Photosynth Res
DOI 10.1007/s11120-015-0149-2

REVIEW

Production of carotenoids by microalgae: achievements and challenges

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Received: 3 February 2015 / Accepted: 21 April 2015

Abstract Carotenoids are a wide group of lipophylic isoprenoids synthesized by all photosynthetic organisms and also by some non-photosynthetic bacteria and fungi. Animals, which cannot synthesize carotenoids de novo, must include them in their diet to fulfil essential provitamin, antioxidant, or colouring requirements. Carotenoids are indispensable in light harvesting and energy transfer during photosynthesis and in the protection of the photosynthetic apparatus against photooxidative damage. In this review, we outline the factors inducing carotenoid accumulation in microalgae, the knowledge acquired on the metabolic pathways responsible for their biosynthesis, and the recent achievements in the genetic engineering of this pathway. Despite the considerable progress achieved in understanding and engineering algal carotenogenesis, many aspects remain to be elucidated. The increasing number of sequenced microalgal genomes and the data generated by high-throughput technologies will enable a better understanding of carotenoid biosynthesis in microalgae. Moreover, the growing number of industrial microalgal species genetically modified will allow the production of novel strains with enhanced carotenoid contents.

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Keywords Carotenoid biosynthesis · Microalgae · Microalgal transformation · Genetic engineering · Abiotic stress · Carotenoid function

Introduction

Carotenoids are the most diverse and widespread pigments found in nature (Sasso et al. 2012). They are a wide group of lipophylic isoprenoids synthesized by all photosynthetic organisms and also by some non-photosynthetic bacteria and fungi. The central C_{40} backbone, made up of eight isoprene units, forms a polyene chain of conjugated double bonds and establishes an extended π-electron system that accounts for its ability to absorb both ultraviolet (UV) radiation and visible light (Grossman et al. 2004). The number of conjugated double bonds within this basic backbone, as well as cyclic and oxygenic modifications, yields a variety of carotenoids whose colours range from yellow to reddish brown. More than 750 carotenoid structures have been isolated from different natural sources (Britton et al. 2004). These carotenoids and the pathways involved in their biosynthesis are frequently used as taxonomic markers within the diverse microalgal phyla (Takaichi 2011). Despite the wide diversity of carotenoids structures, no more than 30 of them play a direct role in photosynthesis of eukaryotic microalgae.

Carotenoids are indispensable in light harvesting and energy transfer during photosynthesis and in the protection of the photosynthetic apparatus against photooxidative damage (Li et al. 2009). Most carotenoids are bound to integral membrane proteins, associated with light-harvesting complexes (LHCs), where they absorb light across a broader range of the spectral region and transfer the energy to chlorophyll, initiating the photochemical events of

Published online: 29 April 2015

photosynthesis (Polívka and Frank 2010). There are also carotenoids located in the core complexes of both photosystems (Pineau et al. 2001; Ferreira et al. 2004), and in the cytochrome b_6f complex (Zhang et al. 1999), where they promote the stability and functionality of the photosynthetic apparatus (Grossman et al. 2004). Strong evidence indicates the existence of a tightly regulated coordination in the biosynthesis of proteins, carotenoids, and chlorophylls integrating the photosystems (Bouvier et al. 2005). In addition, some microalgae have the ability to accumulate carotenoids under unfavourable conditions. In this case, carotenoids are accumulated in lipid globules both extraplastidic, as the accumulation of astaxanthin in the green alga *Haematococcus* (Boussiba 2000), or located within the chloroplast, as seems to occur in β-carotene-accumulating *Dunaliella* species (Ramos et al. 2008; Davidi et al. 2014).

In this review, we will describe the applications of the main commercial natural carotenoids, including their role in prevention of human diseases. We will highlight the progress made in understanding carotenoid biosynthesis pathway and its regulation in microalgae, and we will analyse the main strategies applied to optimize biotechnological production of carotenoids, both by manipulation of the culture conditions and by genetic engineering of the pathway. We will focus mainly on chlorophytes due to their higher similarity to land plants and greater potential for biotechnological production of carotenoids and because their carotenoid biosynthetic pathways are better known.

Importance of carotenoids and their applications

Aside from their importance in light harvesting and stability of the photosynthetic apparatus, carotenoids have a wide range of other roles in nature. They can be used as pigments in scales, feathers or skin of birds, fish, amphibians, and reptiles to advertise fitness (e.g. low parasite load and high caloric intake), attract potential sexual mates, and signal social status (Ruff and Fuller 1975; Blount and McGraw 2008). Moreover, carotenoid pigments may be important for predation avoidance either by announcing toxicity or providing a suitable hue for camouflage (Blount and McGraw 2008; Cazzonelli 2011; Stoddard 2012). In plants, carotenoids seem to play a role in reproduction by attracting pollinators and frugivores for seed dispersion (Blount and McGraw 2008). In animals, reproduction success has been linked to carotenoid intake, most probably because carotenoid-derived metabolites (e.g. vitamin A) are critical in key developmental stages (Dugas et al. 2013; Marri and Richner 2014). There is evidence that accumulation of carotenoids in the outer layers of an organism, a cell or an organelle, is related to the photoprotective effect of these molecules. Carotenoids together with flavonoids appear to prevent UV-induced sunburn in humans with light-sensitive skin containing low levels of melanin (Stahl and Sies 2007). In photosynthetic organisms, carotenoid-based photoprotection may be achieved not only via excess energy dissipation in the thylakoids, but also by creating a sunscreen layer of oil droplets rich in non-oxygenated (i.e. carotenes, e.g. β-carotene) or oxygenated (i.e. xanthophylls, e.g. astaxanthin) carotenoids at the periphery of the chloroplast or cell body (Borowitzka et al. 1984; Ben-Amotz et al. 1988; Bar et al. 1995; Wang et al. 2005). This strategy may be particularly beneficial for algae thriving in environments with high incident solar radiation (e.g. saltworks and alpine regions). Carotenoids absorb mainly blue and green light as well as UV radiation, and more so if they become aggregated in more polar environments upon interaction with anions, phospholipids, other carotenoids, and carotenoid-binding proteins (Hager 1970; Kohn et al. 2008). This is perhaps one of the reasons why carotenogenic *Dunaliella salina* cells can withstand long (up to 30 min) UV radiation treatments without apparent loss of viability when plated on salt-containing solid growth media (J. Varela, unpublished results). It is thus not uncommon for brine and salt harvested in saltworks to acquire a pinkish colour during springtime due to the proliferation of *D. salina* as well as carotenoid-accumulating bacteria and archaea (Oren 2009).

