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Isolation and characterization of a stress-inducible *Dunaliella salina Lcy-β* gene encoding a functional lycopene β-cyclase

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Abstract The halotolerant green alga *Dunaliella salina* accumulates large amounts of β-carotene when exposed to various stress conditions. Although several studies concerning accumulation and biotechnological production of β-carotene have been published, the molecular basis and regulation of the genes involved in carotenoid biosynthesis in *D. salina* are still poorly known. In this paper, we report the isolation and regulation of the lycopene β-cyclase (*Lcy-β*) gene by abiotic stress. The function of this gene was determined by heterologous genetic complementation in *E. coli*. Gene expression and physiological analyses revealed that *D. salina Lcy-β* steady-state transcript and carotenoid levels were up-regulated in response to all stress conditions tested (salt, light and nutrient depletion). The results presented here suggest that nutrient availability is a key factor influencing carotenogenesis as well as carotenoid biosynthesis-related gene expression in *D. salina*.

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Introduction

Carotenoids are an important group of natural pigments that are found in all photosynthetic organisms (plants, algae and cyanobacteria) and several species of non-photosynthetic bacteria and fungi (Goodwin 1980). These isoprenoid pigments play essential roles in photosynthesis, nutrition and protection against photooxidative damage in higher plants. The increasing importance of naturally occurring carotenoids is due to their beneficial effects on human health and nutrition, such as preventive effects on cancer and chronic diseases (Nkondjock et al. 2005).

In recent years, the biotechnological potential of algal biomass as a source of industrially valuable compounds has been exploited (León-Bañares et al. 2004; Del Campo et al. 2007). One of the species subjected to considerable mass culture in several countries is the green alga *Dunaliella salina* (Teodoresco) since it can accumulate high amounts (more than 10% of algal dry weight) of β-carotene when maintained under growth-limiting conditions, namely high salinity, high temperature, high irradiance and/or limiting nutrients (Ben-Amotz and Avron 1983; Borowitzka et al. 1990; Raja et al. 2007).

Carotenoids are synthesized via the general biosynthetic pathway within the chloroplasts of plants and algae and the cyclization of lycopene is an important branch point in this pathway (for review, see Sandmann 2001). Lycopene, the product of the sequential desaturations of phytoene, is

converted into β-carotene by the action of lycopene β-cyclase (Lcy-β; Sandmann 1994). Several genes of this pathway and distinct types of lycopene cyclases have been cloned from prokaryotic and eukaryotic organisms (e.g. *Haematococcus pluvialis* and *Chlamydomonas reinhardtii*; Steinbrenner and Linden 2003; Lohr et al. 2005) and also successfully targeted by genetic engineering (Römer and Fraser 2005). Nevertheless, the regulation of the carotenogenesis still remains poorly understood in either wild-type or recombinant hosts and also in the particular case of *D. salina*.

In other green algae, such as *H. pluvialis* and *C. reinhardtii*, the regulation of carotenoid gene transcript levels seems to be related to the stress (e.g. high light and high salt) response and, in some cases, with carotenoid accumulation (Grünewald et al. 2000; Bohne and Linden 2002). For example, *H. pluvialis Lcy-β* and other carotenoid biosynthesis-related genes were found to be up-regulated in response to light (Steinbrenner and Linden 2003).

Recent evidence obtained in our research group by means of quantitative polymerase chain reaction (qPCR) indicates that high salinity and high light enhance the steady-state transcript levels of phytoene synthase (*Psy*) and phytoene desaturase (*Pds*) carotenogenic enzymes in *D. salina* cells under nutrient deprivation. Conversely, in nutrient-rich media, the effect of salinity and light intensity on gene expression was dampened or abolished altogether (Coesel et al. 2008).

In the present work, we report the isolation and characterization of the *D. salina Lcy-β* (*DsLcy-β*) gene. The regulation of this gene in response to abiotic stress conditions was assessed as well as its ability to convert lycopene to β-carotene in vivo.

Materials and methods

D. salina strain, culture conditions and analytical methods

The green algae *D. salina* strain CCAP 19/30 was obtained from the Culture Collection for Algae and Protozoa (Cumbria, UK). The cells were cultured in modified Walne medium (Walne 1974) containing: 128 μM NaH_2PO_4, 141 μM Na_2–ethylenediamine tetraacetic acid (EDTA), 1.2 mM $NaNO_3$, 2.2 μM $FeCl_3$, 544 μM H_3BO_3, 1.8 μM $MnCl_2$, 154 nM $ZnSO_4$, 78 nM $CuSO_4$, 84 nM $CoCl_2{\cdot}6H_2O$, 56 nM $Na_2MoO_4{\cdot}2H_2O$, 3.7 nM cyanocobalamin and 296 nM thiamine in UV-treated filtered seawater (salinity adjusted to the desired level with NaCl—9% or 18% [*w*/*v*]). *Dunaliella* was cultivated in round glass flasks (1–5 L) under continuous illumination (45 μmol m^{-2} s^{-1}) provided by Phillips cool daylight tubes at 22±2°C and were bubbled continuously with filtered-sterile air. The cell number was determined using a 0.1-mm-deep counting Neubauer chamber. Total nitrate concentration of the media was determined according to Navalho (1997), and it was used to monitor nutrient depletion. The total β-carotene content of cells (500-μL culture samples) was extracted with acetone (1 mL of 80% [*v*/*v*]), and the extinction coefficient ε_{435} nm=0.233 L mg^{-1} cm^{-1} was used for quantification (Goodwin 1980). All the measurements were repeated in triplicate, and their averages were determined. Data were analysed by means of analysis of covariance and a post-hoc comparison test (Fisher's least significant difference method—LSD) with the computational program STATISTICA v. 6.0 (StatSoft). Differences were considered to be significant at $p<0.05$.

