Plant Science 179 (2010) 437–449

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Expressed sequence tag (EST) profiling in hyper saline shocked *Dunaliella salina* reveals high expression of protein synthetic apparatus components

Fadi Alkayal[a], Rebecca L. Albion[b], Richard L. Tillett[b], Leyla T. Hathwaik[b], Mark S. Lemos[b], John C. Cushman[b,*]

[a] *Dasman Center for Research and Treatment of Diabetes, P.O Box 1180, Dasman, Kuwait*
[b] *Department of Biochemistry & Molecular Biology, MS200, University of Nevada, Reno, NV 89557-0200, USA*

ARTICLE INFO

Article history:
Received 19 June 2009
Received in revised form 28 June 2010
Accepted 1 July 2010
Available online 14 July 2010

Keywords:
Salinity
Halotolerance
Salt stress
ESTs
Dunaliella salina

ABSTRACT

The unicellular, halotolerant, green alga, *Dunaliella salina* (Chlorophyceae) has the unique ability to adapt and grow in a wide range of salt conditions from about 0.05 to 5.5 M. To better understand the molecular basis of its salinity tolerance, a complementary DNA (cDNA) library was constructed from *D. salina* cells adapted to 2.5 M NaCl, salt-shocked at 3.4 M NaCl for 5 h, and used to generate an expressed sequence tag (EST) database. ESTs were obtained for 2831 clones representing 1401 unique transcripts. Putative functions were assigned to 1901 (67.2%) ESTs after comparison with protein databases. An additional 154 (5.4%) ESTs had significant similarity to known sequences whose functions are unclear and 776 (27.4%) had no similarity to known sequences. For those *D. salina* ESTs for which functional assignments could be made, the largest functional categories included protein synthesis (35.7%), energy (photosynthesis) (21.4%), primary metabolism (13.8%) and protein fate (6.8%). Within the protein synthesis category, the vast majority of ESTs (80.3%) encoded ribosomal proteins representing about 95% of the approximately 82 subunits of the cytosolic ribosome indicating that *D. salina* invests substantial resources in the production and maintenance of protein synthesis. The increased mRNA expression upon salinity shock was verified for a small set of selected genes by real-time, quantitative reverse-transcription-polymerase chain reaction (qRT-PCR). This EST collection also provided important new insights into the genetic underpinnings for the biosynthesis and utilization of glycerol and other osmoprotectants, the carotenoid biosynthetic pathway, reactive oxygen-scavenging enzymes, and molecular chaperones (heat shock proteins) not described previously for *D. salina.* EST discovery also revealed the existence of RNA interference and signaling pathways associated with osmotic stress adaptation. The unknown ESTs described here provide a rich resource for the identification of novel genes associated with the mechanistic basis of salinity stress tolerance and other stress-adaptive traits.

1. Introduction

The halotolerant green alga, *Dunaliella salina*, and related species within the Chlorophyceae were first described more than 100 years ago [1]. Since that time, *Dunaliella* has become an important model for the commercial production of natural β-carotene [2–6] and other products such as phytosterols [7,8]. Carotenoids, such as β-carotene, are widely used as dyes in the food, feed, cosmetic and nutraceutical industries [3,9]. Carotenoids, such as those produced by *Dunaliella*, are synthesized mainly in the plastid via the non-mevalonate, methylerythritol phosphate (MEP) pathway [10,11]. Carotenoid mixtures have been reported to reduce the risk of cancer and cardiovascular diseases [9,12]. Accumulation of carotenoids and steady-state mRNAs encoding key carotenoid biosynthetic enzymes is regulated mainly by nutrient availability [13–15], but can also be influenced by high light intensity [6]. Green algae, including *Dunaliella*, have also been utilized as feedstocks for biofuel production [16–19] and for CO_2 capture and recycling [20,21]. *D. salina* has also been investigated for its production of extracellular polysaccharide substances for industrial or biotechnological applications [22]. The induction of reactive oxygen-scavenging enzymes by UV-b irradiation, or by high light and low temperature, has also been investigated in *D. salina* [23,24]. Although not as widely studied as the related genetic model species, *Chlamydomonas reinhardtii*, several mutants of *Dunaliella* have been described [25–29].

