The salt-regulated element in the promoter of lycopene β-cyclase gene confers a salt regulatory pattern in carotenogenesis of *Dunaliella bardawil*

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Running title: Salt-regulated element in lycopene β-cyclase promoter

Abstract

In the carotenoid biosynthesis, lycopene β-cyclase (LCYb) is a key regulatory enzyme involved in the conversion of lycopene into β-carotene. Under stress conditions, such as high salinity, high light, and nutrient deprivation, large amounts of β-carotene can be accumulated in *Dunaliella bardawil*. To study on the molecular responses of salt stress in *D. bardawil* is of great significance to reveal the mechanisms of salt tolerance and engineer crop plants to be salt-tolerant. In this study, the full-length coding sequence of *lcyb* from *D. bardawil* (*Dblcyb*, GenBank: KX218392) was isolated by transcriptome sequencing. Then the genomic sequence, promoter and terminator regions of *Dblcyb* were isolated by genome walking. The *Dblcyb* promoter (GenBank: KX218393) contained several typical transcription boxes, multiple light response elements, and a salt-regulated element (SRE, GT1GMSCAM4). *Dbpsy* and *Dblcyb* responsible for β-carotene biosynthesis in *D. bardawil* was shown to be up-regulated under salt stress, and their promoters contained the common SRE. By element deletion analysis and using *Ble-EGFP* as the reporter, the salt-inducible SRE was confirmed to confer salt-induced expression of *Dblcyb* promoter. It was indicated that the salt-regulated expression of *Dblcyb* may be attributed to the salt-responsive element (GT1GMSCAM4) and the GT-rich region in its genomic sequence.

Keywords: *Dunaliella bardawil*; carotenogenic genes; lycopene β-cyclase (LCYb); promoter analysis; salt stress.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/1462-2920.13539

Introduction

Dunaliella bardawil is one of the most highly salt-tolerant organisms, capable to accumulate glycerol, glycine betaine and β-carotene under salt stress, and has been considered as an ideal model organism to reveal the molecular mechanisms of salt stress responses (Ramos *et al.*, 2011). When exposed to stress conditions such as salinity, high irradiance, or nutrient starvation, β-carotene can be accumulated in *D. bardawil*, reaching up to 10% of the dry cell weight (Liang *et al.*, 2015).

Carotenoids not only play an important role in protecting photosynthetic system against photo-oxidative damage, but also act as precursors of abscisic acid, the major hormone that regulates stress responses in plants (McQuinn *et al.*, 2015). The first step of the carotenoids biosynthetic pathway is the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to produce phytoene, catalyzed by phytoene synthase (PSY). Then phytoene is desaturated by phytoene desaturases (PDS) and ζ-carotene desaturases (ZDS) and isomerized by 15-cis-ζ-carotene isomerase (ZISO) and carotenoid isomerase (CRTISO) to form all-*trans* lycopene. Lycopene is converted into β-carotene by the action of lycopene β-cyclase (LCYb) (Liang *et al.*, 2016). Carotenoids accumulation in response to environmental stimuli correlates with induction of some of these enzymes. Nutrient limitation led to steady state transcript levels of *psy* and *pds* correlated with carotenoid accumulation in *Dunaliella salina* under high light and salt stress (Coesel *et al.*, 2008). Gene expression and physiological analyses revealed that *D. salina lcyb* steady state transcript and carotenoid levels were up-regulated in response to salt and light stress, and nutrient depletion (Ramos *et al.*, 2008). Key carotenogenic enzymes of *Dunaliella* genus seem to be regulated at least at the transcriptional level.

It was indicated that *cis*-acting elements and transcription factors can play important roles in plant abiotic stresses (Narusaka *et al.*, 2003; Saibo *et al.*, 2009). In this study, we mainly studied on the *cis*-acting elements in the carotenogenic genes promoter regions under salt stress. Previously, we have analyzed the *cis*-acting elements in the promoters of some carotenogenic genes (such as *Dbpsy* (Lao *et al.*, 2011) and *Dbzds* (Lao *et al.*, 2014)) involved in the carotenoid biosynthesis of *D. bardawil*. It was found that the transcription levsls of some carotenogenic genes in *Dunaliella bardawil* were up-regulated under salt stress (Lao *et al.*, 2014). It was shown that the *Dbpsy* promoter contained a salt-regulated element (SRE) and light-regulated elements involved in stress-responsive gene expression (Lao *et al.*, 2011), and the *Dbzds* promoter had a hypoosmolarity-responsive element (HRE) instead of salt-inducible elements (Lao *et al.*,

2014).

