UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS DO MAR E AMBIENTE

Molecular basis of isoprenoid biosynthesis in *Dunaliella salina*

Doutoramento em Ciências do Mar

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Ana Alexandra Pedrosa Ramos

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Orientador: Prof. Doutor João Carlos Serafim Varela (Universidade do Algarve)

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Aos meus Pais e

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Bases moleculares da biosíntese de isoprenóides em *Dunaliella salina*

RESUMO

A principal fonte de β-caroteno de origem "natural" corresponde à microalga *Dunaliella salina*, sendo esta acumulação uma resposta fisiológica a condições de stress ambiental. Esta capacidade associada aos reduzidos custos de produção e ao aumento significativo a nível mundial da exploração desta microalga potenciam a sua utilização para estudos de engenharia metabólica e também como um importante bioreactor pertencente a um sistema mais vasto de biorefinaria. Na bibliografia encontram-se descritos vários estudos relacionados com as adaptações fisiológicas de *Dunaliella* a condições de stress abiótico contudo, a informação relativa às bases moleculares envolvidas na carotenogénese são escassas e incompletas. Com a presente tese pretendeu-se contribuir para a caracterização dos genes e mecanismos envolvidos na regulação das vias biosintéticas do 2C-metil-D-eritritol-4-fosfato (MEP) e dos carotenóides. Assim, foram isolados (*DsDXS*, *DsMCS*, *DsHDS*) e caracterizados funcionalmente (*DsDXR*, *DsHDR*, *DsLCY-β*) vários genes de ambas as vias. Os resultados obtidos com células cultivadas sob elevada salinidade, elevada luz e deplecção de nutrientes sugerem a existência de um controlo regulatório ao nível transcricional passível de ser induzido por condições de stress abiótico e evidenciam potenciais alvos para a optimização da produção de carotenóides em *D. salina* ou noutros organismos. De modo a investigar os mecanismos celulares envolvidos na acumulação de β-caroteno foi realizado um ensaio de duplo-híbrido em levedura utilizando como "isco" *Ds*LCY-β. Foi obtido um único provável interactor proteico cuja função é desconhecida e como tal será necessário análises subsequentes para validar esta interação e caracterizar este interactor.

PALAVRAS-CHAVE: *Dunaliella salina*, biossíntese de carotenóides, via do mevalonato, expressão genética, regulação da resposta ao stress

Molecular basis of isoprenoid biosynthesis in *Dunaliella salina*

ABSTRACT

Dunaliella salina, a unicellular biflagellate green alga, is the main natural source of β-carotene. The accumulation of this high-value carotenoid occurs in response to several abiotic stress conditions. Large-scale exploitation of *D. salina* has increased in the last years and culture cost-effectiveness makes this alga an interesting target for metabolic engineering purposes and also a valuable bioreactor / cell-factory system. Although numerous studies concerned physiological adaptations and responses of *Dunaliella* to abiotic stress, the molecular basis of β-carotene accumulation has yet to be elucidated. The aim of this thesis was to contribute towards the characterization of genes and the complex mechanisms involved in the regulation of *D. salina* methylerythritol phosphate (MEP) and carotenoid biosynthetic pathways. Several novel genes (*DsDXS*, *DsMCS*, *DsHDS)* were isolated and functionally characterized (*DsDXR*, *DsHDR*, *DsLCY-β*). Results from cells submitted to high salt, high light and nutrient depletion suggested the regulation at the transcriptional level of both pathways may be induced by abiotic stress, thus highlighting some possible targets for the improvement of carotenoid production in *D. salina* or other organisms. To further understand the cellular mechanisms underlying β-carotene overproduction in this microalga, one putative *Ds*LCY-β protein-protein interactor with unknown function was identified via yeast two-hybrid analysis, although future analysis of this protein of interest is necessary.

KEY-WORDS: *Dunaliella salina*, carotenoid biosynthesis, methylerythritol phosphate pathway, gene expression, stress response regulation

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Abbreviations

bp	base pairs
cDNA	complementary DNA
cfu	colony forming unit
CHYB	carotene β-hydroxylase
CHYE	carotene ε-hydroxylase
CMK	4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol kinase
DMAPP	dimethylallyl diphosphate
DNA	deoxyribonucleic acid
DXP	1-deoxy-D-xylulose-5-phosphate
DXS	1-deoxy-D-xylulose-5-phosphate synthase
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
EST	expressed sequence tag
FPP	farnesyl diphosphate
FPS	farnesyl diphosphate synthase
GA3P	glyceraldehyde-3-phosphate
GPP	geranyl diphosphate
GPS	geranyl diphosphate synthase
GGPP	geranyl geranyl diphosphate
GGPS	geranyl geranyl synthase
HMBPP	4-hydroxy-3-methylbut-2-enyl diphosphate
HDR	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
HDS	4-hydroxy-3-methylbut-2-enyl disphosphate synthase
HPLC	high performance liquid chromatography
IPI	isopentenyl pyrophosphate isomerase
IPP	isopentenyl diphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobase pairs
LCY-β	lycopene β-cyclase
MCS	2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase
MCT	2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase
MEP	methylerythritol phosphate
mRNA	messenger RNA
MVA	mevalonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
ORF	open reading frame
PCR	polymerase chain reaction
PDS	phytoene desaturase
PSY	phytoene synthase
PRX	peroxiredoxin
qPCR	quantitative polymerase chain reaction
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SSPE	salt-sodium phosphate-EDTA
TPP	thiamin pyrophosphate
UTR	untranslated region
VDE	violaxanthin deepoxidase

Y2H	yeast two-hybrid
ZDS	ζ-carotene desaturase
ZE	zeoxanthin epoxidase
ZISO	15-cis-ζ-isomerase

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The unicellular green alga *Dunaliella salina* Teod. as a model system for abiotic stress tolerance: Genetic advances and future perspectives

Ramos AA, Polle J, Tran D, Cushman J, Jin E, Varela JC. *Submitted for publication*

ABSTRACT

The physiology of the unicellular green alga *Dunaliella salina* in response to abiotic stress has been studied for several decades. Early *D. salina* research focused on its remarkable salinity tolerance and its ability, upon exposure to various abiotic stresses, to accumulate high concentrations of β-carotene and other carotenoid pigments valued highly as nutraceuticals. The simple life cycle and growth requirements of *D. salina* make this organism one of the large-scale commercially exploited microalgae for natural carotenoids. Recent advances in genomics and proteomics now allow investigation of abiotic stress responses at the molecular level and also the fast progress of microalgal biotechnology. Detailed knowledge of isoprenoid biosynthesis mechanisms and the development of molecular tools and techniques for *D. salina* will allow the improvement of physiological characteristics of algal strains and the use of transgenic algae in bioreactors. Here we review *D. salina* isoprenoid and carotenoid biosynthesis regulation, and also the biotechnological and genetic transformation procedures developed for this alga.

Key-words: carotenogenesis, *Dunaliella salina*, isoprenoid biosynthesis, genomics, transformation

Abbreviations: Acetyl-CoA, acetyl-coenzyme A; CaMV35S, *Cauliflower mosaic virus* 35S promoter; CAT, chloramphenicol acetyltransferase; cDNA, complementary DNA; CRT-ISO, carotenoid isomerase; DCA1, carbonic anhydrase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; ESTs, expressed sequence tags; GGPP, geranylgeranyl diphosphate; HbsAg, hepatitis B surface antigen; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPP, isopentenyl diphosphate; LCY-β, lycopene β-cyclase; LCY-ε, lycopene ε-cyclase; MVA, mevalonate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; NR, nitrate redutase; PAT, phosphinothricin acetyltransferase; PDS, phytoene desaturase; PSY, phytoene synthase; TAG, triacylglycerols Ubil-Ω, ubiquitin; SV40, simian virus 40; Ubil-Ω ubiquitin; ZDS, ζ-carotene desaturase.

INTRODUCTION

Microalgae include a very diverse group of prokaryotic and eukaryotic organisms that play important ecological roles and synthesize a vast array of natural products. Microalgal species have been widely used as simple model systems for research on higher plants due to shared physiological and biochemical reactions (Harris 2001; Hicks *et al.* 2001; Rochaix 2002). With fast-growth rates and low production costs relative to other transgenic expression systems, microalgae provide useful cell factories for the production of valuable chemical compounds and recombinant products (e.g. biofuels, novel carotenoids, vaccines and antibodies). Various aspects of algaculture and the use of microalgae as bioreactors have been reviewed extensively (Chapin 1991; Dunahay *et al.* 1996; Geng *et al.* 2003; Rochaix 2004; Hejazi & Wijffels, 2004; Pulz & Gross 2004; León-Bañares *et al.* 2004; Walker *et al.* 2005a; Chisti 2007, 2008; Raja *et al.* 2008; Rosenberg *et al.* 2008; Wijffels 2008).

Among eukaryotic microalgae, the relatively unique ability to accumulate glycerol and β-carotene in response to osmotic stress has made the halotolerant, unicellular, green alga *Dunaliella salina* (Fig. 1) an ideal model organism for dissecting the molecular mechanism(s) of osmotic stress responses (Cowan *et al.* 1992; Pick 1998). In contrast to the intensively studied chlorophyte *Chlamydomonas reinhardtii* (Lefebvre & Silflow 1999; Harris 2001; Grossman *et al.* 2003, 2007; Hema *et al.* 2007; León *et al.* 2007; Merchant *et al.* 2007), relatively few studies have investigated *D. salina* (Park *et al.* 2006). However, as *D. salina* is one of a few microalgae currently cultivated on an industrial scale, this organism is rapidly becoming the focus of intense fundamental and applied research. The purpose of this review is to survey recent developments into cellular, biochemical, molecular biological and biotechnological studies of *D. salina* with a focus on isoprenoid biosynthesis.

GENUS *DUNALIELLA*

In 1905, Teodoresco established the new algal genus *Dunaliella* (Chlorophyta), which mainly includes halophilic species adapted to hypersaline (0.05-5.0 M NaCl) environments. *Dunaliella* species can be found in euryhaline waters on all continents. In general, cells of *Dunaliella* species are of ovoid form, flagellated, and lack a rigid polysaccharide wall, although they are enclosed by a mucilaginous glycoprotein coat called a glycocalyx (Teodoresco 1905; Avron & Ben-Amotz 1992; Borowitzka & Borowitzka 1988; Oren 2005). The taxonomic organization of this genus, which includes more than 20 species, based on morphological and physiological characteristics, is still controversial due to large intra-species variation in morphology, which depends on growth conditions. Consequently numerous *Dunaliella* isolates were misidentified previously (Borowitzka & Borowitzka 1988; Pick 1998). Such erroneous classification of *Dunaliella* isolates still causes problems, for example, when new molecular data are published, pointing to the need for the establishment of a robust molecular phylogeny within the genus. A recent attempt to update and reorganize the genus based on morphological and physiological characteristics was performed by Borowitzka & Siva (2007). Overall, systematic molecular data (Olmos *et al.* 2000; Cifuentes *et al.* 2001; González *et al.* 2001; Olmos-Soto *et al.* 2002; Gómez & Gonzaléz 2004; Raja *et al.* 2007a) should be extended and used in combination with morphological and physiological markers to re-evaluate species classification.

Some *Dunaliella* strains can accumulate β-carotene and glycerol, properties with economical interest that have led, since 1980s, to the large-scale culture of this algae in several countries such as Australia, China, Israel, and India (Ben-Amotz & Avron 1990; Borowitzka 1999; Pulz 2001; Tseng 2001; Del Campo *et al.* 2007). *D. salina* is the main

natural source of β-carotene (up to 14% of algal dry weight) and also one of the most extensively analyzed strains in terms of physiological abiotic stress adaptations especially under mass culture conditions (Fig. 1; Loeblich 1982; Ben-Amotz & Avron 1983; Cifuentes *et al.* 1996).

In response to several stress or growth limiting conditions (salt, temperature, light and nutrient deficiencies) *D. salina* synthesizes and accumulates β-carotene as lipid globules in the stroma of chloroplasts (Borowitzka *et al.* 1990; Shaish *et al.* 1992; Vorst *et al.* 1994; Katz *et al.* 1995; Bhosale 2004; Coesel *et al.* 2008). This high value compound, produced by *D. salina* under abiotic stress in equal amounts of all-*trans* and 9-*cis* stereoisomers, has a wide range of important economical applications such as human health, cosmetic and aquaculture industries (Jiménez & Pick, 1993; Murthy *et al.* 2005; Spolaore *et al.* 2006; Raja *et al.* 2007b). Consequently, understanding stress-induced carotenoid metabolism and unraveling the regulatory mechanism(s) of stress tolerance in this organism are important biotechnological research goals (Xue *et al.* 2003).

In the past, use of *Dunaliella* species as model organisms had one major disadvantage: lack of established procedures of genetic analysis. However, more recent studies addressed this shortcoming (see below) and it can be expected that genomic technologies will be developed soon. The most widely used model species within the genus of *Dunaliella* was *D. salina*. Therefore, the following sections will concern *D. salina* unless otherwise indicated.

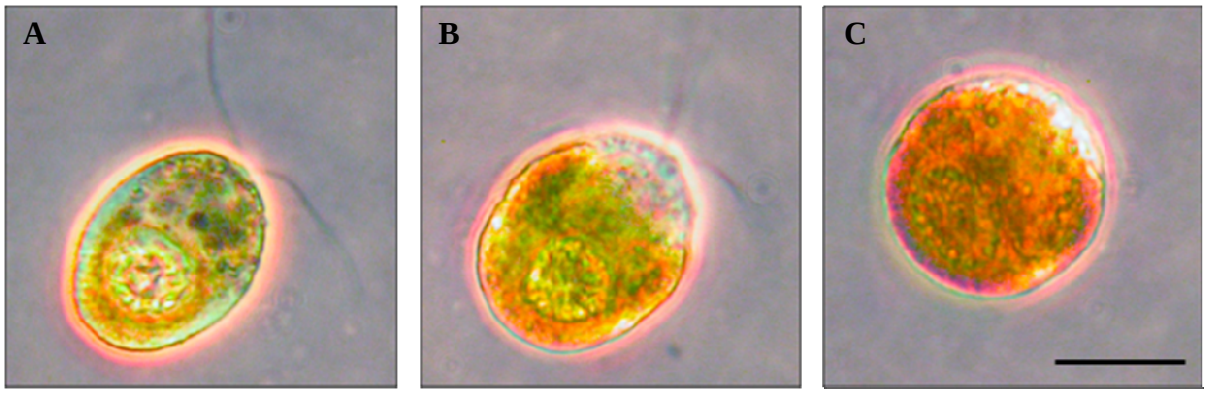

Figure 1. *Dunaliella salina* cells in different culture conditions. **A)** Green cell from a non-stressed culture; **B)** Stressed cell turning orange; **C)** Orange cell from a culture exposed to nutrient stress due to β-carotene accumulation. The bar represents 10 μm.

GENES AND PROTEIN IDENTIFICATION

Complex regulatory and signalling networks of stress response have been uncovered in several model plants and algae using novel genomics, functional, and computational approaches (Cushman & Bohnert 2000; Shrager *et al.* 2003; Grossman 2005; Grossman *et al.* 2007; Jain *et al.* 2007; Montsant *et al.* 2007). This type of data often provides clues first towards identification and then regarding possible functions of unknown encoded proteins in other species. Currently, a genome sequencing project is under way for *D. salina* strain CCAP 19/18 (US DOE Joint Genome Institute). The availability of genomic data will be a major step towards a better understanding of the molecular mechanisms underlying the response of *D. salina* to abiotic stress.

Gene discovery followed by genome-wide expression analysis is the initial step to clarify complex cellular abiotic stress responses. Molecular approaches such as the generation

of expressed sequence tags (ESTs) databases, microarray analysis, deep cDNA sequencing, and/or parallel genomic sequencing can thus provide the required gene expression data. Limited EST data and microarray data are available from a partial number of halophytic plants including *Mesembryanthemum crystallin*um (Cushman *et al.* 2008), *Thellungiella halophila* (Du *et al.* 2008; Taji *et al.* 2008), and *Spartina alterniflora* (Baisakh *et al.* 2008), and can provide novel insights into osmoregulation and responses to stress.

Compared with *C. reinhardtii*, for which more than 200,000 ESTs are stored at NCBI (Jain *et al.* 2007), only about 3,900 ESTs are available for *D. salina* cells subjected to salinity stress. Profiling of about 2,800 ESTs revealed elevated expression of protein synthetic apparatus components in salinity shocked cells. Recent data from J. Varela's laboratory with a subtractive cDNA library obtained from cells submitted to high salinity stress and nutrient depletion showed that several differentially expressed clones corresponded to genes involved in adaptation to stress, but the function of many others was unknown (A. Baumgartner, N. Henriques, A. Ramos and J. Varela, unpublished results). These uncharacterized clones are potential tools for engineered stress tolerance.

In addition to ongoing genomic approaches, proteomic methodologies are also necessary to further characterize cell stress adaptations mechanisms. Only very few studies concerning the protein patterns observed with salinity stressed *D. salina* cells have been described (Liska *et al.* 2004; Katz *et al.* 2007). These studies revealed changes in multiple biochemical pathways in response to salt stress, suggesting that more than one mechanism may be important for the unique capacity of salinity tolerance of *D. salina* cells. Several salt-induced proteins were key enzymes in central metabolic networks such as cell carbon and energy metabolism and also transport activities (e.g. photosynthesis and Calvin cycle, redox energy production, protein biosynthesis and degradation, signal transduction; Liska *et al.* 2004; Katz *et al.* 2007).

ISOPRENOID BIOSYNTHESIS AND ITS REGULATION IN *D. SALINA*

The large group of secondary metabolites known as isoprenoids or terpenoids includes several biological and economical important compounds such as carotenoids (Stahl & Sies 2005). In all living organisms isoprenoids are synthesized from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) obtained from a cytosolic mevalonate (MVA) pathway and/or a plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway which is also known as the non-mevalonate or 1-deoxy-D-xylulose-5-phosphate (DXP) pathway (Rohmer *et al.* 1993; Sacchettini & Poulter 1997; Rohdich *et al.* 2003; Rohmer 2003). In green algae only the plastid localized MEP pathway provides the precursors for the biosynthesis of all isoprenoids regardless of whether they are synthesized in the cytosol, such as squalene, or in the plastid, such as carotenoids, chlorophylls, tocopherols and certain hormones (Chappel 1995; Schwender *et al.* 1996; Lichtenthaler *et al.* 1997; Eisenreich *et al.* 1998, 2001; Bach *et al.* 1999; Lichtenthaler 1999, 2000; Rohmer 1999). In spite of the physical separation of these two pathways in distinct cell compartments, several researchers reported an apparent crosstalk between them (Kasahara *et al.* 2002; Bick & Lange 2003; Hemmerlin *et al.* 2003; Laule *et al.* 2003; Dudareva *et al.* 2005; Hampel *et al.* 2005). However, further research is necessary to understand not only the mechanisms involved in the transport of isoprenoid precursor molecules across membranes, but also to dissect the biological mechanisms of regulatory communication between pathways. In the particular case of *D. salina* the molecular information regarding the isoprenoid biosynthesis pathway is still very limited and incomplete (Ye *et al.* 2008), as discussed in the following sections.

Non-mevalonate or MEP pathway

The MEP pathway was discovered only in the 1980s and since then was identified in several prokaryotic (eubacteria, cyanobacteria) and eukaryotic (algae and higher plants) organisms (Lichtenthaler 1999; Rohmer 1999). Green algae (Chlorophyta) appear to have lost the cytosolic MVA pathway (Schwender *et al.* 2001). In contrast to the cytosolic MVA pathway which requires three molecules of acetyl-coenzyme A (acetyl-CoA) for IPP generation, the MEP pathway uses pyruvate and glyceraldehyde-3-phosphate as substrates and involves eight plastid-localized enzymes (Fig. 2; Hsieh *et al.* 2008), which use different cofactors and metal ions (Hunter 2007). For higher plants all the MEP genes, which are encoded in the nucleus, and the intermediary pathway products have been identified (Rohdich *et al.* 2001; Eisenreich *et al.* 2004). Further, the MEP pathway in plants was reported to contain at least two rate-determining steps at the level of the DXS and the HDR (Botella-Pavía *et al.* 2004; Rodríguez-Concepción 2006). However, the regulatory mechanisms involved in this biochemical pathway require further clarification.

For *D. salina,* MEP pathway genes and their regulatory mechanisms were unknown until recently (Fig. 2). All *D. salina* MEP genes have been identified (partial or full-length) and their respective characterization is now ongoing (A. Ramos and J. Varela, unpublished results; Polle and Tran, unpublished results). Considering the clear importance of this pathway in plant cell biology and the existence of a coordinated regulation between the MEP pathway and other downstream plastidial isoprenoid pathways (Bouvier *et al.* 1998; Lois *et al.* 2000; Rodríguez-Concepción *et al.* 2003; Botella-Pavía *et al.* 2004; Wille *et al.* 2004), identification of genes involved in the MEP pathway in *Dunaliella* represented the first step in further investigation of isoprenoid biosynthesis regulation in this alga.