Abiotic stress growth conditions

Three abiotic stress conditions were tested: high salt, nutrient depletion and high light. All experiments were carried out on exponentially growing cultures at a cell density of approximately 2.5×10^5 cells mL^{-1}. Salt shifts from 9% to 18% (*w*/*v*) NaCl were performed by diluting the cultures with an equal amount of Walne medium (nutrient-supplemented) or water (nutrient-depleted) at a salinity of 27% (*w*/*v*). This method of imposing nutrient and salt stress can be easily reproduced when cultures of large volumes are needed for specific biotechnological applications. High light stress was imposed by increasing light intensity from 45 to 500 μmol m^{-2} s^{-1} by means of a 150-W Massive N. V./S.A. white light lamp.

Isolation of genomic DNA and RNA

Genomic deoxyribonucleic acid (DNA) extraction from cells in the late log phase (10 ml) was performed according to the method described by Long et al. (1989). For preparation of complementary DNA (cDNA) and northern blotting analysis, total ribonucleic acid (RNA) was isolated from $5–10\times10^6$ cells using the TRI REAGENT™ (Sigma) procedure according to the manufacturer's instructions.

Cloning of full-length *DsLcy-β* cDNA

cDNA synthesis and subsequent amplification reactions were performed with the SMART™ rapid amplification of cDNA ends (RACE) cDNA Amplification Kit (BD Biosciences Clontech). Alignment of the Lcy-β polypeptides deposited in GenBank revealed the conserved amino acid stretches and a set of four degenerated primers were designed (Lcyβ1Fw, Lcyβ2Rev, Lcyβ3Fw and Lcyβ4Rev; Table 1). RACE was performed with a series of gene-specific primers in order to

Table 1 Nucleotide sequences of primer pairs used for PCR amplification

Primer	Sequence (5′→3′)
cDNA amplification	
Lcyβ1Fw[a]	TBTGGGTBGAYGAGTTYGAGGCNATG
Lcyβ2Rev[a]	CSCKCTGVGGCAGVACRGGCAGRGG
Lcyβ3Fw[a]	AYAACCCYGGYTAYCAGGGTBGC
Lcyβ4Rev[a]	TRAARGGCATNGCRTACAGRAARGT
Lcyβ5Fw	GGCTCAGAAAAAATTGCTAACTCAC
Lcyβ6Rev	TTTAGGAATCCATCACAAGCCAATACC
Genome-walking PCR	
AP1	GTAATACGACTCACTATAGGGC
GW-Lcyβ1	GTACGGCATCACAGCAGAGGTTGAGTCC
PCR for probe preparation	
LcyβPFw	TTCAACCCAGGGTACCAG
LcyβPRev	CGATAGCGTCCGCAACAAC
Genetic complementation—pQE-80L	
pQE1Fw	CGCGGATCCGCGATGCTTCAAACACTGAGCGGTCGA
pQE2Rev	CGCGTCGACGTCGGCCTATTGCTGCTTTGCAGC

[a] IUBMB codes used for mixed nucleotides: *B* G/T/C, *K* G/T, *N* A/C/G/T, *R* A/G, *S* G/C, *V* G/A/C, *Y* C/T

reach the 5′ and 3′ ends of the gene (data not shown). Furthermore, for the amplification of *DsLcy-β* full-length cDNA, the sense primer Lcyβ5Fw and the anti-sense primer Lcyβ6Rev were used (Table 1). The amplification procedure was as follows: one cycle of 94°C, 120 s; 35 cycles of 94°C, 60 s; 54°C, 60 s; 72°C, 90 s; and one cycle of 72°C, 10 min. All amplified cDNA fragments were cloned into pGEM-T Easy Vector (Promega).

Cloning of *DsLcy-β* genomic DNA

GenomeWalker libraries were constructed from *D. salina* genomic DNA using the Universal GenomeWalker™ Kit (BD Biosciences Clontech) according to the manufacturer's protocol. The initial PCR was carried out using an adaptor primer AP1 and an *Lcy-β* gene-specific sense primer GW-Lcyβ1 designed on the basis of the known *DsLcy-β* cDNA sequence (Table 1). Subsequently, additional gene-specific primers were synthesized for DNA walking (data not shown), and the amplified fragments were cloned as described in the previous section.

DNA sequencing and analysis

Sequences were determined for both strands using an ABI Prism automated sequencing system (PE Biosystems, Macrogen—Korea), and computer analysis of nucleotide and derived amino acid sequences were done using DNASTAR (Lasergene) and Geneious Pro 2.5.3 (Biomatters) software packages. A phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei 1987). The search for putative chloroplast transit peptides was carried out with the ChloroP 1.1 programme (Emanuelsson et al. 1999), and the analysis of intron/exon structure of the *DsLyc-β* gene was performed with Spidey 1.0 programme (Wheelan et al. 2001).