In the carotenoid biosynthetic pathway, LCYb is a key regulatory enzyme involved in the conversion of lycopene into β-carotene, an important dietary precursor of vitamin A beneficial to human nutrition. The *lcyb* genes have been isolated, characterized, and functional identified from various plants, such as citrus fruits (Zhang *et al.*, 2013; Zhang *et al.*, 2012), *Nicotiana tabacum* (Shi *et al.*, 2015), *Lycium chinense* (Jin *et al.*, 2015), *Ficus carica* (Araya-Garay *et al.*, 2011), and *Dunaliella salina* (Ramos *et al.*, 2008). As for the promoter of the *lcyb* gene, it was paid limited attention. To our knowledge, one report was that functional analysis of chromoplast-specific *lycopene β-cyclase* (*CYC-B*) promoter from *Solanum habrochaites* was characterized and a short promoter region was identified to exhibit a higher promoter strength by promoter deletion analysis (Dalal *et al.*, 2010). However, the salt-regulated element (SRE) of the *lcyb* promoter has not been studied. In this study, we first isolated the promoter and terminator of *Dblcyb*, and then use them to construct an expression cassette to express a fusion protein of zeocin resistance protein (Ble) and the enhanced green fluorescent protein (EGFP) in *D. bardawil*. The potential *cis*-acting elements in the *Dblcyb* promoter were identified by PLACE and PlantCARE tools. The SRE of the *lcyb* promoter was confirmed by promoter (element) deletion analysis. The isolation and characterization of the *Dblcyb* promoter can be helpful to understand the regulation of carotenoid biosynthesis pathway in *D. bardawil*.

Results

Identification and characterization of *Dblcyb* coding sequence

The transcriptome sequencing and annotation of *Dunaliella tertiolecta* UTEX LB 999 (NCBI accession number: SRA023642) has been reported (Rismani-Yazdi *et al.*, 2011), which can provide a valuable resource for research on carotenogenic genes in this study. The two documents SRR070441 and SRR070442 of SRA023642 were downloaded in NCBI SRA database. The RNA sequencing reads of *D. tertiolecta* UTEX LB 999 were assembled and clustered into 35,625 unigenes. One unigene annotated as lycopene β-cyclase was found and identified by Blast, PCR (using these two primers: *lcyb* forward, 5'-ATGCAACAAATGCTCAGCAGTC-3'; *lcyb* reverse: 5'-TCAAGCACCAGGGTTGCCACCT-3') and sequencing. It was found that the *lcyb* gene coding sequence from *D. bardawil* FACHB-847 was 100% homology to that from *D. tertiolecta* UTEX LB 999. The complete coding sequence (CDS) of *Dblcyb* was determined as 1,794 bp (GenBank: KX218392) encoding a deduced protein sequence of 597 amino acids, with a theoretical molecular weight of 65.67 kDa. Blast results showed that *Dblcyb* CDS had high

homology to *Dunaliella salina lcyb* (*Dslcyb*) mRNA (GenBank: EU327876.1) (Ramos *et al.*, 2008) with 86% identity, and the predicted protein sequence of DbLCYb had 84% similarity to DsLCYb, and 62~67% homology to *Haematococcus pluvialis* and *Chlamydomonas reinhardtii*. It was predicted that the DbLCYb protein was located in the chloroplast by TargetP tool. Analysis by ChloroP tool indicated that DbLCYb protein has a 55-amino acid transit peptide at the N terminus to target to the chloroplast.

Analyzed by Conserved Domains Search and Pfam tools, it was indicated that the predicted DbLCYb protein belongs to Lycopene_cycl protein family. Amino acid sequences of LCYbs from green algae and higher plants were aligned by ClustalX2, and the result was shown in Figure S1. Several distinct conserved motifs were observed in DbLCYb, such as plant LCYb conserved regions, dinucleotide FAD/NAD-binding domain ($DX_4GXGX_2GX_3A$), cyclase motifs I and II, and charged region. Cyclase motifs were essential domains involved in catalytic activity or substrate recognition (Koc *et al.*, 2015).

Promoters cloning and genomic structures of *Dblcyb* gene

According to the full-length CDS of *Dblcyb*, the promoter region and genomic DNA of *Dblcyb* were isolated by genome walking kit. Sequence analysis by Blastn found that the promoter and terminator regions of *Dblcyb* were 2621 bp (GenBank: KX218393) and 820 bp in length, respectively.