Similar to higher plants, abiotic stress, specifically nutrient limitation and high light, seems to be an important factor in the transcriptional regulation of *DXS* and *HDR* (GenBank

accession numbers FJ469276 and FJ040210; Ramos *et al.* 2009) whereas *DXR* (GenBank acession number FJ469277) is apparently unresponsive to stress (A. Ramos and J. Varela, unpublished results; Tran and Polle, unpublished results).

Carotenoid biosynthesis

The carotenoid biosynthetic pathway (Fig. 2), as an essential biological and biotechnologically relevant process, has been extensively investigated in bacteria, fungi, plants, and some algae. Thus, elucidation and characterization of genes, enzymes, metabolic intermediates and regulatory mechanisms have been reported for several plants and other organisms including algae (Bartley *et al.* 1994; Sandmann 1994; Hirschberg *et al.* 1997; Cunningham & Gantt 1998; Cunningham 2002; Römer & Fraser 2005, Liang *et al.* 2006; Vidhyavathi *et al.* 2008).

In plastids, after the formation of IPP by the MEP pathway as described above, three consecutive condensation reactions, which are catalyzed by prenyltransferases, lead to formation of geranylgeranyl diphosphate (GGPP), which is the precursor of all carotenoids. The first step of carotenoid biosynthesis consists of the condensation of two molecules of GGPP (C_{20}), by the enzyme phytoene synthase (PSY), into phytoene (Sandmann 2001). Subsequent steps then involve several membrane-associated or membrane-integrated enzymes performing a sequence of desaturation (PDS and ZDS) and isomerisation (CRT-ISO and Z-ISO) reactions (Li *et al.* 2007) leading to lycopene. Following ring-formation by cyclases (LCY-ε and/or LCY-β) synthesis of α- and β-carotene and further hydroxylation reactions lead to lutein or violanxanthin from which other end product carotenoids may be formed (Fig. 2; Lichtenthaler 1999, 2000; Bouvier *et al.* 2005).

So far, few sequences were published for genes that code for enzymes involved in the carotenoid biosynthesis pathway. Known genes corresponding to the *PSY* (GenBank acession number: AY601075; Yan *et al.* 2005), *PDS* (AY954517; Zhu *et al.* 2005) and *LCY-β* (EU327876; Ramos *et al.* 2008). The molecular basis of carotenogenesis in *D. salina* is currently under investigation with several remaining pathway genes being fully or partially known (Polle and Cushman, unpublished data). Abiotic stress (nutrient limitation, high light and salt) experiments performed revealed that transcriptional regulation may be an important control step of this pathway (Ramos *et al.* 2009). *PSY*, *PDS* and *LCY-β* presented similar expression patterns and nutrient availability seemed to be the most important inductive factor for β-carotene accumulation in this microalga. Therefore, up-regulation of carotenoid biosynthetic pathway genes may occur in response to abiotic stress conditions (Coesel *et al.* 2008; Ramos *et al.* 2008), although earlier results indicated the contrary (see below; Rabbani *et al.* 1998; Sánchez-Estudillo *et al.* 2006).

The evident complexity of carotenoid biosynthesis observed in other photosynthetic organisms highlights the need for an integrated research effort to further understand the regulation of this pathway in *D. salina* (Lamers *et al.* 2008). Examination of posttranscriptional and metabolic regulatory factors such as redox control (Liska *et al.* 2004), key control pathway enzymes, signalling molecules (e.g. ROS, SOS pathway and transcription factors; Shaish *et al.* 1993; Xiong *et al.* 2002; Aarts & Fiers 2003); is fundamental to dissect the mechanism(s) leading to stress-induced β-carotene over-accumulation in *D. salina* and the development of transgenic strategies for the use of this alga in metabolic engineering of the carotenoid biosynthesis.

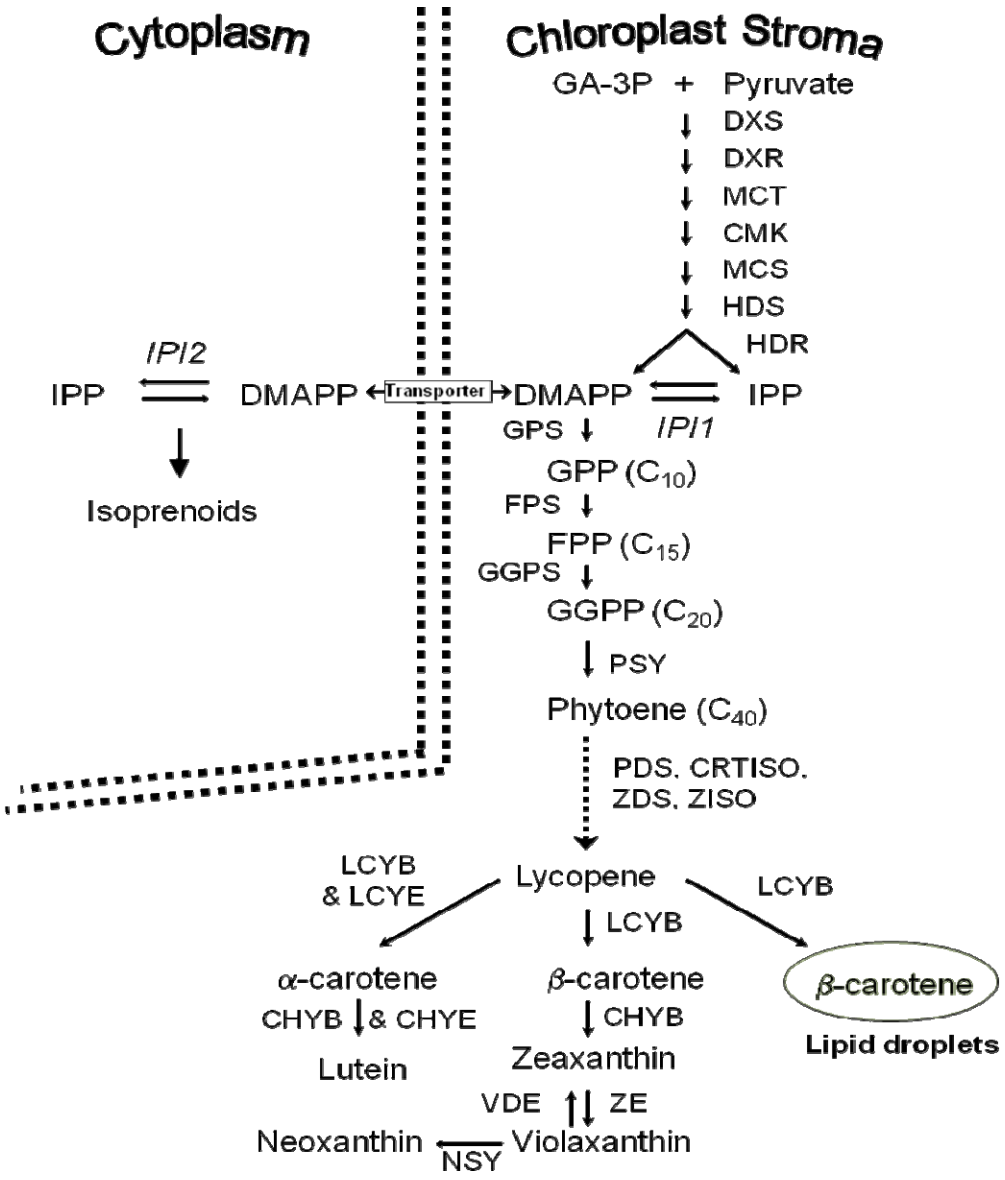

Figure 2. Schematic overview of proposed isoprenoid biosynthesis for the microalga *D. salina* (according to published data on higher plant). The abbreviations used for the intermediate molecules and enzymes are as follows: GA-3P, glyceraldehyde-3-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT, 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol kinase; MCS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; IPI1 & 2, isopentenyl pyrophosphate isomerase; GPS, GPP synthase; GPP, geranyl diphosphate; FPS, FPP synthase; FPP, farnesyl diphosphate; GGPS, GGPP synthase; GGPP, geranyl geranyl diphosphate; PSY, phytoene synthase (catalyses the first committed step of carotenoid biosynthetic pathway); PDS, phytoene desaturase; CRTISO, carotenoid isomerase; ZDS, ζ-carotene desaturase; ZISO, 15-cis-ζ-isomerase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; CHYB, carotene β-hydroxylase; CHYE, carotene ε-hydroxylase; VDE, violaxanthin deepoxidase; ZE, zeoxanthin epoxidase; NSY, neoxanthin synthase.

LIPID BIOSYNTHESIS AND ITS APPLICATION

Dunaliella species have been found to have unusually high contents of total lipids, carotenoids and polyunsaturated fatty acid (PUFA) such as 16:4 as well as 18:3 (Tornabene *et al.* 1980; Ben-Amotz *et al.* 1982; Evans *et al.* 1982, 1984; Mendoza *et al.* 1999). These properties of *Dunaliella* species has prompted an interest in their lipid biosynthesis associated with carotenoids biosynthesis for commercial use.

D. salina exposed to stress conditions, such as high light intensity or nutrient starvation, accumulate β-carotene in plastid lipid globules as the sequestering structure (Ben-Amotz & Avron 1983; Jiménez & Pick 1994; Rabbani *et al.*1998). This mechanism is not unique for *Dunaliella*; a number of microalgae exposed to stress growth conditions (i.e. high irradiance and nitrogen starvation) accumulate intra- or extra-plastidic lipid bodies composed of both triacylglycerols (TAG) and carotenoids (Ben-Amotz & Avron 1983; Jiménez & Pick 1994; Thompson 1996; Rabbani *et al.* 1998; Boussiba 2000). In *D. bardawil* the interrelationship between lipid synthesis, β-carotene accumulation, and chloroplast lipid globule formation was demonstrated (Rabbani *et al.* 1998). In this investigation, carotenoid biosynthetic enzymes, including PSY or PDS, were not enhanced at the transcriptional and translational levels under β-carotene overproducing conditions, whereas the activity of a key lipid biosynthesis regulatory enzyme, acetyl-CoA carboxylase, increased dramatically. Additionally glycerol-3-phosphate acyltransferase was enhanced about 8-fold compared with control conditions. Thus, it is possible that β-carotene synthesis is driven by lipid deposition, or vice versa resulting in the sequestration and storage of carotenoid biosynthetic pathway end products.

Abiotic stress is able to change not only the total content of fatty acid, but also the composition of those in *D. salina*. Increased fatty acid content under stress conditions (e.g. high irradiance, high salt or nitrogen starvation) has been described (Cho & Thompson 1986; Mendoza *et al.* 1999; El-Baky *et al.* 2004). The main PUFA, 18:3(n-3) and 16:4(n-3), comprised almost 70% of the total fatty acids in *D. salina*, whereas the proportion of 16:0 and 18:1 increased at the expense of the PUFAs 16:4 (n-3) and 18:3 (n-3) under high irradiance (Mendoza *et al.* 1999). Whether or not the observed changes in the 16:0/16:4 fatty acid ratio is related to alterations in the balance between storage and photosynthetic related fatty acid during the adaptation to high light, remains unclear. Recently algal-based biofuels are gaining widespread attention and microalgae have been studied for production of hydrogen, oils (triglycerides, for biodiesel) and bioethanol (Ghirardi 2002; Metzger & Largeau 2005; Wu & Miao 2006; Chisti 2007, 2008; Wijffels 2008; Rosenberg *et al.* 2008). The broadest evaluation of algal species was performed by the US Department of Energy's Aquatic Species Program (ASP) to develop microalgae as a source of biodiesel. ASP scientists screened a large number of microalgae for growth rate and oil production as well as composition. Throughout this project, the ASP recognized that species selection, optimal cultivation, and genetic diversity, in addition to metabolic engineering, were critical for commercial viability (Sheehan *et al.* 1998).

D. salina is one candidate as a feedstock for biodiesel or bioethanol production, because mass production systems for this alga have been established already worldwide. *D. salina* can produce high amounts of lipids in the range from 38% to 44% in terms of dry weight (El-Baky *et al.* 2004; Wedly & Hauesemann 2007). *D. salina* can be manipulated easily for either fatty acid content or composition by high irradiance, salinity stress and N-deprivation. However, information about the enzymes related to the biosynthesis,

desaturation and elongation of fatty acids is limited for the rational manipulation of lipid quantity and quality. Furthermore, unlike cells under nutrient stress, exponentially growing *D. salina* displays low lipid levels. Therefore, optimal lipid production should involve a first stage of rapid cell division in hybrid photo-bioreactors followed by imposition of nutritional stress in outdoor ponds.

GENETIC MANIPULATION AND BIOTECHNOLOGICAL APPLICATIONS

Microalgal strains, either novel or genetically improved, are indispensable tools for algal biotechnology companies. Although only non-transgenic strains of *D. salina* have been commercially used to date, recent development of tools and methods for genetic engineering of microalgae has allowed the transformation of several species including *C. reinhardtii* (Kindle *et al.* 1989; Kindle 1990; Dunahay 1993; Shimogawara *et al.* 1998; Kumar *et al.* 2004), *Volvox carteri* (Schiedlmeier *et al.* 1994; Gruber *et al.* 1996), and *Phaeodactylum tricornutum* (Apt *et al.* 1996; Zaslavskaia *et al.* 2000**).** Transformation of other algae species has also been accomplished (25 species in total; Fuhrmann 2002; Walker *et al.* 2005b; Coll 2006; León *et al.* 2007). One species that has recently attracted great attention regarding genetic engineering is *D. salina.*

The development of a stable, efficient and reproducible transformation system depends on several critical factors such as the availability of suitable, highly active promoters, selective markers (dominant or recessive), reporter genes and stable transformation methods (Coll 2006; Hallmann 2007). Regarding *Dunaliella*, the species *D. salina, D. tertiolecta* and *D. viridis* have been the main focus of genetic transformation experiments (Geng *et al.* 2003, 2004; Walker *et al.* 2005c; Sun *et al.* 2006).

Early engineering attempts resulted in stable transformation of *D. salina* with the selective *ble* gene marker (zeocin antibiotic resistance) by glass bead agitation; however, no evidence of genome integration was presented (Jin *et al.* 2001). Subsequently, this problem was overcome by Geng *et al.* (2003, 2004), who reported nuclear transformation of *D. salina* by means of electroporation and stable foreign expression of hepatitis B surface antigen (*HbsAg*) under the control of maize ubiquitin (Ubil-Ω) promoter as well as the chloramphenicol acetyltransferase (*CAT*) selectable gene under the control of simian virus 40 (SV40) promoter.

In addition, the micro-particle bombardment transformation method was used with the *bar* gene providing the selective marker, which encodes for phosphinothricin acetyltransferase (PAT) and confers Basta herbicide tolerance (Walker *et al.* 2005b; Coll *et al.* 2006). Three distinct promoters were tested in different reports: DCA1 (carbonic anhydrase gene; Lü *et al.* 2004), actin (Guo-Zhong *et al.* 2005) and CaMV35S (*Cauliflower mosaic virus* 35S promoter; Tan *et al.* 2005). The latter was the only report without evidence of stable genome incorporation (Tan *et al.* 2005).

Recently, another expression system was described in which the heterologous gene (*bar*) expression could be regulated by an inducible promoter. For this purpose a *D. salina* nitrate redutase (*NR*) gene promoter-terminator cassette with the *bar* gene was constructed. *D. salina* cells were transformed with this cassette by electroporation and the *bar* control expression was observed. Furthermore, gene integration into the algal genome in the stable transformants was confirmed (Li *et al.* 2007).

Development of highly efficient transformation systems for stable gene expression is necessary for commercially interesting algae strains such as *D. salina*, especially for the improvement of carotenoid biosynthesis. Genetic engineering of this pathway with a mutated phytoene desaturase (*PDS*) gene in *H. pluvialis* led to increased carotenoid and rapid

astaxanthin accumulation (Steinbrenner & Sandmann 2006). The *PDS* gene was also used in a double-stranded RNA interference approach for gene expression analysis in *D. salina* electroporated cells. This gene silencing method proved to be useful for understanding gene function analysis in this alga (Sun *et al.* 2008). However, further research in *D. salina* transgenics is essential for the use of this alga as a bioreactor and also as a molecular toolkit.

CONCLUSIONS AND FUTURE PERSPECTIVES

The progress of modern genomics has led in recent years to the complete genome sequencing of about a dozen microalgal species (http://www.jgi.doe.gov) and several EST projects are available or currently in progress for several other algae. Indeed one of the earliest algal genomes sequenced corresponded to *C. reinhardtii*, which served as a reference and a guidepost for further molecular analyses (Grossman *et al.* 2007; Merchant *et al.* 2007). Research on this unicellular green alga and two diatom species, whose genomes have recently been sequenced, are now in the post-genomics stage (Armbrust *et al.* 2004; Montsant *et al.* 2007; Siaut *et al.* 2007). Despite the considerable available molecular data regarding these microscopical photosynthetic organisms, further biotechnology development is necessary for overall algal commercial exploitation.

The USDA-DOE Joint Genome Institute (JGI) is currently sequencing the complete genome of *D. salina* strain CCAP19/18. With the genome of *D. salina* close to being sequenced, it is anticipated that genome-based approaches will become available in the near future. For example, development of microarrays or deep cDNA sequencing methods (Margulies *et al.* 2005) can be expected to follow in short order and allow comparative analysis of the transcriptome of cells exposed to various environmental conditions. These

techniques will allow the study of multiple cellular processes at the molecular level as, for example, the dissection of the regulatory mechanisms involved in carotenogenesis and glycerol metabolism. In comparison to the alga *C. reinhardtii* for its close relative *D. salina* more genomic tool development is necessary. At this time highly efficient transformation systems are not available and genetic crosses cannot be performed on a routine basis. However, with the genome becoming available soon, the alga *D. salina* as a model system is about to undergo a major transformation. The short-term outcome of research on this industrially important species will allow not only an enhanced understanding of complex metabolic networks, but also further development of transgenic algal strains for particular commercial applications (e.g. production of carotenoids and recombinant proteins).

AIM AND OUTLINE OF THE THESIS

The unicellular alga *Dunaliella salina* is one of the most extensively investigated microalgae for β-carotene production. Commercial demand of natural carotenoids has increased as of late due to their widespread biomedical, industrial and biotechnological applications. Beside the unique ability to accumulate large amounts of carotenoids, other physiological features (e.g. growth in high salinity concentrations) make *D. salina* an interesting organism not only for mass-cultivation purposes, but also for its development as a bioreactor / biorefinery system. Nevertheless, the molecular mechanisms involved in carotenoid biosynthesis and other cellular process (e.g. abiotic stress response) are poorly known.

The main objective of the present thesis was to investigate *D. salina* carotenoid biosynthesis and possible regulation mechanisms. Research goals involved the (i) isolation and characterization of MEP and carotenoid biosynthetic pathways genes; (ii) gene expression analysis of cells exposed to abiotic stress, a known carotenogenic condition and; (iii) identification of protein interactors to identify novel enzymes and regulators of the previous pathways.

The outline of this thesis is as follows:

Part I includes submitted or published data regarding isoprenoid biosynthesis in *D. salina*. Green algae (Chlorophyta) use exclusively the plastidial non-mevalonate or 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway for the biosynthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the universal five-carbon precursors of isoprenoids (e.g. carotenoids). In **Chapter 1** and **2** the molecular cloning of genes involved in this pathway, namely 1-deoxy-D-xylulose 5-phosphate synthase (*DsDXS*),

1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DsDXR*) and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*DsHDR*), is described. Furthermore, as the published data regarding the genes involved in carotenoid biosynthesis was limited to the first two biochemical steps, namely phytoene desaturase (*DsPDS*) and phytoene syntase (*DsPSY*), **Chapter 2** describes the isolation and characterization of an additional gene of this pathway: lycopene β-cyclase (*DsLCY-β*). The reaction catalyzed by this enzyme corresponds to the conversion of lycopene into β-carotene, two metabolites with nutraceutical and economical relevance. Moreover, *DsDXR*, *DsHDR* and *DsLCY-β* were functionally characterized. Gene expression analysis of the novel genes in response to abiotic stress conditions (e.g high light, nutrient depletion and high salinity) is presented.

Part II comprises a proteomic approach to analyse *DsLCY-β* protein-protein interactors. Protein-protein interactions, either stable or transient, are essential in every cellular process. The discovery of potential interactions might elucidate, for example, the function of known or novel proteins and complex signal transduction pathways. Several methods are currently used to detect protein-protein interactions namely the yeast two-hybrid system. In **Chapter 4** the single putative *Ds*LCY-β protein-protein interactor obtained with the previous method is described.

