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

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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 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. 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 that set the stage for its future use as a production system.

**Key Words:** abiotic stress; carotenogenesis; *Dunaliella salina*; genomics; isoprenoid biosynthesis; lipid biosynthesis; transformation

**Abbreviations:** Acetyl-CoA, acetyl-coenzyme A; CaMV35S, Cauliflower mosaic virus 35S promoter; CAT, chloramphenicol acetyltransferase; CrtISO, 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-b, lycopene β-cyclase; LCY-e, lycopene ε-cyclase; MEP, 2-C-methyl-D-erythritol 4-phosphate; MVA, mevalonate; NR, nitrate reductase; PAT, phosphinothricin acetyltransferase; PDS, phytoene desaturase; PSY, phytoene synthase; ROS, reactive oxygen species; SV40, simian virus 40; TAG, triacylglycerols; Ubil-Ω, ubiquitin; ZDS, ζ-carotene desaturase; ZISO, 15-cis-ζ-isomerase

**INTRODUCTION**

Microalgae include a very diverse group of prokaryotic and eukaryotic organisms that play important ecological...
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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, Chlorophyceae, Chlamydomonadales, Dunaliellaceae), which mainly includes halophilic species adapted to hypersaline (0.05-5.5 M NaCl) environments (Chen and Jiang 2009, Polle et al. 2009). *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 (size varying from 5-25 µm in length and 3-13 µm width) (Teodoresco 1905, Borowitzka and Borowitzka 1988, Avron and Ben-Amotz 1992, Oren 2005). This genus includes more than 20 species and its taxonomic organization based on cell morphology and physiology is still controversial due to large intra-species variation in morphology, which depends on growth conditions. Consequently numerous *Dunaliella* isolates were misidentified previously (Borowitzka and 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 cell morphology and physiology was performed by...

Some *Dunaliella* strains can accumulate β-carotene and glycerol, properties with economical interest that have led, since the 1980s, to the large-scale culture of these algae in several countries such as Australia, China, Israel, and India with pilot-scale projects attempted in other countries (e.g., Chile, Spain, Iran) (Ben-Amotz and Avron 1990, Borowitzka 1999, Pulz 2001, Tseng 2001, Del Campo et al. 2007). *D. salina* and *D. bardawil*, which might be a variety of *D. salina* (Borowitzka and Borowitzka 1988, González et al. 2009), are the main natural sources of β-carotene (up to about 10-14% of algal dry weight) and also the most extensively analyzed strains in terms of physiological abiotic stress adaptations especially under mass culture conditions (Loeblich 1982, Ben-Amotz and Avron 1983, Cifuentes et al. 1996, Oren 2005).

In response to several stress or growth limiting conditions (e.g., salt, temperature, light, and nutrient deficiencies) *D. salina* synthesizes and accumulates β-carotene in 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, Jin and Polle 2009). This high-value compound, mostly composed of all-trans and 9-cis stereoisomers, has a wide range of important economical applications such as human health, cosmetic and aquaculture industries (Jiménez and Pick 1993, Murthy et al. 2005, Spolaore et al. 2006, Raja et al. 2007a, Oren 2010). 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 and genomic analysis. However, more recent studies have addressed this shortcoming (Polle and Song 2009, Smith et al. 2010) and genomic technologies promise further improvements in such analyses. The most widely used model species within the genus of *Dunaliella* has been *D. salina*. Therefore, the following sections will concern *D. salina* unless otherwise indicated.

**GENES AND PROTEIN IDENTIFICATION**

Complex regulatory and signaling networks of stress response have been uncovered in several model plants and algae using novel genomics, functional, and computational approaches (Cushman and Bohnert 2000, Shragger et al. 2003, Grossman et al. 2007, Jain et al. 2007, Montsant et al. 2007, Urano et al. 2010). These data often provide initial clues towards the identification and possible functions of unknown encoded proteins in other species. Currently, a genome sequencing project is under way for *D. salina* strain CCAP 19/18 (USDA-DOE Joint Genome Institute [JGI]). Part of this effort has resulted in the recent publication of the chloroplast and mitochondrial genomes of *D. salina* (269 and 28.3 Kb, respectively) (Smith et al. 2010). Future availability of more genomic data including the nuclear genome (approximately 300 megabases) (Smith et al. 2010) will be a major step towards a better understanding of the molecular mechanisms underlying the response of *D. salina* to abiotic stress as well as the development of genetic tools, such as nuclear and plastid transformation.

