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ORIGINAL ARTICLE

Isolation, characterization and functional annotation of the salt tolerance genes through screening the high-quality cDNA library of the halophytic green alga *Dunaliella salina* (Chlorophyta)

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Abstract *Dunaliella salina* (Dunal), a unicellular green alga which lacks a rigid polysaccharide cell wall, can thrive in hypersaline environments. To better understand its salt tolerance mechanism, we first constructed a high-quality cDNA library with 55 % novel genes for *D. salina* and then screened this library, isolating 37 unique salt tolerance genes. Eighteen of these newly isolated genes were annotated using the public databases, including a number of conventional salt tolerance genes, such as spermdine synthetase, protein disulfide isomerase, cyclophilin CYP1, F-box protein SKIP5, among others. The other 19 isolated genes were novel salt tolerance genes. Further annotation of these data may help us elucidate the salt tolerance mechanism of *Dunaliella salina*.

Keywords Expressed sequence tags · *Dunaliella salina* · Salt tolerance genes · Cyclophilin CYP1

Introduction

The unicellular green alga *Dunaliella salina* (Dunal), first discovered on the Mediterranean coast in 1838 (Oren 2005), is known for its massive production of β-carotene which is used commercially in cosmetics and food supplements (Ye et al. 2008). Its synthesis of β-carotene can be further enhanced with exposure to UV or heat stress (Jahnke 1999; García et al. 2007; Baird and Delorenzo 2009; Mogedas et al. 2009). *D. salina* also has a great potential in the search for ways to meet the global demand for biodiesel fuels (Gouveia and Oliveira 2009). Under conditions of hyperosmotic stress or nitrogen starvation, *D. salina* can be induced to accumulate raw material for biodiesel production, such as lipid or unsaturated fatty acid (Azachi et al. 2002; Takagi et al. 2006).

Salt stress disrupts the homeostasis in water potential and ion distribution at both the whole plant and cellular level (Zhu 2001). To adapt to the salt stress, *D. salina* synthesizes a large amount of glycerol to maintain the osmotic homeostasis (Amotz and Avron 1973). The carbon source of glycerol biosynthesis is derived from photosynthetic products and the metabolism of stored polysaccharide (Goyal 2007a, b). The enzyme NAD^+-dependent glycerol-3-phosphate dehydrogenase (GPD1) catalyzes the rate-limiting step of glycerol biosynthesis. Under hypersalinity stress, *D. salina* expresses high levels of GPD1 in order to accumulate glycerol (Chen et al. 2011). It is speculated that the mitogen-activated protein kinase (MAPK) cascade initiates the biosynthesis of the stabilizing osmolytes through translational and post-translational modification and that it plays an important role in abiotic stress tolerance mechanisms. In *D. salina*, the level of MAPK protein and its phosphorylated form are upregulated under salt stress (Jimenez et al. 2004). However, to date, neither the substrates of MAPKs nor the underlying salt tolerance mechanism have been identified in *D. salina*. One kind of cyclophilin, named PIN1, is able to discern and isomerize the phosphorylated serine or threonine residues preceding the proline (S/T-P) motif in MAPKs and in turn affect the fate of the target proteins (Lu et al. 2002). Nevertheless, this cyclophilin has not yet been identified in plants.

The adaptation of the halophytes to the salt stress involves a complex adjustment process, and there are still many salt tolerance genes which have not been isolated and identified. Many methods have been used to isolate and analyze the salt tolerance genes of the halophytic alga *Dunaliella* with the aim

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of studying its complicated salt-stress mechanism and discovering more novel salt tolerance genes. The expressed sequence tag (EST) profile of *D. salina*, obtained through characterization of a hypersaline shocked cDNA library, reveals that, under salt stress, this alga expresses components of the translation system at a higher level (Alkayala et al. 2010). The results of a proteomic approach aimed at identifying salt-induced proteins in *Dunaliella* revealed that key enzymes involved in the Calvin cycle, starch mobilization and redox energy production, regulatory factors in protein biosynthesis and degradation and Na^+-redox transporters are upregulated under high salinity stress (Liska et al. 2004).

Although transcriptomic and proteomic analyses of *Dunaliella* have been reported, there are still only a limited number of nucleotide sequences and amino acid sequences in the public databases. The paucity of *Dunaliella* genome sequences is a major limitation to the discovery of novel salt tolerance genes. We therefore have constructed a high-quality cDNA library with 55 % novel genes for *D. salina* which provides a rich resource for other scientists interested in *Dunaliella*. Through screening the cDNA library, we first isolated and then functionally annotated 37 salt tolerance genes, paving the way for further study of the salt tolerance mechanism of *Dunaliella*.

