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Identification of a glucose-6-phosphate isomerase involved in adaptation to salt stress of *Dunaliella salina*

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Abstract The unicellular green alga *Dunaliella salina* is a recognized model for studying plant adaptation to high salinity. To isolate some salt-induced proteins at proteomics levels and to identify their expressions at gene levels, algal cells at logarithmic phase cultured in 1.5 and 3.5 M NaCl media were harvested for protein extraction. Solubilized proteins were applied to two-dimensional gel electrophoresis (2-DE) and analyzed by ImageMaster 2D Platinum software. Twenty-one protein spots whose intensities were elevated threefold to 13-fold at 3.5 M NaCl as compared to 1.5 M NaCl were analyzed by matrix-assisted laser desorption/ionization tandem time of flight mass spectrometry. One salt-induced protein isolated from the 2-DE gels was identified as a glucose-6-phosphate isomerase (GPI) from *D. salina* (DsGPI). A full-length cDNA of DsGPI was obtained using rapid amplification of cDNA end technique, and it was shown by heterologous expression to encode a protein with a molecular weight consistent with the protein spot in the 2-DE gels. Real-time quantitative RT-PCR demonstrated that the mRNA of DsGPI was induced up to eightfold ($P<0.01$) by 2.5 M and 14-fold higher ($P<0.01$) by 3.5 M NaCl than by 1.5 M NaCl, respectively. It is concluded that the protein isolated through 2-DE is indeed DsGPI and that the DsGPI gene may be involved in adaptation to high salinity.

Keywords *Dunaliella salina* · Glucose-6-phosphate isomerase · Halotolerance · Salt stress · Two-dimensional gel electrophoresis

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Introduction

Salt stress severely impairs plant growth and productivity. Recently, knowledge of the halotolerance mechanisms has developed quickly, and improvements of halotolerance in some salt-sensitive species have also been realized (Klähn et al. 2009; Parida and Das 2005; Yang et al. 2009). However, the entire network of plant genes contributing to halotolerance is still unclear as it is a complex process involving a range of physiological responses to osmotic and ionic stresses (Munns 2005; Parida and Das 2005; Tuteja 2007).

Plants cope with salt stress by synergistic activation of biochemical pathways including (1) synthesizing compatible solutes, (2) maintaining the intracellular ion homeostasis, (3) altering photosynthesis intensity, (4) scavenging reactive oxygen species, and (5) modifying gene expression of membrane structure elements and enzymes related to energy metabolism (Parida and Das 2005; Popova et al. 2008).

The unicellular eukaryotic green alga *Dunaliella salina* grows over a wide range of salt concentrations from 0.05 to

5.0 M (Mishra et al. 2008) and has received considerable attention in studies of halotolerance mechanisms (Liska et al. 2004). *Dunaliella salina* cells lack a rigid cell wall and are enclosed solely by a thin plasma membrane and are able to rapidly change their volume and shape in response to changes of salinity, making the membrane an important barrier to shield cells against changes of salinity (Katz et al. 2007; Paz et al. 2007). Components of the plasma membrane including ion transporter iron-binding transferrin-like protein Ttf, signal transduction molecules GTP-binding proteins, molecular chaperones heat shock proteins 70 and 90 (HSP 70 and 90), and antioxidative enzymes Fe superoxide dismutase participate in the processes of halotolerance (Katz et al. 2007; Liska et al. 2004). Another reason for halotolerance is that intracellular accumulation of a large amount of glycerol reaching approximately 7.8 M when the cells are grown in 4 M NaCl medium, serving as compatible solute to balance the osmotic pressures inside and outside the cells (Borowitzka and Brown 1974; Chen and Jiang 2009; Oren 2005). In contrast to salt-sensitive plants that reduce photosynthesis, *D. salina* enhances photosynthesis and oxygen evolution when NaCl concentration is increased (Goyal 2007; Liska et al. 2004). In addition, some proteins such as glucose-6-phosphate dehydrogenase, rubisco large subunit, dihydroxyacetone kinase, and ATP synthase referred to carbon and energy metabolism have been isolated (Chen and Jiang 2009; Liska et al. 2004); however, they seldom have been further studied due to the absence of bioinformatics of the genes mentioned above.