Analysis of the nucleotide sequence of the cloned genomic DNA fragment indicated that *Dblcyb* contains 11 exons interrupted by 10 introns (Figure S2). It was reported that *lcyb* from *Dunaliella salina* also had 11 exons and 10 introns (Ramos *et al.*, 2008). It seemed that the genomic structure of *lcyb* gene from *Dunaliella* genus are much more complicated (more intron numbers and longer average intron length) than that from other algae and plants (Figure S2). One GT-rich region were found in the first intron region of *Dblcyb* genomic sequence. It was previously reported that the GT-rich region in the duplicated carbonic anhydare 1 (*dca1*) promoter in *D. salina* confer salt-induced expression (Li *et al.*, 2010). It was inferred that the salt regulation of *Dblcyb* expression may be associated with this GT-rich region.

Potential *cis*-acting elements in the *Dblcyb* promoter

The *cis*-acting elements of *Dblcyb* promoter was analyzed by PLACE and PlantCARE tools (Table 1). There were several TATA and CAAT boxes that are typical in eukaryotic promoters, multiple light response elements (GATABOX, CGCGBOXAT and SORLIP1AT), ASF1MOTIFCAMV involved in abiotic and biotic stress, and salt-regulated element (SRE, GT1GMSCAM4). Previously, *cis*-acting elements of the promoters of *Dbpsy* (Lao *et al.*, 2011) and *Dbzds* (Lao *et al.*, 2014) have been analyzed (Table 1). *Dbpsy*

and *Dblcyb* promoters contained the same salt-regulated element (SRE), named GT1GMSCAM4. Exceptionally, only *Dbzds* promoter contained a hypoosmolarity-responsive element (HRE).

Verification of the expression activity of the *Dblcyb* promoter

In order to confirm the expression activity of the *Dblcyb* promoter and terminator, the plasmids pET and pLET were transformed in *D. bardawil* by electroporation, respectively. At days 2 and 8 after electrotransformation, only red fluorescence was observed in *D. bardawil* cells transformed with the negative control vector pET. The fluorescence pictures were shown in Figure 2. Some green fluorescence appeared at day 2 after electroporation with pLET vector in *D. bardawil*; however, at day 8 electroporation with pLET, no green fluorescence could be observed. It was indicated that the foreign EGFP protein can be expressed transiently by the *Dblcyb* promoter in *D. bardawil* cells.

Subsequently, pLBET plasmid containing *Ble-EGFP* fusion gene was transformed into *D. bardawil* cells to obtain stable nuclear transformants. Small green algal colonies grown on zeocin selective plates were transferred into liquid medium containing 10 μg/mL zeocin for 21 d. The positive transformants were confirmed by PCR, reverse transcription (RT)-PCR and sequencing to ensure the introduction of the foreign fragment (*Ble-EGFP*) in *D. bardawil*. Brighter green fluorescence could be observed at days 2 and 21 after transformation with pLBET plasmid when zeocin was used for the selective pressure (Figure 2D and 2E). Stable green fluorescence appeared sustainably at day 42 (the second generation of *D. bardawil* cells) (Figure 2F). By contrast, no green fluorescence could be observed in *D. bardawil* cells transformed with the pBET vector. It was obvious that the *Dblcyb* promoter displays strong expression activity, and stable expression of the fusion protein Ble-EGFP can be achieved by the *Dblcyb* promoter.

Identification of the SRE of the *Dblcyb* promoter by element deletion analysis

Previously, the promoter region of *Dbpsy* was isolated and a *cis*-acting element "GT1GMSCAM4" or GT-1 motif with the recognition sequence "GAAAAA" defined as SRE in this study was found, which was potentially involved in the salt-induced expression of *Dbpsy* (Lao *et al.*, 2011). In this study, we also found the GT1GMSCAM4 element (-1367~-1362 bp) in *Dblcyb* promoter as expected. The key carotenogenic genes (*Dbpsy* and *Dblcyb*) responsible for β-carotene biosynthesis in *D. bardawil* was shown to be up-regulated under salt stress (Figure 3A). We supposed that the SRE shared in *Dbpsy* and *Dblcyb* promoters might play a vital role in salt-inducible expression.