Northern blot analysis

Total RNA (6 μg) was denaturated in formaldehyde and formamide, run on a 1% (*w*/*v*) denaturing agarose gel (5% formaldehyde) and transferred to a Hybond™-N nylon membrane (Amersham Biosciences) according to the manufacturer's instructions. Loading of equal quantities of RNA on agarose–formaldehyde gels was confirmed by staining with ethidium bromide. Upon blotting, the membranes and gels were tested for complete transfer under UV light. The membranes were pre-hybridized at 37°C for 1 h in ULTRA-hyb hybridization buffer (Ambion) and hybridized at 42°C for 16 h in the same solution after adding the ^{32}P-labelled cDNA probe. Probed membranes were washed twice at 42°C with 2× salt–sodium phosphate–EDTA (SSPE) containing 0.1% (*w*/*v*) sodium dodecyl sulphate (SDS) for 10 min and twice at the same temperature for 20 min with 0.1× SSPE containing 0.1% (*w*/*v*) SDS. Membranes were subsequently exposed to X-ray film (Kodak Biomax MR), and signal intensities from autoradiographs were quantified using the Quantity One software (Bio-Rad Laboratories) and compared to ribosomal RNA (rRNA) loading. The gene-specific probe was prepared via PCR using the primers LcyβPFw and LcyβPRev (Table 1). Probe labelling was performed with Prime-It II Random Primer Labeling Kit (Stratagene).

Genetic complementation assay

The *DsLcy-β* full-length cDNA was amplified by PCR, cut with *Bam*HI and *Sal*I at sites introduced via the pQE1Fw and pQE2Rev primers (Table 1), purified and cloned into a

similarly digested pQE-80L vector (Qiagen). *E. coli* strain TOP10 (Invitrogen) was co-transformed with pAC-LYC (plasmid harboring carotenoid biosynthesis genes for producing lycopene; Cunningham et al. 1994), and pQE-*DsLcy-β* and cultures were plated on Luria–Bertani (LB) plates containing 50 μg/mL chloramphenicol, 100 μg/mL ampicillin and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Petri plates were incubated at 37°C for 16 h and then at room temperature for 5 days to allow maximum colour development.

Carotenoid extraction and HPLC analysis

For analysis of carotenoids from *E. coli* cells, a 0.5-mL aliquot of an overnight culture was used to inoculate 50 mL LB medium with the respective antibiotics in a 250-mL Erlenmeyer flask. Cultures were grown in the dark at 37°C for 48 h (at an optical density at 600 nm of 0.5–0.6, 1 mM IPTG was added) and harvested by centrifugation, and the pellet was re-suspended in 10 mL acetone. Samples were incubated at 65°C for 15 min and centrifuged, and the supernatant was dried under a stream of N_2 and stored at −20°C until required for analysis. Carotenoids were separated by reverse-phase high-performance liquid chromatography (HPLC) using an analytical reversed-phase column C-18 Nucleosil (250 mm×4 mm, inner diameter, particle size 5 μm, Macherey-Nagel, GmbH KG). Samples of 50-μL acetone-dissolved pigments were injected to a Waters 600 pump. The mobile phase consisted in an isocratic system of acetonitrile–dichloromethane–methanol (65:26:9) and eluted at a flow of 1 mL min^{-1}. Light absorption peaks were detected in the range of 200–600 nm using a Waters 996 photo diode-array detector. All spectra were recorded in the eluting HPLC solvent as was the fine absorbance spectral structure. Carotenoids were identified by their characteristic absorption spectra and their typical retention time, which corresponded to standard compounds of lycopene and β-carotene (Sigma). Peak areas were integrated by the MassLynx™ 4.0 software (Micromass, UK).

Nucleotide sequence accession numbers

The *DsLcy-β* cDNA and genomic DNA sequences were deposited in GenBank under the accession numbers EU327876 and EU327877, respectively.

Results

Isolation and characterization of the *DsLcy-β* gene

Using a combination of degenerated primers, a partial *DsLcy-β* cDNA fragment was isolated; a full-length cDNA clone with 1,910 bp was later obtained by means of RACE PCR. In silico analysis revealed one open reading frame (84 to 1,835 bp) encoding a deduced protein sequence of 584 amino acids, with an estimated molecular weight of 64.5 kDa. The amino acid sequence of DsLcy-β showed the highest overall sequence similarity with other monomeric Lcy-β polypeptides from green algae (*H. pluvialis*—67% and *C. reinhardtii*—64%; GenBank accession numbers AAO64977 and AAX54906, respectively) and several plants (e.g. *Arabidopsis thaliana*, *Citrus sinensis*, *Adonis palaestina*, *Cryptomeria japonica*, *Lycopersicum esculentum*—49–50%).

A phylogenetic analysis of the monomeric lycopene cyclases from bacteria, cyanobacteria and plants is illustrated in Fig. 1a. Lycopene cyclases from bacteria and cyanobacteria are polypeptides of about 400 amino acids, whereas plant Lcy-β sequences have more than 500 amino acids. DsLcy-β is more closely related to the other green algae Lcy-β (*H. pluvialis* and *C. reinhardtii*) and the cluster formed by these organisms is also closely related with plant enzymes.

Several distinct conserved patterns in the amino acid sequences already identified in other lycopene cyclases from bacteria and plants were also observed in DsLcy-β, namely one potential dinucleotide-binding region (Fig. 1b; Krubasik and Sandmann 2000; Sandmann 2002). Analysis with the ChloroP program identified a putative chloroplast transit peptide at the N terminus suggesting that DsLcy-β is targeted to the chloroplast.