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Part I

Isoprenoid biosynthesis

Chapter 1

Expression and molecular characterization of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), the initial enzymes of *Dunaliella salina* methylerythritol phosphate pathway

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ABSTRACT

The methylerythritol phosphate (MEP) pathway for the biosynthesis of plastid isoprenoids is currently being elucidated for the microalga *Dunaliella salina*. The initial steps of this pathway involve namely 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). Using a degenerate primer approach, full-length *DsDXS* (2262 bp) and *DsDXR* (1448 bp) cDNA clones encoding *D. salina* DXS and DXR homologues, respectively, were isolated and characterized. Partial cDNA clones coding for *D. salina* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*DsMCS*) and 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (*DsHDS*) were also obtained. The deduced *DsDXS* and *DsDXR* open reading frames code for polypeptides of 753 and 436 amino acids, respectively. Phylogenetic analysis indicated a bacterial gene origin for *DsDXS* in contrast to the observed cyanobacterial *DsDXR* ancestral. In opposition to *DsDXR* and *DsMCS* results, gene expression transcript levels accumulation was observed under light stress conditions for other MEP pathway genes and also lycopene β-cyclase (*DsLCY-β*) gene from the carotenoid biosynthetic pathway. Evidence of a coordinated transcript regulation and carotenoid cell production indicates a possible involvement of these MEP pathway enzymes in the flux control of the carotenogenic process in *D. salina*.

Keywords: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; 1-deoxy-D-xylulose 5-phosphate synthase; *Dunaliella salina*; isoprenoid biosynthesis; methylerythritol phosphate pathway

Abbreviations: CMK, 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol kinase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; HDS, 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HMBPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; IPP, isopentenyl diphosphate; LCY-β, lycopene β-cyclase; MCS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MCT, 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase; MEP, methylerythritol phosphate or 2-*C*-methyl-D-erythritol-4-phosphate; MVA, mevalonate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; ORF, open reading frame; RACE, rapid amplification of cDNA ends; TPP, thiamin pyrophosphate; UTR, untranslated region.

INTRODUCTION

Two distinct pathways are involved in the production of the key isoprene units for the biosynthesis of the largest group of secondary metabolites known as isoprenoids. Comprising numerous important biological functions (e.g. photosynthesis, photoprotection, regulation of growth and development; Bouvier et al., 2005) and biotechnological applications (e.g. pigments and drugs; Walker et al., 2005; Rao and Rao, 2007), these compounds are all derived from two universal precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) produced from the cytosolic mevalonate (MVA) or plastidial methylerythritol phosphate (MEP) pathways (Rohmer et al., 1993; Rohmer, 1999; Kuzuyama, 2002). However, in the particular case of green algae (Chlorophyta), available evidence suggests evolutionary loss of the MVA pathway (Schwender et al., 2001).

The MEP pathway, also known as the non-mevalonate or 1-deoxy-D-xylulose-5-phosphate (DXP) pathway, involves seven enzymatic steps (Lichtenthaler, 1999; 2000; Rohdich et al., 2001; Hunter, 2007). The initial reaction concerns the condensation of glyceraldehyde 3-phosphate and pyruvate to form DXP, catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS, EC: 4.1.3.37; Sprenger et al., 1997; Lange et al., 1998). In the subsequent step DXP is converted to 2-*C*-methyl-D-erythritol-4-phosphate or methylerythritol phosphate (MEP) by the NADPH-dependent 1-deoxy-D-xylulose 5-phosphate reductoisomerase enzyme (DXR, EC: 1.1.1.267). This is considered the first committed step of the MEP pathway (Kuzuyama et al., 1998; Lange and Croteau, 1999; Schwender et al., 1999). The following steps involve four enzymatic reactions which result in the conversion of MEP to 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) by the consecutive biochemical activity of 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase

(MCT, EC: 2.7.7.60), 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol kinase (CMK, EC: 2.7.1.148), 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate synthase (MCS, EC: 4.6.1.12) and 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS, EC: 1.17.4.3). Finally, HMBPP is converted to IPP and DMAPP by the recently discovered 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR, EC: 1.17.1.2; Eisenreich et al., 2001; Rodríguez-Concepción and Boronat, 2002; Bouvier et al., 2005).

Initial studies in *Escherichia coli* allowed the identification of the genes and enzymes of this pathway and in the last decade data regarding similar steps in higher plants increased rapidly (Hoeffler et al., 2002; Eisenreich et al., 2004). Although the regulatory mechanisms involved in metabolic flux control of this pathway and coordination with downstream pathways need further investigation, current knowledge indicates that several enzymes might be involved in this regulation. For example, DXS and HDR might be key regulators, as they seem to participate in rate-determining steps (Botella-Pavía et al., 2004; Rodríguez-Concepción, 2006). Additionally, constitutive DXS and DXR overexpression resulted in accumulation of downstream MEP-derived isoprenoids such as chlorophylls, carotenoids and also essential oils in plant cells (Estévez et al., 2001; Mahmoud and Croteau, 2001; Carretero-Paulet et al., 2006; Morris et al., 2006, Muñoz-Bertomeu et al., 2006).

Insufficient MEP knowledge and scarce molecular data is evident not only in higher plants but also in other plant-like organisms with biotechnological research interest such as the green alga *Dunaliella salina* (Chlorophyta; Ye et al., 2008). This organism presents some unique physiological characteristics such as high salinity tolerance and the ability of accumulating large amounts of β-carotene under abiotic stress growth conditions (Loeblich, 1982; Ben-Amotz and Avron, 1983; Coesel et al., 2008). The high market value of this antioxidant compound has favoured the large-scale culture of its main natural source, *D.*

salina, in several countries including Israel, USA and Australia (Ben-Amotz and Avron, 1990; Pulz, 2001; Del Campo et al., 2007).

β-carotene is an isoprenoid synthesized in the plastids via the MEP pathway and evidence of a coordinated control between this biosynthetic pathway and carotenogenesis has been described for several plants (e.g *Arabidopsis thaliana*, *Lycopersicum esculentum*, *Capsicum annuum*; Bouvier et al., 1998; Lois et al., 2000; Rodríguez-Concepción et al., 2003; Botella-Pavia et al., 2004, Wille et al., 2004). Therefore, characterization of *D. salina* MEP pathway enzymes and research on its regulatory mechanisms are required for potential biotechnological applications, such as improvement of carotenoid biosynthesis efficiency (León-Bañares et al., 2004; Spolaore et al., 2006).

Recently, an initial insight in *D. salina* MEP pathway was described regarding *HDR* isolation, characterization and abiotic transcriptional regulation (Ramos et al., 2009). To gain further knowledge, by means of a degenerate primer approach, we were able to clone other genes from this metabolic route. In the present study, we describe the cloning and characterization of full-length cDNA clones encoding the two early potential control points of the MEP pathway, *DsDXS* and *DsDXR*. Expression analysis of several MEP genes and carotenogenic *DsLCY-β* in response to light stress was also investigated.

MATERIAL AND METHODS

D. salina growth conditions and analytical methods

The carotenogenic *D. salina* strain CCAP 19/30 was obtained from the Culture Collection for Algae and Protozoa (Cumbria UK) and grown according to standard laboratory conditions: modified Walne medium (9% NaCl w/v) and continuous illumination (45 μmol m^{-2} s^{-1}; Ramos et al., 2008). Experimental parameters (cell number, medium nitrate concentration, cell β-carotene content) and statistical analysis (STATISTICA v. 6.0, StatSoft) were performed as described previously (Ramos et al., 2008).

Experimental design concerned two abiotic stress conditions namely nutrient limitation and high light. Log phase *Dunaliella* cultures (density of 2.5 x 10^5 cells mL^{-1}) were used to inoculate distinct flasks (500 mL final culture volume) containing either Walne medium (nutrient-supplemented) or sterilized water (nutrient-depleted), both at 9% NaCl (w/v). Light intensity increment, from standard 45 to 500 μmol m^{-2} s^{-1}, was obtained with a supplementary white lamp (150-W Massive N.V/S.A).

Cloning of *D. salina DXS* and *DXR*

Total RNA from 5-10 x 10^6 *D. salina* cells for cDNA synthesis and also northern blot analysis (see following sections) was isolated with TRI REAGENT™ (Sigma) according to the described manufacturer procedure. SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech) was used for cDNA synthesis and MEP pathway gene PCR amplifications. At first, degenerate primers (DXS1Fw and DXS2Rev, DXR1Fw and DXR2Rev; Table 1) were constructed based on the available Viridiplantae sequences and

partial *DsDXS* and *DsDXR* cDNA clones were obtained. 5' and 3' RACE PCR amplification (data not shown) allowed the cloning of the full-length *DsDXS* and *DsDXR*. Primers used for these amplifications are described in Table 1 (DXS3Fw and DXS4Rev; DXR3Fw and DXR4Rev) and PCR conditions were as follows: 1 cycle of 94 ºC, 2 min; 30 cycles of 94 ºC - 60 s, 60 ºC – 60 s, 72 ºC – 90 s; and 1 cycle of 72 ºC – 10 min. All RACE amplification products were cloned into pGEM-T Easy vector (Promega) and sequenced (Genetic Analyzer-Applied Biosystems, CCMAR Portugal).

Bioinformatic and phylogenetic analysis

Sequence analysis (nucleotide and derived amino acid) was performed with DNASTAR (Lasergene) and Geneious Pro 2.5.3 (Biomatters). ChloroP 1.1 and TargetP 1.1 were used to identify possible chloroplast transit peptides (Emanuelsson et al., 1999; 2000).

M-coffee option of the T-Coffee program was used for the amino acid sequences alignments (Notredame et al., 2000; Moretti et al., 2007). These alignments were edited with Gblocks version 0.91b using the default parameters in order to exclude gaps and other non-conserved regions (Castresana, 2000; Talavera and Castresana, 2007). This yielded a final data set of 501 (39% of the original 1261 positions) and 294 (54% of the original 535 positions) amino acids for DXS and DXR, respectively. Selection of the best-fit empirical model of protein evolution was performed with ProtTest v1.2 software (Abascal et al., 2005), according to the Akaike information criterion. Phylogenetic analysis was inferred with the maximum likelihood method implemented in PhyML v3.0 (Guindon and Gascuel, 2003) and to obtain bootstrap support values (1000 replicates). Estimated ProtTest parameters were used and the analysis started with the BIONJ tree. Phylogenetic trees were drawn with TreeDyn software (Chevenet et al., 2006).

cDNA sequence data for the genes reported in the present paper have been deposited in GenBank under the following accession numbers: *DsDXS*, FJ469276; *DsDXR*, FJ469277; *DsMCS* (partial), FJ469279; *DsHDS* (partial), FJ469278.

Northern blot analysis

For northern blot preparation 6 μg of total RNA were used and analysis was performed as described in Ramos et al. (2008). PCR probe amplification was performed with the gene-specific primers described in Table 1.

Complementation of *Escherichia coli dxr* mutant

pBADM1 was generated by removing the *NdeI* (position 4926) by site-directed mutagenesis of the original pBAD-GFPuv (Clontech Laboratories; Lois et al., 2000). A full-length *DsDXR* cDNA fragment was PCR amplified with the primers DXR7Fw and DXR8Rev (Table 1). This fragment was cut with *Nde*I and *Eco*RI and cloned into plasmid pPADM1 previously digested with the same restriction enzymes. Plasmid pBAD-*DsDXR*, under the control of pBAD promoter which is induced with L-arabinose (L-ara), was used to transform *E. coli* mutant strain EcAB4-10 (MG1655 *dxr::CAT* MVA^+) (Rodríguez-Concepción et al., 2000; Campos et al., 2001). Transformants were plated in Luria-Bertani medium containing 17 μg mL^{-1} chloramphenicol for the mutant strain, 100 μg mL^{-1} ampicillin to select for the pBAD plasmid, 1 mM MVA for the MVA^+ operon (prepared from mevanolactone, Sigma) and 0.2 % (w/v) L-ara to induce expression of pBAD-*DsDXR*. *DsDXR* activity was confirmed by growth of transformed mutant cells on mevalonate-free media (Rodríguez-Concepción et al., 2000; Campos et al., 2001).

Table 1. Primer sequences used in this study.

Primer	Sequence (5'→ 3')
Degenerate PCR	
DXS1Fw[a]	TACTACATYGGCCCYGTBGAYGG
DXS2Rev[a]	AARGTSACKGCGTGCTGCTCVGC
DXR1Fw[a]	GTSGTSACCGGCATYGTSGGCTGCGC
DXR2Rev[a]	ATSGGCARGCGCATVTCRGGCCAGCC
Full-length amplification	
DXS3Fw	TTCTCAAGTAAGCCGGTGTCTAGCCCA
DXS4Rev	CCGACAAGACCACCAAGCAACTGTTG
DXR3Fw	AAATGCAGCAGCAACTCCGCAAGC
DXR4Rev	ACCACCATGTCCGTTTGCTAGATAG
Probe amplification	
DXS5Fw	CACCTCAGCGCGAGCCTGG
DXS6Rev	CTCGTAGCCATGGCCCTTCTCC
DXR5Fw	CTGCCTACTGTGGCCGCGATT
DXR6Rev	GCTGAGCGATCACTGAGGAGT
MCS1Fw	ACGGTCACGGATGCCGTG
MCS2Rev	TGGCCTTCAGGTTAACCACG
HDS1Fw	GTGGGTATGCCCTTTAAGGAT
HDS2Rev	GTCCATTGACGATGCAGCCCAT
HDR1Fw	TGATGTTGTCCAACAGCTTC
HDR2Rev	CCGGGTTGTGGATGATTTCGTTGGT
LCY-β1FW	TTCAACCCAGGGTACCAG
LCY-β2Rev	CGATAGCGTCCGCAACAAC
Functional assay	
DXR7Fw	CGCCATATGCGCATGCAGCAGCAACTCCGCA
DXR8Rev	ACGAATTCATTACACGGTGACAAAAACCT

[a]IUBMB codes: B=G,T,C; K=G,T; R=A,G; S=G,C; Y=C,T; V=G,A,C

RESULTS AND DISCUSSION

Cloning and sequence analysis of *DsDXS* and *DsDXR*

Although *DXS* and *DXR* have been identified and characterized from bacteria (e.g *E. coli*; Lois et al., 1998; Radykewicz et al., 2000) and several other organisms, namely plants (e.g *A. thaliana*; Estévez et al., 2000; Carretero-Paulet et al., 2002) no information regarding *Dunaliella salina* homologues was present in the available databases. Therefore, a degenerate primer approach based on the conserved sequence regions of plant and *C. reinhardii* was attempted. Degenerate primer PCR (Table 1) resulted in the amplification of partial *DsDXS* and *DsDXR* cDNA fragments 353 and 520 bp long, respectively. Nucleotide sequence data of these partial clones were used to design specific primers for 5' and 3' end RACE PCR (not shown).

The 2334-bp *DsDXS* full-length cDNA clone contained an open reading frame of 2262 bp, flanked by a 35 and 37 bp 5' and 3' untranslated region (UTR), respectively. The deduced polypeptide of 753 amino acids had an estimated molecular weight of 81.0 kDa. Amino acid sequence analysis revealed that *Ds*DXS presented higher identity with *C. reinhardtii* (77%) and other Viridiplantae (<67%) homologues, whereas a lower value was obtained with Eubacteria sequences (<59% and <46% for bacteria and cyanobacteria, respectively). Multiple sequence alignment of Viridiplantae and bacteria DXS (Figure 1) revealed the presence of a conserved histine residue (residue 137), probably involved in proton transfer by transketolases (Lois et al., 1998) and a conserved motif corresponding to a consensus thiamine pyrophosphate (TPP) binding domain located in amino acid positions 237-269 (Souret et al., 2002; Krushkal et al., 2003). Also a plant conserved glutamate residue (position 485), possibly necessary for transketolase and transketolase-like activity was identified (Bouvier et al., 1998).

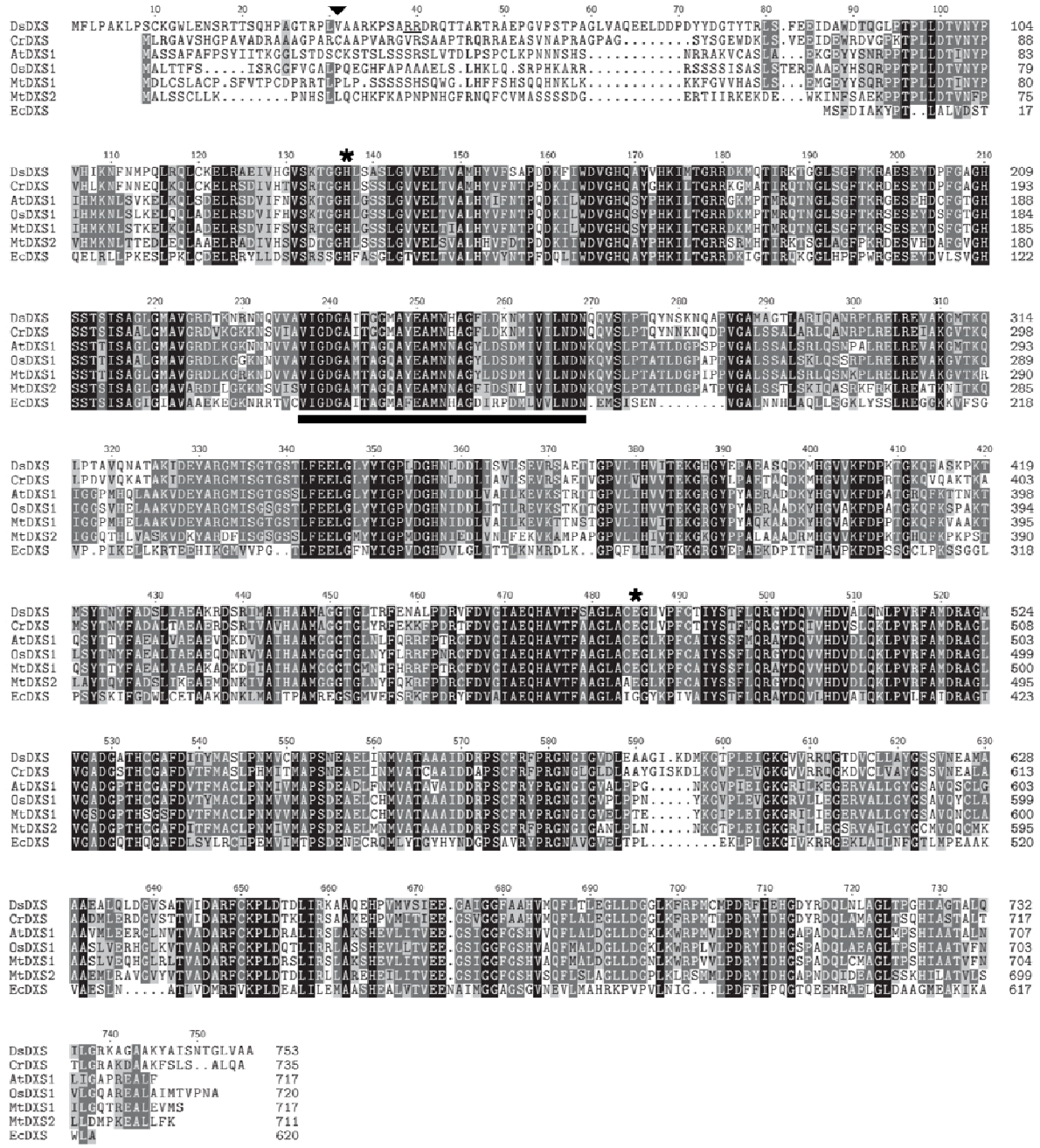

Figure 1. Alignment of DXS sequences from *Dunaliella salina* (DsDXS; GenBank accession no. FJ469276), *Chlamydomonas reinhardtii* (CrDXS, CAA07554), *Arabidopsis thaliana* (AtDXS1, Q38854), *Oryza sativa* (OsDXS1, O22567), *Medicago truncatula* (MtDXS1, CAD22530; MtDXS2, CAD22531), *Escherichia coli* (EcDXS, A1A890). Identical residues present in all sequences are indicated in black background. Arrow indicates *Ds*DXS putative plastid transit peptide cleavage site. Asterisk indicates the conserved histidine and glutamate residues involved in proton transfer and transketolocase activity, respectively. Horizontal line indicates the conserved thiamin pyrophosphate (TPP) binding domain. In the N-terminal region are underlined the arginine residues observed in thylacoid ΔpH-dependent pathway.

The entire coding region of *Ds*DXR was also amplified. The full-length cDNA sequence (1448 bp) encoded a deduced 436 amino acid protein (5' and 3' UTR of 70 and 67 bp, respectively) with a calculated molecular mass of 47.2 kDa. Comparative analysis of *Ds*DXR with other available protein sequences showed a higher identity values with higher plants (<69%) and algae (69% for *C. reinhardtii*). In contrast to *Ds*DXS, higher identity percentages were obtained for cyanobacterial species (<64%) when compared with other bacterial taxa (<59%). In Figure 2 is represented the alignment of *Ds*DXR with other plant, algae and bacterial DXR protein sequences. Bacteria and eukaryotic sequences presented a highly and important conserved amino acid motif (GSTGSIG, 91-97 positions) of a reduced nicotinamide adenine dinucleotide phosphate (NADPH) binding site (Schwender et al., 1999; Kuzuyama et al., 2000) and several putative key catalytic activity residues (aspartic acid - 233 position; histidine - positions 236, 292 and 340; glutamic acid - position 235, 314 and 317 (Reuter et al., 2002; Mac Sweeney et al., 2005).