Most *Dunaliella* species can adapt to an exceptionally wide range of salt concentrations from 0.05 to 5.5 M NaCl [30,31]. This remarkable osmotic adaptability is mediated primarily by the massive *de novo* synthesis of the compatible solute, glycerol, following salt

* Corresponding author. Tel.: +1 775 784 1918; fax: +1 775 784 1650.
E-mail address: jcushman@unr.edu (J.C. Cushman).

0168-9452/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2010.07.001

stress, which can accumulate to about 8 M or 55% of cell weight under saturating NaCl conditions [32,33]. Glycerol synthesis is derived mainly from photosynthesis and the hydrolysis of starch reserves [34,35]. Intracellular Na^+ concentrations are maintained at around 10–30 mM over a wide range of extracellular NaCl concentrations indicating an active export system across the *Dunaliella* plasma membrane; however, the exact mechanisms of Na^+ extrusion are not fully understood [36]. Salinity tolerance is thought to be mediated by the activity of two distinct Na^+ extrusion systems in the plasma membrane: a Na^+-ATPase and an NADH-driven electron transport Na^+ pump [36]. Although amiloride-sensitive Na^+/H^+ antiporter activity has been identified in plasma membrane vesicle preparations of *D. salina* [37,38], this antiporter activity is apparently involved in pH regulation, as it is activated upon internal acidification [39] or it catalyzes Na^+ uptake as it is induced by hyperosmotic shock [40].

Although the mechanistic basis of salinity tolerance, namely glycerol accumulation and the active elimination of Na^+ ions, are both constitutive processes [36], a number of studies have documented the accumulation of specific plasma membrane-localized proteins associated with adaptation to very high NaCl concentrations including a Na^+/H^+ antiporter [38], a transferrin-like protein that mediates iron uptake [41,42], a plasma membrane-localized carbonic anhydrase [43], and a fatty acid elongase [44]. The crystal structure of the *D. salina* carbonic anhydrase revealed features that allow the enzyme to retain conformational stability and solubility from low to high salt concentrations and predicted the unexpected halotolerance of a mammalian homolog functioning in the kidney [45,46]. Two-dimensional gel electrophoresis and mass spectrometry (MS) approaches identified 76 salinity-induced proteins from *D. salina* cells grown continuously in either 0.5 or 3.0 M NaCl [47]. Salinity stress was found to increase the relative abundance of enzymes in the Calvin cycle, starch mobilization, and redox energy production, regulation of protein synthesis and degradation, and bacteria Na^+-redox transporter homologue [47]. A more comprehensive analysis of the plasma membrane proteome from salinity-stressed *D. salina* cells was also performed using two-dimensional blue native/SDS-PAGE that identified 55 proteins by nano-liquid chromatography (nLC)–MS/MS of which about 60% were integral membrane or membrane-associated proteins [48]. Among the 20 proteins that showed increased relative abundance under high salinity were proteins implicated in protein and membrane stabilization and signal transduction [48]. More recently, capillary high performance liquid chromatography (cHPLC) coupled with tandem MS was used to identify 520 distinct proteins from isolated *D. salina* flagella [49].

Although proteomic analyses of *D. salina* cells have been conducted, there is very limited information about the transcriptome of this organism. Suppression–subtraction hybridization (SSH) had been used previously to isolate differentially expressed cDNAs in hyperosmotically shocked *D. salina*, but only a very limited number of clones were characterized [50]. More recently, a limited number of ESTs (778) were used to fabricate a printed cDNA microarray to analyze the differential mRNA abundance changes associated with growth of *Dunaliella* sp. under hypo (0.08 M) and hyper saline (4.5 M) conditions [51]. The complete organellar genomes (e.g., mitochondria and chloroplast) of *D. salina* CCAP 19/18 have also been reported [52]. In order to investigate the molecular basis of salinity tolerance in a halotolerant algal species, we characterized 2831 ESTs from a cDNA library constructed from *D. salina* undergoing salinity shock comprising 1401 unique transcripts. Analysis of those *D. salina* ESTs for which functional assignments could be made, revealed that 42.5% of annotated ESTs had functions associated with protein synthesis, folding, modification, and degradation, indicating that *D. salina* invests heavily in the production and maintenance of protein production under conditions of salinity shock. The ESTs described here provide a rich resource for the identification of novel genes associated with salinity stress tolerance and may provide important clues into halotolerance mechanisms.