Material and methods

Alga material and salt stress treatment

The green alga *D. salina* strain (435) was provided by the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China. Cells were cultured in DM medium [NaCl, 87.69 g (1.5 M); $NaNO_3$, 0.42 g; $NaH_2PO_4 \cdot 2H_2O$, 0.156 g; $NaHCO_3$, 0.084 g; KCl, 0.074 g; $MgSO_4 \cdot 7H_2O$, 1.23 g; $CaCl_2 \cdot 2H_2O$, 0.044 g; 1 % ferric citrate, 1 mL; A_5 solution, 0.5 mL; in 1 L distilled water]. The pH value of the DM medium was adjusted to 7.2. The A_5 solution contained (in 100 mL distilled water) H_3BO_3, 286 mg; $MnCl_2 \cdot 4H_2O$, 181 mg; $ZnSO_4 \cdot 7H_2O$, 22 mg; $CuSO_4 \cdot 5H_2O$, 7.9 mg; $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 3.9 mg. *D. salina* cells were cultivated at 26 °C and 2,600 lx provided by cool/white fluorescent lamps, under a 12/12 h light/dark cycle in an intelligent lighting incubator (GXZ-1000A; Dongnan Instrument Co. Ltd. Ningbo, Zhejiang, China). Cells in the exponential phase (approx. $2–10 \times 10^9$ cells L^{-1}) were centrifuged at 6,000 rpm for 5 min at 4 °C using the Sorvall Legend Micro 17 microcentrifuge (Thermo Electron, Pittsburgh, PA). To facilitate detection of the transcript level of the cyclophilin *Dscyp1* under the salt shock condition, the algal pellets were suspended in fresh medium containing 4.5 M NaCl. Cultures were harvested at 0, 1, 2, 4, 12, 24, 36, 48 and 72 h.

Construction and sequencing of the *D. salina* cDNA library

For construction of the cDNA library, total RNA was extracted from *D. salina* in the exponential growth phase (1.5 M) using TriZol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA). Poly(A+) RNA was extracted from the total RNA using the PolyATtract® mRNA Isolation System (Promega, Madison, WI). Using 0.75 ng mRNA as templates, we adopted the long-distance PCR method to synthesize cDNAs using the SMART™ cDNA Library Construction kit (Takara Biotechnology Co., Ltd., Dalian, China). The cDNAs were then digested by proteinase K and SfiI enzyme and size-fractionated using CHROMA SPIN™-400 columns (Clontech, Takara Biotechnology Co., Ltd., Mountain View, CA) to remove the cDNAs smaller than 500 bp. The selected longer fractions were ligated with the SfiI-digested λTriplEx2 vector, packaged by MaxPlax™ Lambda Packaging Extracts (Epicentre Biotechnologies, Madison, WI). Randomly selected plaques from the unamplified library were manipulated using touchdown PCR with the insert screening primers 5′-TAATACGACTCACTATAGGGC-3′ and 5′-CTCCGAGA TCTGGACGAGC-3′. The positive plaques with cDNA inserts were then sequenced.

Screening of the *D. salina* cDNA library for salt tolerance genes

The unamplified cDNA library was massively converted in vivo, resulting in pTriplEx2-hosted *Escherichia coli* strain BM25.8. The conversion product was plated on LB agar plates containing carbenicillin (50 gL^{-1}) and then incubated overnight at 37 °C. The plasmid (250 ng) isolated from the bacteria eluted from the plates was electro-transformed into the electrocompetent cells XL1-Blue by a single voltage pulse at 1.5 kV using the ECM® 399 Electroporation System (Harvard Apparatus, Holliston, MA). The recoverable electro-transformed product was plated on LB soft agar (0.7 %) plates containing carbenicillin (50 gL^{-1}), isopropyl β-D-1-thiogalactopyranoside (100 mM) and 6 % NaCl (salt-stress) and incubated at 37 °C for 3 days. All of the colonies which grew on the 6 % salinity plates were characterized by colony PCR using the insert screening primers. The positive colonies were sequenced.

Analysis and functional annotation of *D. salina* Uniseqs

The cDNA clones so identified (i.e. the randomly picked clones and salt tolerance clones) were sequenced from the 5′ terminal using a reverse sequencing primer (5′-TAATACGA CTCACTATAGGGC-3′) by the Shanghai Biological Technology Co. Ltd. (Shanghai, China). The raw data so retrieved were cleaned to remove any vector contamination and chimeric sequences (Lee et al. 2005). The vector

contamination was trimmed using the VecScreen program (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). The 3′ poly(A) [or poly(T)] tails were removed by TRIMST (http://www.bi.up.ac.za/cgi-bin/emboss.pl?_action=input&_app=trimest). The low-complexity sequences were removed using the default values of the DUST program (http://harlequin.jax.org/tools/faProcess.html). All of the cleaned ESTs for the same gene were assembled into contigs using the CAP3 program (Huang and Madan 1999; Blair et al. 2011). Finally, all deduced amino acid sequences of Uniseqs were functionally annotated by searching the public databases, including all non-redundant (NR) protein databases and the PFARM database (Aron and Bryant 2004; Aron et al. 2009, 2011), with BLASTP2.2.26 (Altschul et al. 1997, 2005). Those sequences which were hits were further annotated with the Gene Ontology (GO) analysis (Ashburner et al. 2000). The trimmed and cleaned salt tolerance ESTs were also compared to the *Dunaliella*'s EST database with the aim to collect other parts of those identified salt tolerance ESTs using the MegaBLASTn program (Zhang et al. 2000).