In fact, the protective role of carotenoids appears to be so essential to photosynthetic organisms that gene mutations or use of chemicals (e.g. norflurazon) causing the abrogation of coloured carotenoid biosynthesis can prevent recovery from photoinhibition and, in some cases, be lethal in the presence of light and oxygen (Sistrom and Clayton 1964; Ben-Amotz et al. 1987). Carotenoids seem to promote the integrity of membranes, photosystems, and antenna complexes through their ability to quench highly reactive species, such as singlet oxygen ($^1O_2$*) and triplet (^{3}Chl*) chlorophyll (Fig. 1). The resulting triplet state carotenoids (^{3}Car*) can safely release their excess energy as heat (Telfer et al. 2008). In addition, in chlorophytes and most stramenopiles algae, the xanthophylls violaxanthin and zeaxanthin are involved in a set of protective mechanisms that dissipate excess energy via heat release or non-photosynthetic quenching (NPQ; Müller et al. 2001; Erickson et al. 2015; Goss and Lepetit 2015). However, the interconversion of diadinoxanthin to diatoxanthin and back appears to modulate NPQ in diatoms, dinoflagellates, and haptophytes (Goss and Lepetit 2015). Although not yet fully understood, NPQ lowers the levels of ^{1}Chl*, which decreases the likelihood of formation of ^{3}Chl via intersystem crossing (Fig. 1). As a result, the formation of $^1O_2$*, one of the most damaging forms of reactive oxygen species (Jahns and Holzarth 2012), is largely prevented (Erickson

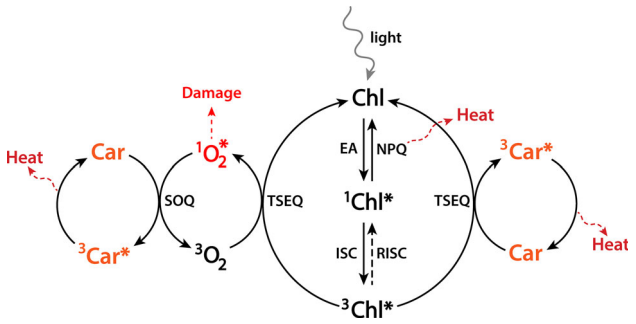

Fig. 1 Production and quenching of singlet oxygen ($^1O_2$*), singlet (^{1}Chl*), and triplet (^{3}Chl*) chlorophyll and the protective role of carotenoids (Car). Light can excite chlorophyll into a state known as ^{1}Chl* via direct energy absorption (EA) or singlet–singlet energy transfer from other light-harvesting molecules. In turn, ^{1}Chl* can give rise to ^{3}Chl* through a radiationless interconversion process known as intersystem crossing (ISC). Triple-state energy quenching (TSEQ) of ^{3}Chl* by ground-state molecular oxygen ($^3O_2$) can generate $^1O_2$*. Singlet oxygen is one the most reactive oxygen species (ROS), leading to cell damage. Carotenoids can prevent this by singlet-oxygen quenching (SOQ) or by deactivating ^{3}Chl* via TSEQ. Both processes produce triple-state carotenoids (^{3}Car*), which can safely return to ground state by releasing heat. ^{3}Chl*-to-^{1}Chl* conversion by reverse ISC (RISC) may happen, but it is a rare event. Oxygen-containing carotenoids seem also to play an essential role in several protective mechanisms that result in thermal dissipation of excess energy or non-photochemical quenching (NPQ). NPQ enables microalgae and other photosynthetic organisms to lower excess ^{1}Chl* levels and thus decrease the formation of ^{3}Chl and $^1O_2$*

et al. 2015). Moreover, carotenoids have been reported to be potential scavengers of peroxyl radicals (ROO•) with concomitant formation of carotenoid radicals (Chisté et al. 2014). Subsequently, carotenoid radicals can be deactivated by ascorbate (vitamin C) and tocopherol (vitamin E), breaking the chain of radical formation (Tsuchihashi et al. 1995).

Carotenoid biosynthesis appears to be important for microorganisms to withstand low temperatures as well (Dieser et al. 2010). The authors suggest that xanthophylls may improve membrane integrity by stabilizing the lipid bilayer. This property might be crucial for survival to cold stress, because ice nucleation may disrupt the plasma membrane and cause cell death. In addition, this stabilization should counterbalance increased membrane fluidisation due to higher lipid unsaturation, a usual cellular response designed to prevent membrane rigidity caused by cold-induced tighter packing of molecules. A similar stabilizing role has been suggested for de-epoxidated xanthophylls (e.g. zeaxanthin) for organisms under heat and salt stress, two environmental conditions known to influence membrane fluidity (Los and Murata 2004; Ashraf and Harris 2013).