Although the genome of *D. salina* is not available, proteome analysis is an efficient technique to detect and characterize proteins from *D. salina* (Katz et al. 2007; Liska et al. 2004). In the present study, we analyzed changes of proteins between algal cells cultured at 1.5 or 3.5 M NaCl. Among the salt-induced proteins, one which increased by 5.7-fold was identified as glucose-6-phosphate isomerase from *D. salina* (DsGPI) by matrix-assisted laser desorption/ionization tandem time of flight (MALDI-TOF/TOF) mass spectrometry (MS). Also, a complete cloned cDNA of DsGPI was strongly induced when algal cells were transferred from 1.5 to 2.5 M or 3.5 M NaCl.

Material and methods

Dunaliella salina UTEX-LB-1644 was obtained from the UTEX Culture Collection USA. Cells of *D. salina* were cultured in a modified medium (Ben-Amotz and Avron 1990) comprising 50 mM $NaHCO_3$, 10 mM KNO_3, 5 mM $MgSO_4$, 0.2 mM KH_2PO_4, 6 μM EDTA, 2 μM $FeCl_3$, 0.2 mM $CaCl_2$, 7 μM $MnCl_2$, 1 μM $ZnSO_4$, 1 μM Co $(NO_3)_2$, 1 μM $CuSO_4$, and 1.5 M NaCl (lower salinity) or 3.5 M NaCl (higher salinity) at 26°C with a 12-h light/day under light intensity of 60 μmol photons m^{-2} s^{-1}.

Protein extraction and two-dimensional gel electrophoresis

Algal cells were broken by ultrasonication in lysis buffer containing 6 M urea, 2 M thiourea, 0.02 M dithiothreitol (DTT), 2% 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonate (CHAPS), 0.2% Bio-lyte (pH 3–10), 1% Triton-X 100, and a protease inhibitor cocktail (Sigma). Proteins precipitated by TCA-actone were dissolved in a buffer containing 6 M urea, 2 M thiourea, 0.02 M DTT, 2% CHAPS, 0.2% Bio-lyte (pH 3–10), and 0.05% IPG buffer, and protein concentrations were determined using the Bradford method (Kao et al. 2008).

A total of 400 μg proteins was loaded onto 24 cm Immobiline™ DryStrips (pH 3–10, linear; GE Healthcare), and programs of isoelectric focusing on IPGphor were as follows: at 30 V for 12 h, at 200 V for 1 h, at 500 V for 1 h, and at 1,000 V for 1 h; linear gradient from 1,000 to 8,000 V and then at 8,000 V for 10 h with a current limitation of 50 μA/strip. After focused gel strips were equilibrated in sodium dodecyl sulfate (SDS) equilibration buffers, loaded on top of prepared 12.5% polyacrylamide gels and covered with 0.5% agarose, SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was run at 20°C and 40 mA for 40 min and then at 60 mA for 5 h.

Identification of proteins

The two-dimensional gel electrophoresis (2-DE) gels stained with Coomassie Brilliant Blue (CBB) R-250 were scanned using Image-Scanner (GE Healthcare), and the intensities of protein spots from the 3.5 M NaCl medium were compared to those from the 1.5 M NaCl medium (control) using ImageMaster 2D Platinum software (version 5.0, Amersham). Each sample was replicated at least three times, and protein spots for comparative analyses were detected on all of the gels. The pixel intensity of each spot was normalized against the total pixel density arising from all the protein spots on the gels. After spots of proteins up-regulated by more than three times from the 2-DE gels were digested by trypsin, the resulting peptides were extracted and analyzed using MALDI-TOF/TOF MS (Applied Biosystems). MS data were collected and protein identification was searched against the database of plants in NCBI using the MASCOT search engine. An identified protein was matched with the known protein(s) in the database only when its score is greater than 65 ($P<0.05$).