Characterization of ***D. salina***'s cyclophilin

The subcellular localization of the cyclophilin DsCYP1, which is encoded by one of the salt tolerance genes, was predicted using the PSORT program (http://psort.hgc.jp/). The theoretical isoelectric point (pI) and molecular weight (Mw) of this protein was predicted using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The amino acids sequences of DsCYP1 were aligned with AtCYP18-3 (*Arabidopsis thaliana*; NP_195585.1), ThCYP1 (*Thellungiella halophila*; AY392408), CrCYP1 (*Chlamydomonas reinhardtii*; XP_001700372.1), OsCYP2 (*Oryza sativa* L.; NP_001045717.1) and CcCYPA (*Cajanus cajan*; ADB04247.1) using default parameters (protein weight matrix, Gonnet; gap open, 10; gap extension, 0.1) of ClustalW2. The neighbor-joining tree inferring the evolutionary distance of these CYP1s was derived using default parameters of Mega 4.1. In order to analyze the expression level of *Dscyp1* under the salt stress condition (4.5 M), relative quantitative PCR was carried out with the Dscyp1F (5′-TGGAGGCCTTGGGATCCT-3′) and Dscyp1R (5′-CGGCGATGGTGATGTTCTT-3′) primers, following the instructions for the GoTaq® qPCR Master Mix (Promega) and using an ABI 7900 Fast Real-Time PCR System (Applied Biosystems, Foster, CA). Another pair of primers, ActinF (5′-ACCACACCTTCTTCAACGAG-3′) and ActinR (5′-GGATGGCTACATACATGGCA-3′), was used as the endogenous control (Chen et al. 2011). Similar experiments were repeated three times, and the statistical significance of the samples at different treatment times was analyzed using one-way analysis of variance (ANOVA) (Li et al. 2005).

Results

Characterization of ***D. salina*** cDNA library

Using the SMART cDNA technique, we successfully constructed a cDNA library with *D. salina* grown in medium containing 1.5 M NaCl. The original titer of the primary library was 4×10^9 plaque-forming units per liter ($PFUL^{-1}$). The library was then amplified to a titer of 2.6×10^{12} $PFUL^{-1}$; when the titer of an unamplified library is $>1.7\times10^8$ $PFUL^{-1}$, the library contains the low-abundance transcripts. A total of 633 randomly selected colonies were verified by the colony PCR; of these, the 501 cDNA inserts larger than 200 bp were ultimately sequenced, generating 501 raw reads with an average length of 695 bp. After trimming the vector contamination, the low-complexity sequences and 3′ poly(A) tails, 501 cleaned reads were clustered and assembled by the CAP3 program into 55 contigs and 369 singletons, representing 424 Uniseqs. The majority of Uniseqs (127, 30 %) were >800 bp. The size of 55 contigs ranged from 123 to 1,524 bp, with an average length of 844 bp. Among these 55 contigs, 44 (80 %) Uniseqs were >800 bp, and 39 contigs contained two Uniseqs, 11 contigs contained three Uniseqs, four contigs contained four Uniseqs and one contig contained five Uniseqs.

Functional annotation of ***D. salina*** Uniseqs

The deduced amino acid sequences of the 424 Uniseqs were BLASTed against all non-redundant protein databases using BLASTP2.2.26, with an alignment score of≥80. Of these Uniseqs, 190 (45 %) showed significant matches to known proteins of other species, including algae, higher plants, animals and bacteria. From those 190 hits, 144 (76 %) Uniseqs displayed a significant match with the algae *Chlamydomonas* (61 Uniseqs), *Volvox carteri f. nagariensis* (37 Uniseqs), *Dunaliella* (22 Uniseqs), *Coccomyxa subellipsoidea* C-169 (13 Uniseqs), as well as with *Chlorella variabilis*, *Griffithsia japonica*, *Micromonas*, *Helicosporidium* sp. ex, *Ostreococcus tauri*, *Pyrobotrys stellata*, *Rhodomonas salina* and *Ulva fenestrata* (11 Uniseqs combined). An additional 24 (13 %) Uniseqs were significantly matched with higher plants: *Arabidopsis thaliana*, *Cicer arietinum*, *Cucumis sativus*, *Elaeis guineensis*, *Glycine max*, *Medicago truncatula*, *Nicotiana tabacum*, *Oryza sativa* Indica Group, *Pisum sativum*, *Physcomitrella patens* subsp., *Zea mays* and *Triticum aestivum*. Twelve (6 %) Uniseqs matched with bacteria, such as *Verticillium albo-atrum* VaMs.102, *Actinosynnema mirum* DSM 43827, *Metarhizium acridum* CQMa 102, *Bacillus* sp. m3-13, *Serinicoccus profundi* MCCC 1A05965, *Escherichia coli*, *Paenibacillus* sp. JDR-2, *Enterococcus faecium* 1,231,502, *Collinsella aerofaciens* ATCC 25986 and *Listeria grayi* DSM 20601. Ten (5 %)

Uniseqs showed a high similarity with animals, such as *Branchiostoma belcheri tsingtauense*, *Brugia malayi*, *Ciona intestinalis*, *Mustela putorius furo*, *Simulium jonesi*, *Schistosoma japonicum*, *Trichinella spiralis* and *Trichosurus vulpecula*. The remaining 234 (55 %) Uniseqs [Electronic Supplementary Material (ESM) Table 1] displayed no match or scored <80. The latter are likely unique genes for *D. salina* or novel genes for all genera.

Screening for ***D. salina***'s salt tolerance genes

The genes of *Dunaliella* which are active in providing its remarkable tolerance to high salinity were expected to endow the converted *E. coli* with the ability to survive the high salinity stress. The principle of this screening method is based on the functional expression of the *Dunaliella* genes in the host cell *E. coli* (Miyasaka et al. 2000). Following the plating of all converted *E. coli* in 6 % NaCl plates, about 100 colonies survived 72 h later. All of these were picked continuously and verified by the colony PCR, resulting in a positive PCR ratio of 70 %. Of the 70 sequencing attempts, 39 salt tolerance sequences were successfully obtained. To confirm the salt tolerance of these isolated salt tolerance genes, four salt tolerance clones, labeled ST-2, ST-52, ST-58 and ST-83, were individually cultured on the solid LB plates containing a higher salt concentration (7 or 8 % NaCl). Compared with the empty vector (pTriplEx2), all survived the 7 % salt stress (Fig. 1) but failed the 8 % salt stress. In addition, clones ST-83 and ST-52 formed much shallower colonies than ST-2 and ST-58 and showed poor growth at the 7 % salt stress.