It has also been attributed to carotenoids a role in photoreception and phototaxis in microalgae. Some flagellate microalgal species, such as chlorophytes, haptophytes, heterokontophytes, dinoflagellates, and euglenoids, possess a photosensitive eyespot. This apparatus is able to detect variations of light intensity for a microalga to determine the direction it should swim for optimal light for photosynthesis or predation avoidance (Kreimer 2009). The photoreceptors are usually membrane-bound rhodopsins, which are in the vicinity of layers of photoprotective/reflective carotenoid-rich globules (Britton 2008; Kreimer 2009). In turn, rhodopsin is composed of an opsin apoprotein and a covalently bound molecule of retinal, a highly photosensitive carotenoid-derived chromophore (Ernst et al. 2014).

Interestingly, both photoprotective carotenoids and retinal-containing rhodopsins have also been found to be crucial for human vision (Demmig-Adams and Adams 2002; Vílchez et al. 2011; Álvarez et al. 2014). For example, β-carotene is often called provitamin A because its cleavage yields retinal, which can then be converted into retinol (vitamin A) and retinoic acid (Tang and Russell 2009). As all these metabolites play important roles in photoreception and/or signal transduction and most animals are unable to synthesize them, low levels of dietary carotenoids can lead to xerophthalmia, night blindness, and in severe cases to the keratinization of the conjunctiva and cornea (Britton 2009).

Vitamin A deficiency has also been linked to higher susceptibility to infection-related medical conditions (e.g. AIDS, measles, and dysentery), most likely due to an impaired immune response. The role of vitamin A in all these ailments seems to be confirmed by the fact that many of their symptoms and consequent mortality rate can be alleviated by supplemental doses of this vitamin (Britton 2009). Carotenoids might also modulate the immune system and prevent several forms of cancer, diabetes, cataracts, macular degeneration, neuroinflammation, neurodegenerative, and cardiovascular diseases (Paiva and Russell 1999; Vílchez et al. 2011; Ambati et al. 2014; Chinembiri et al. 2014; Yin et al. 2014; Zhang et al. 2014).

Because of the health-promoting benefits of carotenoids and their vibrant colours, strong demand for these molecules has become apparent over the last few years. In 2010, the world market for carotenoids has been estimated to be about 1.2 billion USD with β-carotene, lutein, and astaxanthin accounting for 60 % of the total market value (Borowitzka 2013). They have been applied as colourants, antioxidants, nutritional supplements, and nutraceuticals by the aquaculture, cosmetic, food, and ornamental fish industries (Gouveia and Empis 2003; Christaki et al. 2012; Borowitzka 2013). As microalgae remain one of most important natural sources of these pigments (Borowitzka 2013), knowledge of how these microorganisms synthesize and accumulate carotenoids will be key to any research programme or entrepreneurial venture designed to improve their production.

Carotenogenic environmental cues

The biosynthesis, cellular accumulation, and storage of carotenoids are highly regulated. One of the model organisms often used to study carotenogenesis is the chlorophyte *D. salina*, an alga able to accumulate carotenoids up to 14 % of its dry weight (Ramos et al. 2011). These extreme levels of mainly all-*trans*- and 9-*cis*-*β*–carotene protect the single chloroplast of the microalgal cell from the deleterious effects of high insolation typically found in saltworks, its most common habitat (Borowitzka et al. 1984; Ben-Amotz et al. 1988). As the water column drops and salinity increases upon water evaporation for salt crystallization and harvesting, nutrients become unavailable due to precipitation or nutrient uptake inhibition (Oren 2009). Simultaneously, light intensity can reach such high levels that photoinhibition and light-induced destruction of the photosynthetic apparatus might lead to cell death (Ben-Amotz et al. 1989; Smith et al. 1990). This high light intensity is due to not only lower light attenuation caused by decreased water depth (Kirk 1988), but also due to increased surface albedo from crystallizing salt (Oroud 2001; Fujimaki et al. 2003; Fig. 2). All these types of abiotic stress are environmental cues for *D. salina* to induce carotenogenesis, including the expression of genes encoding several enzymes of the carotenoid biosynthetic pathway as well as of those feeding biochemical precursors into it (see below). However, low levels of nutrients (e.g. nitrate and sulphate) were found to be essential for carotenoid accumulation in *D. salina* (Rabbani et al. 1998), as supplementation with fresh medium inhibited both high light- and salt-induced carotenogenesis (Coesel et al. 2008a).

Similar results have been found for *H. pluvialis* and *Chromochloris zofingiensis*. Unfortunately, the latter species is often called *Chlorella zofingiensis* for historical reasons, but recent phylogenetic data have shown that this species should not be included in the *Chlorella* genus (Fučikova and Lewis 2012). Nevertheless, both species are two chlorophytes that can be grown photo-, hetero-, and mixotrophically for the production of astaxanthin (Kobayashi et al. 1992; Liu et al. 2014). Moreover, similarly to *D. salina*, carotenogenesis in *H. pluvialis* and *C. zofingiensis* is induced by high light and salt stress (Borowitzka et al. 1991; Harker et al. 1996; Liu et al. 2014). However, unlike in the halophile *D. salina*, the salt concentrations leading to faster carotenoid accumulation can also result in cell death in freshwater species (Harker et al. 1996).