In order to test the SRE activity in the *Dblcyb* promoter, the two vectors, pLBET and SRE-deleted

vector pSDBET, were transformed into *D. bardawil*, respectively The positive transformants were grown in liquid medium containing different NaCl concentrations (0 M, 1.0 M, 2.0 M, 3.0 M and 4.5 M) for quantitative real-time analysis. Then qRT-PCR analysis found that the transcription levels of *Ble-EGFP* was increased with the salt concentration in the transformant with pLBET plasmid, i.e. driven by the normal *Dblcyb* promoter (Figure 3B). By contrast, loss of salt-induced expression of *Ble-EGFP* driven by the SRE-deleted promoter in the pSDBET plasmid resulted in no obvious changes in transcription levels of *Ble-EGFP* (Figure 3B). It was indicated that the salt-regulated expression of *Dblcyb* may be attributed to the salt-responsive element (GT1GMSCAM4).

Discussion

Lycopene β-cyclase (LCYb) is one of the crucial enzymes for carotenoid biosynthesis. Recently, the *lcyb* gene from *Nicotiana tabacum* (*Ntlcyb*) was considered not only a cyclase involved in β-carotene accumulation but also confering salt and drought stress tolerance (Shi *et al.*, 2015). It was displayed that *Ntlcyb* was induced by drought, salt, and exogenous abscisic acid treatments. It was reported that the salt tolerance of *lcyb* from *Lycium chinense* (*Lclcyb*) might be ascribed to the enhanced carotenoid content for its ROS scavenging ability, photoprotection and membrane stabilization (Jin *et al.*, 2015). It was indicated that overexpression of the *lcyb* gene from *Salicornia europaea* in transgenic *Arabidopsis* improved plant salt tolerance by increasing synthesis of carotenoids, which impairs reactive oxygen species (ROS) and protects the photosynthesis system under salt stress (Chen *et al.*, 2011). Until now, the function of LCYb in salt stress is not well studied, and the mechanism of the response of *lcyb* gene under salt stress remains to be a crucial research field. One of the strategies to understand the regulation of *lcyb* expression under salt stress is to characterize the promoter of *lcyb* gene. In this study, we isolated and characterized the *Dblcyb* promoter from *D. bardawil*, and several stress-responsive *cis*-acting elements involved in the *Dblcyb* promoter were analyzed, especially the salt-induced element, i.e. GT1GMSCAM4, which was also quite common found in the promoters of stress-inducible genes or in gene promoters from salt-tolerant plants (Guo *et al.*, 2010; Lao *et al.*, 2011; Trivedi *et al.*, 2013). SRE deletion experiment showed that the transcription profiles of exogenous *Ble-EGFP* driven by the *Dblcyb* promoter (pLBET) were the same as that of the endogenous *Dblcyb* transcripts, while the salt-inducible expression trend of *Ble-EGFP* expressed by the SRE-deleted promoter (pSDBET) was disappeared (Figure 3). It was indicated that the salt-responsive element (GT1GMSCAM4) can play a vital role in the salt-regulated expression of *Dblcyb*.

By analysis of the *Dblcyb* genomic sequence, a GT-rich region were found in in the first intron region. GT-rich regions were discovered and confirmed to be salt inducible in the duplicated carbonic anhydrase1 (*DsDCA1*) promoter from *D. salina* (Li *et al.*, 2010). It was indicated that the salt-regulated expression of *Dblcyb* may be attributed to the salt-responsive element (GT1GMSCAM4) and the GT-rich region in the *Dblcyb* genomic sequence.

Salt stress is one of the major environmental factors limiting the productivity and distribution of major crops worldwide (Yamaguchi and Blumwald, 2005). To reveal the molecular mechanisms of salt stress tolerance in plants, especially in haloduric plants, is very urgent. The stress-induced components involved in plant salt stress tolerance, such as metabolites, proteins, genes and miRNAs have been widely studied with the help of 'omics' (metabolomics, proteomics, transcriptomics, and RNomics) analyses (Lu *et al.*, 2011; Urano *et al.*, 2010). It seems that more than one mechanism may play a role in plant salt stress tolerance (Ramos *et al.*, 2011). And the basic mechanisms of salt tolerance for algae are often considered to be similar to that of higher plants. Nowadays, with the development of transcriptomics, biologists and researchers can have access to vast valuable resources for gene discovery and gene isolation. The transcriptome sequencing and annotation of *Dunaliella tertiolecta* UTEX LB 999 has been reported (Rismani-Yazdi *et al.*, 2011), which can be a great help to isolation the *Dblcyb* gene in this study.