The cleaned 39 salt tolerance sequences as queries were compared to *Dunaliella*'s EST database using the MegaBLASTn program. A BLAST hit with an alignment score of >80 was considered to indicate significant similarity. A total of 91 *Dunaliella* ESTs from five cDNA libraries (ESM Table 2) were found with significant matches to 18 salt tolerance ESTs identified in our study. The queries and the corresponding BLAST hits retrieved from the *Dunaliella* EST database were then combined into raw clusters (RCs). A total of 16 RCs were finally identified based on the clustering of four cleaned salt tolerance ESTs for the same gene into two RCs (RC 4, RC 14). The distribution of RC coverage ranged from 2 to 26 (Fig. 2a), whereas the remaining 21 salt tolerance ESTs had no match in the *Dunaliella* EST database and they may therefore be new genes that have not yet been found in *D. salina* (ESM Table 3).

Functional annotation of the ***D. salina*** salt tolerance genes

A total of 16 RCs were assembled into contigs and singletons using the CAP3 algorithm. Among these 16 RCs, four were completely assembled, while three could not be assembled

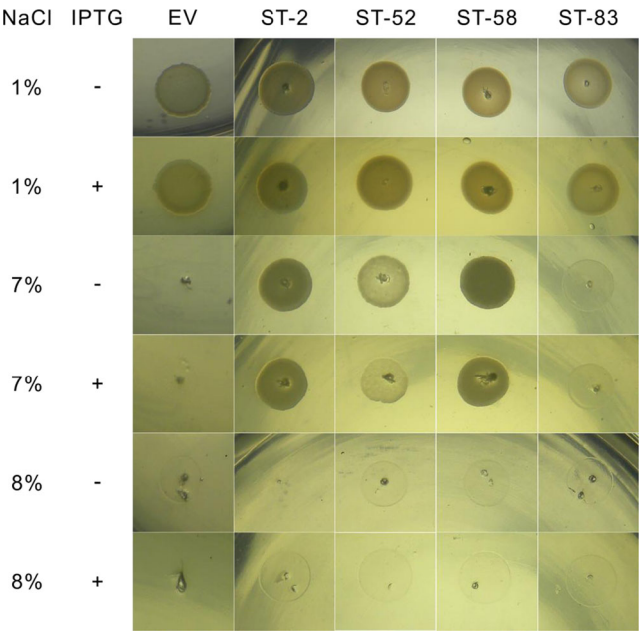


Fig. 1 Confirmation of the salt tolerance of the genes isolated through screening the *Dunaliella salina* (Dunal) cDNA library. Four salt tolerance clones (*ST-2*, *ST-52*, *ST-58*, *ST-83*) and the negative control (empty vector, *EV*) were cultured on solid LB plates containing a high salt concentration (7 or 8 % NaCl), induced by isopropyl β-D-1-thiogalactopyranoside. The growth of these four salt tolerance clones was measured after 18 h. Compared with EV, clones ST-2, ST-52, ST-58 and ST-83 survived the 7 % salt stress condition, but died at 8 % salt stress. Clones ST-83 and ST-52 also formed much shallower colonies than ST-2 and ST-58 and showed poor growth at 7 % NaCl

and found to be singletons. The remaining RCs were assembled into contigs and singletons, even though they displayed some degree of similarity, suggesting the possibility that they may be orthologous genes that differ in some regions (Zhao et al. 2011). For example, the two contigs in RC 2 displayed a high match with the Rieske iron-sulfur subunit of the cytochrome b6f complex protein in *Chlamydomonas reinhardtii*. However, one of the singletons showed a significant match with a hypothetical protein in *Chlamydomonas* sp. HS-5, which is salt inducible.

All RCs and 21 unique salt tolerance ESTs (UESTs) were then inspected for the best matches with known functional proteins from Chlamydomonadales or other vacuolated plants, with a cutoff score of≥80. Five RCs and 16 UESTs had a score of <80 after running BLASTp (ESM Table 1) and may therefore be novel salt tolerance genes for all plant genera. The remaining 16 salt tolerance genes were found with significant BLASTP hits in the non-redundant protein database (Table 1). Of these salt tolerance ESTs, RC 8 and two UESTs were found with significant similarity to the hypothetical proteins (*Volvox carteri f. nagariensis* and *Chlamydomonas reinhardtii*) and the F-box SKIP5-like protein (*Brachypodium distachyon*). The remaining salt tolerance ESTs showed a significant match to *Chlamydomonas* (five RCs and two UESTs), *Volvox* (three RCs) and higher plants