N-terminal region of DXS and DXR of higher plants and algae, as observed in Figure 1 and 2, is poorly conserved and is not present prokaryotic sequences. Computational analysis revealed the presence of a putative plastid transit peptide in *Ds*DXS and *Ds*DXR sequences (residue 31 and 54, respectively) which is consistent with the plastidial localization of MEP pathway (Rodríguez-Concepción and Boronat, 2002; Hsieh et al., 2008). Although regarding the specific location of the following important motifs this prediction might not be precisely accurate and *in situ* data may be needed for further clarification. In the case of *Ds*DXS sequence (Figure 1) the presence of twin arginine, although with some differences in the described domain residues, suggests involvement of the ΔpH-dependent translocation pathway in the importation of this polypeptide into the thylacoids (Mori and Cline, 2001). In

the N-terminal region of plant and algal DXR sequences (Figure 2) a proline-rich motif was identified (positions 64 to 72; Carretero-Paulet et al., 2002).

As observed in other plant species (Walter et al., 2002; Phillips et al., 2007; Seetang-Nun et al., 2008), Southern blot analysis did not exclude the possibility of the occurrence of more than one gene copy encoding *DsDXS* and *DsDXR* (data not shown).

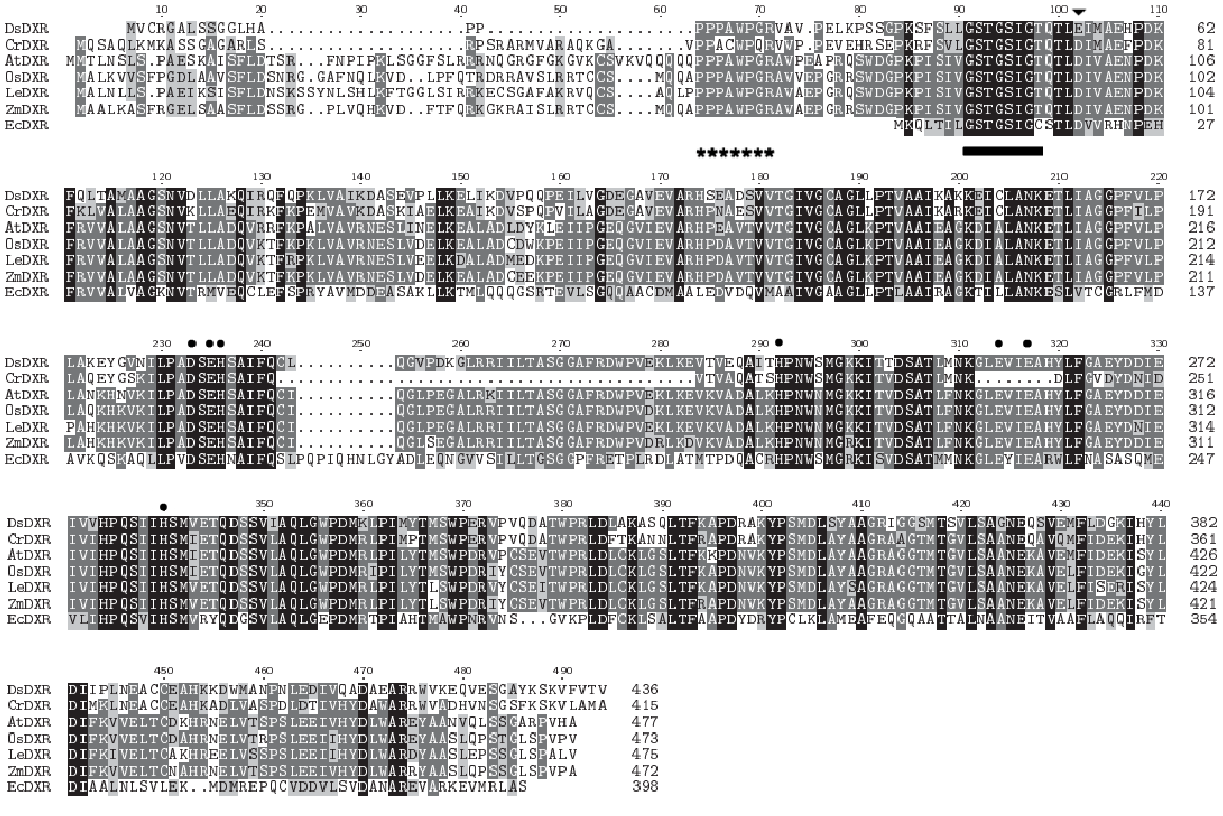

Figure 2. Alignment of DXR sequences from *Dunaliella salina* (DsDXR, GenBank accession no. FJ469277), *Chlamydomonas reinhardtii* (CrDXR, XP_001693958), *Arabidopsis thaliana* (AtDXR, AAF73140), *Oryza sativa* (OsDXR, Q8W250), *Lycopersicon esculentum* (LeDXR, AAK96063), *Zea mays* (ZmDXR, NP_001105139), *Escherichia coli* (EcDXR, NP_414715). Identical residues present in all sequences are indicated in black background. Arrow indicates *Ds*DXR putative plastid transit peptide cleavage site. Horizontal line indicates the conserved NADPH binding motif. Dots designate important catalytic activity residues. Residues representing the plant N-terminal Pro-rich motif are marked with asterisks.

Phylogenetic analysis

Phylogenetic relationships concerning DXS and DXR from *D. salina* and other algae (Chlorophyta), plant (Streptophyta) and bacterial species were performed using a maximum likehood approach. Initially, Gblocks was used to remove ambiguous parts (poorly aligned positions and divergent regions) of the amino acid sequence alignments. After these analysis a final data set of 501 (39% of original 1261 positions) and 294 (54% of the original 535 positions) amino acids for DXS and DXR, respectively, were used in phylogenetic tree constructions. The best-fitting models of protein evolution were WAG+I+G+F (-lnL of 14318.85, gamma shape with 4 rate categories of 1.088 and a proportion of invariable sites of 0.085) and RtREV+G+F (-lnL of 8254.05, gamma shape with 4 rate categories of 0.860 and a proportion of invariable sites of 0.089) for DXS and DXR, respectively. In Figure 3 and 4 are represented the DXS and DXR phylogenetic trees obtained with PhyML reconstruction method and rooted with malaria parasite, *Plasmodium falciparum,* sequences. As observed, these two MEP proteins from green algae (Chlorophyta) and higher plants (Streptophyta) seem to have different evolutionary ancestors. Viridiplantae DXRs were more closely related to cyanobacterial (Cyanophyta) homologues (Figure 4) in contrast to the apparent bacterial origin of DXS (Figure 3). These results are in agreement with the proposed prokaryotic lateral gene transfer origin of MEP pathway genes (DXS) and the described exceptions presenting a cyanobacterial gene origin: DXR and HDR (Lange et al., 2000; Ramos et al., 2009).

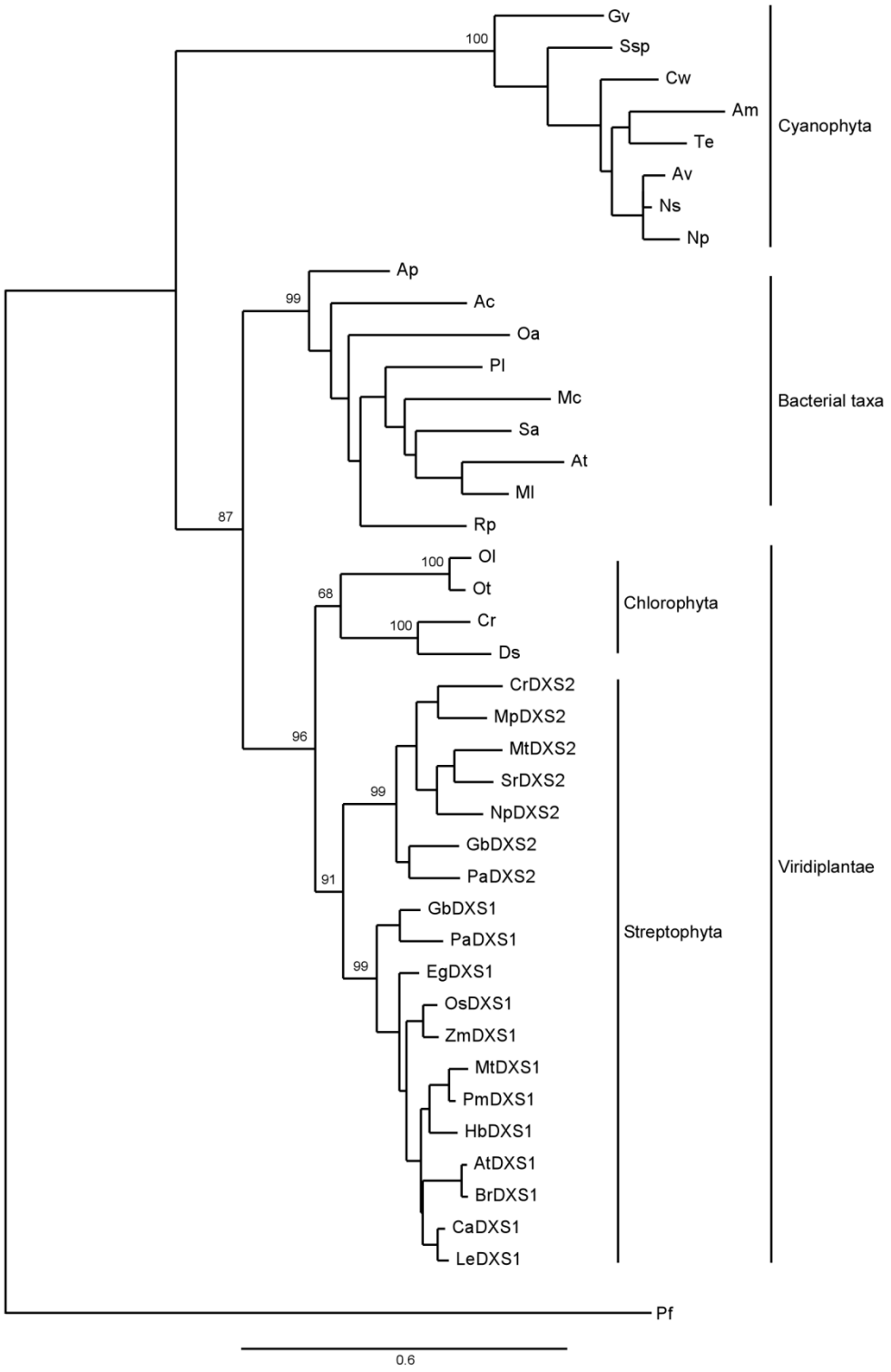

Figure 3. Maximum likelihood phylogenetic tree of Viridiplantae and bacteria DXS. The tree was obtained with PhyML (501 amino acids) using WAG+I+G+F model of protein evolution. Bootstrap analysis was done with 1000 replicates (numbers indicated on nodes in percentage) and branch lengths are drawn to scale. The tree was

rooted with DXS sequence from malaria parasite *Plasmodium falciparum* (Pf). Species and sequence accessions (GenBank) are as follows: *Gloeobacter violaceus* (Gv), Q7NP63; *Synechococcus* sp. (Ssp), YP_478194; *Crocosphaera watsonii* (Cw), EAM53007; *Acaryochloris marina* (Am), B0C8J3; *Trichodesmium erythraeum* (Te), Q10ZY2; *Anabaena variabilis* (Av), ABA24130; *Nodularia spumigena* (Ns), ZP_01629368; *Nostoc punctiforme* (Np), YP_001868716; unclassified Alphaproteobacteria (Ap), ZP_02191748; *Acidiphilium cryptum* (Ac), YP_001234954; *Oceanicaulis alexandrii* (Oa), ZP_00957752; *Parvibaculum lavamentivorans* (Pl), YP_001412061; *Methylobacterium chloromethanicum* (Mc), ZP_02057846; *Stappia aggregata* (Sa), ZP_01546679; *Agrobacterium tumefaciens* (At), AAP56243; *Mesorhizobium loti* (Ml), NP_107784; *Rhodopseudomonas palustris* (Rp); YP_571423; *Ostreococcus lucimarinus* (Ol), XP_001416190; *Ostreococcus tauri* (Ot), CAL52263; *Chlamydomonas reinhardtii* (Cr), CAA07554; *Dunaliella salina* (Ds), FJ469276; *Catharanthus roseus* (CrDXS2), ABI35993; *Mentha piperita* (MpDXS2), AAC33513; *Medicago truncatula* (MtDXS2), CAD22531; *Stevia rebaudiana* (SrDXS2), CAD22155; *Narcissus pseudonarcissus* (NpDXS2) CAC08458; *Ginkgo biloba* (GbDXS2), AAR95699; *Picea abies* (PaDXS2), ABS50520; *Ginkgo biloba* (GbDXS1), AAS89341; *Picea abies* (PaDXS1), ABS50518; *Elaeis guineensis* (EgDXS1), AAS99588; *Oryza sativa* (OsDXS1), O22567; *Zea mays* (ZmDXS1), ABP88134; *Medicago truncatula* (MtDXS1), CAD22530; *Pueraria montana* (PmDXS1), AAQ84169; *Hevea brasiliensis* (HbDXS1), BAF98288; *Arabidopsis thaliana* (AtDXS1), Q38854; *Brassica rapa* (BrDXS1), ABE60813; *Capsicum annuum* (CaDXS1), CAA75778; *Lycopersicon esculentum* (LeDXS1), AAD38941; *Plasmodium falciparum* (Pf), CAD52509.

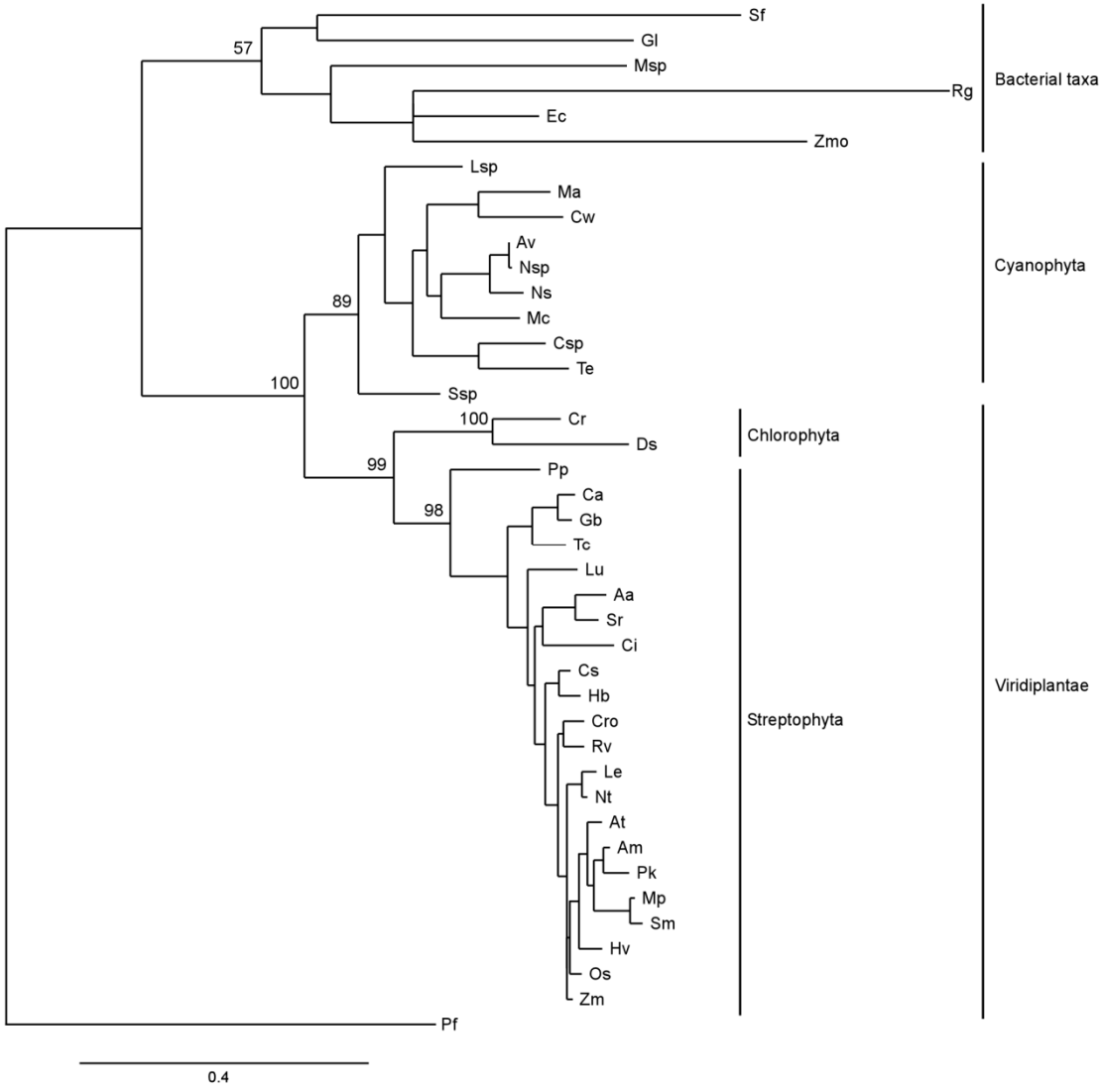

Figure 4. Maximum likelihood phylogenetic tree of Viridiplantae and bacteria DXR. The tree was obtained with PhyML (294 amino acids) using RtREV+G+F model of protein evolution. Bootstrap analysis was done with 1000 replicates (numbers indicated on nodes in percentage) and branch lengths are drawn to scale. The tree was rooted with DXR sequence from malaria parasite *P. falciparum* (Pf). Species and sequence accessions (GenBank) are as follows: *Syntrophobacter fumaroxidans* (Sf), ABK17471; *Geobacter lovleyi* (Gl), ACD96427; *Magnetococcus* sp. (Msp), ABK44354; *Rickettsiella grylli* (Rg), ZP_02062407; *Escherichia coli* (Ec), NP_414715; *Zymomonas mobilis* (Zmo), Q9X5F2; *Lyngbya* sp. (Lsp), ZP_01623647; *Microcystis aeruginosa* (Ma), YP_001660045; *Crocosphaera watsonii* (Cw), ZP_00515416; *Anabaena variabilis* (Av), ABA20924; *Nostoc* sp. (Nsp), NP_488391; *Nodularia spumigena* (Ns) ZP_01629982; *Microcoleus chthonoplastes* (Mc), EDX71261; *Cyanothece* sp. (Csp), EDY11432; *Thermosynechococcus elongatus* (Te), BAC08593; *Synechococcus* sp. (Ssp), EDX86332; *Chlamydomonas reinhardtii* (Cr), XP_001693958; *Dunaliella salina* (Ds), FJ469277; *Physcomitrella patens* (Pp), XP_001763690; *Camptotheca acuminata* (Ca), ABC86579;

Ginkgo biloba (Gb), AAR95700; *Taxus cuspidata* (Tc), AAT47184; *Linum usitatissimum* (Lu), CAF22092; *Artemisia annua* (Aa), AAD56391; *Stevia rebaudiana* (Sr), CAD22156; *Cistus incanus* (Ci), AAP56260; *Croton stellatopilosus* (Cs), ABO38177; *Hevea brasiliensis* (Hb), AAS94121; *Catharanthus roseus* (Cro), AAF65154; *Rauvolfia verticillata* (Rv), AAY87151; *Lycopersicon esculentum* (Le), AAK96063; *Nicotiana tabacum* (Nt), ABH08964; *Arabidopsis thaliana* (At), AAF73140; *Antirrhinum majus* (Am), AAW28998; *Picrorhiza kurrooa* (Pk), ABC74566.1; *Mentha piperita* (Mp), AAD24768; *Salvia miltiorrhiza* (Sm), ABJ80680; *Hordeum vulgare* (Hv), CAE47438; *Oryza sativa* (Os), Q8W250; *Zea mays* (Zm), NP_001105139; *Plasmodium falciparum* (Pf), AAD03739.

Functional analysis of *DsDXS* and *DsDXR*

Disruption of any *E. coli* MEP pathway enzyme is lethal although rescue is possible with metabolic engineered synthetic operons and heterologous genes. An *E. coli* defective *DXR* gene mutant EcAB4-10 (MG1655 *dxr::CAT* MVA^+) was transformed with the plasmid pBAD-*DsDXR* and with the empty pBADM1 plasmid as an expression control (Rodríguez-Concepción et al., 2000; Campos et al., 2001). Upon induction, only pBAD-*DsDXR* transformants were able to grow in media containing L-ara (without MVA). These results indicate that the isolated *D. salina* cDNA fragment encodes a functional DXR.