Cloning and heterologous expression of the DsGPI gene

A pair of degenerated PCR primers "GARTTYGGNATH GAYCC" (sense) and "NACNCCCCAYTGRTCRAA" (antisense) corresponding to the highly conserved amino acid residues "EFGIDP" and "FDQWGV" was used to

amplify a cDNA fragment of the DsGPI, and then 5′- and 3′-RACEs were performed using a First Choice RLM-RACE Kit (Ambion). The putative 5′- and 3′-RACE cDNAs and the partial sequences cloned using the degenarate primers were organized to form a cDNA contigue.

The DsGPI cDNA was subcloned into the prokaryotic protein expression vector pET28a(+) (Novagen) with *Nde*I and *Eco*RI sites, and expressed in *Escherichia coli* BL 21 to produce fusion DsGPI proteins with 6× His-tags. Transformants were cultured in Luria–Bertani medium until the cells reached an A_{600} of 0.6, and then 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to induce the expression of fusion proteins. The proteins collected from logarithmic phase cells and those purified by Ni-NTA His·Bind column (Novagen) were subjected to 12% SDS-PAGE.

Analysis of DsGPI gene expression

To confirm whether the DsGPI gene responds to salt stress, after algal cells at logarithmic phase cultured in 1.5 M NaCl were transferred to fresh 1.5, 2.5, or 3.5 M NaCl media for 1, 2, 4, 8, 12, 16, 20, and 24 h, respectively, all of the harvested cells were applied to RNA extraction and cDNA synthesis.

Real-time quantitative RT-PCR was performed using SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instructions. Primers "CAGTGTCCACGAACCT CAAGC" (sense) and "CCTCCATCAAGTCAAAGCC ATA" (antisense) were used to amplify the DsGPI gene to create a 153-bp product, while "CAAGTTCTCCGCCGA TGTGA" (sense) and "GAACACGCCTGTGCCCTCAA" (antisense) were used for amplification of the glyceraldehyde-3-phosphate dehydrogenase gene (internal standard) to create a 147-bp product. The program was monitored by 7300 Real-Time PCR System (Applied Biosystems) and relative gene expression data were analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

The data were processed by one-way analysis of variance using SPSS version13.0 (SPSS, USA). Summary statistics were expressed as means ± standard deviations. In all statistical analyses, a P value of <0.05 was considered statistically significant.

Results

Proteomic changes of *D. salina* in adaptation to high salinity

Algal cells grown in 1.5 M NaCl were used as control to identify salt-induced proteins in 3.5 M NaCl. For each concentration, gels were run at least three times in p*I* ranging from 3 to 10 and then stained with CBB R-250. More than 500 protein spots were reproducibly detected in each CBB-stained gel (Fig. 1). Intensity quantification of protein spots revealed that intensities of a total of 21 protein spots were increased by over threefold at 3.5 M NaCl as compared to control gels.

Identification of salt-induced proteins

The selected 21 protein spots digested by trypsin were applied to MALDI-TOF/TOF MS. According to the search results by MASCOT, there was not a good match (score <65) in nine spots while 12 spots had a good match (score >65). Of the 12 spots, one protein spot was identified as DsGPI (Figs. 2 and 3), whose total amount was increased by 5.7-fold higher in 3.5 M NaCl than in 1.5 M NaCl (Fig. 1), two spots were unknown proteins, and the nine proteins have been identified as follows: Fe superoxide dismutase, ATP synthase, heat shock protein 70B, 30S ribosomal protein S4, kinetochore protein, dynein 1-alpha heavy chain (flagellar inner arm), G-protein, calcium-dependent protein kinase, and alcohol dehydrogenase (Liska et al. 2004; Katz et al. 2007; Wang et al. 2008; Parida and Das 2005; Popova et al. 2008).

Cloning and prokaryotic expression of the DsGPI gene

PCR amplification with degenerated primers resulted in a specific PCR product of 771 bp long, and a full-length sequence of 2,338 bp (GenBank accession number FJ 210719) containing an ORF of 1,980 bp was deduced when the 5′ and 3′-RACE results were taken together. Blast analysis indicated that the deduced amino acids from the ORF were very similar to the known GPIs from *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, etc.