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, transcriptome analysis, and/or parallel genomic sequencing can thus provide the required gene expression data. Partial EST, microarray and cDNA data are available from a limited number of halophytic plants including *Mesembryanthemum crystallinum* (Kore-eda et al. 2004, Cushman et al. 2008), *Thellungiella halophila* (Amtmann 2009, Taji et al. 2010), 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 4,100 ESTs (as of January 2011) are available for *D. salina* cells subjected to salinity and light stress (Park et al. 2006, Alkayal et al. 2010). Profiling of about 2,800 ESTs revealed elevated expression of protein synthetic apparatus components in salinity shocked cells (Alkayal et al. 2010).

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 (e.g., signal transduction, redox energy production, protein synthesis, membrane stabilization) 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. In addition, a more recent study investigated the flagellar proteome (Jia et al. 2010). A total of 520 groups of proteins from *D. salina* flagella have been found by shotgun proteomics, but only a limited number of these proteins were identified due, in part, to the lack of a fully sequenced reference genome.

**Fig. 2.** Diagram of the proposed cellular response mechanisms of *Dunaliella salina* to abiotic stress, in which a single, large chloroplast occupying most of the cellular volume is shown. The nucleus (Nuc) and mitochondria (M) are drawn in light blue and brown, respectively. Cell adaptation to carotenogenic conditions such as salt upshifts (A) involves an earlier, fast response (drawn in red) to changes in osmolarity that apparently does not need active transcription to be induced. This response results in the immediate accumulation of compatible solutes such as glycerol. A second, slower response to salt stress (A), nutrient limitation and high light (B) involves signal perception by primary sensors and the activation of signaling-transduction pathways on the plasma membrane and photosynthetic apparatus, which in turn alters gene expression in the nucleus and cytoplasm. Translated gene products (e.g., enzymes) are imported into the chloroplast leading to increased neutral lipid and isoprenoid production, the latter via the plastidial 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The concomitant induction of the carotenoid biosynthetic pathway results in the accumulation of carotenoids in multiple lipid droplets in the stroma, chiefly in the vicinity of thylakoids. There is evidence that kinases, transcription factors (TF), reactive oxygen species (ROS), and changes in the redox state of the cell (e.g., over-reduction of the plastoquinone [PQ] pool upon exposure to high light) along with unknown factors (X) may be part of the molecular network regulating the response of *Dunaliella salina* to abiotic stress. A more detailed explanation of the model and respective references is given in the text.

**SALT STRESS AND ITS REGULATION IN DUNALIELLA SALINA**

Algae of the genus *Dunaliella* appear to be unique in their ability to grow in saturated brine solutions as well as withstand drastic changes in salinity due to use of glycerol and glycine betaine as intracellular osmotic metabolites (Ben-Amotz and Avron 1973, Avron 1986, Chitlaru and Pick 1991, Mishra et al. 2008, Chen and Jiang 2009). Consequently, various species of *Dunaliella* (e.g., *D. parva*, *D. viridis*, *D. tertiolecta*, *D. salina* and *D. bioculata*) were used in the past to elucidate the cellular response to changes in medium osmolarity (Ben-Amotz and Avron 1973, Borowitzka and Brown 1974, Ahmed and Zidan 1986).
The adaptation of *D. salina* to salt stress (Fig. 2) can be divided into two stages: i) rapid adjustment of the intracellular concentration of glyceral and glycine betaine, resembling the osmoregulatory mechanisms common in fungi and higher plants, respectively; and ii) a long-term response that might include neutral lipid biosynthesis and carotenoid over-accumulation. The early rapid response was demonstrated to be independent of *de novo* protein biosynthesis (Sadka et al. 1989). Fluctuations in medium osmolarity are assumed to result in changes of plasma membrane lipid order, thus triggering activation of a protein kinase cascade similar to that found in fungi (Pick 1998) and ultimately leading to conversion of starch into glyceral in the chloroplast. At this time it remains unknown how the signal is transduced from the cytosol into the chloroplast. In contrast, long-term acclimation to higher salinities is thought to include changes in gene expression events through an as yet unidentified transcription factor or factors followed by *de novo* protein biosynthesis (Lers et al. 1990). Known salt stress-induced gene products include transcripts and proteins involved in carbon and iron assimilation as well as carotenoid biosynthesis (Fisher et al. 1997, 1998, Coesel et al. 2008, Ramos et al. 2008, 2009). Salt stress-induced homologues of proteins playing a role in amino acid and pyrimidine turnover, protein biosynthesis and degradation, as well as Na⁺ extrusion have also been identified (Liska et al. 2004, Katz et al. 2007). These results suggest that the late responses of *D. salina* to high salinity are geared towards its survival in these extreme environmental conditions, as lack of CO₂ and iron are thought to be two major impediments for growth in aquatic environments (Fisher et al. 1997, 1998). On the other hand, concomitant carotenoid accumulation may provide cross-protection against other forms of abiotic stress (e.g., high light) (Wang et al. 2003) common in saltworks, a natural habitat of this alga.