In the case of *Ds*DXS, several pBADM1 plasmid constructions with the isolated full-length or partial (without the N-terminal region) cDNA were used to transform the *E. coli* mutant strain EcAB4-2 (MG1655 *dxs::CAT* MVA^+; Rodríguez-Concepción et al., 2000; Campos et al., 2001). So far all attempts to obtain stable *DsDXS* transformants have failed. Further expression systems will be used in order to characterize *Ds*DXS.

Expression analysis of *DsDXS* and *DsDXR* in response to light stress

Carotenoid biosynthesis in *D. salina* can be induced by several abiotic conditions and earlier results in our laboratory suggested not only a transcriptional regulation of carotenogenic genes but also a possible coordination between this process and MEP pathway (Coesel et al., 2008; Ramos et al., 2008; 2009). To investigate the possible regulatory role of *DsDXS* and *DsDXR* a light stress experiment was performed (Figure 5) since up-regulation of some MEP pathway genes by light has also been described in plants (Souret et al., 2002; Botella-Pavía et al., 2004; Kim et al., 2008). Gene expression analysis obtained by northern blot analysis of *Dunaliella* MEP pathway genes is represented in Figure 5D. In comparison with control cells (+N LL), an increase in steady-state mRNA transcript levels of all the investigated genes, with the exception of *DsDXR*, was observed in response to high light (HL) conditions either at 3 or 24 h. Furthermore, a coordinated up-regulation of *DsDXS*, *DsHDS*, and *DsHDR* was noticeable (Figure 5D). In this particular experiment, high light rather than nutritional limitation appeared to be the main gene expression inductive factor. Although the incident light intensity was the same as described in previous experiments (Coesel et al., 2008; Ramos et al., 2008; 2009), the effect of self-shading was lower due to smaller culture volumes (0.5 L instead of 1.0 L) used during this experiment. This result suggests that high light can become the factor with the highest impact on MEP pathway transcript up-regulation if the effect of self-shading is decreased by lowering culture volumes. To test whether the same held true for genes encoding carotenoid biosynthetic enzymes, lycopene β-cyclase (*DsLCY-β*) transcript levels were also determined. Indeed, steady-state *DsLCY-β* mRNA levels were higher in cells under high light when compared to control cells, suggesting that a majority of MEP pathway and carotenogenic enzymes are co-ordinately induced by high light in *D. salina* (Figure 5D). In these conditions, the nutrient status of the culture seems to be a less important regulatory factor if self-shading is reduced to a minimum. Nevertheless, highest steady-state

DsHDS and *DsHDR* transcript levels were observed after 24 h induction in conditions of high light (HL) and nutritional depletion (-N; Figure 5D). Conversely, no observable changes in *DsDXR* steady-state mRNA transcript levels were detected in the tested conditions (Figure 5D). After 24 h light up-shift, β-carotene accumulation was higher in cells exposed to high light conditions (HL +N, 5.05 μg mL^{-1}, 9.72 pg $cell^{-1}$; HL –N, 5.34 μg mL^{-1}, 10.51 pg $cell^{-1}$; Figure 5B, C). Significant differences in β-carotene (μg mL^{-1}) content (Fisher's LSD test) between cells submitted to low (LL, +N and –N) and high light (HL, +N and –N) conditions were obtained (Figure 5 B, C).

Thus, plastidial isoprenoid end product synthesis, such as carotenoids, seems to involve increased *DsDXS*, *DsHDS*, *DsHDR* and *DsLCY-β* transcript levels in this microalga. Similar correlation of gene up-regulation and carotenoid accumulation were obtained for *DXS* and *HDR* during tomato fruit ripening (Lois et al., 2000; Rodríguez-Concepción et al., 2003; Botella-Pavía et al., 2004). Interestingly, *DsHDS* transcript accumulation differs from the described non-limiting proposed role of tomato *HDS* for carotenoid biosynthesis (Rodríguez-Concepción et al., 2003). DXR role in MEP regulation is still unclear; the stable *DsDXR* transcript levels throughout the imposition of carotenogenesis-inducing abiotic stress conditions are in agreement with the observation that DXR may play an insignificant role in the metabolic flux control of the MEP pathway demonstrated not only during tomato fruit ripening carotenoid accumulation (Rodríguez-Concepción et al., 2001), but also in the biosynthesis of volatile terpenes in snapdragon flowers (Dudareva et al., 2005). However other plant system experimental results suggest a contradictory DXR role (Veau et al., 2000; Carretero-Paulet et al., 2002; Mayrhofer et al., 2005).

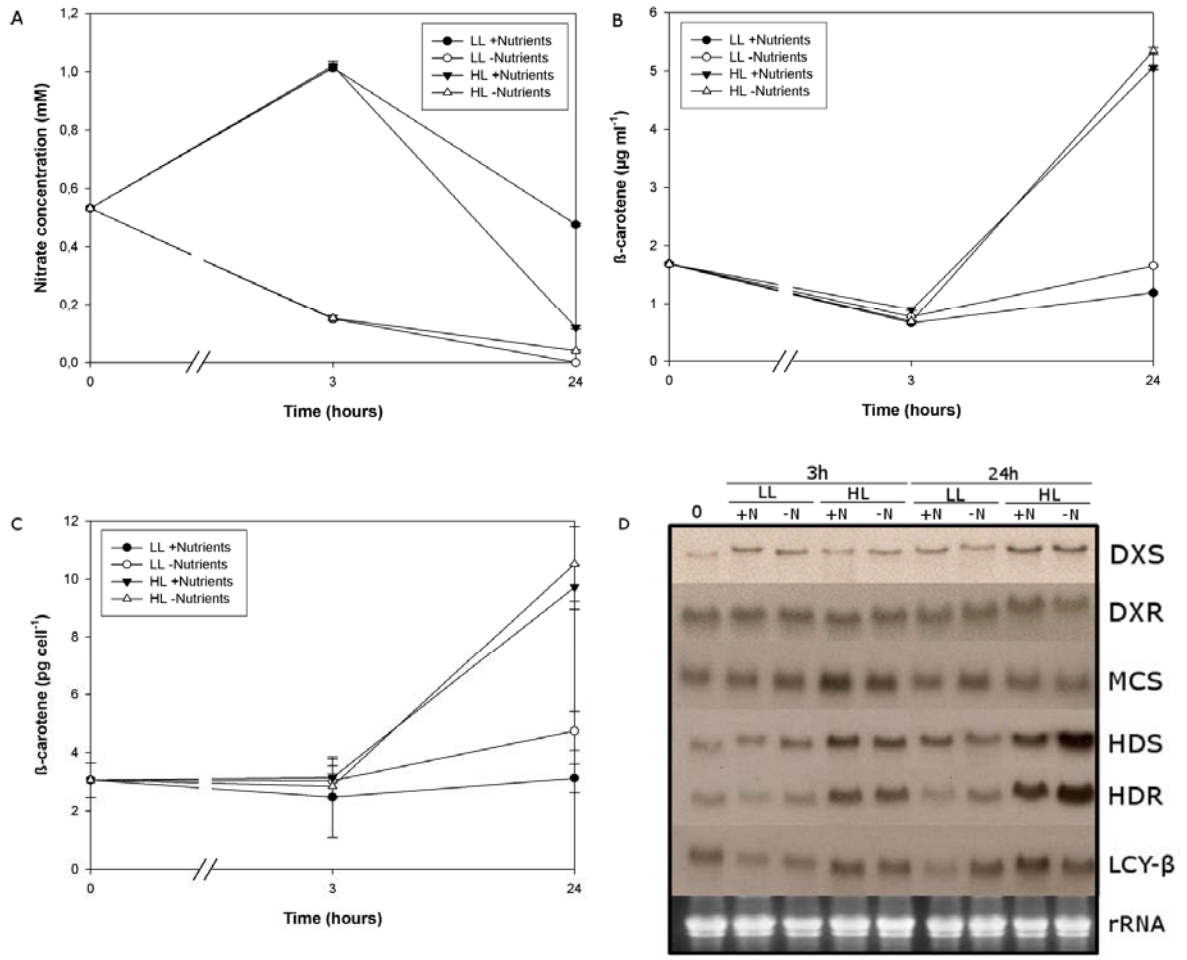

Figure 5. Analysis of light stress induction on steady-state mRNA levels of several *D. salina* MEP (*DXS*, *DXR*, *MCS*, *HDS* and *HDR*) and carotenoid biosynthetic (*LCY-β*) pathways genes (**D**). Cells pre-adapted to Walne medium (9% NaCl) and low-light conditions (LL) were transferred to a nutrient-supplemented (+N) or depleted (-N) medium at the same salinity and high-light conditions (HL). Samples were collected before (0) and 3 and 24 h upon the stress onset. (**A**) Nitrate concentration (mM) in the medium; (**B**) and (**C**) represent accumulation per unit volume (µg mL^{-1}) and intracellular concentration (pg $cell^{-1}$) of β-carotene in *D. salina* cultures. Data are the means of 3 replicates ±SD.

Although post-transcriptional modulation of MEP pathway occurs (Laule et al., 2003; Guevara-García et al., 2005; Sauret-Güeto et al., 2006), the data obtained in this and prior *Dunaliella* studies (Ramos et al., 2009) suggest that this metabolic process is likely regulated at transcription level and a correlation between mRNA transcript levels of *DsDXS*, *DsHDS*, *DsHDR* and carotenoid biosynthesis occurs. Further research on *D. salina* MEP pathway in order to characterize the remaining unknown genes and to elucidate flux control contribution of each specific enzyme is thus required for potential biotechnological purposes. Also feedback control mechanisms and specific transcriptional regulators that might control several steps of this pathway should be considered in future studies. Understanding the factors involved in the regulation of isoprenoid biosynthesis is required for the productivity improvement of commercially valuable carotenoids by metabolic engineering in genetically modified organisms.

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Chapter 2

Molecular and functional characterization of a cDNA encoding 4-hydroxy-3-methylbut-2-enyl diphosphate reductase from *Dunaliella salina*

Ramos AA, Marques AR, Rodrigues M, Henriques N, Baumgartner A, Castilho R, Brenig B, Varela J. J Plant Physiol 2009. doi:10.1016/ j.jplph.2008.11.008

SUMMARY

In green algae, the final step of the plastidial methylerythritol phosphate (MEP) pathway is catalyzed by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR; EC: 1.17.1.2), an enzyme proposed to play a key role in the regulation of isoprenoid biosynthesis. Here we report the isolation and functional characterization of a 1959-bp *Dunaliella salina HDR (DsHDR)* cDNA encoding a deduced polypeptide of 474 amino acid residues. Phylogenetic analysis implied a cyanobacterial origin for plant and algal *HDR* genes. Steady-state *DsHDR* transcript levels were higher in *D. salina* cells submitted to nutritional depletion, high salt and/or high light, suggesting that *DsHDR* may respond to the same environmental cues as genes involved in carotenoid biosynthesis.

Keywords: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; carotenogenesis; *Dunaliella salina*; isoprenoid biosynthesis; methylerythritol phosphate pathway

Abbreviations: DMAPP, dimethylallyl diphosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; HMBPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; IPP, isopentenyl diphosphate; LCY-β, lycopene β-cyclase; MEP, methylerythritol phosphate; MVA, mevalonate; PDS, phytoene desaturase; PSY, phytoene synthase; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

INTRODUCTION

Isoprenoids (also called terpenoids) comprise the largest group of natural products and play essential roles in all organisms. Plants isoprenoids are involved in several biological processes (e.g. photosynthesis, membrane fluidity, growth regulation, cell division, communication and defense responses; Bouvier et al., 2005) and have numerous commercial and industrial purposes (e.g. carotenoids applied to human health; Rao and Rao, 2007).

Two distinct pathways are involved in the biosynthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the universal C_5 precursors of isoprenoids: the cytosolic mevalonate (MVA) pathway, present in most organisms, but apparently absent in Chlorophyta, and a plastidial pathway known as methylerythritol phosphate (MEP) pathway which, occurs in eubacteria, cyanobacteria, apicomplex parasites, algae and higher plants (reviewed in Lichtenthaler et al., 1997; Rohmer, 1999; Schwender et al., 2001; Rodríguez-Concepción and Boronat, 2002; Liu et al., 2005). Cross-talk between these two pathways occurs (Laule et al., 2003; Dudareva et al., 2005) and the complex regulation of this non-mevalonate pathway, which involves eight identified enzymes, still remains unclear (Rohmer, 2003; Hunter, 2007). It has been suggested that several enzymes may regulate the metabolic flux through the MEP pathway and two rate-determining enzymes have been identified, namely 1-deoxy-D-xylulose 5-phosphate synthase (DXS, EC: 4.1.3.37) and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR, EC: 1.17.1.2). DXS is involved in the initial step, while HDR is responsible for the conversion of 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) to IPP and DMAPP in the final step of the MEP pathway (Lichtenthaler, 1999; Eisenreich et al., 2004).

Recently, selective gene knockout has demonstrated not only that HDR activity is essential in *E. coli* (Cunningham et al., 2000; Altincicek et al., 2001), but also this enzyme plays a role in plant isoprenoid biosynthesis (Page et al., 2004). Beside transcription and post-

transcriptional events modulating the MEP pathway (Guevara-García et al., 2005; Rodríguez-Concepción, 2006), evidence of coordinated control between this and other downstream pathways (e.g. carotenoid biosynthetic pathway) has been shown (Lois et al., 2000; Rodríguez-Concepción et al., 2003; Wille et al., 2004). Moreover, overexpression of several MEP pathway enzymes in *Arabidopsis thaliana*, *Mentha piperita* and *Lycopersicon esculentum* resulted in higher accumulation of plastid isoprenoids (Estévez et al., 2001; Mahmoud and Croteau, 2001; Botella-Pavía et al., 2004; Carretero-Paulet et al., 2006).

Plastidial isoprenoid end-products such as carotenoids have been commercially exploited for numerous market applications. An example of these is β-carotene, a carotenoid possessing well-known antioxidant and immunomodulatory activities (Murthy et al., 2005; Raja et al., 2007a) and whose main natural source is the green alga *Dunaliella salina* (Del Campo et al., 2007; Raja et al., 2007b). This microalga can accumulate more than 10% of algal dry weight of β-carotene under particular abiotic conditions (Ben-Amotz and Avron, 1983; Borowitzka et al., 1990; Cifuentes et al., 1996; Coesel et al., 2008). Therefore, understanding the regulation of isoprenoid biosynthesis in this halophilic organism is important for potential biotechnological and metabolic engineering purposes (León-Bañares et al., 2004).

To date, the enzymes and the regulatory mechanisms involved in MEP pathway in *D. salina* (Ye et al., 2008) and other green algae remain largely unknown and recent evidence suggests a major role of HDR in controlling the flux of MEP-derived precursors in plants (Botella-Pavía et al., 2004). By means of a subtractive cDNA library generated with cells under abiotic stress (nutrient and salt stress) we were able to isolate a stress-inducible *D. salina HDR* homolog (*DsHDR*). In this study, we report the cloning of a full-length *DsHDR* cDNA encoding the first enzyme of *D. salina* MEP pathway to be described and characterized. Biotechnological applications of this study are discussed.

MATERIAL AND METHODS

Alga growth conditions and analytical methods

Dunaliella salina strain CCAP 19/30 was obtained from the Culture Collection for Algae and Protozoa (Cumbria, UK). The cells were maintained and cultured in modified Walne medium (Walne, 1974) in conditions described previously (9% salinity [w/v] and continuous illumination – 45 μmol m^{-2} s^{-1}; Ramos et al., 2008) and salinity levels were adjusted with NaCl (9 or 18% w/v). Cell number, total nitrate concentration of the media and total β-carotene content of the cells were determined as described previously (Ramos et al., 2008). Covariance analysis (ANCOVA) and a post-hoc comparison test (Fisher's least significant difference method - LSD) were performed with the computational program STATISTICA v. 6.0 (StatSoft). Differences were considered to be significant at a probability of 5% ($p<0.05$).

Carotenogenesis inductive experiments were performed with exponentially growing *Dunaliella* cultures (2.5 x 10^5 cells mL^{-1}) and concerned three abiotic stress conditions: high salt, nutrient depletion and high light. Cultures were diluted in equal amount of either Walne medium (nutrient-supplemented) or water (nutrient-depleted) at a salinity of 27% (w/v) to obtain a NaCl concentration of 18% (w/v). High light stress was performed with an additional white lamp (150 W Massive N.V/S.A) which increased the light intensity levels from 45 to 500 μmol m^{-2} s^{-1}.

Total RNA isolation

Standard methods (Sambrook et al., 1989) were used unless otherwise indicated. Total RNA was extracted from 5-10 x 10^6 cells using the TRI REAGENT™ (Sigma) method according to the supplier's protocol.

Cloning of *DsHDR*

Dot-blot analysis of a *D. salina* subtractive cDNA library constructed with cells subject to abiotic stress (high salinity and nutrient depletion) allowed the isolation of several cDNA clones differentially expressed under the tested conditions, including a partial one with homology to published plant *HDR* sequences (data not shown; GenBank accession number EG591728).

Several rapid amplifications of cDNA ends (RACE) in order to reach the 5' and 3' ends of this gene (data not shown) were performed using the SMART^{TM} RACE cDNA Amplification Kit (BD Biosciences Clontech). The full-length *DsHDR* cDNA was amplified with the sense primer HDR1Fw and the anti-sense primer HDR2Rev (Table 1). The procedure is as follows: 1 cycle of 94 ºC, 2 min; 30 cycles of 94 ºC - 1 min, 57ºC – 1 min, 68ºC – 3 min; and 1 cycle of 68ºC – 10 min. All RACE products were cloned into pGEM-T Easy vector (Promega) and provided for sequencing.

Sequencing and bioinformatic analysis

The nucleotide sequences were determined for both strands using an ABI Prism automated sequencing system (PE Biosystems, Macrogen-Korea). Nucleotide and derived amino acid sequence analysis was performed using DNASTAR (Lasergene) and Geneious Pro 2.5.3 (Biomatters). Multiple alignments of amino acid sequences were performed using the M-coffee option of the T-Coffee program (Notredame et al., 2000; Moretti et al., 2007). Gaps and poorly aligned sequences were trimmed from alignments using Gblocks version 0.91b (Castresana, 2000; Talavera and Castresana, 2007). The default parameters used by Gblocks (minimum number of sequences for a conserved position, 12; minimum number of sequences for a flanking position, 19; maximum number of contiguous non-conserved positions, 8;

minimum length of a block, 10) yielded a final data set of 347 (61% of the original 561 positions). The best-fitting model of evolution was selected by ProtTest v1.2 (Abascal et al., 2005), following the Akaike information criterion. The selected model was used in all phylogenetic reconstructions. Maximum likelihood analyses were carried out with PHYML v2.4.4 (Guindon and Gascuel, 2003) starting from the BIONJ tree, and fixing the proportion of I and Γ to the value estimated by ProtTest. Bootstrap values were based on 500 replicates. The tree was rooted using the midpoint rooting algorithm in PAUP version 10b (Swofford, 2002). Prediction of putative chloroplast transit peptides was made with the ChloroP 1.1 program (Emanuelsson et al., 1999). The full-length *DsHDR* cDNA was deposited in GenBank under the acession number FJ040210.

RNA gel blot analysis

Total RNA samples (6 μg) were denaturated and electrophoresed under denaturing conditions on a 1% (w/v) agarose gel containing formaldehyde (5%) and transferred to a Hybond^{TM}-N nylon membrane (Amersham Biosciences) according to the manufacturer's instructions. Probe labeling, hybridization, washes and signal detection were performed as described in Ramos et al. (2008). A 459-bp *DsHDR* probe used for blot analysis was amplified by PCR with the gene-specific primers HDR3Fw and HDR4Rev (Table 1).

Genetic complementation assay

DsHDR full-length cDNA was digested with *Sph*I and *Hin*dIII at sites introduced via PCR primers (HDR5Fw and HDR6Rev; Table 1), and cloned into a similarly cut pQE-80L expression vector (Qiagen). The pQE-*DsHDR* plasmid was transformed in the *Escherichia*

coli mutant strain MG1655 *ara<>ispH* (McAteer et al., 2001) and selected on LB plates containing 50 mg/mL kanamycin, 50 mg/mL ampicillin, 0.2% glucose (Glc), and 0.5 mM IPTG. As a negative control the empty pQE-80L was transformed into the previous *E. coli* mutant strain and plated on LB plates as described above. Cells transformed with the empty pQE-80L were unable to grow on LB medium containing 0.2% glucose.

Table 1. Oligonucleotides used in the present study.