After competent cells of *E. coli* BL21 transformed with the pET28a(+)-DsGPI were induced by IPTG, the predicted abundant fusion proteins with a molecular weight of ~75 kDa were identified by SDS-PAGE, while null or trace of the protein band appeared in control *E. coli* BL21 cells that were untransformed or transformed but without induction. Purification of the fusion proteins using Ni-NTA spin column revealed that the heterologous expression of DsGPI had the 6× His-tags (Fig. 4).

Responses to high salinity in DsGPI expression

As shown in Fig. 5, when the algal cells were transferred to higher NaCl (2.5 and 3.5 M) from lower (1.5 M) concentrations, DsGPI expressions were abruptly reduced at the first 2 h but then rapidly increased by up to eightfold or 14-fold in 2.5 or 3.5 M NaCl, respectively.

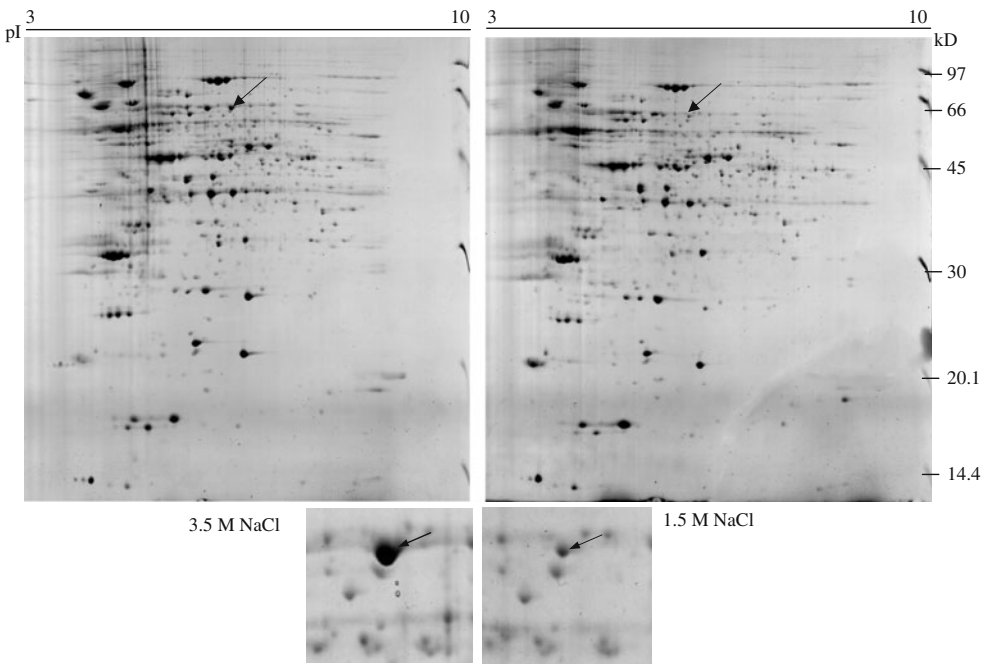

Fig. 1 Two-dimensional gel electrophoresis (*2-DE*) of total proteins from *D. salina*. The gels were stained with Coomassie Brilliant Blue R-250, and 21 of the total 509 protein spots having more than threefold higher intensity in 3.5 M NaCl than that in 1.5 M NaCl were excised for MALIDI-TOF/TOF MS. The *arrow*-indicated spots (magnified in the *bottom views*) with a theoretical p*I* of 6.52 and molecular weight of 70.5 kDa were identified as DsGPI