Lastly, light quality seems also to influence carotenogenesis. For example, exposure to UV-A can enhance carotenoid accumulation in *D. salina* (var. *bardawil*) growing under photosynthetically active radiation (Salguero et al. 2005). In addition, blue light has been shown to promote carotenoid biosynthesis gene expression in both

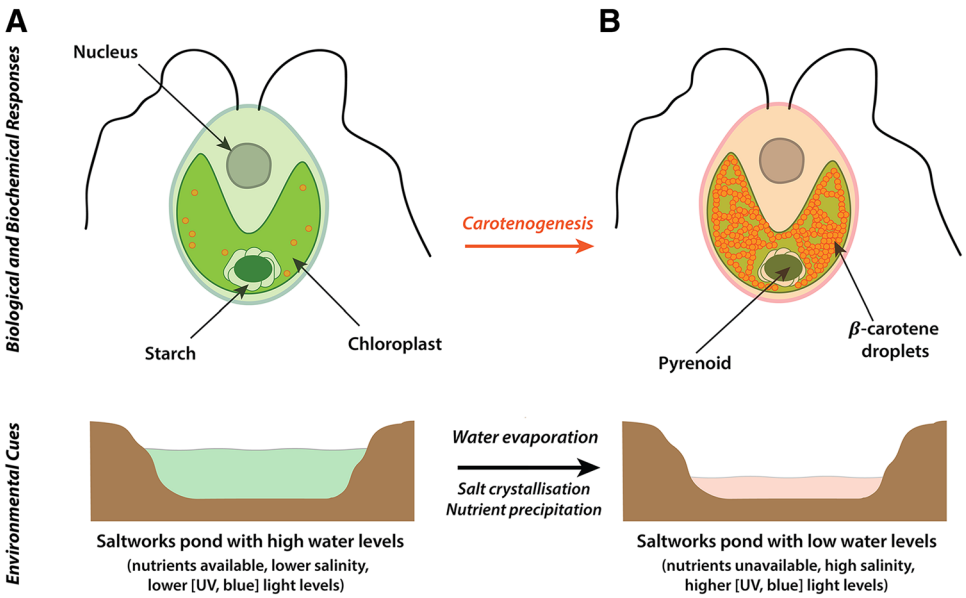

Fig. 2 Carotenogenic environmental cues for *Dunaliella salina*. Nutrient depletion, salt stress, and high light are indicators that the habitat is about to change. As the water levels of the saltworks pond drop as a result of evaporation (A), nutrient availability decreases due to nutrient precipitation or nutrient uptake collapse (B). Simultaneous exposure to increased salinity and light intensity takes place. Higher photon flux densities are due to lower light attenuation caused by decreasing water depth and enhanced surface albedo owing to the deposition of salt crusts. Carotenogenic microalgae respond by accumulating photoprotective carotenoid-rich droplets at the periphery of their chloroplasts or cell bodies, so that photoinhibition is kept at a minimum and survival is ensured

chlorophytes (Bohne and Linden 2002; Kianianmomeni 2014) and diatoms (Coesel et al. 2008b). The Mn-containing oxygen-evolving complex (OEC) of photosystem II (PSII) seems to be particularly vulnerable to both UV and blue light. Both wavelength ranges can elicit PSII photoinhibition via a mechanism involving Mn loss from the OEC (Hakala et al. 2005). Taken together, these results strongly suggest that carotenogenesis plays an essential role in photoinhibition prevention, highlighting once more the importance of carotenoids in photosynthetic organisms, particularly those adapted to habitats exposed to intense solar radiation.

Another common thread leading to carotenogenesis in microalgae is that environmental cues that induce carotenoid accumulation are, for the most part, identical to the ones inducing storage neutral lipids (mostly in the form of triacylglycerols; TAGs), as has been demonstrated for *Dunaliella salina* (Ben-Amotz et al. 1982) or *H. pluvialis* (Chekanov et al., 2014). However, the existence of a common regulation for these two processes remains to be elucidated (Rabbani et al. 1998). Classical studies carried out by Ben-Amotz and co-workers showed that in *Dunaliella* β-carotene is accumulated in carotenoid-rich plastidial globules, composed of practically only neutral lipids, more than half of which was β-carotene (Ben-Amotz et al. 1982). Recently Davidi et al. (2014) have demonstrated that, beside these β-carotene-rich plastoglobuli, under nitrogen deprivation *Dunaliella bardawil* also accumulates cytoplasmatic lipid droplets composed by more than 90 % TAGs, with no chlorophyll or carotenoids. Similar lipid droplets have been found in *Chlamydomonas reinhardtii* (Goold et al. 2015). Nonetheless, carotenoid-accumulating oil droplets seem to be pervasive in many species, including snow microalgae, land plants, carotenogenic fungi, and vertebrates (Bidgare et al. 1993; Britton 2008; Kohn et al. 2008).

Understanding the carotenoid biosynthesis pathway in microalgae

Our current understanding of carotenoid metabolism and its regulation in microalgae is still somewhat limited and mainly inferred from the knowledge acquired for higher plants. Carotenoids are synthesized in the chloroplast by the action of a series of nuclear-encoded membrane proteins. These gene products are synthesized in the cytoplasm as precursor polypeptides containing amine-terminal extensions that target them to the chloroplasts. The biosynthesis of carotenoids has been exhaustively reviewed by Cunningham and Gantt (1998) and more recently by Botella-Pavía et al. (2004) or Farré et al. (2010). However, reviews focusing on the carotenoid biosynthesis in algae are scarce (Lohr 2009; Takaichi 2011; Paniagua-Michel et al. 2012).

