Algal Research 23 (2017) 135–149

Contents lists available at ScienceDirect

Algal Research

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

De novo transcriptomic profiling of *Dunaliella salina* reveals concordant flows of glycerol metabolic pathways upon reciprocal salinity changes☆,☆☆

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ARTICLE INFO

Article history:
Received 22 August 2016
Received in revised form 26 December 2016
Accepted 15 January 2017
Available online xxxx

ABSTRACT

Regulation of glycerol contents is vital for adaptation to salinity changes in *Dunaliella salina*. However, transcriptional regulation of genes encoding enzymes involved in glycerol metabolisms upon salinity changes remains controversial. To address this issue, we de novo assembled *D. salina* transcriptome by using Illumina PE90 strategy with an average 700-fold coverage. Transcriptomic profiling analysis of *D. salina* cells in response to reciprocal salinity changes within the range of optimal growth conditions reveals 330 and 553 differentially transcribed ESTs out of a total of 6700 annotated ones upon salinity increase and decrease, respectively. We found 130 common differentially-transcribed ESTs under both conditions, nearly all of which display salinity-correlated transcriptional response. Based on 33 enzymes involved in metabolisms of glycerol and its potential carbon sources, 8 out of 10 common differentially-transcribed ESTs appear to encode enzymes at key sites. Cluster analysis indicated that 28 out of 33 enzymes exhibit salinity-correlated transcriptional profiles in response to reciprocal salinity changes. Transcriptional profiles of the enzymes are in full agreement with the flow of glycerol metabolisms upon salinity changes. This result is consistent with the observation that glycerol and starch contents are positively and negatively correlated with salinity. Furthermore, it indicates that photosynthetic sugar preferentially avails upon salinity decrease, suggesting that photosynthetic sugar is a preferential carbon source for starch accumulation but not glycerol synthesis. Taken together, our analyses demonstrate that transcriptional profiling in response to reciprocal salinity alterations within the range of optimal growth conditions greatly enriches for salinity-specific responsive ESTs in *D. salina*. Under these conditions, we are able to show that transcriptional regulation plays a clear role in controlling enzymatic activities involved in metabolisms of glycerol and its potential carbon sources in *D. salina*. We propose that transcriptional regulation of osmobalancing is likely to be evolutionarily conserved in other halotolerant unicellular organisms.

Abbreviations: AMY-A, alpha-amylase; AMY-B, beta-amylase; DHAK, dihydroxyacetone kinase; DHAR, glycerol 2-dehydrogenase (NADP+); ESR, environmental stress responsive genes; FBPA, fructose-bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphae dehydrogenase; GluPAT, glucose-1-phosphate adenylyltransferase; GPDH-c, soluble glycerol-3-phosphate dehydrogenase; GPDH-m, membrane-associated glycerol-3-phosphate dehydrogenase; GPDH, glycerol-3-phosphate dehydrogenase; GPP, glycerol-3-phosphate phosphatase; HK, hexokinase; PGK, phosphoglycerate kinase; RuBisCO, ribulose-biphosphate carboxylase; RuPK, ribulose-5-phosphate kinase; Sg, granules; Sp, starch plates; SS, starch synthetase; TAG, triacylglycerol.

☆ Competing interests: The authors declare that they have no competing interests.

☆☆ Authors' contribution: JL and XC planned and designed the research, LF, SQ, and JH performed experiments, ZX, WW, XC and JL analyzed data, JL wrote the manuscript.

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http://dx.doi.org/10.1016/j.algal.2017.01.017
2211-9264/

1. Introduction

A number of species in the *Dunaliella* genus are halotolerant, including the well-studied *Dunaliella salina* that is capable of survival within a wide range of salinities from as low as 0.05 M NaCl to as high as 5.5 M NaCl [1–3]. Optimal growth salinity ranges from 0.5 M to 2 M NaCl [4,5]. Cell wall-less *D. salina* cells shrink and expand by up to ~50% of their original volume within seconds to a few minutes (or short-term response) after salinity increase (i.e., hyperosmotic stress) and salinity decrease (i.e., hypoosmotic stress), respectively [6]. *D. salina* cells regain its original cell volume in a few hours to a day (or long-term response) after salinity change by adjusting cellular content of the osmolyte glycerol [7–9].

It is believed that the drastic change of carbohydrate metabolism is triggered by alteration of the surrounding osmotic strength. While the Na^+/H^+ antiporter, Na^+-ATPase, and K^+ carrier may play a role in osmoregulation [10–12], stretch-activated Ca^{2+} channel plays a key role in signaling the glycerol metabolism upon osmotic stresses in *Dunaliella* species [13]. Various ion channels may serve as sensors to change of surrounding osmotic strength. By using radioactive isotope $^{14}CO_2$, salt stress-induced glycerol biosynthesis is found to attribute to both the breakdown of starch and sugars derived from photosynthetic carbon fixation when light avails [14,15]. While glycerol contents in cell of *Dunaliella* species positively correlate with salinities, starch contents are found to inversely correlate with salinities [6,14].