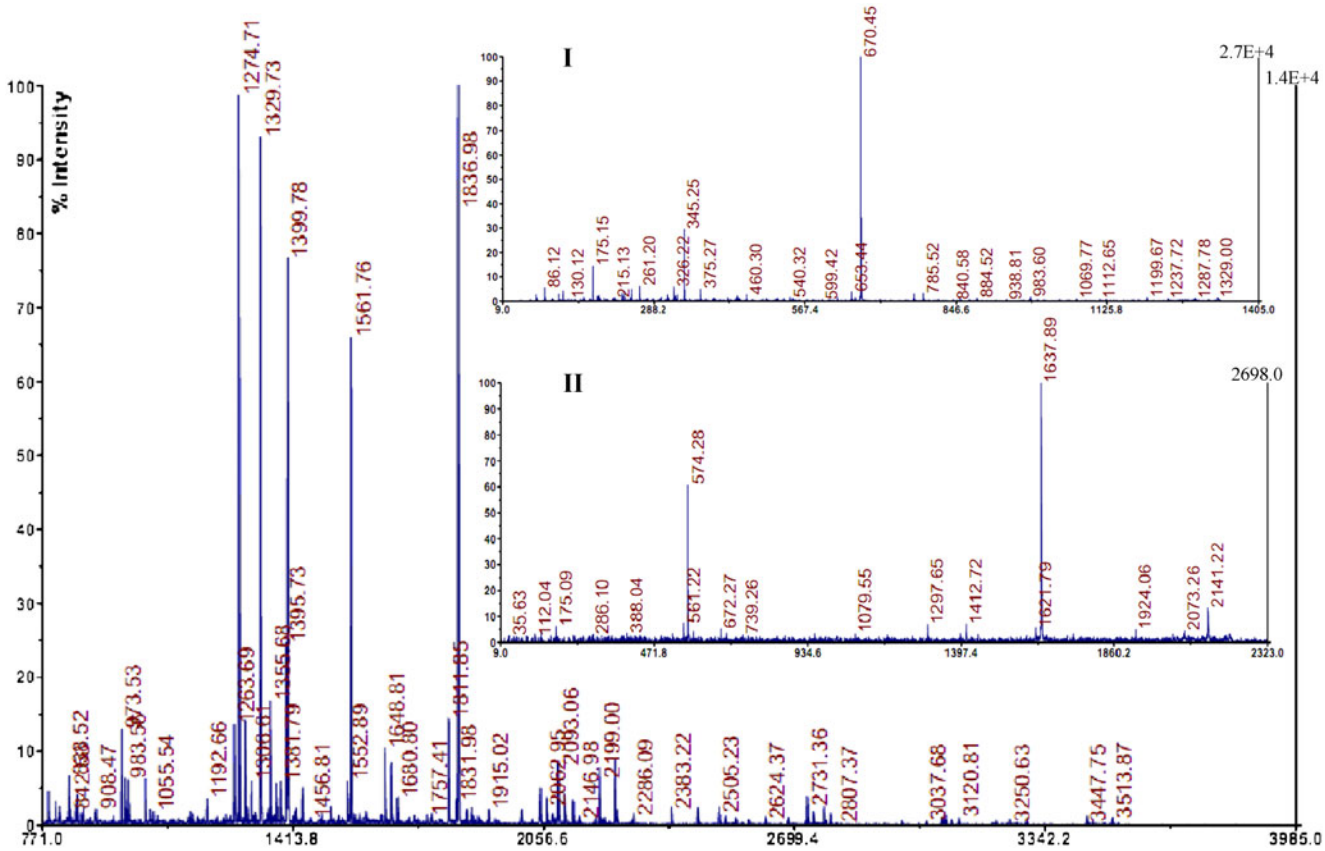

Fig. 2 MALDI-TOF/TOF MS of DsGPI. The *bottom left panel* shows the MALDI-TOF MS of the trypsin-digested DsGPI, and the *top right panel* shows the MALDI-TOF/TOF MS of the precursors (*I*) 1,329.73 (*m*/*z*) and (*II*) 2,199.00 (*m*/*z*) in the MALDI-TOF MS