All carotenoids are formed from a C_5 building block, common precursor of all isoprenoids, the isopentenyl pyrophosphate IPP, via the plastidial 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway (Schwender et al. 2001). Differently from higher plants, where both the cytosolic mevalonate (MVA) and the plastidial MEP, also known as non-MVA, pathways supply IPP to the carotenoid biosynthetic pathway, in chlorophytes the cytosolic MVA pathway appears to be absent (Capa-Robles et al. 2009).

The first committing step of carotenoid biosynthesis is the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to yield the first uncoloured carotenoid, phytoene, catalyzed by phytoene synthase (PSY). PSY is one of the most important regulatory enzymes in the pathway (Table 1; Fig. 3). It has been found to be up-regulated during stress conditions in several chlorophyte microalgae, such as *Haematococcus pluvialis* (Vidhyavathi et al. 2008), *Chlamydomonas reinhardtii* (Bohne and Linden 2002; Couso et al. 2012), or *D. salina* (Coesel et al. 2008a). In the genomes of *C. reinhardtii*, *Volvox carteri,* or *Chlorella vulgaris* (Lohr et al. 2005), a unique PSY-encoding gene has been found, but in other chlorophytes such as *D. salina* two classes of *PSY* gene families are present (Ye et al. 2008), probably due to the need of a differentially regulated response to stress conditions. This enzyme has been the main target for genetic engineering approaches to increase carotenoid content by overexpression of the corresponding gene as detailed below.

From this step on, phytoene is subjected to four sequential desaturations catalyzed by phytoene (PDS) and ς-carotene (ZDS) desaturases, resulting in the formation of pro-lycopene, which is isomerised by a specific isomerase (CRTISO) to all-*trans* lycopene, the first coloured carotenoid. PDS and ZDS are structurally related, indicating that they could have evolved from a common ancestor by gene duplication (Grossman et al. 2004). They follow the same reaction mechanism as other dehydrogenases with plastoquinone as the hydrogen acceptor, connecting in this way the photosynthetic electron transport chain with carotene desaturation. This is other important regulatory point of the pathway and is the target of the bleaching herbicide norflurazon, which blocks the synthesis of carotenoids, inhibiting PDS by competition with its cofactors (Breitenbach et al. 2001). The inhibitory effect of this herbicide has been used to induce the accumulation of phytoene in *Dunaliella* (León et al. 2005) and to propose an alternative selectable marker for genetic transformation of *C. reinhardtii* (Liu et al. 2013) and *H. pluvialis* (Steinbrenner and Sandmann 2006).

At the level of lycopene, the pathway splits into two branches. In one branch, lycopene is cyclised at both ends

Table 1 Main enzymes involved in the biosynthesis of carotenoids in plant and bacteria

Enzyme	Plants	Bacteria	Function
Phytoene synthase	PSY	CRTB	Condensation of two GGPP
Phytoene desaturase	PDS	CRTI	Desaturation of phytoene
ζ-carotene isomerase	Z-ISO	–	Isomerization of ζ-carotene
ζ-carotene desaturase	ZDS	CRTI	Desaturation of ζ-carotene
Carotenoid isomerase	CRTISO	–	Isomerization of pro-lycopene
Lycopene β-cyclase	LCYB	CRTY	Introduction of β rings
Lycopene-ε-cyclase	LCYE	–	Introduction of ε rings
Carotene β-hydroxylase	CHYB	CRTZ	Hydroxylation of β rings
Carotene β-hydroxylase	CYP97A5	–	Hydroxylation of β rings
Carotene ε-hydroxylase	CYP97C3	–	Hydroxylation of ε rings
Violaxanthin de-epoxidase	VDE	–	De-epoxidation of β rings
β-carotene (C4) ketolase	BKT	CRTW	Introduction of a keto group in the C4 position of β rings.

