0067  | -1.53                      |
| 132      | 169           | 0.0082  | 1.82                       |
| 133      | 1457          | 0.0089  | -1.59                      |
| 134      | 1902          | 0.0092  | 2.18                       |
| 135      | 1219          | 0.01    | 1.66                       |
| 136      | 655           | 0.012   | 1.52                       |
| 137      | 293           | 0.013   | 1.65                       |
| 138      | 356           | 0.013   | -1.59                      |
| 139      | 1845          | 0.013   | 1.9                        |
| 140      | 965           | 0.017   | -1.7                       |
| 141      | 1742          | 0.022   | -1.64                      |

Table 1 - cont.
Expression of this protein was increased by exposure to high salinity in comparison to low salinity. This finding confirmed that obtained by MALDI-TOF/TOF MS after DIGE analysis. GS plays an essential role in nitrogen metabolism. Previous studies showed that overexpression of chloroplast GS could enhance tolerance to salt stress in transgenic rice (Hoshida et al., 2000) and may potentially be used to enhance the use of nitrogen, light and photorespiration in transgenic crop plants (Oliveira et al., 2002). The elevated expression of GS seen here in D. salina may play an important role in alleviating late-occurring salt stress and in maintaining the carbon-nitrogen metabolic balance during normal cell development and growth, as described by Bao et al. (2015).

Some of the proteins identified here were associated with photosynthesis and the Calvin cycle, including light harvesting protein of photosystem II, chlorophyll a-b binding protein, Rubisco and SBPase. Salt stress has been shown to inhibit photosynthesis in halophytes and non-halophytes, with the degree of inhibition being positively correlated with the salt concentration (Xu et al., 2000). For maximum efficiency, plants and green algae use chlorophyll a/b-binding proteins that can switch between being light-harvesting antenna for two photosystems (photosys-

### Table 2 - Summary of 24 differentially expressed protein spots identified by MALDI-TOF/TOF MS after DIGE analysis.

| Protein spot no. | Protein name                                      | Accession no. | Protein score | Protein score (CI%) | Mr (Da)  |
|------------------|--------------------------------------------------|---------------|---------------|---------------------|----------|
| 548              | ATP synthase subunit beta                        | gi|231586        | 288              | 100                  | 60221.3  |
| 549              | Mitochondrial F-1-ATPase subunit [Zea mays]       | gi|162462751     | 284              | 100                  | 59066.9  |
| 551              | Putative ATP synthase beta subunit [Oryza sativa]| gi|56784991      | 281              | 100                  | 45879.8  |
| 558              | Beta subunit of mitochondrial ATP synthase       | gi|159466892     | 271              | 100                  | 61783    |
| 721              | ATP synthase beta-subunit [Astrephomene]         | gi|4519320       | 375              | 100                  | 26225.6  |
| 754              | ATP synthase CF1 alpha subunit [Chlamydomonas]   | gi|41179050      | 203              | 100                  | 54717.7  |
| 746              | ATP synthase CF1 alpha subunit [Chlamydomonas]   | gi|41179050      | 151              | 100                  | 54717.7  |
| 759              | ATP synthase CF1 alpha subunit [Chlamydomonas]   | gi|41179050      | 245              | 100                  | 54717.7  |
| 782              | Beta tubulin 2 [Chlamydomonas reinhardtii]       | gi|159471706     | 299              | 100                  | 49586.8  |
| 1976             | Beta tubulin 2 [Chlamydomonas reinhardtii]       | gi|159471706     | 496              | 100                  | 49586.8  |
| 788              | Alpha-tubulin [Chloromonas sp. ANT3]             | gi|2625154       | 402              | 100                  | 49536.6  |
| 789              | Alpha-tubulin [Chloromonas sp. ANT3]             | gi|2625154       | 325              | 100                  | 49536.6  |
| 979              | Glutamine synthetase [Dunaliella tertiolecta]     | gi|3869304       | 89               | 99.979               | 22550.9  |
| 1143             | Aspartate aminotransferase Asp2 [Arabidopsis thaliana] | gi|22135928 | 67               | 96.233               | 22042.1  |
| 1206             | Sedoheptulose-1 7-bisphosphatase precursor [Oryza] | gi|27804768 | 104              | 100                  | 42218.1  |
| 1365             | Chlorophyll a-b binding protein of LHClII         | gi|115828       | 140              | 100                  | 29089.4  |
| 1387             | Chlorophyll a-b binding protein of LHClII         | gi|115828       | 237              | 100                  | 29089.4  |
| 1401             | Light-harvesting protein of photosystem II        | gi|159471686    | 107              | 100                  | 26633.6  |
| 1422             | Light-harvesting protein of photosystem II        | gi|159471686    | 128              | 100                  | 26633.6  |
| 1457             | Major light-harvesting chlorophyll a/b protein 3 | gi|123316054    | 165              | 100                  | 27794.2  |
| 1506             | Major light-harvesting complex II protein m7      | gi|19423289     | 96               | 99.996               | 27936.3  |
| 1886             | Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit | gi|44890111 | 73               | 98.986               | 21145.6  |

Note: Protein scores with a CI% ≥ 95 were considered significant (p < 0.05) under the established criterion. Theoretical Mr (Da) are based on the amino acid sequences of the identified proteins.
tem I and photosystem II) thereby providing an optimal balance in excitation (Kargul and Barber, 2008). Rubisco, a key enzyme involved in photosynthetic CO₂ assimilation (Wang et al., 2015), is highly regulated in response to fluctuations in the environment, including changes in irradiance (Grabsztunowicz et al., 2015). SBPase is the most important factor for ribulose-1,5-bisphosphate (RuBP) regeneration in the Calvin cycle. An increase in the SBPase content of chloroplasts had a marked positive effect on photosynthesis (Tamoi et al., 2006). In the present study, the upregulated synthesis and activities of proteins related to photosynthesis and stress defense in *D. salina* may contribute to the priming effects that allow the cells to cope with salt stress. In addition to proteins related to photosynthesis, the up- or down-regulation of proteins involved in biochemical metabolism such as carbon and nitrogen metabolism in *D. salina* under salt stress, indicated that these processes were also differentially regulated.

In this work, we used a precipitation/resolubilization protocol for protein extraction. Theoretically, it is possible that the differences observed in the expression of certain proteins between low and high salt conditions could have reflected the inefficient resolubilization of some proteins, with the result that the insoluble residue was unintentionally eliminated. Close monitoring of resolubilization is therefore a critical step in sample preparation in order to ensure that all proteins are recovered. As shown elsewhere (Davidi et al., 2015), the insoluble pellet can be reextracted with 1% SDS, or other methods of purification that do not involve precipitation can be used for comparative proteomic analyses.

In summary, the level of many proteins in *D. salina* was altered in response to environmental salt stress. These proteins may be involved in maintaining intracellular osmotic pressure, cellular stress responses, physiological changes in metabolism, the continuation of photosynthesis, and other aspects of salt stress. These findings extend our understanding of the changes in protein expression associated with salt stress and provide new insights into the mechanisms of halotolerance in *D. salina*.

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