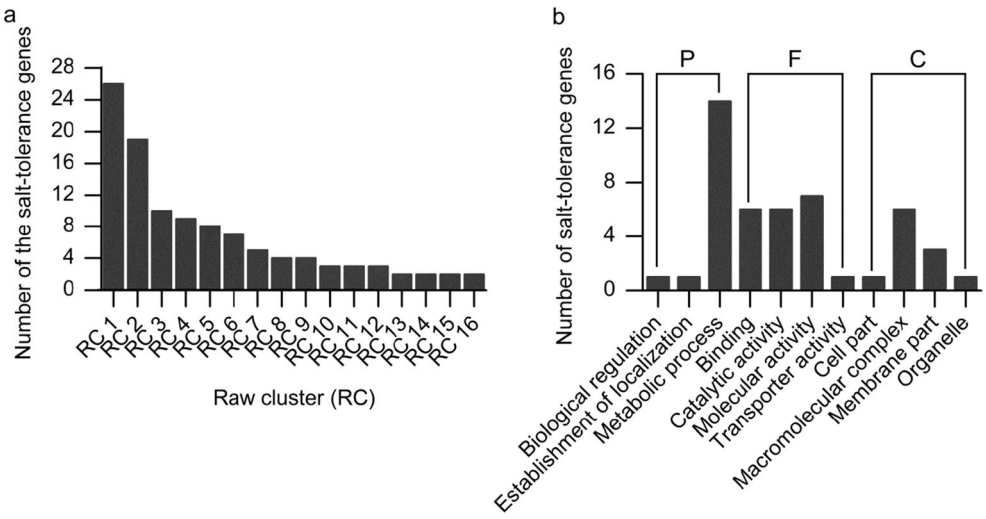


Fig. 2 Abundance and GO annotation of *D. salina* salt tolerance genes. **a** Number of salt tolerance genes covered by the raw clusters (*RCs*). The more expressed sequence tags (ESTs) contained by the RCs, the more abundant the corresponding salt tolerance genes under salt stress. **b** GO annotation of *D. salina* salt tolerance genes. About 14 salt tolerance ESTs of *D. salina* were assigned into 47 GO terms; these were divided into three categories, such as biological processes (*P*), molecular functions (*F*) and cellular components (*C*)

(two RCs and one UEST) (e.g. *Triticum aestivum* and *Arachis hypogaea*). A search of the PFAM databases using a cut-off E-value of $\leq 1e^{-5}$ resulted in the identification of 17 salt tolerance ESTs possessing the conserved domain and belonging to 16 superfamilies, including ribosomal protein superfamilies (e.g. L18e, L14e, RPL 43A, S10, L24e and S6 superfamily), the Rieske superfamily, YMF 19 superfamily, cyclophilin superfamily, thloredoxin-like superfamily, elongation-factor superfamily, class II-HisRS-like-core superfamily, F-box superfamily and AdoMet-Mtase superfamily (ESM Table 4).

Using BLAST hit gene identifiers and gene accessions, the NR- and PARM-annotated salt tolerance ESTs were further annotated using GO analysis, revealing 14 unique salt tolerance ESTs assigned into 47 GO terms. Based on the electro-annotation, all of the GO terms could be divided into three categories (Fig. 2b) as follows: biological processes (P; 16 GO terms, 14 salt tolerance ESTs), molecular functions (F; 20 GO terms, 13 salt tolerance ESTs) and cellular components (C; 11 GO terms, 9 salt tolerance ESTs). The P category contained the GO terms of "Metabolic process" (14 GO terms), "Biological regulation" (1 GO term) and "Establishment of localization" (1 GO term). The F category included the GO term of "Molecular activity" (7 GO terms), "Binding" (6 GO terms), "Transporter activity" (1 GO term) and "Catalytic

Table 1 Functional annotation of the salt tolerance genes of *Dunaliella salina* (Dunal) with a cutoff score of ≥80

Raw cluster and UEST	Top BLAST hit		
	Accession ID	Best match (Genus)	Score
RC 1	XP_002956593.1	Component of cytosolic 80S ribosome and 60S large subunit (*Volvox carteri f. nagarensis*)	245
RC 2	XP_001698786.1	Rieske iron-sulfur subunit of the cytochrome b6f complex, chloroplast precursor (*Chlamydomonas reinhardtii*)	270
RC 3	XP_002949644.1	Ribosomal protein L14 (*Volvox carteri f. nagariensis*)	157
RC 4	XP_001696616.1	Ribosomal protein L37a (*Chlamydomonas reinhardtii*)	152
RC 5	XP_002949809.1	Component of cytosolic 80S ribosome and 40S small subunit (*Volvox carteri f. nagariensis*)	155
RC 6	XP_001698368.1	Ribosomal protein S24 (*Chlamydomonas reinhardtii*])	224
RC 7	ABA01112.1	Chloroplast ATP synthase subunit II (*Chlamydomonas incerta*)	201
RC 8	XP_002951128.1	Hypothetical protein (*Volvox carteri f. nagariensis*)	162
RC 9	AEV66153.1	Cyclophilin (*Arachis hypogaea*)	278
RC 13	XP_001699351.1	Protein disulfide isomerase (*Chlamydomonas reinhardtii*)	172
RC 15	AAP80650.1	Elongation factor (*Triticum aestivum*)	241
HO709975	XP_001702843.1	Spermidine synthetase (*Chlamydomonas reinhardtii*)	99.4
HO709980	AAD10242.1	Histidyl-tRNA synthetase (*Triticum aestivum*)	188
HO709983	XP_003559852.1	F-box protein SKIP5-like (*Brachypodium distachyon*)	114
HO709985	XP_001697927.1	Hypothetical protein (*Chlamydomonas reinhardtii*)	115
HO709989	XP 001702823.1	Mitochondrial ribosomal protein S6 (*Chlamydomonas reinhardtii*)	107

UEST, Unique salt tolerance expressed sequence tag

activity" (6 GO terms). The C category included the GO term of "Macromolecular complex" (6 GO terms), "Cell part" (1 GO term), "Organelle" (3 GO terms) and "Membrane part" (1 GO term).