Here we mainly focus on the molecular mechanisms of salt stress tolerance in *Dunaliella bardawil*. Previously, the osmoregulatory response of *Dunaliella* at high salinity has been well elucidated (Chen and Jiang, 2009; Zhao *et al.*, 2013). And the acclimation to high salinity of *Dunaliella* is associated with the activation of expression of salt-induced genes and proteins (Katz *et al.*, 2007; Liska *et al.*, 2004). In addition, isolation and characterization of promoters of stress-responsive genes also can help understand the mechanisms of salt regulation in *Dunaliella*. Under the conditions of high salinity, high light, and nutrient deprivation, β-carotene can be accumulated up to 10% dry weight in *D. bardawil* (Liang *et al.*, 2016). To Isolate and characterize the promoters of carotenogenic genes can be helpful to reveal the regulation of carotenoid biosynthesis pathway in *Dunaliella*. Unexpectedly, a hypoosmolarity-responsive element (HRE), instead of salt-inducible elements, was found in the *Dbzds* promoter. The HRE of the *Dbzds* promoter was confirmed to be responsible for hypoosmotic expression by element deletion analysis, and the *Dbzds* gene was not salt-inducible (Lao *et al.*, 2014). It may be a phenomenon that the regulation of key carotenogenic genes is partly attributed to related transcriptional regulatory elements in their promoter

regions.

In this study, the expression levels of *Dbpsy* and *Dblcyb* were increased with the salt concentration (Figure 3A). And *Dbpsy* and *Dblcyb* promoters contained the common salt-regulated element (SRE) (Table 1), i.e. GT1GMSCAM4, which has be confirmed to play a vital role in the salt-regulated expression in *D. bardawil* in this study. It was reported that some carotenogenic genes (*psy2* and *lcyb*) were induced by NaCl, and the productions of β-carotene and lutein were enhanced in *Solanum nigrum* (Abdallah *et al.*, 2016). Dalal *et al.* (2010) have isolated and analyzed the chromoplast-specific *lcyb* (*CYC-B*) promoter from *Solanum habrochaites*, and discovered a short promoter region (436 bp) with a higher promoter strength by promoter deletion analysis and using β-glucuronidase (*GUS*) as a reporter gene. Here we mainly focused on the salt-inducible element in *Dblcyb* promoter and used *Ble-EGFP* as the reporter gene. While the foreign EGFP protein was expressed transiently by the *Dblcyb* promoter in *D. bardawil* cells, stable expression of the fusion protein Ble-EGFP could be achieved by the *Dblcyb* promoter (Figure 2), which may be useful in genetic engineering of carotenoid production in *Dunaliella*.

All in all, we have isolated and functionally characterized the *Dblcyb* promoter. It contained several typical boxes, multiple light response elements, and salt-regulated element. By element deletion analysis, the salt-inducible *cis*-acting element was confirmed to confer salt-induced expression to *Dblcyb* promoter. And it is a practicable and convenient means of using *EGFP* as a reporter gene to identify the promoter activity. To study on the molecular mechanisms of salt stress responses in *D. bardawil* is of great significance to reveal the mechanism of salt tolerance and engineer crop plants to be salt-tolerant. It is possible to use the stress-responsive genes from *Dunaliella* to improve the salt tolerance of other plants and microorganisms by genetic engineering and biotechnology.

Experimental procedures

Strains, plasmids and cultivation conditions.

The green alga *Dunaliella bardawil* strain FACHB-847 was obtained from the Institute of Hydrobiology, Chinese Academy of Science. *D. bardawil* cells were grown in a *Dunaliella* medium (Zhu *et al.*, 2007) at 26 °C under a 16/8 h light/dark cycle with shaking at 96 rpm for about 14 days (14 d). For semisolid culture, semisolid medium was made by adding 0.7 g/L Phytagel (Sigma) to 25 mL of liquid medium; cells were then plated and cultured for about 14 d under the normal condition as above but without shaking. The treatment of salt stress in *D. bardawil* has been mentioned by Lao *et al.* (2014). *D. bardawil* cells cultivated

in the normal liquid medium were harvested by centrifugation at 5,000 g for 8 min at 26°C, transferred to 100 mL of fresh medium containing 0.5 M, 1.0 M, 1.5 M, 2.0 M, 2.5 M, 3.5 M, and 4.5 M NaCl, and cultivated for about 21 d (log or late log phase) under the normal condition. *E. coli* DH5α was used as the host for the multiplication of plasmids. pCR2.1 vector was purchased from Invitrogen. Plasimd pcDNA6 myc-his-EGFP B was kindly provided by Yi-Cheng Cao, School of Biological Science and Engineering, South China University of Technology.