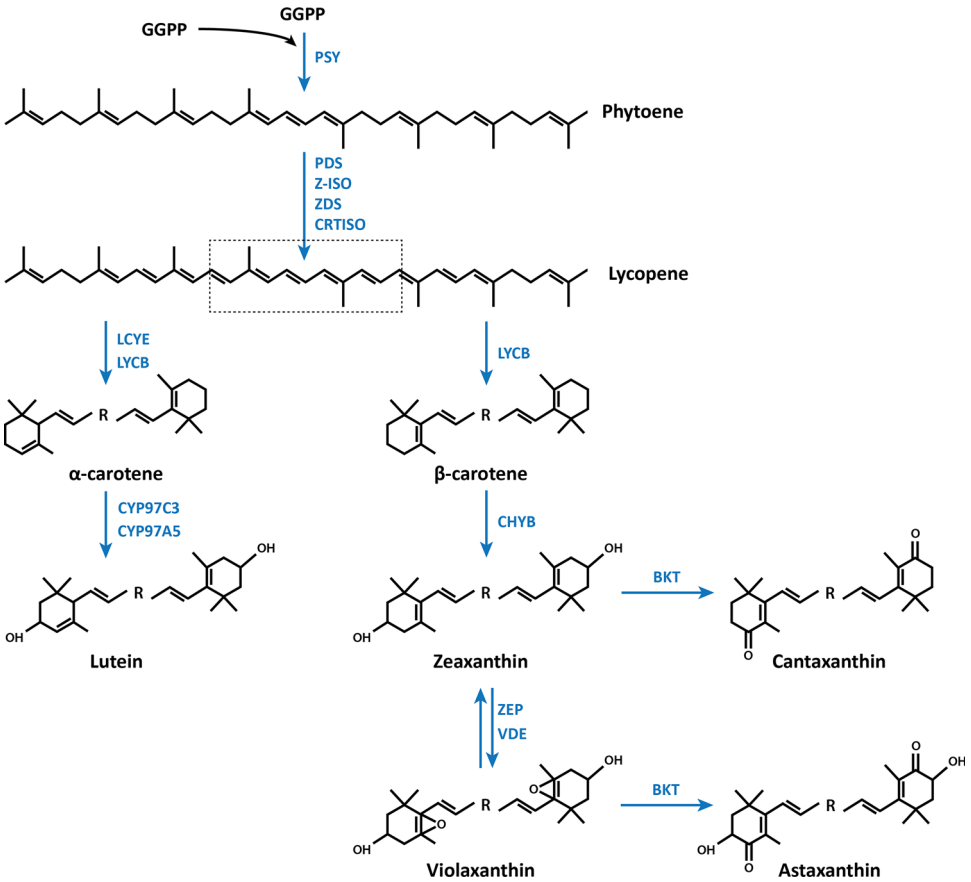

Fig. 3 Carotenoid biosynthesis pathway in chlorophytes. GGPP, geranylgeranyl pyrophosphate; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LCYE, lycopene ε-cyclase; LCYB, lycopene β-cyclase; CYP97C3, cytochrome P450 ε-hydroxylase; CYP97A5, cytochrome P450 β-hydroxylase; CHYB, carotene β-hydroxylase; BKT, β-carotene oxygenase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase

by lycopene β-cyclase (LCYB), yielding β-carotene with two β–ionone end groups. These can be further hydroxylated by a non-heme di-iron hydroxylase, β-carotene hydroxylase (CHYB) to yield zeaxanthin. Accumulation of transcripts corresponding to both genes under stress conditions has been reported in *D. salina* (Ramos et al. 2008), *C. reinhardtii* (Sun et al. 2010), and *C. zofingiensis* (Cordero et al. 2010), among other microalgae. LCYB contains a characteristic Rossmann or dinucleotide-binding fold, constituted by a β–sheet–α-helix-loop–β-sheet, present both in higher plants (Cunningham et al. 1996) and in microalgae (Cordero et al. 2010).

In the other branch, the concerted action of LCYB and ε-cyclases (LCYE) results in the formation of α-carotene.

The relative activities of LCYE and LCYB determine the proportion of carotenoids directed to each branch of the pathway. LCYB and LCYE are structurally very similar, as has been demonstrated for *C. zofingiensis*, with amino acid sequence similarities higher than 50 % (Cordero et al. 2012). Hydroxylation of α-carotene catalyzed by two heme-containing cytochrome P450 monoxygenases, a carotene *β*-hydroxylase (CYP97A5) and a carotene *ε*-hydroxylase (CYP97C3) leads to the formation of lutein. These two hydroxylases, evolutionary unrelated to the non-heme BCH responsible for the hydroxylation of *β*-carotene, have been well characterized in *Arabidopsis* (Tian et al. 2003; Kim and DellaPenna 2006). In *Chlamydomonas* genome, candidate genes encoding both cytochrome-dependent hydroxylases have been found (Lohr et al. 2005), but their functional characterization has not been performed as yet. The expression of the genes encoding these two hydroxylases was significantly induced under high light stress in the chlorophyte *C. reinhardtii* (Couso et al. 2012).

Zeaxanthin is converted to violaxanthin by zeaxanthin epoxidase (ZEP), which inserts two epoxy groups at positions C-5,6 and C-5′,6′. The reaction is oxygen dependent and involves NADPH, FAD, and ferredoxin as cofactors (Büch et al. 1995). Under high-light conditions, or other stress situations, violaxanthin can be de-epoxidated back to zeaxanthin by violaxanthin de-epoxidase (VDE). ZEP-encoding genes have been cloned in *Chlamydomonas* (Baroli et al. 2003) or *C. zofingiensis* (Couso et al. 2012) and assigned by homology with these enzymes in other microalgae with sequenced genomes. Surprisingly, the VDE-encoding gene has not been found in the *Chlamydomonas* genome, which has been fully sequenced. A gene coding for a putative violaxanthin de-epoxidase-related protein (VDR1) has been found in the genome of this microalga (Merchant et al. 2007). However, this assignment is yet to be ascertained experimentally. The reversible interconversion of zeaxanthin and violaxanthin, known as the violaxanthin cycle, is essential for photoprotection. This was first demonstrated by Demmig-Adams and co-workers (Demmig-Adams et al. 1989) in higher plants and now is widely accepted as a mechanism for NPQ in green algae (Fig. 1; Goss and Jakob 2010). This violaxanthin cycle has also been shown to be present in the Eustigmatophyceae, Chrysophyceae, Phaeophyceae, and Raphidophyceae (Lohr and Wilhelm 1999; Goss and Lepetit 2015). However, despite belonging to the same Stramenopiles-Alveolata-Rhizaria (SAR) megagroup as the former microalgae, diatoms and dinoflagellates have an alternative photoprotective xanthophyll cycle. In this case, the xanthophyll diadinoxanthin is converted to diatoxanthin by depoxidation. Both zeaxanthin and diatoxanthin are viewed as photoprotective rather than light-harvesting pigments. This photoprotection may be linked to a xanthophyll-dependent conformational change of the PSII antenna system that promotes NPQ instead of light harvesting (Bertrand 2010; Goss and Lepetit 2015).

VDE and ZEP are the first reported lipocalin proteins identified in plants and only the second example of lipocalin proteins with enzymatic activity (Hieber et al. 2000). Lipocalins are characterized as having three motifs of structurally conserved regions within a β-barrel structure; both the de-epoxidase and epoxidase enzymes exhibit longer N- and C-terminal regions when compared to other lipocalins and it is realistic to predict that those enzymes have the same tertiary structure, as they both function on the same intermediate: antheraxanthin. However, apart from the lipocalin core structure, there are no common sequences that can predict any homology between these two enzymes.