In summary, among the 37 unique salt tolerance ESTs, 14 were annotated by BLASTP, CD and GO annotation simultaneously; two were annotated by CD annotation, but not by BLASTP and GO annotation. Likewise, one of these was annotated by GO and BLASTP but not by CD; one was annotated by CD and BLASTP but not by GO annotation. In brief, a total of 18 unique salt tolerance ESTs were annotated by these three public databases, whereas the remaining 19 were novel salt tolerance genes.

Characterization and expression profile of ***D. salina***'s cyclophilin under salt stress

Interestingly, the salt tolerance gene, *Dscyp1*, isolated through screening of the cDNA library, was found to contain an intact open reading frame. This gene encodes a protein of 172 amino acids with a related molecular mass of 18.3 kDa and pI of 8.55 predicted by the Compute pI/Mw tool. The PSORT program predicted that *Dscyp1* would be expressed in the cytoplasm.

The deduced amino acid sequences of the cyclophilin DsCYP1 were aligned with AtCYP18-3, ThCYP1, CrCYP1, OsCYP2 and CcCYPA (Fig. 3a) using the default parameters of ClustalW2. All of the sequences shared the same active sites (H-61, R-62, F-67, Q-118, F-120 and W-128). W-128 is a cyclosporin binding site, while H-61, R-62 and F-67 are the three important catalytic residues of the cyclophilin A-, B- and H-like cyclophilin-type peptidyl-prolyl isomerase (PPIase), respectively. The neighbor-jointing tree derived using default parameters of Mega 4.1 (Fig. 3b) showed a genetic relationship between *D. salina* and the green algae *Chlamydomonas reinhardtii* and with the higher plants *Arabidopsis thaliana*, *Thellungiella halophila*, *Oryza sativa L.*, among others.

In order to study the expression profile of *Dscyp1* under salt stress, we analyzed the transcript level of *Dscyp1* under the hyperosmotic shock condition (4.5 M NaCl) for 72 h (Fig. 4). Compared to the sample shocked for 0 h (control),