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  1 MQTQLRAPCV SHKTRINGVP CSPKVVPRVA RVPSGSAPEA PVASPEPTAA
 51 QRNVVAQVAK MIHAQTTTNK KGALVSELPA WQALKEHVNE IEKTHLRDLL
101 HDEARCMSLI KECEGIYGDF TRQRVTQKTL ELLFELAEQS NLRGKINAMF
151 NGEHINSTED RAVLHIATRA HRNQKIFVDG KDVVPDVWEV LDKIKAFSDK
201 VRNGEWLGVT GKPLKNVVAI GIGGSFLGPL FVHTALRTEP VAMRCSRDRS
251 LRFLANVDPI DVARALDGLD PEETLVVVVS KTFTTAETML NARTVRSWLT
301 SRLGPDSVAK HMVAVSTNLK LVKEFGIDPE NAFGFWDWVG GRYSVCSAVG
351 MLPLSLQYGF DLMQEFLAGA NNIDEHFKNQ PYQDNLPVLM GLTSLWNVSF
401 LGHGAKAILP YCQALSKLAP HIQQVDMESN GKGVDINGVR LPFETGEIDF
451 GEPGTNGQHS FYQLIHQGRV VPCEFIGIVR SQQSVYLKGE VVSNHDELMC
501 NFFAQADALA YGKTPEQLRS DNVPDYLIPH RVFTGNRPSM SIMLPSCTAY
551 TVGQLLAIYE HRIAVQGFIW NINSFDQWGV ELGKVLASKV RTVMNSARTR
601 DRKILPQDGF NYSTTRMINK YLEGKTQVLY PEGHDTFPID MLRGGH
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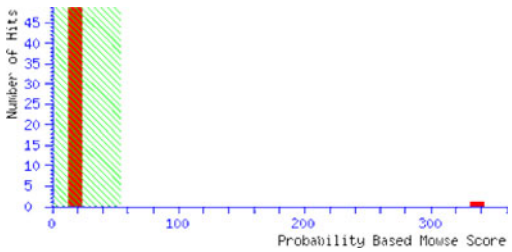

Fig. 3 MASCOT search results of DsGPI MALDI-TOF/TOF MS in NCBI. The result shows that the protein score of DsGPI is 337(scores greater than 54, $P<0.05$) against the sequences of GPI from *C. reinhardtii*. The matched peptides are shown in *italic bold* and *highlighted*

Discussion

In recent years, proteomics approaches have been applied to detect salt-induced proteins in *D. salina* (Katz et al. 2007; Liska et al. 2004). Unlike previous studies that mainly focused on subcellular structures such as chloroplast and plasma membrane, in this study, total proteins of the whole cells were extracted and subjected to 2-DE to globally detect protein changes in high salinity.

As the genome and protein sequences of *D. salina* in databases are very limited, in this study most of the identified proteins by MALDI-TOF/TOF MS have been demonstrated to contribute to halotolerance by being involved in physiological responses to salt stress (Parida and Das 2005; Popova et al. 2008). For example, Fe superoxide dismutase is an antioxidative enzyme that scavenges reactive oxygen species (Chen et al. 2009; Jithesh et al. 2006; Liska et al. 2004); calcium-dependent protein kinase is involved in membrane signal transduction (Katz et al. 2007; Ray et al. 2007); 30S ribosomal protein S4 is related to chloroplast which enhances photosynthesis during salt adaptation (Liska et al. 2004; Stepien and Johnson 2009); ATP synthase and alcohol dehydrogenase are key enzymes in energy and carbon metabolism(Katz et al. 2007; Liska et al. 2004; Wang et al. 2008). Enzymes involved in energy metabolism and glucose metabolism, such as glucose-6-phosphate dehydrogenase, ATP synthase, and phosphoribulokinase, have also been isolated in salt stress, but they have not been paid more attention. In this study, DsGPI was isolated and studied because metabolism-related enzymes have been seldom further studied although they are involved in halotolerance (Chen and Jiang 2009; Liska et al. 2004; Mishra et al. 2008).

Wang et al. (2003) reported that GPI gene quickly responded to nitrate stress in *Arabidopsis*. In this study, the pixel intensity of DsGPI spots in the 2-DE gels showed that it was induced 5.7-fold higher in 3.5 M NaCl than in 1.5 M NaCl. The results of real-time quantitative RT-PCR demonstrated that DsGPI gene expression was increased even up to eightfold higher in 2.5 M NaCl and 14-fold higher in 3.5 M NaCl than in 1.5 M NaCl, respectively, suggesting that the DsGPI gene is strongly induced by salinity, i.e., the higher the salinity, the