**ISOPRENOID BIOSYNTHESIS AND ITS REGULATION IN DUNALIELLA SALINA**

The large group of secondary metabolites known as isoprenoids or terpenoids includes several biological and economical important compounds such as carotenoids (Stahl and Sies 2005). In all living organisms isoprenoids are synthesized from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) obtained from either 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-MVA or 1-deoxy-D-xylulose-5-phosphate (DXP) pathway, respectively (Rohmer et al. 1993, Sacchettini and 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 (Chappell 1995, Schwender et al. 1996, Lichtenthaler et al. 1997, Eisenreich et al. 1998, 2001, Bach et al. 1999, Lichtenthaler 1999, 2000, 2010, Rohmer 1999). In spite of the physical separation of these two pathways in distinct cell compartments, several researchers have reported an apparent crosstalk between them (Kasahara et al. 2002, Bick and Lange 2003, Hemmelin et al. 2003, Laule et al. 2003, Dudareva et al. 2005, Hampel et al. 2005). With only the MEP pathway operating in *D. salina* (Capa-Robles et al. 2009) to provide the precursor metabolites IPP and DMAPP for further isoprenoid biosynthesis, crosstalk is essential to balance the metabolic needs of different cellular compartments. Further, crosstalk is necessary not only between the cytosol and plastids, but also between isoprenoid and lipid metabolic pathways (Rabbani et al. 1998), as secondary carotenoids in *D. salina* are sequestered in lipid globules within the chloroplast, whereas triacylglycerides are stored in cytosolic oil bodies (Katz et al. 1995). Currently, the mechanisms underlying the interactions between carotenoid and lipid biosynthesis as well as synthesis of proteins required to stabilize the carotene-containing lipid globules in *D. salina* are unknown. Consequently, further research is necessary not only to understand the mechanisms involved in the transport of isoprenoid precursor molecules across membranes, but also to dissect the biological mechanisms of regulatory communication between these pathways (Rodríguez-Concepción 2010). 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. Hence, an integrated genomics, proteomics and metabolomics approach will be required not only to unravel the stress-induced over-accumulation of carotenoids, but also to study the overall response of *D. salina* to different abiotic stresses.

**MEP pathway**

The MEP pathway was discovered only in the late 1980s and since then was identified in several prokaryotic (eubacteria, cyanobacteria) and eukaryotic (algae and high-
er plants) organisms (Lichtenthaler 1999, 2010, Rohmer 1999). Green algae (Chlorophyta), including *D. salina*, appear to have lost the cytosolic MVA pathway (Schwender et al. 2001, Capa-Robles et al. 2009). 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. 3) (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 and two key enzymes, at the level of the 1-deoxy-D-xylulose 5-phosphate synthase (DXS) / 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) and DXS / 1-deoxy-D-xylulose 5-phos-

Fig. 3. Schematic overview of proposed isoprenoid biosynthesis for the microalga *Dunaliella salina* (according to published data on higher plants). 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; MDS, 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; IPPI & 2, isopentenyl pyrophosphate isomerase; GPP, geranyl diphosphate; GPS, GPP synthase; FPP, farnesyl diphosphate; FPS, FPP synthase; GGPP, geranyl geranyl diphosphate; GGPS, GGPP synthase; PSY, phytolene synthase; PDS, CRTISO, ZDS, ZISO, 15-cis-ζ-isomerase; LCY-b, lycopene β-cyclase; LCY-e, lycopene ε-cyclase; CHY1, carotene β-hydroxylase; CHY2, carotene ε-hydroxylase; VDE, violaxanthin deepoxidase; ZE, zeaxanthin epoxidase; NSY, neoxanthin synthase.
phate reductoisomerase (DXR), respectively (Estévez et al. 2001, Botella-Pavía et al. 2004, Rodríguez-Concepción 2006, Zulak and Bohlmann 2010). 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. All *D. salina* MEP genes have been identified (partial or full-length) and their respective characterization is now ongoing (Ramos and 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, Rodríguez-Concepción 2010, Sun et al. 2010), identification of genes involved in the MEP pathway in *Dunaliella* represent the first step in further investigation of isoprenoid biosynthesis regulation in this alga.