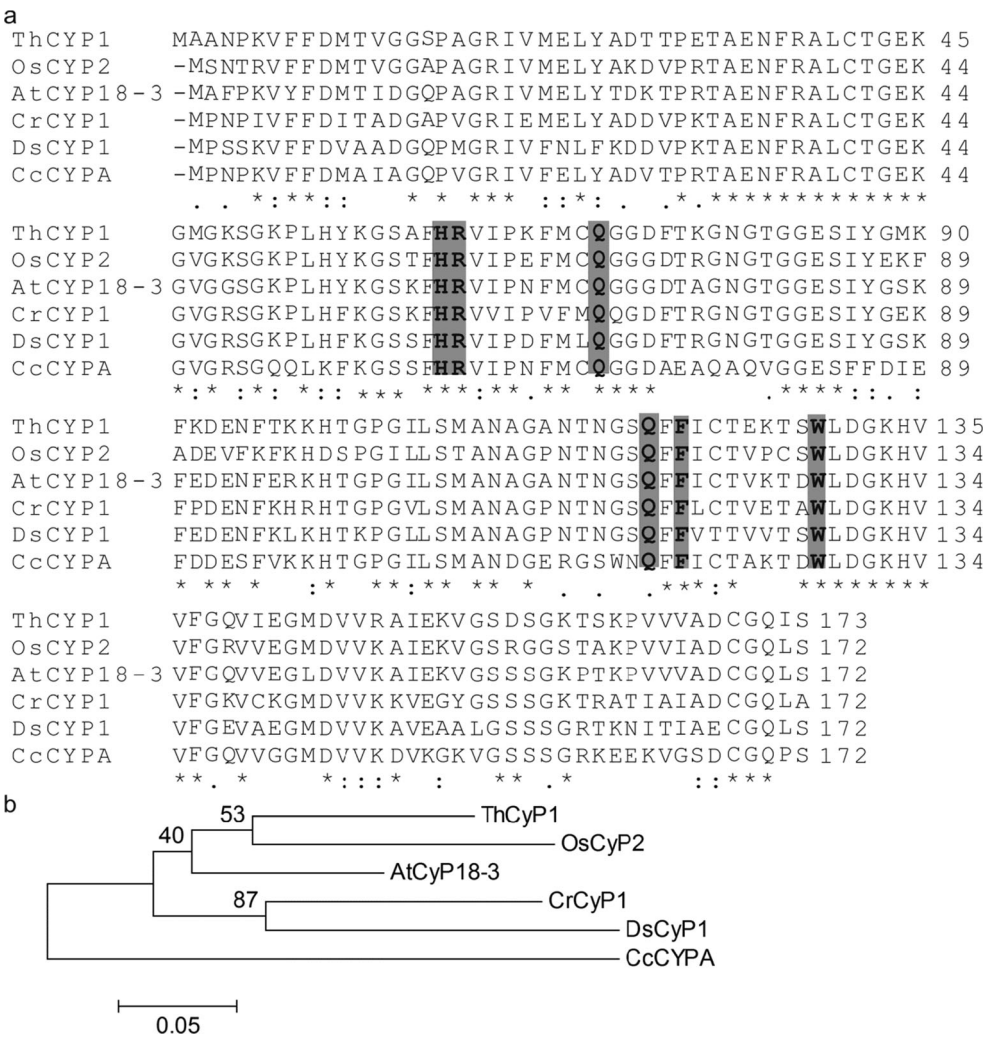


Fig. 3 Multiple alignment of cyclophilin CYP1s from different sources. **a** Multiple sequence alignment of *D. salina* CYP1 (*DsCYP1*) with cyclophilins of various plant species. Asterisk (*) indicates positions which has a single, fully conserved residue; colon (:) indicates conservation between groups of strongly similar properties (scoring > 0.5) in the Gonnet PAM 250 matrix; square (.) indicates conservation between groups of weakly similar properties (scoring ≤ 0.5) in the Gonnet PAM 250 matrix. Amino acids colored in gray are the PPIase catalytic resides. **b** The neighbor-jointing tree inferring the evolutionary distance of CYP1s with *Cajanus cajan* CcCYPA as outlier. Bootstrap values shown are based on 1,000 replicates. *Distance bar*: 0.05 base changes/base. *AtCYP18-3 Arabidopsis thaliana* (NP_195585.1), *ThCYP1 Thellungiella halophila* (AY392408), *OsCYP2 Oryza sativa* L. (NP_001045717.1), *CrCYP1 Chlamydomonas reinhardtii* (XP_001700372.1), *CcCYPA Cajanus cajan* (ADB04247.1)

Dscyp1 was upregulated by about fourfold at 4 h following initial exposure to 4.5 M NaCl (maximum expression level), following which time its expression began to decline gradually until it returned to the basal level within 72 h. The one-way ANOVA was performed for the statistical analysis and the results are shown in Fig. 4. The transcript level of *Dscyp1* in algal samples stressed for 4 h was significantly different ($P<0.05$) from that in samples shocked for 36 h and extremely significantly different ($P<0.01$) from that in samples treated for 1, 48 and 72 h, respectively.

Discussion

Novel genes identified in ***D. salina***

The genome sequencing project of *D. salina* has not yet been completed. Indeed, only a limited number of nucleotide sequences of *D. salina* have been deposited in the NCBI GenBank. In our study, we obtained 424 Uniseqs by sequencing 501 raw reads, of which 234 (55 %) have not yet been annotated, likely because they are novel genes for all genera or unique for *Dunaliella* (ESM Table 1). The remaining 190 Uniseqs were annotated. These data supplement currently available data on the *Dunaliella* genome.

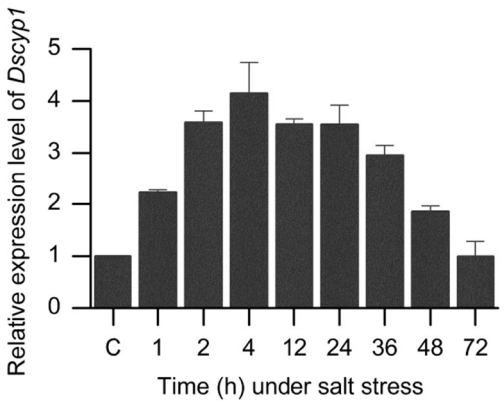
Genes related to salt tolerance in ***D. salina***

In order to isolate the salt tolerance genes and to better explain the salt tolerance mechanism in *D. salina*, we identified 37 unique salt tolerance genes based on the functional screening strategy. Following the bioinformatics analysis, 18 unique salt tolerance ESTs were annotated, of which eight, coding for the components of the translational machinery, were found in our study (Table 1). These encode ribosome proteins, elongation factors and histidyl-tRNA synthetase. The components of the translational machinery are usually regulated by salt (Pang et al. 2010) and play an important role during salt stress (Sahia et al. 2006). Miyasaka et al. (2000) and Tanaka et al. (2002) adopted this strategy and identified many ribosomal proteins by screening *Chlamydomonas* and *Avicennia marina*, respectively. Therefore, it can be concluded that the components of the translational machinery are crucial in the adaptation of halophytes to high salt stress.

Dunaliella can synthesize a large amount of glycerol to resist high salinity. We report here for the first time that polyamines were synthesized by *Dunaliella* to overcome the salinity stress. Polyamines are universal organic polycations, such as diamine putrescine (Put), triamine spermidine (Spd) and tetramine spermine (Spm), and are implicated in abiotic stress tolerance. Spd is synthesized by the successive attachment of aminopropyl to Put, through catalysis by spermdine synthetase (SPDS) (Groppa and Benavides 2008). It has been reported that overexpression of the exogenous SPDS in *Arabidopsis* (Kasukabe et al. 2004), *Ipomoea battas* (Alca'zar et al. 2006) and *Pyrus communis* (Wen et al. 2008) can enhance the plants to tolerate multiple environmental stresses, such as salt, osmosis and heavy metal stress. The substrate of SPDS and other polyamines could play an important role in enabling *D. salina* to overcome the salt stress.

A high concentration of sodium is toxic to an organism and affects protein proper folding. Using the functional screening strategy, we identified a *D. salina* gene coding for protein disulfide isomerase (DsPDI) (Table 1). PDI, an oxidoreductase catalyzing the formation and breakage of the disulfide bonds, is associated with maintenance of proper protein folding in the endoplasmic reticulum (Frand and Kaiser 1998). In *Saccharomyces cerevisiae*, PDI is essential for the formation of native disulfide bonds in carboxypeptidase Y and, therefore, cell viability (Lamantia and Lennarz 1993). In *Arabidopsis*, PDI would be expressed in the first instance to encode proteins that create the optimal polypeptide-folding environment response to stress (Martínez and Chrispeels 2003). Therefore, *E. coli* harboring DsPDI should be able to survive under high salinity stress.