Designation	**Sequence (5'→ 3')**
HDR1Fw	TGAAGGCACTCATCGTCATTCGAAG
HDR2Rev	AAACGTTGGCAAGTGGATTAGATTAGC
HDR3Fw	TGATGTTGTCCAACAGCTTC
HDR4Rev	CCGGGTTGTGGATGATTTCGTTGGT
HDR5Fw	CCGGCATGCGAGATGATGTTGTCCAACAGCTTC
HDR6Rev	CCCAAGCTTGGGTTAGTGGGTGGGCAGGAC

RESULTS AND DISCUSSION

Cloning and characterization of *DsHDR*

A partial *DsHDR* cDNA clone with a high degree of sequence homology to plant *HDR* genes was identified upon the screening of a subtractive cDNA library enriched for abiotic stress-induced sequences. This result allowed the design of specific primers for RACE PCR amplification of the full-length *DsHDR* cDNA (data not shown).

The full-length 1959 bp cDNA clone encoded a deduced protein sequence of 474 amino acids with an estimated molecular weight of 53.2 kDa. The 5' untranslated region (UTR) consisted of 27 bp upstream of the start codon and a 3' UTR of 507 bp downstream from the stop codon.

*Ds*HDR showed the highest sequence similarity with other Chlorophyta (*Chlamydomonas reinhardtii* - 67% and *Ostreococcus lucimarinus* - 64%), plant and cyanobacteria (>53%) homologues, confirming previous observations that MEP pathway enzymes are highly conserved (Lange et al., 2000). Multiple sequence alignment of the deduced *Ds*HDR amino acid sequence with other plant and bacterial enzymes is represented in Figure 1. The Viridiplantae (*D. salina* included) and *Synechococcus elongatus* sequences contained the four conserved cysteine residues that are known to be essential for the catalytic activity of HDR (Wolff et al., 2003; Gräwert et al., 2004). In *E. coli* one cysteine residue (position 272 of the alignment) is not conserved; however, the cysteine residue observed at position 421 might be involved in the [4Fe-4S] coordination. Moreover, two fully conserved histidine residues (position 156 and 246), suggested to be involved in proton-transfer reactions, were also observed (Adam et al., 2002). The predicted cleavage site of the putative chloroplast transit peptide of *Ds*HDR is indicated in Figure 1. This signal sequence at the N-terminal, absent in bacterial sequences, was identified in other plant HDR proteins, which is

consistent with the demonstrated chloroplast subcellular localization of all *Arabidopsis* MEP pathway enzymes (Rodríguez-Concepción and Boronat, 2002; Hsieh et al., 2008).

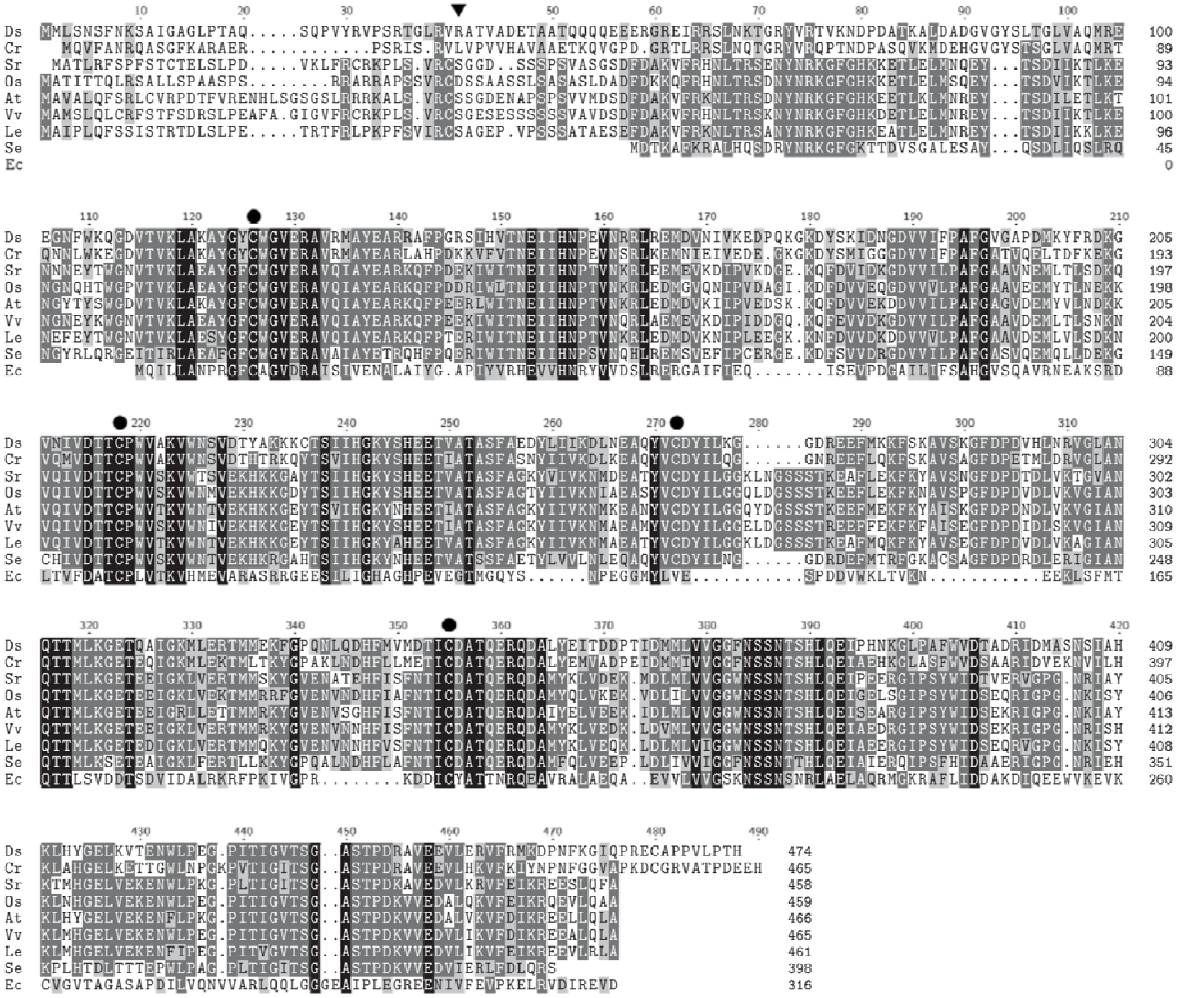

Figure 1. Alignment of HDR sequences from *Dunaliella salina* (Ds; GenBank accession no. FJ040210), *Chlamydomonas reinhardtii* (Cr, EDO97597), *Oryza sativa* (Os; AAT77894), *Arabidopsis thaliana* (At; AAN87171), *Synechococcus elongatus* PCC 7942 (Se; ABB56105), *Stevia rebaudiana* (Sr; ABB88836), *Escherichia coli* (Ec; AAL38655), *Lycopersicon esculentum* (Le; TC Annotator: TC182023) and *Vitis vinifera* (Vv; TC53222). Conserved amino acids when present in all sequences are indicated in black background. Arrow indicates the putative cleavage site of the plastid-targeting peptides for *Ds*HDR. Conserved cysteine residues are marked with dots.

Depending on the plant species, single (e.g. *A. thaliana*) or multiple copies (e.g. *Ginkgo biloba*) of the *HDR* gene were identified (Rodríguez-Concepción and Boronat, 2002; Kim et al., 2008). In the particular case of *D. salina,* DNA gel blot analysis (data not shown) suggested the existence of a single *DsHDR* copy in its genome since the genomic blot pattern showed one band in all the analysed samples (high and low stringency conditions).

Phylogenetic analysis

A phylogenetic analysis was performed using the deduced amino acid HDR sequences from bacterial and non-bacterial organisms (Viridiplantae; Figure 2). Initially, from the 561 amino acid positions in the original alignment of the *DsHDR* fragment, 214 were identified by Gblocks as gapped or poorly aligned and were not considered in subsequent analyses. Thus, the final protein data set contained 61% of the original data set, i.e. 347 amino acids. The RtREV +I+Γ+F model (Dimmic et al., 2002) was selected as best-fitting model among the 96 models tested with an -lnL of 9753.72, gamma shape with 4 rate categories of 0.923 and a proportion of invariable sites of 0.049. This unrooted phylogenetic tree (Figure 2) can be divided into several groups that include Streptophyta (higher plants), Chlorophyta (green algae), Cyanophyta (cyanobacteria) and Eubacteria. Higher plants and green algae HDR are closely related and possibly share a common cyanobacterial ancestral.

Evolution of MEP pathway genes in different eukaryotic species has been explained by lateral gene transfer between prokaryotes (Lange et al., 2000). However, it has been suggested that 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*) and, recently, plant *HDR* genes may have a cyanobacterial origin (Lange et al., 2000; Guevara-García et al., 2005; Wang et al., 2008). Therefore our data, which included green algae in phylogenetic analyses of HDR sequences for the first time, supports the endosymbiotic origin with a cyanobacterial ancestral for eukaryotic *HDR* genes.

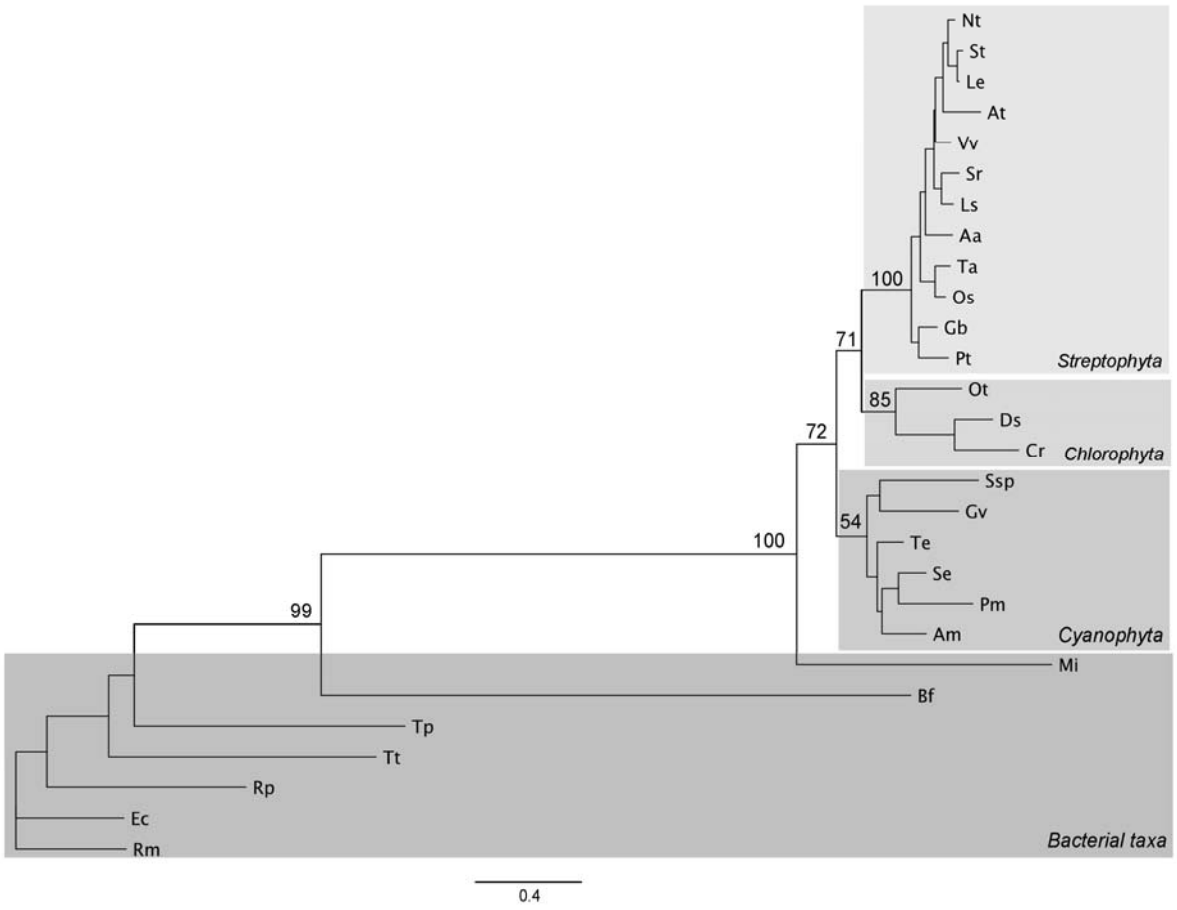

Figure 2. Phylogenetic tree of bacterial and non-bacterial HDR sequences. Unrooted tree was constructed using 28 HDR sequences (347 amino acids) and can be divided in the following groups: bacterial taxa, Cyanophyta (cyanobacteria), Chlorophyta (green algae) and Streptophyta (higher plants). Branch lengths are drawn to scale and the numbers at respective nodes indicate the bootstrap values in percentage (n=500). Species and sequence accessions (GenBank or TC annotator) are as follows: *Ralstonia metallidurans* (Rm), YP_585010; *Escherichia coli* (Ec), AAL38655; *Rhodopseudomonas palustris* (Rp), NP_949607; *Thermus thermophilus* (Tt), Q5SMC8; *Theileria parva* (Tp), XP_762798; *Bacteroides fragilis* YCH46 (Bf), YP_101024; *Methylokorus infernorum* (Mi), YP_001940770; *Acaryochloris marina* (Am), ABW29921; *Prochlorococcus marinus* (Pm), YP_396763; *Synechococcus elongatus* PCC 7942 (Se), ABB56105; *Thermosynechococcus elongatus* (Te), NP_681832; *Gloeobacter violaceus* PCC 7421 (Gv), NP_926245; *Synechococcus* sp. JA-3-3Ab (Ssp), Q2JVA7; *Chlamydomonas reinhardtii* (Cr), XP_001701302; *Dunaliella salina* (Ds), FJ040210; *Ostreococcus tauri* (Ot), Q013S6; *Pinus taeda* (Pt), ABO26587; *Ginkgo biloba* (Gb), DQ251631; *Oryza sativa* (Os), AAT77894; *Triticum aestivum* (Ta), TC232642; *Adonis aestivalis* (Aa), Q9FEP0; *Lactuca sativa* (Ls), TC12240; *Stevia rebaudiana* (Sr), ABB88836; *Vitis vinifera* (Vv), TC53222; *Arabidopsis thaliana* (At), AAN87171; *Lycopersicon esculentum* (Le), TC182023; *Solanum tuberosum* (St), ABB55395; *Nicotiana tabacum* (Nt), TC7319.

DsHDR expression profile under carotenogenesis inductive conditions

To examine the expression pattern of *DsHDR* mRNA levels under different abiotic stress, conditions known to involve high β-carotene production rates in this microalga (Coesel et al., 2008), two experiments were performed (Figures 3 and 4; Tables 2 and 3). In both experiments, significant differences in β-carotene (μg mL^{-1}) content (Fisher's LSD test) in cells exposed to diverse forms of abiotic stress and control conditions were obtained.

Salinity up-shift (9-18% NaCl) and/or nutrient depletion (-N) resulted in an increase, 36h upon the stress onset, of *DsHDR* steady-state mRNA transcript levels as observed in Figure 3. In fact, maximum transcript levels resulted from nutritional stress (9%-N and 18%-N; Figure 3) although the highest accumulation of intracellular β-carotene was observed in cells subjected to both stresses (3.14 μg mL^{-1}, 15.50 pg $cell^{-1}$; Table 2). Similar results were observed in the experiment with low light (LL) or high light (HL) and/or nutrient depletion (-N) conditions (Figure 4). The highest *DsHDR* steady-state mRNA transcript and β-carotene levels were obtained in *D. salina* cells (24h) submitted to nutritional stress (LL –N and HL –N) and both stresses (HL –N; 3.96 μg mL^{-1}, 9.30 pg $cell^{-1}$; Table 3), respectively.

Table 2. Nitrate concentration (mM) in the medium and β-carotene accumulation (μg mL^{-1}; pg $cell^{-1}$) in *D. salina* cultures. Cells pre-adapted to 9% salinity were transferred to a nutrient-supplemented medium at 9% (9%+N) or 18% (18%+N) salinity and to nutrient-depleted medium at both salinities (9%-N, 18%-N). Data are the means of 3 replicates ±SD.

Time (h)	Medium	Nitrate concentration (mM)	β-carotene	
			(μg mL^{-1})	(pg $cell^{-1}$)
0	9% +N	0.44±0.02	2.17±0.12	4.44±1.13
36	9% +N	1.01±0.04	1.57±0.04	3.07±0.37
36	18% +N	1.57±0.04	1.59±0.10	7.02±2.26
36	9% -N	0.00±0.00	1.76±0.13	4.34±1.72
36	18% -N	0.00±0.00	3.14±0.29	15.50±3.36

Table 3. Nitrate concentration (mM) in the medium and β-carotene accumulation (μg mL^{-1}; pg $cell^{-1}$) in *D. salina* cultures. Cells pre-adapted to low light conditions (LL) and 9% salinity were submitted to high light conditions (HL) and either nutrient-supplemented medium (+N) or nutrient-depleted medium (-N). Data are the means of 3 replicates ±SD.

Time (h)	Medium	Nitrate concentration (mM)	β-carotene	
			(μg mL^{-1})	(pg $cell^{-1}$)
0	LL+N	0.32±0.01	1.02±0.16	2.21±0.88
24	LL+N	0.53±0.02	0.74±0.09	1.56±0.51
24	LL-N	0.06±0.03	1.15±0.11	3.61±0.83
24	HL+N	0.17±0.05	3.10±0.30	4.73±2.14
24	HL-N	0.12±0.02	3.96±0.55	9.30±5.14

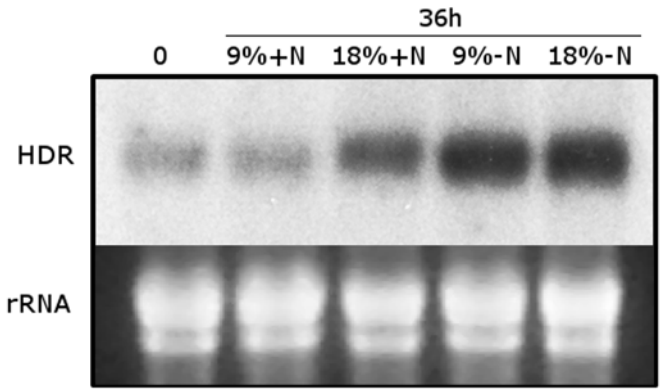

Figure 3. Effect of salinity increase (9-18%) and nutrient availability (supplemented +N or depleted –N) on *DsHDR* steady-state mRNA levels. For RNA gel blot analysis RNA samples were collected before (0) and 36h after the stress onset.

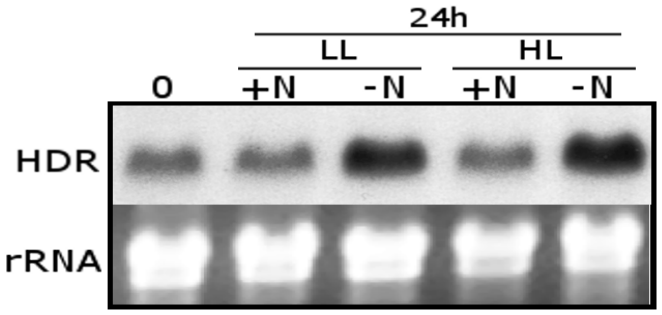

Figure 4. Effect of light stress (HL) and nutrient availability (supplemented +N or depleted –N) on *DsHDR* steady-state mRNA levels. For RNA gel blot analysis RNA samples were collected before (0) and 24h after the stress onset.

DsHDR expression patterns were similar to those observed in the described stress conditions for the downstream carotenoid biosynthetic pathway genes namely phytoene synthase (*PSY*), phytoene desaturase (*PDS*) and lycopene β-cylase (*LCY-β*; Coesel et al., 2008; Ramos et al., 2008). Carotenoid accumulation in this microalga was regulated primarily by nutritional medium limitation and this factor seems to be important in the metabolic control of MEP biosynthetic pathway as well. Indeed, if cell growth is severely limited by nitrate deficiency, gene expression related to carotenoid biosynthesis may be reduced. For instance, Sánchez-Estudillo et al. (2006), using a heterologous probe, have reported that *D. salina DXS* mRNA levels were higher in control cells as compared to nitrate-limited cultures. Nevertheless, additional stress factors known to increment carotenogenic genes transcript levels (high light intensity and high salt) might also regulate MEP genes. In several plants, other factors such as light are known to upregulate some key MEP pathway genes, including *HDR* (Botella-Pavía et al., 2004; Kim et al., 2008). However, in the tested time line, which included additional time points (3 and also 48h; data not shown), light up-shift did not influence steady-state *DsHDR* transcript levels significantly (as observed in 2-

week-old *Arabidopsis* seedlings; Hsieh and Goodman, 2005). Thus, analysis of recent published data concerning *PSY*, *PDS* and *LCY-β* transcript levels (Coesel et al., 2008; Ramos et al., 2008) and *DsHDR* current observations suggest the existence of a coordination of both pathways in *D. salina* under abiotic stress, with particular emphasis on nutrient stress. Interestingly, a similar coordination of MEP with downstream pathways such as the enzymes involved in carotenoid biosynthesis has been observed during tomato fruit ripening and *A. thaliana* seedling de-etiolation (Lois et al., 2000; Botella-Pavía et al., 2004).