Many studies have focused on analyses of various enzymatic activities involved in glycerol, carbon fixation, and starch metabolisms [14,16]. Dihydroxyacetone phosphate or glycerone phosphate is a key intermediate connecting the glycerol metabolism to carbon fixation and starch metabolism [14]. Activities of enzymes involved in glycerol metabolism such as glycerol-3-phosphate dehydrogenase (GPDH), glycerol-3-phosphate phosphatase (GPP), glycerol 2-dehydrogenase ($NADP^+$) (DHAR), and dihydroxyacetone kinase (DHAK) have been demonstrated in *Dunaliella* species [5,17–21]. Enzymatic activities such as ribulose-5-phosphate kinase (RuPK), ribulose-bisphosphate carboxylase (RuBisCO), phosphoglycerate kinase (PGK), and glyceraldehyde-3-phosphae dehydrogenase (GAPDH) involved in photosynthetic carbon fixation have been shown [17,19,22,23]. Furthermore, activities of enzymes such as starch synthetase (SS), alpha/beta-amylase (AMY-A/B), glucose-1-phosphate adenylyltransferase (GluPAT), hexokinase (HK), and fructose-bisphosphate aldolase (FBPA) involved in starch metabolism have been determined [22,24–26]. Gene sequences of some enzyme isoforms such as soluble GPDH (or GPDH-c), membrane-associated GPDH (or GPDH-m), and FBPA have been cloned in *Dunaliella* species [27–30].

Positive correlation of enzymatic activities such as GPP, DHAR, and GHAK, but not GPDH involved in glycerol metabolism has been established in salinity-adapted *D. salina* cells [31]. A similar study on enzymatic activities such as GPI and FPBA involved in starch metabolisms upon salt stress has concluded that change of glycerol contents is a result of concerted efforts of change of various enzymes involved in the pathways [32]. On the other hand, transcriptional regulation of these enzymes is controversial. While transcription levels of the GPDH-c encoding EST are found to be increased upon salinity increase in some studies [32–35], it is decreased in the other [36]. Additionally, transcription levels of FBPA are shown to be increased by several folds in some studies [36,37] but increased by just a few percent in the other [32]. These discrepancies are likely to be a result of different strengths of salinity, methods of detection, and choices of isoform applied. Measurement of transcriptional changes to all available isoforms of the relevant enzymes using a common technique should address this issue.

Though the *Dunaliella salina* genome draft v1.0 was recently released by the *Dunaliella salina* Genome Sequencing Project (http://phytozome.jgi.doe.gov/), the analysis of the genome has not been published and the usage of the data is somewhat limited. Prior to its release, thousands of expression sequence tags (ESTs) derived from cDNA libraries have been generated for identification of gene sequences involved in osmotic stresses [36,38,39]. Based on ~800 EST sequences, Kim et al. [34] build a *D. salina* cDNA microarray to analyze transcriptional profiling of cells in response to salinity changes from optimal salinity of 1.5 M NaCl to suboptimal salinities of 0.08 M NaCl as water stress and 4.5 M NaCl as salt stress. They identified ~140 ESTs exhibiting 2-fold change or greater upon water and salt stresses, nearly 40% of which were induced or repressed regardless of water or salt stresses [34]. No single EST displays correlated transcriptional alteration with salinity, raising a question whether or not salinity-specific responsive genes would exist in *D. salina*.

Large-scale transcriptional profiling is widely used in analysis of environmental stress responses [40,41]. A set of environmental stress responsive (ESR) genes is found to be common to many different stress factors. This raises an issue whether or not ESR genes would be required for growth fitness under the tested stress conditions. Growth assays using barcoded deletion strains show no correlation between stress responsive genes and growth fitness requirement under the tested stress conditions [42]. Later, careful analysis of cell growth reveals that most of the ESR genes are related to slow growth, indicating that transcription regulation of metabolic enzymes is closely associated with the growth rate [43,44]. Hence, responsive transcripts upon salinity changes could be populated with the slow growth genes, when suboptimal growth conditions apply.

Advances of next generation sequencing technology and de novo short-read assembly algorithm have made high-throughput short-read sequencing a useful tool for genome-wide profiling of transcriptomes in non-model organisms [45]. Hence, we want to perform a genome-wide transcriptional profiling of *D. salina* cells to address whether transcription would play a role in regulation of enzymes involved in metabolisms of glycerol and its potential carbon sources upon salinity changes.

In this study, we report the analyses of transcriptional profiling of *D. salina* cells in response to reciprocal salinity changes from 0.5 M (or 2 M) to 2 M (or 0.5 M) NaCl to avoid suboptimal growth conditions. Based on the deep sequencing-based assembly of the transcriptome, we show that differentially transcribed ESTs upon salinity change greatly enrich for ESTs with salinity-correlated transcriptional profiles or salinity-specific ESTs. Based on the transcriptional profiles, we show that transcriptional regulation of ESTs encoding enzymes involved in metabolisms of glycerol and its potential carbon sources is concordant to the flow of metabolic pathways. Our results indicate that transcriptional regulation play a role in modulation of enzymatic activities involved in metabolisms of glycerol and its carbon sources upon salinity changes. Transcriptional regulation of osmobalancing is likely to be evolutionary conserved in other unicellular microorganisms.

2. Methods

2.1. Algal strain and culture manipulations

The *Dunaliella salina* strain CCAP 19/3 was obtained from the Culture Collection of Algae and Protozoa (CCAP) at UK (www.ccap.ac.uk) and grown in sterile ATCC-1174 DA medium (or DA medium) (www.atcc.org) supplemented with 2.0 M (high salt) or 0.5 M (low salt) NaCl. Cells in shaking flasks were grown under continuous illumination of 50 μmol photon m^{-2} s^{-1} at 25 °C, aerated with 2% CO_2. Cell density was estimated by counting number of cells per ml using a Neubauer hemocytometer (Marienfeld, Germany). Cells were grown in low and high salt media for 3 days or longer were designated as high salt and low salt-adapted cells. In glycerol and starch content analyses, samples were collected in triplicate at 4 h and 24 h after salinity changes for analysis. Decrease of salinity was achieved