In accordance with the finding that *de novo* protein biosynthesis is required for carotene over-accumulation (as discussed above), abiotic stresses, specifically nutrient limitation and high light, seem to be important factors in the transcriptional regulation of *DXS* and *HDR* (GenBank accession numbers: FJ469276 and FJ040210) (Ramos et al. 2009). Similarly to its close relative, *C. reinhardtii* (Lohr et al. 2005), *D. salina* appears to have only one nuclear gene coding for each of *DXS* and *HDR*. In contrast to *DXS* and *HDR*, *DXR* (GenBank accession number: FJ469277) is apparently unresponsive to stress and also only represented by one gene in the genome (Ramos and Varela unpublished results, Tran and Polle unpublished results).

**Carotenoid biosynthesis**

The carotenoid biosynthetic pathway (Fig. 3), 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 and Gantt 1998, Cunningham 2002, Römer and 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 (Fig. 4), lead to the 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<sub>20</sub>), 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 (phytoene desaturase [PDS] and ζ-carotene desaturase [ZDS]) and isomerisation (carotenoid isomerase [CrtISO] and 15-cis-ζ-isomerase [ZISO]) reactions (Li et al. 2007) leading to lycopene. Following ring-formation by cyclases (lycopene ε-cyclase [LCY-e] and / or lycopene β-cyclase [LCY-b]) synthesis of α- and β-carotene and further hydroxylation reactions lead to lutein or violaxanthin from which other end-product carotenoids can be formed (Lichtenthaler 1999, 2000, Bouvier et al. 2005). As previously demonstrated for other green algae such as *C. reinhardtii* (Lohr et al. 2005), it is expected that the ongoing USDA-DOE genome sequencing project for *D. salina* will show that basic carotenoid biosynthesis in *D. salina* is similar to that in higher plants. However, regardless of this expected similarity of the basic biosynthesis pathway, it remains unknown how 9-cis β-carotene is produced by *D. salina* (Ben-Amotz and Shaish 1992).

To date, few sequences have been published for genes that code for enzymes involved in the carotenoid biosynthesis pathway in *D. salina*. Known genes are *PSY* (GenBank accession number: AY601075) (Yan et al. 2005), *PDS* (GenBank accession number: AY954517) (Zhu et al. 2005) and *ZDS* (GenBank accession number: HM754265) (Ye and Jiang 2010), *LCY-b* (EU327876) (Ramos et al. 2008). The molecular basis of carotenogenesis in this alga is currently under investigation with several remaining pathway genes being fully or partially known as part of the current genome sequencing effort.

Abiotic stress (nutrient limitation, high light and salt) experiments performed revealed that transcriptional regulation is likely to be an important control step of this pathway (Ramos et al. 2009). *PSY, PDS* and *LCY-b* present similar expression patterns and nutrient availability appears to be a crucial 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 (Coese et al. 2008, Ramos et al. 2008), although earlier results indicated the contrary (see below; Rabbani et al. 1998, Sánchez-Estudio et al. 2006). Previous conflicting results that were published regarding the regulation of *PSY* and *PDS* at the transcript and protein levels might be explained by the
finding that, in contrast to other known chlorophytes, the alga *D. bardawil* contains multiple *PSY* genes (Tran et al. 2009). As in some higher plants (Li et al. 2008a, 2008b) differential expression of multiple *PSY* genes might regulate biosynthesis of carotenoids in *Dunaliella*.

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* (Lammers et al. 2008). Results of previous work indicated that stress-induced carotenoid biosynthesis is regulated at least at the level of transcription (Lers et al. 1990). However, comprehensive examination of transcriptional, posttranscriptional and metabolic regulatory factors such as redox control (Liska et al. 2004), key control pathway enzymes, and signaling molecules (e.g., reactive oxygen species [ROS], SOS pathway and transcription factors) (Shaish et al. 1993, Xiong et al. 2002, Aarts and Fiers 2003), is fundamental to dissecting the mechanism(s) that lead 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.