To obtain the full-length *DsLcy-β* genomic sequence, a genome-walking approach was performed. Analysis of the full-length *DsLcy-β* cDNA and genomic sequences revealed the presence of 11 exons and 10 introns in the *DsLcy-β* gene nucleotide sequence. Exon size ranged between 77 (exon III) and 377 bp (exon I), and intron size reached 1.8 kb (intron V). Overall, approximately 21% (1.9 of 9 kb) of the *DsLcy-β* gene corresponds to the exon sequences (Fig. 2). In some introns, the consensus GT donor and AG acceptor sequences at the 5′ and 3′ termini were found. The 5′ donor junctions and the 3′ acceptor sequences were also similar to those of higher plants (C(A) AG/GTAAGT and TGCAG/G, respectively; Brown 1986) and also to the canonical eukaryotic consensus sequences (Mount 1982). Furthermore, Southern analysis at high and low stringency conditions did not alter the hybridization signal of a single band (data not shown), suggesting the absence of additional copies of the *Lcy-β* gene in the *D. salina* genome.

Expression patterns of *DsLcy-β*: abiotic stress

The effect of hyper-saline shock (9% to 18% NaCl) and/or nutrient depletion (−N) in the steady state of *DsLcy-β* messenger RNA (mRNA) levels is illustrated on Fig. 3d.

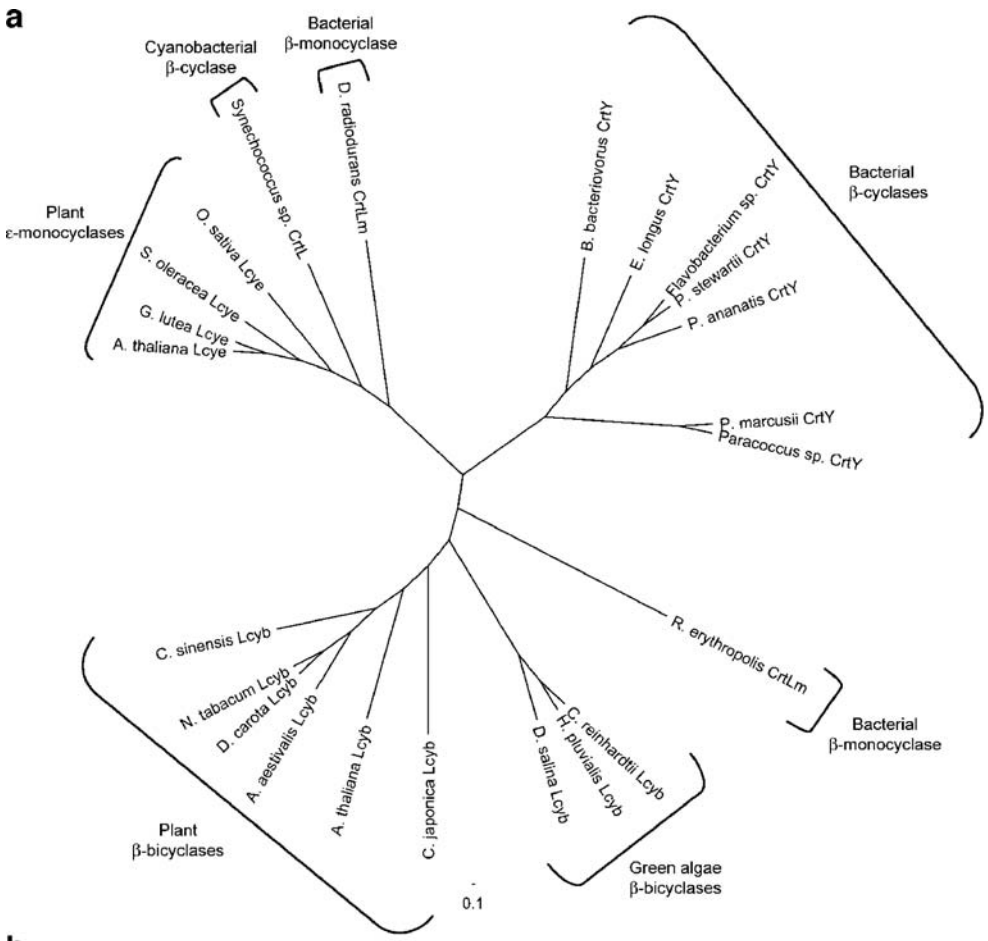

Fig. 1 Unrooted phylogenetic tree of monomeric lycopene cyclases (**a**) and amino acid sequence alignments of Lcy-β, corresponding to the five conserved regions from bacteria, cyanobacteria and plant (first domain corresponds to a putative dinucleotide-binding region) (**b**). The accession numbers for the corresponding sequences are as follows: *Synechococcus* sp. CrtL, Q3AIA0; *Deinococcus radiodurans* CrtLm, Q9RW68; *Rhodococcus erythropolis* CrtLm, Q6T3U7; *Bdellovibrio bacteriovorus* CrtY, Q6MMA6; *Erythrobacter longus* CrtY, O06756; *Flavobacterium* sp. CrtY, P94791; *Pantoea stewartii* CrtY, Q8GCS2; *Pantoea ananatis* CrtY, P21687; *Paracoccus marcusii* CrtY, Q9RLH5; *Paracoccus* sp. CrtY, P54974; *C. reinhardtii* Lcy-β, Q4VKB6; *H. pluvialis* Lcy-β, Q330P8; *D. salina* Lcy-β, EU327876; *C. sinensis* Lcy-β, Q8LPP7; *Nicotiana tabacum* Lcy-β, Q43578; *Daucus carota* Lcy-β, Q2VEX7; *Adonis aestivalis* Lcy-β, Q9AXL1; *A. thaliana* Lcy-β, Q38933; *C. japonica* Lcy-β, Q76J03; *Oryza sativa* Lcy-ε, Q8LJ81; *Spinacea oleracea* Lcy-ε, Q8VWR6; *Gentiana lutea* Lcy-ε, Q1XIT2; *A. thaliana* Lcy-ε, Q38932

After 36 h of growth, an increase in steady-state *DsLcy-β* transcript levels was observed in cells submitted to nutrient (9% −N) or salt (18% +N) stress or the combination thereof (18% −N), when compared to the non-stressed control (9% +N). Maximum transcript levels were found in cells submitted to a salinity up-shift and nutrient depletion (18% −N). Similar results were found at 72 h upon stress onset (data not shown).