Functional analysis of *DsHDR*

A complementation assay with a lethal *E. coli* mutant defective in the *HDR* gene (strain MG1655; McArteer et al., 2001) was performed in order to test whether the *Ds*HDR polypeptide showed enzymatic activity similar to that in its *E. coli* counterpart. The *HDR* gene is essential for the survival of this *E. coli* and therefore no growth was observed in the absence of arabinose (Figure 5). Upon transformation with the constructed vector harbouring the *DsHDR* gene (pQE-*DsHDR*), the lethal phenotype of the mutant strain was rescued and cells were able to grow in medium with glucose (Figure 5D). The opposite was observed for cells transformed with the empty pQE-80L vector (Figure 5C). Therefore, the enzymatic mechanisms involved in the synthesis of the isoprenoid precursors between *Ds*HDR and *E. coli* HDR might be similar.

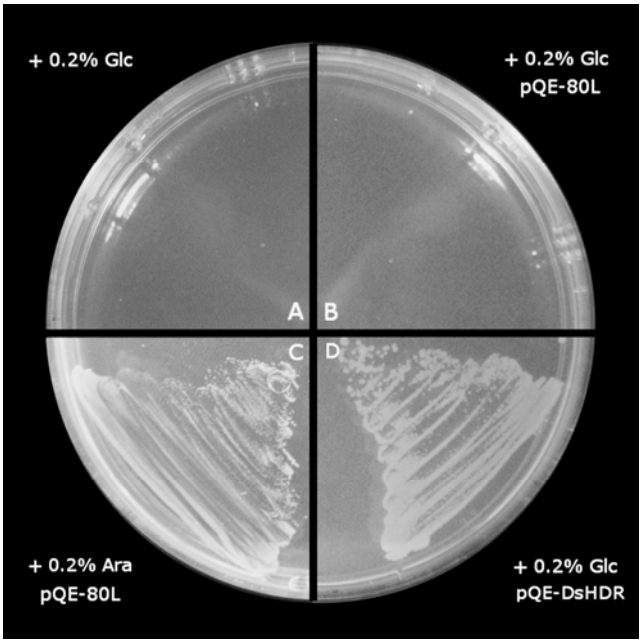

Figure 5. Complementation of *E. coli hdr* mutant (MG1655 *ara<>ispH*) with *D. salina HDR*. The *E. coli* mutant strain was not able to grow on LB plates containing 0.2% glucose (Glc) as expected (**A**). This strain was transformed with the empty expression plasmid pQE-80L (**B**, **C**) and the plasmid containing *D. salina HDR* (pQE-*Ds*HDR; **D**). After transformation, only the *E. coli* transformants containing the pQE-*Ds*HDR were able to grow in the presence of Glc and absence of arabinose (Ara; **D**).

CONCLUSIONS

Since the discovery about a decade ago, of the plastidial MEP pathway numerous studies in plants led to the characterization of the respective enzymes, in contrast to the scarce data concerning the regulatory mechanisms controlling the synthesis of the different pathway compounds. Carotenoid biosynthesis is dependent on the supply of the MEP-derived precursors and therefore *D. salina,* which holds a unique capacity of β-carotene accumulation, is a valuable model organism to better comprehend the complex regulation of isoprenoid biosynthesis (Ben-Amotz et al. 1982; Ye et al. 2008). *DsHDR* seems to be regulated at the transcriptional level in response to environmental changes but further research should also concern post-transcriptional regulation analysis of the MEP pathway. Understanding *D. salina* metabolic flux through the MEP pathway, since several enzymes could act as control points, might also provide the basis for metabolism engineering for massive accumulation of β-carotene and other carotenoids in this alga. Indeed, the biotechnological potential of *D. salina* has recently been enhanced by the development of DNA transformation methods (reviewed by Coll, 2006; León et al., 2007) and the current sequencing genome efforts (Liolios et al., 2006). The combination of genomics and gene expression data will facilitate future prospects of turning this microalga into a highly valuable cell factory system (Lamers et al., 2008).

ACKNOWLEDGMENTS

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Chapter 3

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

Keywords: carotenoid biosynthesis, *Dunaliella salina*, lycopene β-cyclase, gene expression, stress response regulation

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 upregulated 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.

MATERIAL 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 μMH_3BO_3, 1.8 μM $MnCl_2$, 154 nM $ZnSO_4$, 78 nM$CuSO_4$, 84 nM $CoCl_2$•$6H_2O$, 56 nM Na_2MoO_4•$2H_2O$, 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 2.5×10^5cells 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 5-10×10^6 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 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 ULTRAhyb 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.

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
Lcyβ4Fw[a]	GGCTCAGAAAAAATTGCTAACTCAC
Lcyβ5Rev[a]	TTTAGGAATCCATCACAAGCCAATACC
GenomeWalking 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

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 chromatogra-

phy (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 *Ds*Lcy-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. *Ds*Lcy-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 *Ds*Lcy-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 *Ds*Lcy-β is targeted to the chloroplast.

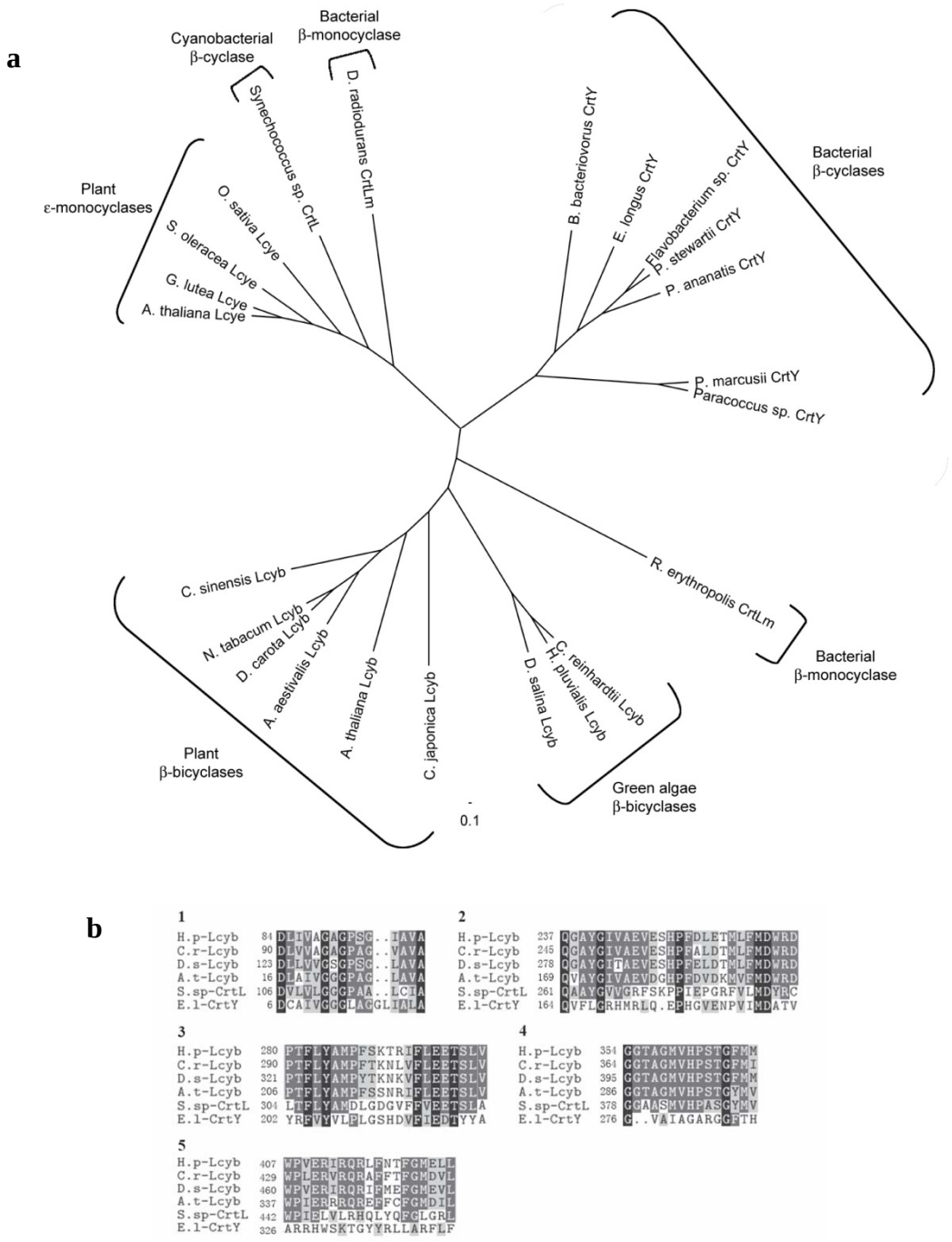

Figure 1. Unrooted phylogenetic tree of monomeric lycopene cyclases (**a**) and amino acid sequence alignments of Lcy-β, corresponding to the five conserved regions from bacteria, cyanobacteria and plant (first domain corresponds to a putative dinucleotide-binding region) (**b**). The accession numbers 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.

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.

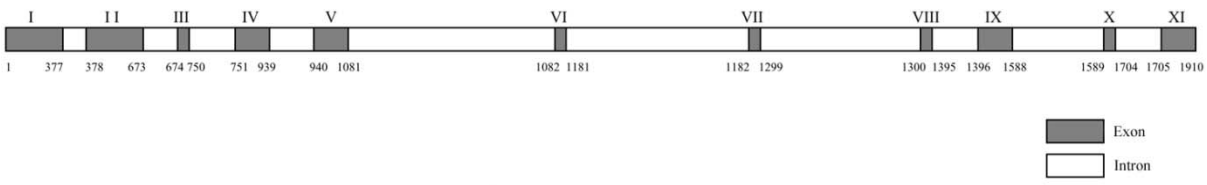

Figure 2. *D. salina Lcy-β* gene organization (accession number EU327877). The diagram showed that *Lcy-β* consisted of 11 exons (I-XI) and 10 introns. Numbers represent the cDNA coordinates (bp).

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. 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 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).

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

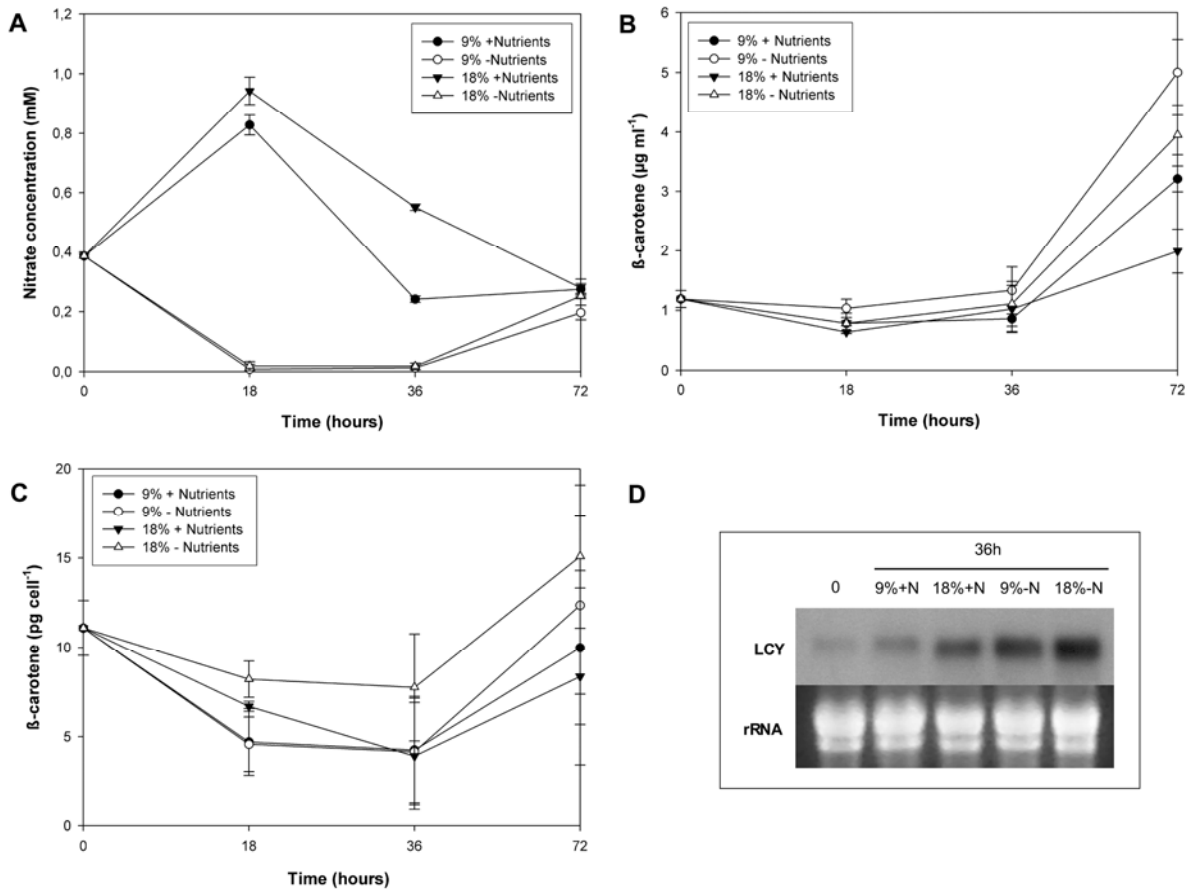

Figure 3. Effect of hyper-saline shock and/or nutrient supplementation on **a** nitrate consumption, **b**, **c** β-carotene concentration and **d** steady-state *DsLcy-β* mRNA levels. *D. salina* cells pre-adapted to Walne medium 9% salinity and low light conditions. Cells were transferred to a nutrient-supplemented 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.

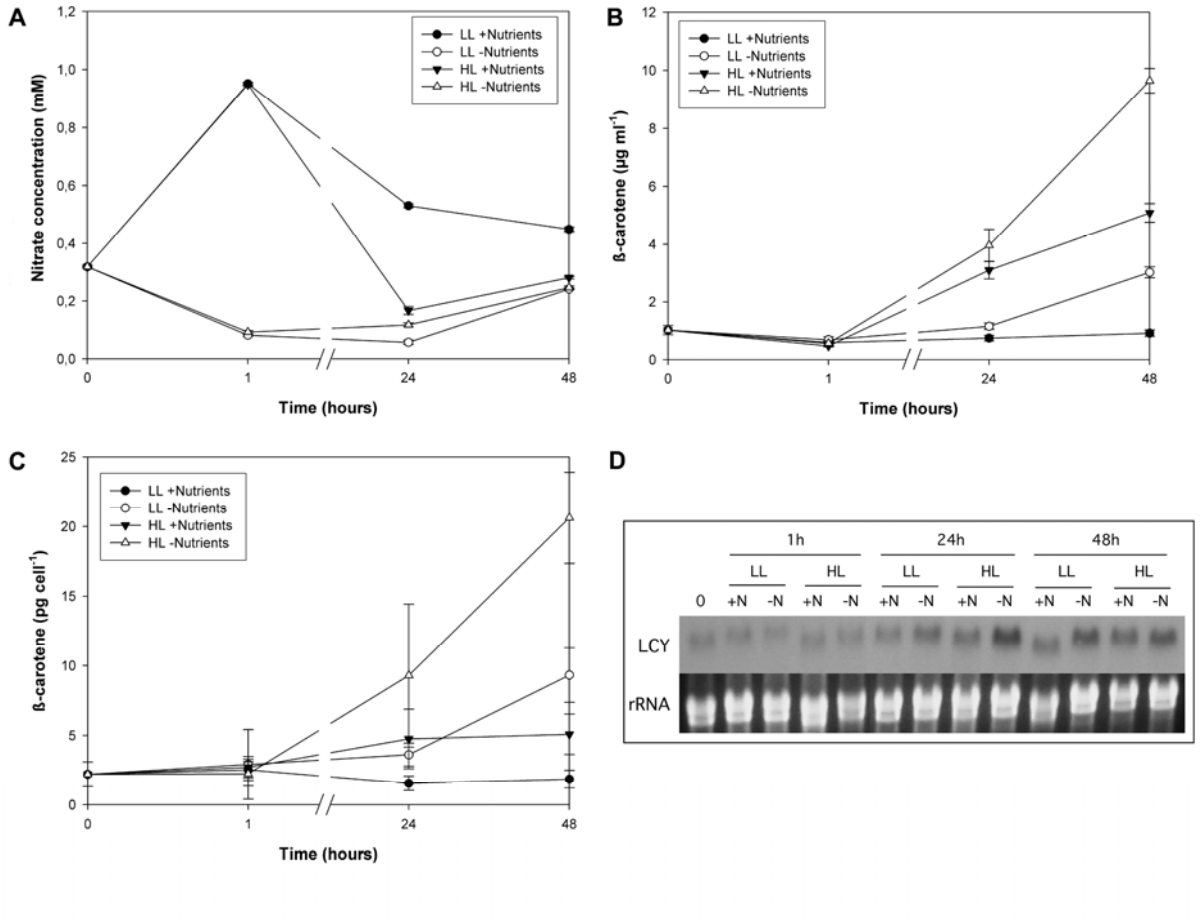

Figure 4. Effect of light stress on **a** nitrate consumption, **b**, **c** β-carotene concentration in *D. salina* cells and **d** steady-state *DsLcy-β* mRNA levels. Cells pre-adapted to Walne medium 9% salinity and low light conditions (LL) were subjected to a light intensity up-shift to 500 µmol 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.

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*.

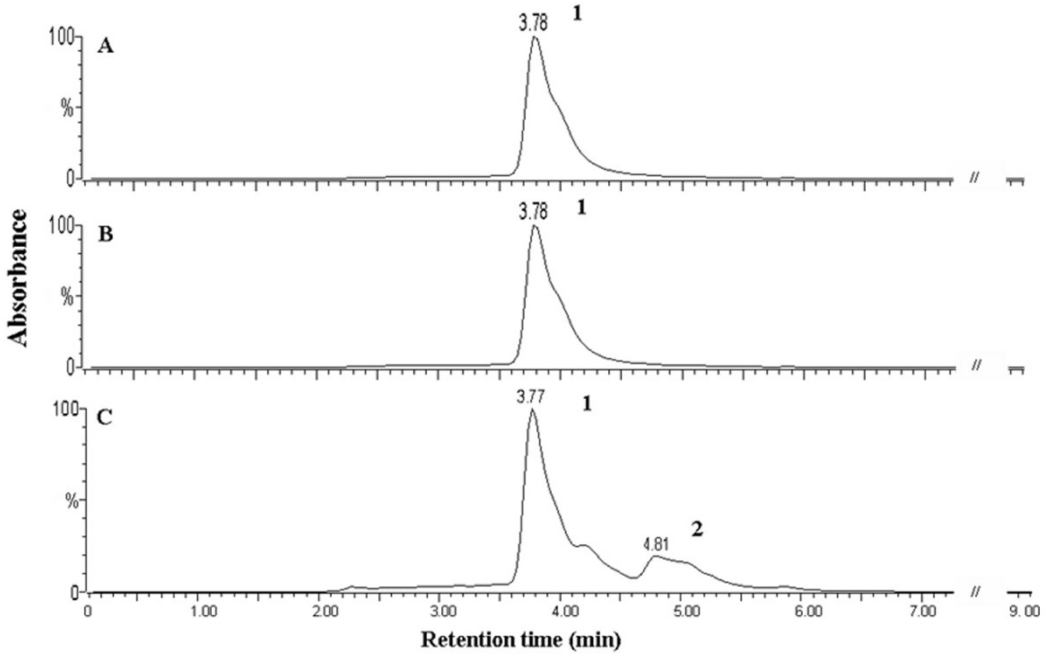

Figure 5. HPLC elution profiles of carotenoid pigments extracted from cultures of *E. coli* carrying plasmids pAC-LYC (**a**), pAC-LYC/pQE-empty (**b**) and pAC-LYC/pQE-*DsLcy-β* (**c**). Carotenoids were identified by their absorption spectra (lycopene - 474 nm; β-carotene - 454 nm) and their retention 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.

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-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.

ACKNOWLEDGEMENTS

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Part II

Protein-protein interaction analysis

Chapter 4

Dunaliella salina lycopene ß-cyclase putative protein interactor identified by yeast two-hybrid screening

ABSTRACT

Lycopene β-cyclase (LCY-β), which catalyzes the conversion of lycopene into β-carotene, is a key enzyme in the complex carotenoid biosynthesis pathway. The main source of natural β-carotene is the mass-cultured green alga *Dunaliella salina* which is also an excellent system to analyse the molecular mechanisms associated with abiotic stress response. Recent studies provided the initial insight on the transcriptional regulation of carotenoid biosynthesis in *D. salina.* However, proteomic-based data on signal transduction pathways triggering carotenogenesis is scarce. To elucidate the components of signalling and regulation of this cellular process a yeast two-hybrid assay (ProteinLinks, Inc.) was performed using full-length and partial *LCY-β* baits. One uncharacterized putative positive LCY-β interactor (full-length bait) with 308 amino acids and a putative chloroplast signal peptide was identified. This protein was only found in other green algae, cyanobacteria and higher plant genomes. Further analysis in order to confirm this interaction and to elucidate the functional and metabolic role of this putative protein-interactor will be performed in future works.