**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, Evans and Kates 1984, Mendoza et al. 1999). These properties of *Dunaliella* species have prompted an interest in the accumulation of lipids (including sterols) associated or not with carotenoid 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 and Avron 1983, Jiménez and Pick 1994, Rabbani et al. 1998). This mechanism is not unique to *Dunaliella*, as 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 and Avron 1983, Jiménez and 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, β-carotene synthesis might be 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 the fatty acids in *D. salina*. Increased fatty acid content under stress conditions (e.g., high irradiance, high salt or nitrogen starvation) has been described (Cho and Thompson 1986, Mendoza et al. 1999, Abd El-Baky et al. 2004, Lammers et al. 2010). 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 the production of hydrogen, oils (triglycerides, for biodiesel) and bioethanol (Ghirardi et al. 2000, Metzger and Largeau 2005, Miao and Wu 2006, Chisti 2008, Rosenberg et al. 2008, Wijffels 2008, Greenwell et al. 2010). 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).

*Dunaliella* species, including *D. salina*, are candidate feedstocks for biofuel production (Huesemann and Benemann 2009), 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 (Abd El-Baky et al. 2004, Weldy and Huesemann 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 might 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 used commercially to date, recent development of tools and methods for genetic engineering (e.g., electroporation, glass beads, silicon carbide whiskers, particle bombardment, *Agrobacterium tumefaciens*-mediated gene transfer) of microalgae has allowed the stable transformation of more than 30 different strains including several Chlorophyta (e.g., *C. reinhardtii*, *Volvox carteri*, *Chlorella kessleri*) (Fuhrmann 2002, Walker et al. 2005b, Coll 2006, León et al. 2007, Radakovits et al. 2010). 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, Sun et al. 2005, 2006, Tan et al. 2005, Walker et al. 2005a, Feng et al. 2009, Polle and Song 2009).

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 (*Ubi-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 2006). Three distinct promoters were tested in different reports: carbonic anhydrase gene (DCA1) (Lü et al. 2004), actin (Jiang et al. 2005) and *Cauliflower mosaic virus* 35S promoter (CaMV-35S) (Tan et al. 2005). The latter was the only report without evidence of stable genome incorporation. Additionally, 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 reductase (*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).

Recently, three *D. salina* transformation methods were tested, namely glass beads, particle bombardment and electroporation, using GUS (coding for beta-glucuronidase) gene expression detection. The glass bead transformation method presented many advantages (simplicity, low expenses and short transformation time) and the highest transformation efficiency and reproducibility (Feng et al. 2009). 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 *PDS* gene in *Haematococcus pluvialis* led to increased carotenoid and rapid astaxanthin accumulation (Steinbrenner and 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 improvement of this alga as a bioreactor and also as a molecular toolkit. The recent elucidation of the plastid genome (Smith et al. 2010) is a significant step towards this goal and it can be predicted that stable and routine chloroplast transformation will be reported soon.

**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 and a number of genome projects are currently in progress for several other algae (http://www.jgi.doe.gov). Indeed one of the earliest algal genomes sequenced, C. reinhardtii’s, serve as an important 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 (Armburst et al. 2004, Montsant et al. 2007, Siaut et al. 2007). Despite the considerable available molecular data regarding these microscopic photosynthetic organisms, further biotechnology development is necessary for overall algal commercial exploitation.

With the recent publication of the mitochondrial and plastid genomes of D. salina (Smith et al. 2010) and the forthcoming release of the nuclear genome by JGI, genome-based and other approaches will become possible in the near future. For example, development of microarrays or deep eDNA 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. Furthermore, combining genomics of D. salina with proteomics will facilitate analysis of the structure and function of the unique glycoalxys and the plasma membrane of this halotolerant organism. In comparison to the alga C. reinhardtii, more genomic tool development is still necessary for its close relative, D. salina. At this time highly efficient transformation systems are not available and genetic crosses cannot be performed on a routine basis. However, with the genome sequence 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).

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REFERENCES


Coesel, S. N., Baumgartner, A. C., Teles, L. M., Ramos, A.