This basic pathway is conserved in most microalgae, although some species are able to accumulate unusual carotenoids via specific biosynthetic routes. Most of the genes encoding enzymes of the carotenoid biosynthetic pathway have been isolated and characterized by functional complementation experiments in higher plants (Sandmann et al. 2006; Cunningham and Gantt 2007). In microalgae, some genes have also been isolated but many others, especially those encoding the enzymes catalyzing the last steps of the pathway have simply been assigned by homology with the corresponding higher plants orthologues (Lohr et al. 2005).

In a limited number of organisms, including the green algae *H. pluvialis*, *C. zofingiensis* (Huang et al. 2006), or species of the *Scenedesmus* genus (Pirastru et al. 2011), zeaxanthin can be epoxidated to yield astaxanthin, a pigment highly appreciated in aquaculture. This xanthophyll has the ability to provide a characteristic pinkish-red colour to salmon, trout, and crustaceans. As these species are unable to synthetize this ketocarotenoid, astaxanthin must be included in their diet for them to acquire their typical reddish hue (Chekanov et al. 2014).

This reaction is catalyzed by *β*-carotene ketolase (BKT). Different types of *β*-carotene ketolase proteins are known, commonly referred to as the BKT (in plants and algae), CRTW (in bacteria, including cyanobacteria), and CRTO types (found mainly in cyanobacteria and some other bacteria as well). In general terms, using *β*-carotene as a substrate, BKT and CRTW ketolases predominantly produce the di-keto carotenoid canthaxanthin, whereas CRTO ketolases produce preferentially the mono-keto compound echinenone. BKT and CRTW ketolases share significant levels of sequence identity with a family of proteins that includes fatty acid desaturases and non-heme di-iron monooxygenases (BCH) (Masamoto et al. 1998). BKT and CRTW ketolases contain four histidine-rich motifs, which

may be useful for the coordination of iron atoms, as is the case with β-hydroxylases (Kajiwara et al. 1995).

Keto-carotenoids are rare in higher plants, but are abundant in cyanobacterial and some microalgal species. Recent evidence that *BKT* genes occur in the genome of *Chlamydomonas*, whose vegetative cells lack keto-carotenoids, was a surprising discovery (Lohr et al. 2005). The presence of keto-carotenoids in zygospores of *Chlamydomonas* may explain the presence of BKT-encoding genes in *Chlamydomonas*, and most probably in other chlorophytes.

Genetic engineering of carotenoid biosynthesis pathway in microalgae

Microalgae are not only an excellent model for the study of the carotenogenesis in plant cells, but they can also be ideal hosts for the expression of genes coding for enzymes belonging to the carotenoid biosynthetic pathway of other species in order to produce novel carotenoids with higher productivities than those of the wild-type strains. Microalgae, besides presenting more physiological and genetic analogies with plant cells than bacteria, have almost the same advantages than these with regard to simplicity of culture and growth rate. Furthermore, many microalgal species have an active central terpenoid metabolism that would guarantee enough supply of precursors and possess high capacity for carotenoid storage. Nevertheless, reports about the manipulation of the carotenogenic pathway in eukaryotic microalgae are scanty.

Modification of microalgae to obtain strains able to produce novel and/or higher levels of pre-existing carotenoids can be carried out by the inactivation or overexpression of endogenous genes or by the expression of genes from other species (Table 2). This would ideally allow to obtain genetically modified microalga with increased or knockdown enzymatic activities and able to accumulate the desired carotenoids. A high number of genes involved in carotenogenic pathways of bacteria, fungi, higher plants, cyanobacteria, and microalgae have been cloned and sequenced (Cunningham and Gantt 1998). Others have been inferred from sequenced genomes and assigned by similarity with the sequences of genes from related species (Grossman et al. 2004; Lohr et al. 2005). They constitute an invaluable genetic portfolio for engineering the production of carotenoids.

The main genes of the carotenogenic pathway have been successfully expressed in *E. coli* (Albrecht et al. 1999; Sandmann 2001) with exception of the gene encoding zeaxanthin epoxidase. This enzyme catalyzing the formation of violaxanthin needs reduced ferredoxin, which is absent in *E. coli*. In addition, the synthesis of carotenoids has also been engineered in non-carotenogenic fungi (Kimura and Shimada 1988; Misawa and Shimada 1998). However, bacteria and fungi have a limited supply of the common isoprenoid precursors, such as dimethylallyl pyrophosphate (DMAPP) and GGDP, which are needed for carotenoid biosynthesis (Schmidt-Dannert et al. 2000). The supply of precursor terpenoids has been partially improved by the overexpression of the initial enzymes of the MEP pathway, such as 1-deoxy-D-xylulose-5-phosphate synthase or isopentenyl pyrophosphate isomerase (Albrecht et al. 1999) or suppressing the use of prenyl-phosphates in other competitive pathways (Kimura and Shimada 1988).