Keywords: *Dunaliella salina*, lycopene β-cyclase, yeast two-hybrid analysis

Abbreviations: EST, expressed sequence tag; LCY-β, lycopene β-cyclase; PCR, polymerase chain reaction; PRX, peroxiredoxin; ORF, open reading frame; RACE, rapid amplification of cDNA ends; UTR, untranslated region; Y2H, yeast two-hybrid

INTRODUCTION

Carotenoids are a special group of natural pigments since they are essential to all living systems. In photosynthetic organisms they play a critical role in several processes such as photoprotection and light harvesting (Demming-Adams et al. 1996; Frank and Cogdell 1996; Niyogi 1999). Animals are incapable of synthesise carotenoids *de novo* but they can incorporate these compounds from their diet. In fact, human dietary carotenoids have beneficial effects such as provitamin A and antioxidant activity, immune-system enhancers and inhibition of development of certain types of cancer and coronary heart diseases (Bartley and Scolnik 1995; Armstrong 1997; Johnson 2002; Fraser and Bramley 2004; Stahl and Sies 2005; Rao and Rao 2007). Therefore, due to their widespread use and potential applications in medicine, an increase in recent years of the commercial demand of natural and synthetic carotenoids (e.g lycopene, β-carotene, astaxanthin, lutein, capxanthin) has been registered.

The application of metabolic engineering and genetics to the biotechnological exploitation of carotenoid biosynthesis allowed the improvement of the nutritional value of several crop plants such as the Golden Rice (Ye et al. 2000; Galili et al. 2002; Sandmann et al. 2006). These progresses also allowed the production of carotenoids by transgenic non-carotenogenic organisms namely bacteria, cyanobacteria and yeast (Misawa and Shimada 1998; Schmidt-Dannert 2000; Sandmann 2001). However, in order to overcome the problems and the undesirable collateral effects of these metabolic modifications a deeper knowledge of the mechanisms underlying the intricate isoprenoid and carotenoid biosynthetic pathways is necessary. Elucidation of metabolic fluxes, key control genes, signalling mechanisms and transcriptional regulators can thus provide a novel insight for the improvement of plant foods and feeds (Naik et al. 2003; Liu et al. 2004; Ducreux et al. 2005; Römer and Fraser 2005).

The biochemical composition and content of accumulated carotenoids by green algae is similar to higher plants with some exceptions. In particular, the main natural sources of β-carotene and astaxanthin are the algae *Dunaliella salina* and *Haematococcus pluvialis* (Chlorophyta, Volvocales), respectively (Lorenz and Cysewski 2000; Spolaore et al. 2006; Del Campo et al. 2007). *D. salina* (Teodoresco, 1905) has been exploited in several countries since the 1980s and presents a remarkable capacity of accumulate large amounts of β-carotene in oil globules in the interthylakoid spaces of its single chloroplast. This accumulation occurs in response to high light and salt, temperature stress and nutrient limitation conditions (Ben-Amotz and Avron 1983; Borowitzka et al. 1990; Lers et al. 1990; Cowan et al. 1992; Raja et al. 2007). Currently, initial studies regarding the molecular basis underlying carotenogenesis in this model organism are emerging; nevertheless, the available genomic and proteomic data regarding *Dunaliella* is still limited as well as restricted to public access (Liska et al. 2004; Yan et al. 2005; Coesel et al. 2008; Ramos et al. 2008, 2009; Ye et al. 2008).

Protein-protein interactions are intrinsic to all biological processes and cellular functions (Alberts 1998). Although the large-scale genomic sequencing data and gene expression analysis undertaken with several plant species (e.g *Arabidopsis thaliana* and *Oryza sativa*; The *Arabidopsis* Initiative 2000; Yu et al. 2002) provided the molecular bases of cellular operations, the next challenge is to understand and establish the complete network of protein interactions (Sharan and Ideker 2006). Numerous techniques have been developed to analyse protein-protein interactions such as physical methods (e.g immunoprecipitation, affinity chromatography and blotting, cross-linking) or genetic *in vivo* approaches (e.g yeast and bacterial two-hybrid systems). The yeast two-hybrid (Y2H) screening method offers several advantages and has been widely used in several plant researches (Fields and Song 1989; Phizicky and Fields 1995; Causier and Davies 2002; Morsy et al. 2008).

In order to understand the regulation of carotenoid biosynthesis in *D. salina* and particularly the specific protein-protein interactions that may occur with lycopene β-cyclase (LCY-β), a pathway key enzyme responsible for the conversion of the linear lycopene into bicyclic β-carotene (Cunningham and Gantt 1998; Ramos et al. 2008), an initial approach with a bacterial two-hybrid system (BacterioMatchII Two-Hybrid System, Stratagene) was attempted in our laboratory (Dove et al. 1997; Dove and Hochschild 1998). Although all the protocol procedures were accomplished with success (full-length and partial bait construction and validation, *D. salina* cDNA library construction and screening), no putative positive clones were obtained in the final validation step. Furthermore, many technical problems regarding the kit components were detected.

In the present study we report a single *D. salina* LCY-β (*Ds*LCY-β) putative protein-protein interactor (*Ds*Pi) with unknown function obtained via yeast two-hybrid system (ProteinLinks). Since no *Ds*Pi homologue proteins were characterized until now a profound analysis is necessary. Therefore, future research goals will include the *in vitro* confirmation of the observed protein-protein interaction and a detailed biochemical characterization of this hypothetical peroxiredoxin (PRX).

MATERIAL AND METHODS

Strains and growth conditions

D. salina CCAP 19/30, from the Culture Collection for Algae and Protozoa (Cumbria, UK), was grown in Walne medium (9% NaCl) in the conditions described previously (Ramos et al. 2008). *E. coli* DH5α was used for standard cloning procedures (Table 1; Sambrook et al. 1989). For the Y2H analysis, *Saccharomyces cerevisiae* strains used were: Y304 (TetR-based screening), Y338 (LexA-based screening) and EGY42 (Table 1; ProteinLinks).

Bait construction

DsLCY-β was previously described (Ramos et al. 2008) and sequence analysis revealed the presence, at the N-terminus, of two transmembrane domains (residues 488 to 510 and 532 to 554; Hirokawa et al. 1998). Two distinct baits, with (FL) and without (P) the predicted transmembrane domains, were used since these hydrophobic regions may prevent the proteins from reaching the nucleus, where the interaction in two-hybrid systems occurs. The baits were cloned in the expression plasmids pCWX200 and pLexA for the TetR and LexA-based screenings, respectively.

The inserts were PCR amplified, cut with *Eco*RI and *Xho*I at sites introduced via specific primers (LCYβFw and LCYβFLRev or LCYβPRev; Table 2), purified and cloned into similarly digested plasmids. Sequencing of the resulting plasmids was performed in order to confirm the correct DNA insertion (Genetic Analyzer - Applied Biosystems, CCMAR Portugal).

Bait plasmids were transformed into the yeast strain Y304 or Y338 and several control experiments were performed to determine whether these baits were suitable for the

Y2H assay (e.g self-activation, protein expression and toxicity to the yeast cells; ProteinLinks).

Library construction

Previously a representative *D. salina* cDNA library containing more than $1x10^6$ cfu was constructed in the pTRG plasmid – BacterioMatch II Two-hybrid system (Stratagene) according to manufacturer's instructions. Colony PCR analysis revealed that the insert size ranged from 0.5 to 3 kb (data not shown).

Library conversion on to pJG4-5 vector for the Y2H screening was performed by PCR and homologous recombination approaches (ProteinLinks). In the PCR reaction (with LCFw and LCRev primers – Table 2; 100 µl final volume) 1 µg of the former cDNA library (pTRG vector; Stratagene) was used as a template. PCR conditions were as follows: one cycle of 95 ºC, 5 min; 30 cycles at 95 ºC, 1 min; 55 ºC, 1 min; 72 ºC, 3 min; and one final cycle of 72 ºC, 10 min. PCR products and Y2H vector pJG4-5 (digested with *Eco*RI and *Xho*I) were introduced into the yeast strain EGY42 by the modified LiOAc/PEG method (Gietz et al. 1992). The transformation efficiency was determined and a *D. salina* cDNA library (EGY42) with $2.2x10^6$ transformants was obtained.

Screening and validation of putative positive clones

Library screening was performed by mating the bait strain Y304 or Y338 with the library strain EGY42. About $1x10^7$ independent *D. salina* cDNA library clones were screened on the selective medium (lacking leucine, histidine, trypthophan and uracil). All the potential interactors were tested in order to identify false positives clones and a final validation step involving the isolation and re-transformation of yeast strains was performed. Selected clones that activate both reporters (*URA*3 or *LEU*2 and *Lac*Z) were sequenced **(**ProteinLinks).

Cloning and bioinformatic analysis of the full-length putative *DsPi*

Total RNA was isolated from 5-10x10^6 *D. salina* cells with TRI REAGENT™ (Sigma) method according to manufacturer's instructions. cDNA synthesis and PCR amplifications were performed with the SMART™ rapid amplification of cDNA ends (RACE) cDNA Amplification Kit (BD Biosciences, Clontech). Several RACE PCR amplifications with gene-specific primers were performed in order to reach the 5' and 3' ends of the gene (data not shown). The full-length PCR amplification conditions of the putative *DsPi* with the sense primer *Ds*PiFLFw and the anti-sense primer *Ds*PiFLRev (Table 2) were as follows: one cycle of 94 ºC, 5 min; 35 cycles at 94 ºC, 1 min; 54 ºC, 1 min; 72 ºC, 1 min; and one cycle of 72 ºC, 10 min. All amplified cDNA fragments were purified, cloned into pGEM-T Easy Vector (Promega) and sequenced (Genetic Analyzer-Applied Biosystems, CCMAR Portugal).

Sequence analysis was done using Geneious Pro 2.5.3 (Biomatters) software package and the identification of putative chloroplast transit peptides was carried out with ChloroP 1.1 programme (Emanuelsson et al. 1999). Homologous sequence searches were performed in several DNA, EST and protein databases (e.g National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/; ExPASy Proteomics Server, http://www.expasy.org/; The Institute for Genomic Research, http://www.tigr.org/; The *Arabidopsis* Information Resource, http://www.arabidopsis.org/) by means of BLAST analysis (Altschul et al. 1990). Multiple sequence alignments of the deduced amino acid sequences were created using the program ClustalW (Thompson et al. 1994). In addition, available protein interactions databases were examined for possible *Ds*LCY-β and *Ds*Pi curated or predicted interactors (e.g IntAct, http://www.ebi.ac.uk/intact/site/index.jsf; AtPID, http://atpid.biosino.org/; DIP, http://dip.doe-mbi.ucla.edu/; STRING, http://string.embl.de/; BioGRID, http://www.thebiogrid.org/).

Table 1. Genotypes of bacterial and yeast strains.

Strain	Genotype
Bacterial	
DH5α	F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1
Yeast	
Y304	*MATα ura3 his3 trp1 leu2 lys2delk::TetOp-URA3 TetOp-LacZ*
Y388	*MATα ura3 his3 trp1 leu2 LexAop LEU2 LexOp-LacZ*
EGY42	*MATa ura3 his3 trp1 leu2 lys2*

Table 2. Primers used in the present study (FL-full-length; P-partial/without the transmembrane domain; *Ds*Pi-putative interactor).

Primers	Sequence 5' → 3'
Bait cloning	
LCYβFw	CGCGAATTCCGGATGCTTCAAACACTGAGCGGTCGA
LCYβFLRev	CCGCTCGAGGGACTATTGCTGCTTTGCAGC
LCYβPRev	CCGCTCGAGGGATTACGTCTGTTGGAGGTTGAGGGA
Library conversion	
LCFw	GCCTCCTACCCTTATGATGTGCCAGATTATGCCTCTCCC GAATTCGGATCCGCGGCCGCAAGAATTC
LCRev	TGATTGGAGACTTGACCAAACCTCTGGCGAAGAAGTCC AAAGCTTAGTTCATTAATTAATTAATTACTCGAG
Putative interactor PCR	
*Ds*PiFLFw	ATTTTCACAGTTAAACTGTGCAG
*Ds*PiFLRev	GCTATCAAGCTAGCCGGGCCTCACCA

RESULTS AND DISCUSSION

Up to now, no Y2H or other protein-protein interactions studies concerning *D. salina* were described. In the present study, Y2H screening and validation was performed in the conditions optimized by ProteinLinks (Xu et al. 1997).

Two distinct *DsLCY-β* baits (with and without the transmembrane domains) were screened against approximately $1x10^7$ clones present in a representative *D. salina* cDNA library (four rounds of selection). Initially, 476 preliminary interacting clones were obtained but further validation steps resulted in 25 verified positive putative clones interacting with *DsLCY-β* full-length baits: 8 and 17 from the TetR and LexA based screenings, respectively. No positive clones were obtained with the partial *DsLCY-β* baits (without the transmembrane domains) assays. Sequence analysis confirmed that all the previous clones corresponded to a single partial gene.

Based in the previous data we designed specific primers to isolate the full-length *DsPi* with 1035 bp. The open reading frame of this cDNA encodes a deduced protein of 308 amino acids with a molecular mass of 33.5 kDa. The coding region is flanked by 5' and 3' UTR of 25 and 84 bp, respectively. Furthermore, a putative chloroplast transit peptide at the N-terminal was predicted by computational methods (ChloroP). BLAST similarity analysis revealed the presence of an unknown conserved protein, just recently discovered. The only evidence regarding *Ds*Pi function was the detection of a conserved peroxiredoxin (PRX)-like 2 family domain. However, in *Ds*Pi as well as in other hypothetical family protein members, the characteristic redox active CXXC motif of thioredoxins was not identified (Marchler-Bauer et al. 2007).

This deduced protein was present only in other green algae (e.g *Chlamydomonas reinhardtii*), cyanobacteria (e.g *Synechococcus* sp. CC9605) and higher plants (*Arabidopsis thaliana*; 55%, 37% and 36% identity with *Ds*Pi, respectively). Figure 1 shows the multiple sequence alignment of *Ds*Pi homologous proteins.

An extensive database search resulted in a small number of predicted LCY-β protein functional partners. These included, in organisms such as *A. thaliana, Prochlorococcus marinus* and *Synechococcus elongatus*, other carotenoid biosynthesis enzymes (phytoene desaturase, lycopene ε-cyclase β-carotene hydroxylase and zeta-carotene desaturase), photosynthetic enzymes (photosystem I and II) as well as uncharacterized membrane or hypothetical proteins.

Plant genomes contain several groups of PRXs known to be involved in the protection of cells from oxidative stress, regulation of gene expression, signalling and also enzyme activity control (Arnér and Holmgren 2000; Wood et al. 2003; Rouhier and Jacquot 2005; Dayer et al. 2008). Previous studies, identified DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase) and HDS (4-hydroxy-3-methylbut-2-enyl disphosphate synthase), enzymes involved in plastidial isoprenoid biosynthesis as linked to thioredoxin (Balmer et al. 2003). Salinity stress was also able to induce known antioxidant proteins namely thioredoxin peroxidase in *D. salina* cells (Liska et al. 2004). Considering this data, the multiplicity of thioredoxin genes and biochemical LCY-β oxireductase activity, the evidence of a possible PRX LCY-β target is conceivable. Nevertheless, validation of this interaction (e.g GST pull-down assay) and *Ds*Pi characterization experiments (biochemical, enzyme activity, expression patterns) will be accomplished. Other methods developed specifically for plants cells or that present higher sensibility for particular baits/interactions than normal Y2H should be also considered in research on *D. salina* interactomes (Piehler 2005; Vignols et al. 2005; Morsy et al. 2008).

Figure 1. Multiple sequence alignment of *Ds*Pi homologous proteins. Species names and GenBank accession numbers are as follows: *Chlamydomonas reinhardtii* (Cr) XP_001691801; *Nostoc* sp. PCC 7120 (No) NP_486668; *Synechococcus* sp. CC9605 (Sy) YP_382196; *Prochlorococcus marinus* str. MIT 9303 (Pm) YP_001017782; *Trichodesmium erythraeum* IMS101 (Te) YP_723640; *Arabidopsis thaliana* (At) NP_201385; *Vitis vinifera* (Vv) CAO23915; *Oryza sativa* (Os) NP_001051154. Arrow indicates the *D. salina* (Ds) deduced chloroplast transit peptide and black background indicates a conserved amino acid in all sequences.

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Conclusions and future perspectives

CONCLUSIONS AND FUTURE PERSPECTIVES

Since the description of the genus *Dunaliella*, more than one hundred years ago, numerous investigations described the physiological and adaptation capabilities of these microalgae (Ben-Amotz and Avron 1983; Borowitzka et al. 1990; Loeblich 1982; Cifuentes et al. 2001; Oren et al. 2005). One of these species, *Dunaliella salina,* has been used as a model system for the study of plant cell responses to abiotic stress, as it is able to grow in environments of extreme salinity and high light intensity, probably due to its ability to accumulate large amounts of intracellular glycerol and β-carotene. In addition to this scientific interest, *D. salina* has been commercially exploited world-wide in response to the increasing market demand for natural carotenoids (Cowan et al. 2002; Del Campo et al. 2007; Ye et al. 2008). As outlined in the state of the art, the underlying cellular and molecular mechanisms of isoprenoid biosynthesis in *D. salina* were virtually unknown. Therefore, the main purpose of the present thesis was to contribute to the elucidation of carotenoid metabolism in this alga.

The lack of genomic information and stable DNA transformation methods regarding *D. salina* has limited the research and development efforts to elucidate the molecular response of this alga to abiotic stress, in particular the accumulation of high levels of carotenoids upon high light and nutrient depletion (Coll 2006; Liolios et al. 2006). Until now, no other relevant data concerning its plastidial MEP pathway, responsible for the biosynthesis of the basic precursors (IPP and DMAPP) for carotenoid production, has been published (Schwender et al. 2001; Hunter 2007; Ye et al. 2008). Additionally, only two-carotenogenic genes were previously known (*DsPSY* and *DsPDS*; Coesel et al. 2008). Therefore, improvement of *D. salina* biotechnological potential as a cell-factory entails a better understanding of both metabolic pathways (Naik et al. 2003; Lamers et al. 2008).

Cultivation of *D. salina* cells under abiotic stress conditions (mainly high light and nutrient depletion) resulted in intracellular accumulation of β-carotene and changes in MEP and carotenoid biosynthetic pathways gene expression patterns at the physiological and molecular levels, respectively (Coesel et al. 2008; Ramos et al. 2008, 2009). Carotenogenic gene-expression data on *DsPSY*, *DsPDS* (Coesel et al. 2008) and *DsLCY-β* (Chapter 3; Ramos et al. 2008) described an up-regulation of the steady-state mRNA levels in *D. salina* cells grown under the described stress conditions. Additionally, two important MEP regulatory steps might correspond to *DsDXS* and *DsHDR* (Chapter 1, 2; Ramos et al. 2009). Thus, transcriptional regulation seems to be an important control factor of both pathways in *D. salina.* These novel genes are potential targets for the improvement of carotenoid production in future silencing engineering strategies or overexpression studies not only in *D. salina* but also in other recombinant systems (Ravanello et al. 2003; Enfissi et al. 2005; Carretero-Paulet et al. 2006; Diretto et al. 2006; Hallmann 2007). Nevertheless, clarification of the remaining unknown steps and analysis of the distinct regulation levels (e.g. post-transcriptional) is mandatory for carotenoid production optimization in this microalga. Other regulatory elements such as transcription factors, signalling molecules or other possible flux control metabolites should also be considered in future investigations (Aarts and Fiers 2003; Lamers et al. 2007; Giuliano et al. 2008).

Elucidation of complex cellular processes involves the identification of protein-protein interactions (Cho et al. 2004; Stelzl and Wanker, 2006). Despite an unsuccessful initial attempt (BacterioMatch Two-hybrid system; *DsLCY-β* and *DsHDR* baits tested), one putative *Ds*LCY-β interactor with unknown biological function, but also present in other algae and plants, was obtained by a yeast-two hybrid analysis (Chapter 4). Further interaction validation and characterization of this putative *Ds*LCY-β protein-interactor is necessary. However, the presence of a conserved peroxiredoxin (PRX)-like 2 family domain suggests

that carotenogenic enzymes may be regulated by the redox state of the cell and / or play a role in the protective cellular mechanisms against reactive oxygen species.

In conclusion, the complete knowledge of the complex regulatory mechanisms of environmental stress response and β-carotene accumulation in *D. salina* will ultimately allow the use of this microalga for several metabolic engineering purposes and its exploitation as a cell-factory organism (e.g. novel carotenoids). In this context, the present work has provided additional insights for future genetic and biotechnological advances.

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