β-Carotene accumulation (per millilitre and per cell) was higher in cultures subjected to nutrient depletion at 72 h after the stress onset, and the highest value (per cell) was obtained under the combination of salt stress and nutrient depletion (18% −N) at 36 and 72 h. In the control culture (9% +N), when nitrate concentrations decreased (36–72 h), β-carotene accumulation was also observed (Fig. 3a–c). Significant differences (Fisher's LSD test) in β-carotene

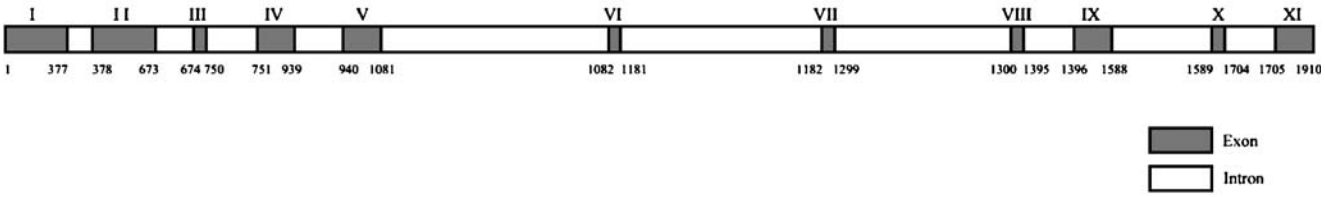

Fig. 2 *D. salina Lcy-β* gene organization (accession number EU327877). The diagram showed that *Lcy-β* consisted of 11 exons (*I–XI*) and 10 introns. *Numbers* represent the cDNA coordinates (bp)

accumulation (μg mL^{-1}) were also found between control (9% +N) and nutrient-depleted (9%-N) cells as well as the latter and salt-challenged cells (18% +N).

The regulation of the *DsLcy-β* steady-sate transcript levels was analyzed following the induction of carotenogenesis by high light (Fig. 4). Upon induction, the highest *DsLcy-β* steady-state mRNA levels were obtained in cells submitted to high (500 μmol m^{-2} s^{-1}) or low (45 μmol m^{-2} s^{-1}) light combined with nutrient depletion at 24 and 48 h, respectively (Fig. 4d). A similar pattern was observed in cellular β-carotene content (per ml and per cell), and accumulation was faster in the case of exposure to high light conditions at 24 h (Fig. 4b, c). After 48 h of induction, the highest values of β-carotene accumulation (per cell) were observed in cells under nutritional stress at both light intensities. However, under these conditions, the cells exposed to high light accumulated about twofold the value obtained in cells exposed to low light conditions (Fig. 4c).