2. Materials and methods

2.1. Algal material

D. salina cells were grown in 2×-artificial sea water (ASW) medium under a photon flux density of 150 $\mu mol\, m^{-2}\, s^{-1}$ (18 h light (26 °C)/6 h dark (18 °C) cycle with bubbling under 3% CO_2 to late-exponential phase at a cell density of ~0.35 (OD_{680})). For cDNA library construction, cells were adapted in 2.5 M NaCl via an incremental series from 1.7 to 2.0, 2.25, 2.5 M NaCl and then salt-shocked by growth in 3.4 M NaCl for 5 h by the addition of media containing 4.6 M NaCl. For quantitative real-time RT-PCR analysis, algal cells were grown in ASW medium containing 1.2 M or 2.5 M NaCl. Algal cultures were salt-shocked by increasing NaCl concentration to 3.5 M by the addition of ASW medium containing 4.6 M NaCl. Control and salt-shocked samples were harvested in parallel at 0, 1, 3, and 5 h following commencement of salt shock. Algal cells were collected by centrifugation at 2800 × *g* for 10 min, flash frozen under liquid N_2, and stored at −80 °C until use.

2.2. cDNA library construction

For cDNA library construction, total RNA was extracted from whole cells using Trizol® reagent (Invitrogen, Carlsbad, CA). Poly(A+) RNA was purified from the total RNA using a poly(A+) Quick mRNA isolation kit (GE Healthcare Life Sciences, Piscataway, NJ). RNA integrity was verified visually by running RNA samples on 1.2% agarose gels. A directionally cloned (*Eco*RI/*Xho*I) cDNA library was generated from poly(A+) RNA using a Lambda Uni-Zap-XR cDNA synthesis kit according to manufacturer's instructions (Stratagene, La Jolla, CA). The unamplified phage cDNA library was mass-excised *in vivo* and the resulting plasmids (pBluescript II KS-) propagated in the *Escherichia coli* SOLR host strain according to manufacturer's instructions (Stratagene, Inc.). The bacteria were then plated at low density on Luria–Bertani (LB) agar plates containing ampicillin (150 mg/l), X-Gal (25 mg/l) and IPTG (25 mg/l), and the plates were incubated overnight at 37 °C. Randomly selected white colonies were grown in 96-well Deep Well culture blocks (Edge BioSystems, Inc., Gaithersburg, MD) containing 1 ml sterile LB broth or Terrific Broth (TB) per well supplemented with ampicillin (100 mg/l). Replica plates were grown in the same media with the addition of 15% (v/v) glycerol for long-term storage at −80 °C. The initial titer of the primary cDNA library was 1.6×10^4 plague forming units (PFU)/ml and the titer of the amplified library was 1.5×10^8 PFU/ml at the time of *in vivo* mass excision.

2.3. DNA sequencing

Double-stranded plasmid DNA from individual cDNA clones was isolated with a R.E.A.L. Prep 96 BioRobot Kit (Qiagen, Inc., Valencia, CA) in conjunction with the Qiagen BioRobot 3000 according to manufacturer's instructions. Recovered plasmid DNA was resuspended in 50 μl sterile 10 mM TRIS buffer (pH = 8.0) and sequenced from the 5′ end of each cDNA insert with a T3 (5′-GGGAAATCACTCCCAATTAA-3′) primer using the dideoxy chain-termination method on an Applied Biosystems 373A-XL Stretch or 3700 automated DNA sequencing system using the Prism™ Ready Reaction Dyedeoxy™ Terminator Cycle Sequencing kit (Applied Biosystems Division, Perkin-Elmer, Foster City, CA).