Cyclophilin, an ubiquitous protein present in all subcellular compartments, could catalyze *cis–trans* isomerization of the disulfide bonds (Chou and Gasser 1997). It plays a crucial role



Time	36	1	48	72
4	*	**	**	**
2	—	**	**	**
12	—	**	**	**
24	—	**	**	**
36	—	—	*	**
1	—	—	—	**
48	—	—	—	**

Fig. 4 Transcript level of *Dscyp1* under salt stress (4.5 M). The statistical significance of the samples at different treatment times was analyzed using one-way analysis of variance (shown *below* the histogram). *Significant difference ($P<0.05$), **E extremely significant difference ($P<0.01$)

in mRNA processing, protein folding, protein degradation and signal transduction during stress responsiveness (Romano et al. 2004). Based on the functional screening method, we found that *Dscyp1* (Table 1) conferred ability of salt resistance to *E. coli*. Indeed, this gene was upregulated by fourfold (maximum expression) after 4 h of salt stress (4.5 M), following which time its transcription level declined gradually until returning to the basal level at 72 h (Fig. 4). These results demonstrate that *Dscyp1* was inducible by salt stress. Similarly, the expression level of *cyp1* in other genera is also upregulated during salt, heat, cold and other abiotic stresses, such as *Thcyp1* (Chen et al. 2007), *CccypA* (Sekhar et al. 2010) and *Oscyp2* (Ruan et al. 2011). Both ThCYP1 and CcCYPA are predominantly expressed in the nucleus, while their expression in the cytoplasm is weak (Chen et al. 2007; Sekhar et al. 2010). We predicted that DsCYP1 would be expressed in the cytoplasm. Our results indicate that, after being transported from the cytoplasm to the nucleus, CYP1 could regulate the expression of abiotic-related genes through interacting with nucleus proteins. DsCYP1, ThcCYP1, CcCYPA and OsCYP2, as well as two other CYPs (CrCYP1 and AtCYP18-3), were aligned using the ClusterW2 (Fig. 3a). All of these six CYPs shared the same PPIase catalytic resides (R, F and H) and they belonged to cyclophilin A-, B- and H-like cyclophilin-type PPIase. PPIase activity is enhanced under the salt stress condition (Sekhar et al. 2010). Hence, when *Dunaliella* receives a signal of salt stress, DsCYP1 is predicted to interconvert *cis*- and *trans*-conformations in order to regulate the abiotic tolerance genes through the MAPK signal pathway. In this situation, *Dscyp1s* will be highly expressed immediately and transported to the nucleus to regulate the expression of salt tolerance genes in different ways. One possible mechanism is to change the conformation of the inhibitors of the salt tolerance genes, thereby targeting the respective gene for ubiquitination and proteolysis (Freeman 2001); another possibility is to activate the transcription of the corresponding salt tolerance genes through the conformational switch (Hunter 1998). There must be many other mechanisms by which CYPs can initiate the salt tolerance mechanisms, and these need to be clarified. Therefore, CYP1 might be a key regulator in the salt tolerance mechanism.

Another salt tolerance gene displayed a high similarity with F-box protein SKIP5 of *Brachypodium distachyon* (Table 1). SKIP5 is able to interact with SKP1/ASK1, a conserved SCF ubiquitin ligase subunit, to form the ubiquitin/proteasome system (Farras et al. 2001). The ubiquitin/proteasome system plays an important role in the defense against biotic threats (Dreher and Callis 2007). Therefore, DsSKIP5 is very essential to the survival of *E. coli* under the salt stress condition. Another two salt tolerance genes were found to possess the NADH:flavorubredoxin oxidoreductase domain and CY domain (ESM Table 4). Proteins with the former domain are able to efficiently scavenge reactive oxygen species generated under oxidative stress (Di Matteo et al. 2008), and high salinity stress is usually accompanied by oxidative stress. The overexpression of two cysteine proteinase inhibitors which possess the CY domain, AtCYSa and AtCYSb, in yeast and *Arabidopsis* increases resistance to high salt, drought, oxidative and cold stresses (Zhang et al. 2008). In addition, we identified 19 novel salt tolerance genes which need durther effort to annotate; these would further enrich our knowledge of the salt tolerance mechanism in *Dunaliella*.

Conclusion

This study presents another high-quality cDNA library of *D. salina*, with 55 % novel genes which have not yet been functionally annotated. By screening this library, we isolated 37 unique salt tolerance genes which can be used in a comprehensive analysis and further study of the salt tolerance mechanism. Those encoding DsPDI and DsCYP1 were found to be implicated in protein folding under salt stress. We speculate that *Dscyp1* regulates the expression of the downstream salt tolerance genes. The components of the translation system, F-box protein SKIP5 and the proteins possessing the NADH:flavorubredoxin oxidoreductase domain and CY domain were also found to be related to salt stress tolerance. No only glycerol but also polyamines were synthesized in *D. salina*, based on the identification of DsSPDS. We isolated many novel salt tolerance ESTs, all of which conferred different levels of salt tolerance to *E. coli*. Further annotation of these data increases our understanding of the salt tolerance mechanism of *Dunaliella salina*.

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