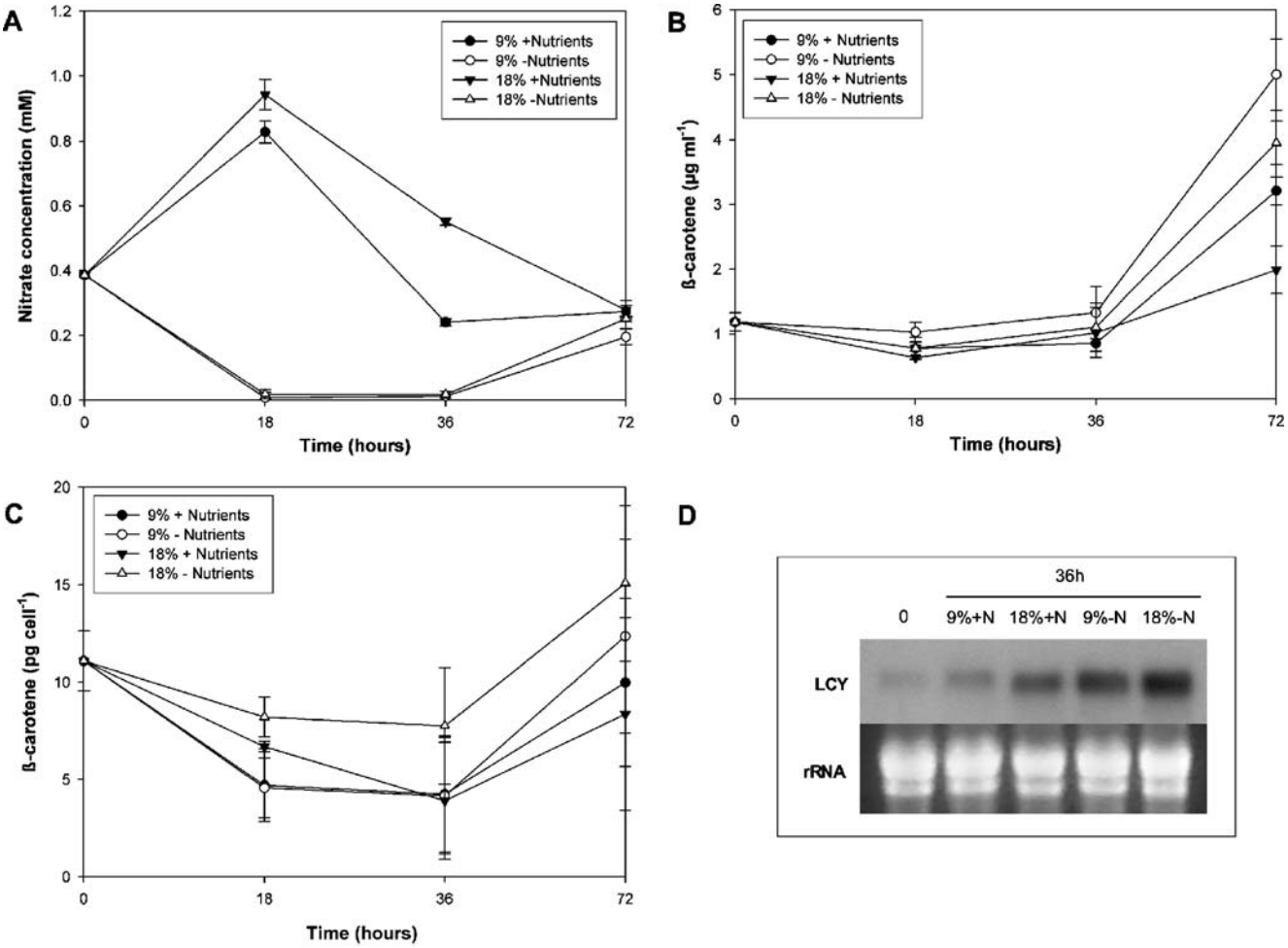

Fig. 3 Effect of hyper-saline shock and/or nutrient supplementation on a nitrate consumption, **b**, **c** β-carotene concentration and **d** steady-state *DsLcy-β* mRNA levels. *D. salina* cells pre-adapted to Walne medium 9% salinity and low light conditions. Cells were transferred to a nutrient-supplemented medium at 9% (*9%+N*) or 18% (*18%+N*) salinity and to nutrient-depleted medium (*9%−N* and *18%−N*). Plotted data are the average±SD of three replicates. For northern blot analysis, RNA samples were collected before (*lane 0*) and 36 h upon the onset of stress, and a *DsLcy-β*-specific cDNA was used as a probe. For comparison, total RNA was stained with ethidium bromide