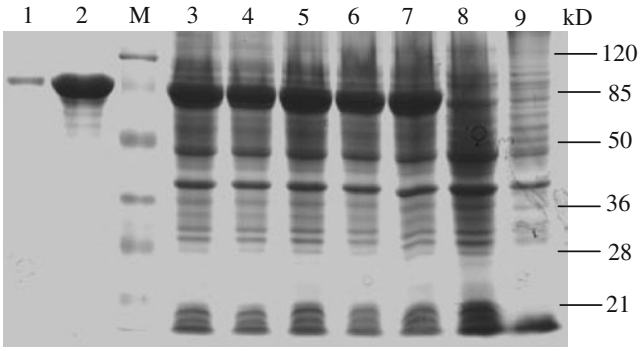

Fig. 4 Expression and purification of DsGPI in *E. coli* BL21. *Lane 1* purified DsGPI fusion proteins; *lane 2* inclusion bodies; *M* molecular weight marker; *lanes 3–7* inducing periods of 5, 4, 3, 2, and 1 h, respectively; *lane 8* without inducement; and *lane 9 E. coli* BL21 cells

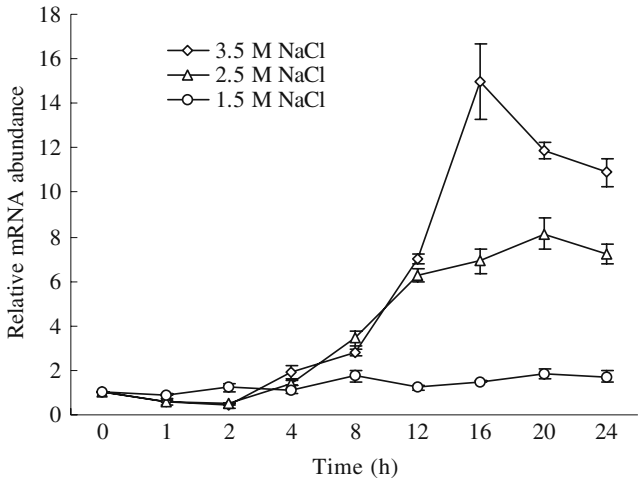

Fig. 5 Induced expression of the DsGPI gene by salinity. In 1.5 M NaCl (control), expression of the DsGPI gene was relatively steady, but the relative proportion of DsGPI mRNA were increased rapidly after the adaptation period of the first 2 h with the highest levels up to eightfold higher ($P<0.01$) at 24 h in 2.5 M and 14-fold higher ($P<0.01$) at 16 h in 3.5 M NaCl, respectively

stronger the effects of induction, and it may play an important role in halotolerance.

During salt stress, glycerol as the major compatible solute is massively accumulated (Borowitzka and Brown 1974; Chen and Jiang 2009; Oren 2005). The carbon sources for glycerol come partially from enhanced photosynthetic CO_2 fixation at late stages of hyperosmotic adaptation or from starch reserve breakdown at early stages of adaptation (Chen and Jiang 2009; Goyal 2007). The possible pathway of glycerol synthesis from starch is that glucose hydrolyzed from starch is converted to fructose-1,6-diphosphate in which GPI is a key enzyme and next to dihydroxyacetonephosphate (DHAP) and to glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase. Finally, gycerol-3-phosphate is converted to glycerol by glycerol-3-phosphate phosphatase (Chen and Jiang 2009). Additionally, excess glycerol is converted to dihydroxyacetone (DHA) by glycerol dehydrogenase (Ben-Amotz and Avron 1973; Borowitzka et al. 1977) and to DHAP by dihydroxyacetone kinase. Thus, cells of *D. salina* in high salinity have to up-regulate the expression of DsGPI not only at gene but at protein levels for massive synthesis of glycerol to adapt the stress environment.

In summary, proteonomic analysis revealed that adaptation of salt stress is a complex interaction network requiring the coordination of a large number of genes in *D. salina*. One salt-induced protein isolated from the 2-DE gels was identified as DsGPI, and the DsGPI gene also has been shown to be strongly induced by high salinities, suggesting that DsGPI is involved in responding to salt stress not only at the gene level but also at the protein level.

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