Genomic DNA isolation and promoter cloning

Genomic DNA extraction from *D. bardawil* in the log or late log phase was performed according to the OMEGA HP Plant DNA Kit. Genome walking was implemented with genome walking kit (Takara). The gene-specific primers for isolation the promoter and genomic structure of *Dblcyb* were not shown.

Bioinformatics analysis

Sequence similarity analysis was performed using BLAST program (http://blast.ncbi.nlm.nih.gov/). The sequence alignment was generated using ClustalX2 software. Conserved domains within a protein or coding nucleotide sequence were detected using the Conserved Domains Search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The translation prediction was performed by using the Translation tool (http://web.expasy.org/translate/). The open reading frame (ORF) of a nucleotide sequence was predicted by ORF Finder on NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Subcellular localization of protein was predicted by TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/). Chloroplast transit peptides was predicted by ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/). Gene structure was displayed by GSDS2.0 (http://gsds.cbi.pku.edu.cn/). Promoter analysis was performed with the PLACE (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?lang=en&pj=640&action=page&page=newplace) and PlantCARE websites (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Construction of plasmids

In order to confirm the expression activity of the *Dblcyb* promoter, an exogenous EGFP protein was used as a reporter to express in *D. bardawil*. The Construction of the plasmids used in this study was shown in Figure 1. First, the fragment of the *Dblcyb* promoter with *Asc*I site was ligated into pCR2.1 vector (Invitrogen) to construct the plasmid plcybPro. The *EGFP* fragment was amplified with *Asc*I and *Sac*II sites, and then inserted at the *Asc*I and *Sac*II sites in the plcybPro vector to construct the plasmid pLE. And

the fusion fragment *Ble-EGFP* were amplified with *Asc*I and *Sac*II sites by overlap PCR, and then ligated into the plcybPro vector to construct the plasmid pLBE. The fragment of the *Dblcyb* terminator was amplified with *Sac*II and *Xba*I sites, and then inserted into the pLE and pLBE vector to construct the pLET and pLBET vectors, respectively. The pET and pBET vectors were constructed by TA cloning strategy for negative expression. In order to verify the salt-regulated expression of *Dblcyb*, SRE-deleted vector pSDBET where the SRE of *Dblcyb* promoter was deleted was constructed from the pLBET vector by overlap extension-PCR. The primers used in the construction of plasmids were listed in Table S1.

Nuclear transformation of *D. bardawil* and selection of positive transformants

The method for expression of foreign genes in *D. bardawil* cells was used by electroporation (Lao *et al.*, 2014; Sun *et al.*, 2005). After electrotransformation, the cells was cultivated in liquid medium for 12 h at 25°C in the dark, and then transferred in the normal cultivation condition. The procedure for zeocin resistance selection of transgenic *D. bardawil* was mentioned by Lao *et al.* (2014). The positive transformants were grown in liquid media containing different NaCl concentrations (0 M, 1.0 M, 2.0 M, 3.0 M and 4.5 M) for quantitative real-time PCR analysis.

Total RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

The total RNAs were prepared from *D. bardawil* cells cultivated with different NaCl concentrations in the late log phase using RNAiso Plus Reagent (Takara), respectively. qRT-PCR was performed with a 7500 Real-Time PCR System (Applied Biosystems) using the PrimeScript RT Reagent Kit with gDNA Eraser and the SYBR Green PCR Kit (Takara). Primers for quantitative PCR analysis were listed in Table S2. All the manipulations of qRT-PCR were on the basis of the reference by Liang *et al.* (2016). All reactions were set up in triplicate, and every sample was replicated in parallel three times to ensure statistical relevance. Results were normalized to the glyceraldehyde-3-phosphate dehydrogenase gene from *D. bardawil* (*Dbgapdh*). The data of the transcription levels were expressed as mean ± SD of three parallel measurements (*t* test, $p<0.05$). A *p*-value less than 0.05 was considered significant.

Acknowledgements

This project was supported by the National Natural Foundation of China (31171631 and 31571773), Guangdong Province Science and technology plan project (2011B031200005), and Guangdong Provincial Bureau of ocean and fishery science and technology to promote a special (A201301C04).