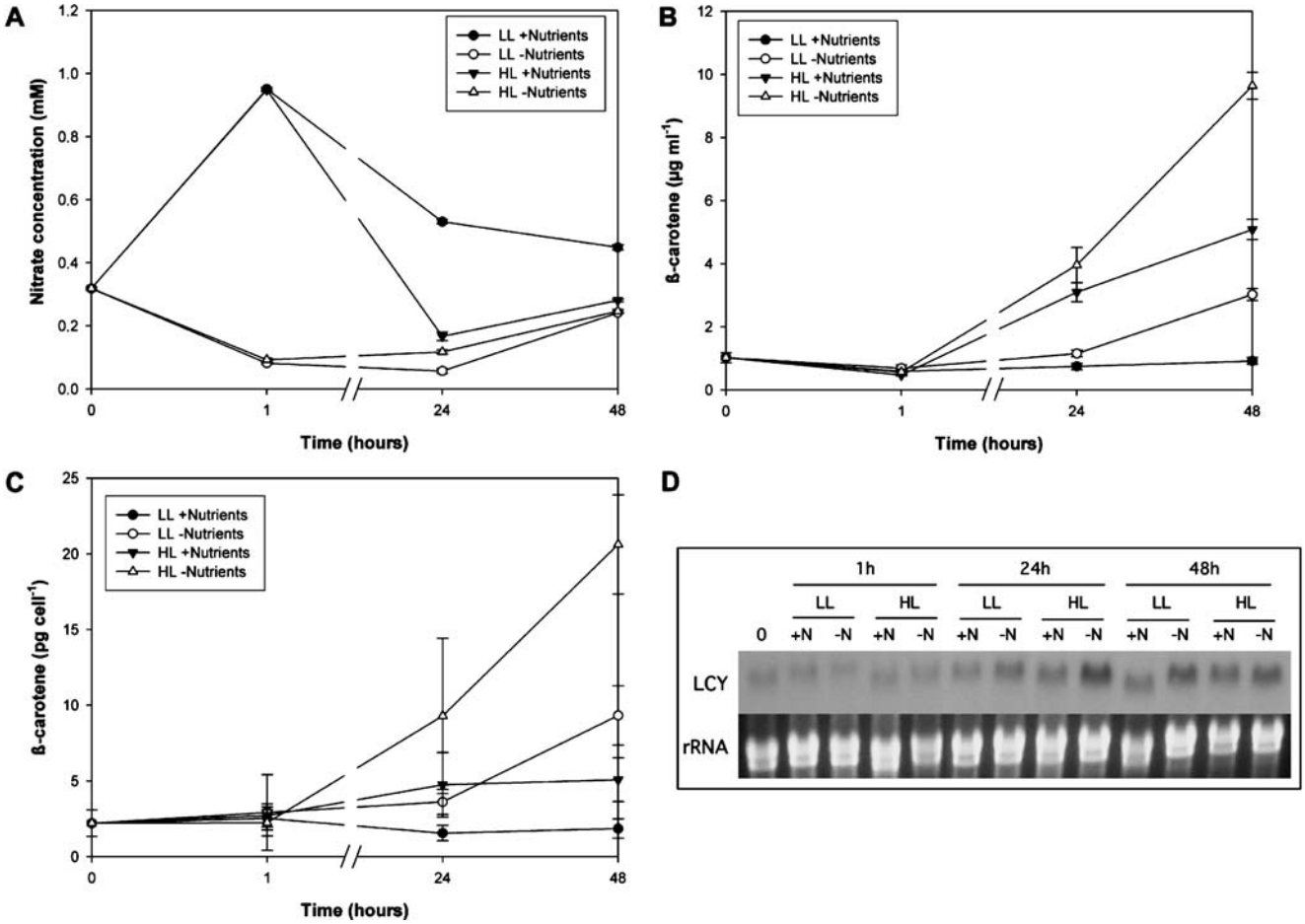

Fig. 4 Effect of light stress on **a** nitrate consumption, **b**, **c** β-carotene concentration in *D. salina* cells and **d** steady-state *DsLcy-β* mRNA levels. Cells pre-adapted to Walne medium 9% salinity and low light conditions (*LL*) were subjected to a light intensity up-shift to 500 µmol m^{-2} s^{-1} (*HL*) and transferred to either nutrient-supplemented medium (+*N*) or nutrient-depleted medium (−*N*), both at 9% salinity. Plotted data are the average±SD of three replicates. For northern blot analysis, RNA samples were collected before (*lane 0*) and 3, 24 and 48 h upon the onset of stress, and a *DsLcy-β* cDNA was used as the gene-specific probe. For comparison, total RNA was stained with ethidium bromide

Statistically significant differences (Fisher's LSD test) of β-carotene accumulation (µg mL^{-1}) between all the tested conditions were found.

Functional expression of *DsLcy-β* in *E. coli*

The use of *E. coli* cells as a heterologous system for carotenoid biosynthesis has proven to be an efficient method for identification and functional characterization of carotenoid biosynthetic genes (Cunningham et al. 1994). HPLC analysis of TOP10 cell extracts showed that the strain containing pAC-LYC produced lycopene, while cells with both pAC-LYC and pQE-*DsLcy-β* accumulated β-carotene. As a negative control, cells co-transformed with pAC-LYC, and empty pQE-80L accumulated lycopene (Fig. 5). These results indicate that the polypeptide encoded by the *DsLcy-β* cDNA clone is a functional Lcy-β able to convert the acyclic lycopene into β-carotene in *E. coli*.

Discussion

Despite the publication of extensive studies on physiology, ecology and commercial applications of *Dunaliella* spp., the molecular basis of stress-induced intracellular β-carotene accumulation in this photosynthetic organism is still poorly known (Oren 2005). Additionally, only a few genes of isoprenoid and carotenoid biosynthetic pathways have been described for this unicellular green alga (e.g. 1-deoxyxylulose-5-phosphate synthase-*Dxs*, *Psy* and *Pds*; Yan et al. 2005; Sánchez-Estudillo et al. 2006).

In the present report, we describe the isolation and expression of a novel gene of this pathway. Functional complementation in *E. coli* revealed that the isolated gene codes for a bona fide Lcy-β. Furthermore, sequence similarity analysis with other Lcy-β from different organisms revealed a close relationship with other green algae β-cyclases and also between this cluster and plant β-

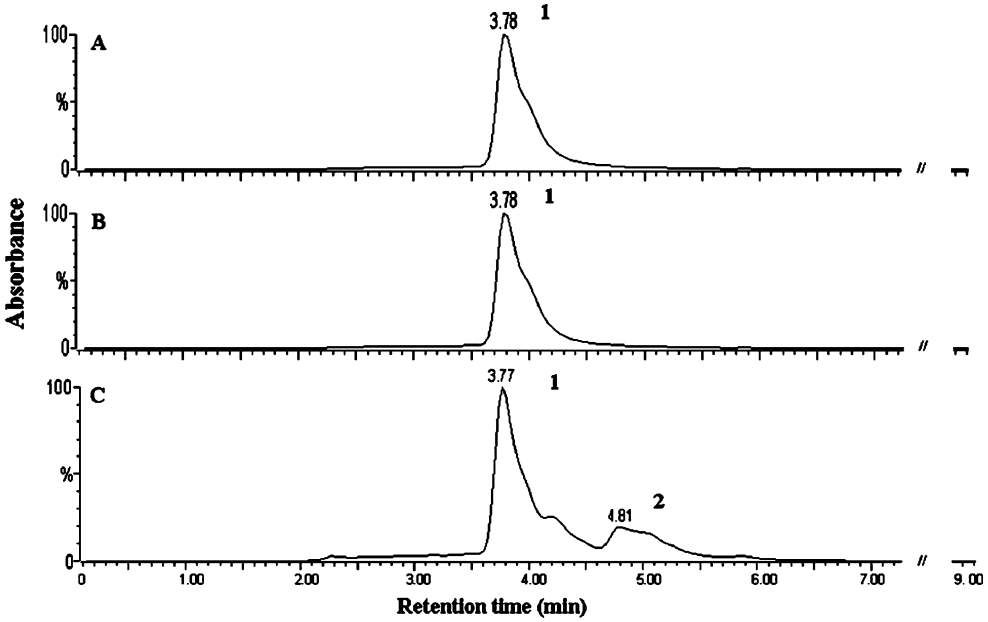

Fig. 5 HPLC elution profiles of carotenoid pigments extracted from cultures of *E. coli* carrying plasmids pAC-LYC (**a**), pAC-LYC/pQE-empty (**b**) and pAC-LYC/pQE-*DsLcy-β* (**c**). Carotenoids were identified by their absorption spectra (lycopene—474 nm; β-carotene—454 nm) and their retention times as compared to standards. Peaks *1* and *2* correspond to lycopene and β-carotene, respectively. The peak between peaks 1 and 2 (retention time=4.21) most probably corresponds to γ-carotene, an intermediate product of lycopene cyclization

cyclases. As described by other authors, the eubacterial lycopene β-monocyclases (*Synechococcus* sp. CrtL, *Deinococcus radiodurans* CrtLm and *Rhodococcus erythropolis* CrtLm) are closely related to plant Lcy-β and -ε cyclases and thus might correspond to an evolutionary link between these and bacterial CrtY-type lycopene cyclase (Sandmann 2002; Tao et al. 2004).

To our knowledge, this is also the first report concerning the genomic organization of *Lcy-β*, and no published information regarding this analysis in other eukaryotic organisms was found. The genomic sequence of the *DsLcy-β* gene revealed the presence of large introns, and Southern blot analysis suggests the existence of only one copy of the *Lcy-β* gene in the *D. salina* genome.

In *Dunaliella* species, several abiotic factors are known to be inductive conditions of carotenogenesis such as low levels of nitrate and sulphate, high salinity and high light (Loeblich 1982; Borowitzka et al. 1990; Vorst et al. 1994; Cifuentes et al. 1996). These factors retard cell division and increase the β-carotene-to-chlorophyll ratio (Ben-Amotz et al. 1982).

Under stress-inductive conditions, β-carotene intracellular accumulation was higher in cells in low-nutrient media (usually with nitrate levels lower than 50 μM). This effect was enhanced by high light and, to a lesser degree, by high salt conditions. Similar results were described for other *Dunaliella* strains (Loeblich 1982; Ben-Amotz and Avron 1983). Furthermore, high light intensity coupled with nutrient deficiency revealed the highest accumulation values of this carotenoid and supports the role of β-carotene in light harvesting and photoprotection (Ben-Amotz 1987; Peñuelas and Munné-Bosch 2005).

Carotenoid formation is known to be highly regulated and the previous environmental stress conditions were used to investigate the transcriptional regulation of *Lcy-β* gene expression in *D. salina*. Steady-state *DsLcy-β* mRNA levels increased when *D. salina* cells were submitted to abiotic stress conditions, and the highest levels were obtained in the case of salt and high light combined with nutrient depletion. Thus, our results suggest that the effect of high light and salinity on carotenogenesis and *DsLcy-β* gene expression is highly dependent on low nutrient levels. It is interesting to note that although high salt and high light by themselves were able to induce slightly higher *DsLcy-β* steady-state transcript levels, compared to control cells, low nutrient availability seems to be essential for β-carotene accumulation in this microalga. Recent data from our laboratory have shown that *D. salina Psy* and *Pds* are similarly regulated (Coesel et al. 2008).

Taken together with the fact that transcriptional inhibitors block carotenoid accumulation in *D. salina* (Lers et al. 1990), these results suggest that transcriptional regulation may be a key factor in carotenogenesis induction in *D. salina* and other carotenogenic organisms (Almeida and Cerdá-Olmedo 2008). Several studies in other algae, namely *H. pluvialis* and *C. reinhardtii*, described the up-regulation of other carotenogenesis-related genes in response to stressful conditions (Grünewald et al. 2000; Steinbrenner and Linden 2001, 2003; Bohne and Linden 2002). Moreover, regulatory control at the transcriptional level of carotenoid biosynthesis seems also to be evident during the development of fruit and flowers in higher plants (Giuliano et al. 1993; Bouvier et al. 1998; Moehs et al. 2001; Bramley 2002; Zhu et al. 2002). However, it is unlikely that the observed simultaneous upregulation of genes encoding the first three carotenoid biosynthetic enzymes to be the only mechanism controlling this

pathway. Additional regulatory factors involving posttranscriptional, translational and metabolic regulation should be considered (Welsch et al. 2003; Römer and Fraser 2005), such as feedback regulation by metabolites (e.g. retinol and other β-ring-containing compounds) or end-products (Lois et al. 2000; Bramley 2002; Cunningham 2002; Almeida and Cerdá-Olmedo 2008) and redox control (Steinbrenner and Linden 2003; Woitsch and Römer 2003).

As transcriptional regulation seems to be a key regulatory step, recent developments in genetic manipulation and genome-wide approaches will allow the use of *D. salina* as a molecular toolkit for several metabolic engineering applications (León-Bañares et al. 2004; Tan et al. 2005). Molecular techniques such as gene silencing of the isolated *DsLcy-β* gene may provide a way of enhancing lycopene content in this organism (Diretto et al. 2007). Recently, transgenic *Halomonas elongata* has been suggested as an alternative source of carotenoids in halophilic growth conditions. However, the use of genes from a non-halophilic species (*Pantoea agglomerans*) may have led to reduced carotenoid production under high salinity (Rodríguez-Saíz et al. 2007). Thus, heterologous gene expression of carotenogenic enzymes from halophilic species such as *D. salina* may be a better approach for metabolic engineering of organisms suitable for carotenoid production in marine environments.

In conclusion, the present study provides a novel insight on the regulation of carotenogenesis in *D. salina*. Complementary studies are necessary to understand the regulatory mechanisms of carotenoid biosynthesis in this microalga and enhance the biotechnological and commercial potential of these natural compounds.

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