Supporting information

Figure S1 Multiple alignment of amino acid sequences of DbLCYb and other algal and plant LCYbs. The following sequences were used for comparison: DbLCYb, LCYb from *D. bardawil* in this study; DsLCYb, *D. salina* (ACA34344.1); CzLCYb, *Chromochloris zofingiensis* (CBH31264.1); CrLCYb, *Chlamydomonas reinhardtii* (AAX54906.1); ApLCYb, *Auxenochlorella protothecoides* (KFM24907.1); IpLCYb, *Ipomoea batatas* (AGL44392.1); SlLCYb, *Solanum lycopersicum* (CAA60170.1); LcLCYb, *Lycium chinense* (AKO73675.1); AtLCYb, *Arabidopsis thaliana* (AEE74874.1).

Figure S2 Distribution of exons and introns in the genomic DNA of *lcybs*. The sequences are used for analysis as followed: *lcyb* from green algae: *Dblcyb* (in this study); *Aplcyb*, *Auxenochlorella protothecoides* strain 0710 (NW_011934213.1); *Cslcyb*, *Coccomyxa subellipsoidea* C-169 (NW_005178037.1); *Mnlcyb*, *Monoraphidium neglectum* (NW_014012103.1); *lcyb* from higher plants: *Rclcyb*, *Ricinus communis* (NW_002994790.1); *Sllcyb*, *Solanum lycopersicum* (NM_001316759.1); *Vvlcyb*, *Vitis vinifera* (NC_012014.3).

Table S1 Primers for construction of plasmids.

Table S2 Primers for qRT-PCR used in this study.

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Table 1 Comprehensive analysis of some stress-responsive *cis*-acting elements of seven gene promoters involved in carotenoid metabolism in *D. bardawil*.

Promoters	*cis*-Acting elements	Functions	References
Dbpsy promoter	GT1GMSCAM4	NaCl-induced expression	(Lao *et al.*, 2011)
	GT1CONSENSUS, BOXIIPCCHS	Light regulation	
	ASF1MOTIFCAMV	Abiotic and biotic stress	
	W-box	Elicitor-responsive element	
Dbzds promoter	PREATPRODH, GBF5BS	Hypoosmolarity-responsive element (HRE)	(Lao *et al.*, 2014)
	GATABOX	Light regulation	
	ASF1MOTIFCAMV	Abiotic and biotic stress	
	W-box	Elicitor-responsive element	
Dblcyb promoter	GT1GMSCAM4	NaCl-induced expression	In this study
	GATABOX, CGCGBOXAT , SORLIP1AT	Light regulation	
	ASF1MOTIFCAMV	Abiotic and biotic stress	
	W-box	Elicitor-responsive element	

Figure legends

Figure 1 Constructions of plasmids used to confirm the expression activity of the *Dblcyb* promoter and the salt-regulated expression of *Dblcyb*.

Figure 2 Fluorescence analysis after transformation (400 ×). (A) Only red fluorescence in *D. bardawil* cells transformed with the negative control vector pET; (B) Some green fluorescence in *D. bardawil* cells transformed with pLET on day 2; (C) No green fluorescence in *D. bardawil* cells transformed with pLET on day 8; (D) Some green fluorescence in *D. bardawil* cells transformed with pLBET on day 2; (E) Strong green fluorescence in *D. bardawil* cells transformation with pLBET on day 21; (F) Strong green fluorescence in the second generation (42 days) transformants with pLBET. All these pictures were obtained under a merge of all fluorescence. The green and red colors in the pictures represent the expression of EGFP and the autofluorescence of *D. bardawil* cells, respectively.

Figure 3 qRT-PCR analysis of carotenogenic genes under salt stress and SRE of *Dblcyb* promoter. (A) Transcriptional differentiation of *Dblcyb* and *Dbpsy* in response to salt stress; (B) Transcriptional analysis of *Ble-EGFP* driven by the *Dblcyb* promoter (pLBET) and the SRE-deleted *Dblcyb* promoter (pSDBET).

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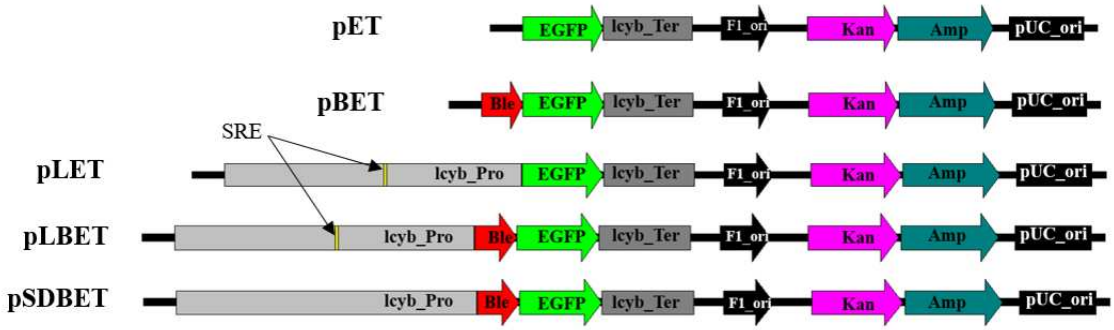

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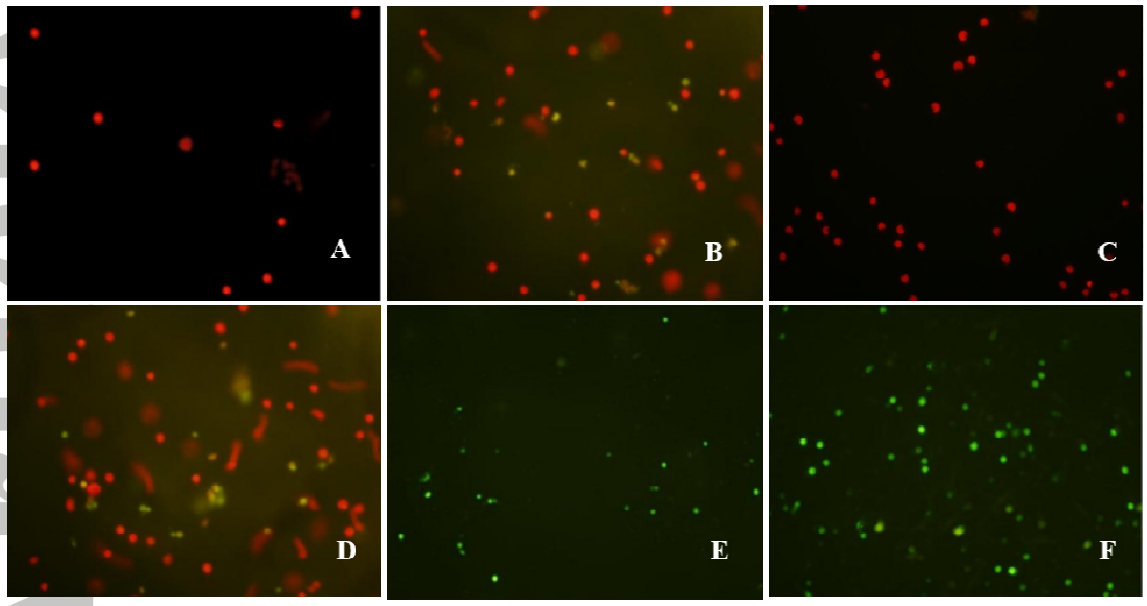

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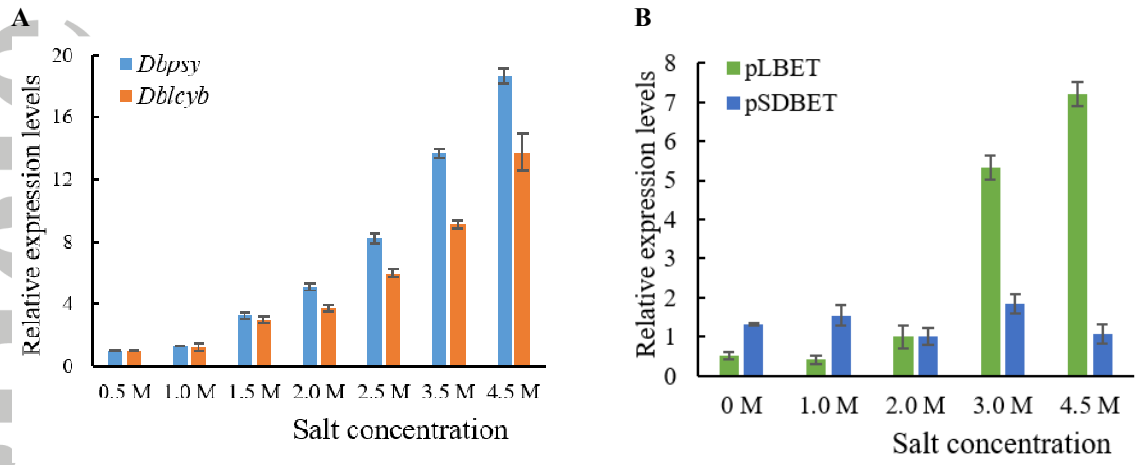

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