There are also many examples of transgenic higher plants expressing exogenous genes of the carotenogenic pathway (Giuliano et al. 2008). Nevertheless, the lack of information about many of the aspects of regulation of the route limits its genetic manipulation and causes problems of low productivity and unwanted side effects, such as plants of small size or reduced content in chlorophyll. Many of these difficulties have been solved. For example, altered synthesis of abscisic acid and phytol, also synthesized from precursors coming from the terpenoid biosynthetic pathway, has been avoided by targeting PSY to the chromoplasts of the fruit of transgenic tomato plants. This fruit-specific expression was achieved using the tomato polygalacturonase promoter and an optimized transit peptide (Fraser et al. 2002). Moreover, the low productivity of β-carotene in the endosperm of the original golden rice has been partially overcome by replacing the phytoene synthase of daffodil with that of maize in the original genic construction (Paine et al. 2005). Another example would be

Table 2 Examples of metabolic engineering strategies for altering the content of various carotenoids in microalgae

Carotenoid	Strategy	Microalga	Reference
Keto-carotenoids	Expression of exogenous BKT	*C. reinhardtii*	León et al. (2007)
Violaxanthin and lutein	Expression of exogenous PSY	*C. reinhardtii*	Cordero et al. (2011), Couso et al. (2011)
No carotenoids	Silencing of the *PSY* gene	*C. reinhardtii*	Molnar et al. (2009)
Astaxanthin	Mutant version of PDS	*H. pluvialis*	Steinbrenner and Sandmann (2006)
Various carotenoids	Mutant version of PDS	*C. reinhardtii*	Liu et al. (2013)

the remarkable increase in β-carotene content of potato tubers by the selection of optimal promoters (Diretto et al. 2007). Lastly, the bottlenecks observed in maize carotenoid pathway have been addressed using a multigene engineering strategy (Farré et al. 2013).

Although microalgae are one of the main sources of natural carotenoids, genetic manipulation of the pathways involved in carotenoids biosynthesis in these microorganisms has been hindered by the fact that genetic transformation is well established only for a limited number of species, which are not necessarily the best from an applied point of view (León-Bañares et al. 2004; León and Fernández 2007). Although model microalgae, such as the chlorophyte *C. reinhardtii* and the diatom *Phaeodactylum tricornutum*, have been routinely transformed over the last few decades, an increasing interest in microalgae has stimulated research on the genetic transformation of species with applied interest. An example of this is the successful stable transformation of *Nannochloropsis* sp. (Kilian et al. 2011), *Nannochloropsis gaditana* (Radakovits et al. 2012; Jinkerson et al. 2013), *Scenedesmus obliquus* (Guo et al. 2013), or *Tetraselmis chuii* (Úbeda-Mínguez et al. 2014). There have also been some reports about the genetic transformation of chlorophycean microalgae *D. salina* (Sun et al. 2005; Tan et al. 2005; Feng et al. 2009) and *H. pluvialis* (Teng et al. 2002; Kathiresan et al. 2009), which are specially important for the production of β-carotene and astaxanthin, respectively. However, long-term trangenesis is not stable and in some of the strains expression levels are usually low.

Despite all these difficulties, there are some interesting examples of the possibilities of engineering the carotenoid biosynthetic pathway in microalgae. Expression of a mutant version of the *PDS* gene in *H. pluvialis* led to isolation of norflurazon-insensitive transformants showing increased astaxanthin levels (Steinbrenner and Sandmann 2006). A similar strategy allowed the isolation of transgenic *Chlamydomonas* with increased resistance to norflurazon and enhanced biosynthesis of carotenoids (Liu et al. 2013). In *C. reinhardtii*, expression of the *H. pluvialis BKT* gene led to the isolation of transgenic strains able to synthesize ketocarotenoids not present in the wild strain (León et al. 2007). In addition, *Chlamydomonas* strains overexpressing exogenous *PSY* genes were able to display a 2-fold increase in carotenoids levels (Cordero et al. 2011; Couso et al. 2011).

A very interesting alternative to overexpressing key genes is the selective inactivation of genes encoding enzymes catalyzing certain steps of the pathway. Differently from prokaryotes, in the nuclear genome of microalgae and other eukaryotes, foreign DNA is preferentially integrated by non-homologous recombination (Zorin et al. 2009). This has impeded metabolic studies via reverse genomics until the discovery of RNA interference (RNAi). The RNAi technology allows the inhibition, at least partially, of specific genes by mechanisms that are now starting to be understood (Cerutti et al. 2011). There are some examples of microalgae in which the silencing of the expression of genes of the carotenoid biosynthetic pathway has been achieved. Transformants of *Chlamydomonas* with low expression levels of PDS (Vila et al. 2008) and PSY (Molnar et al. 2009) were obtained by RNA interference. A similar strategy allowed transient silencing of the *PDS* gene in electroporated *D. salina* (Sun et al. 2008).

Despite the initial achievements in genetic engineering of microalgal carotenogenesis here described, much work is still necessary to achieve high productivity and stability of the transformants. Besides improving expression of endo- and exogenous proteins in microalgae, a better understanding of the regulation of the carotenoid biosynthesis and its interrelation with other processes is urgently needed.

Conclusions and future trends

Carotenoids are a wide group of isoprenoids with essential physiological functions in both photosynthetic and non-photosynthetic organisms and with a high commercial value. There is much interest to produce novel carotenoids and/or higher levels of the pre-existing ones, and genetic engineering represents a promising approach to achieve this aim. However, this approach has been limited by the lack of information about many aspects of the regulation of the biosynthetic pathways and by the small number of microalgal species for which an optimized and stable transformation protocol has been established.

In the last few years, the number of novel microalgal species genetically transformed in a stable and efficient way has started to increase and important insights into the carotenoid biosynthetic pathway and its regulation have been obtained. Nonetheless, despite these achievements in understanding and engineering carotenogenesis, many aspects remain to be elucidated. Carotenoid biosynthesis and accumulation in microalgae is a complex process, involving not only carotenoids biosynthesis, but also coordinated synthesis of lipids and proteins necessary for the assembly of the carotene-rich oil droplets (Davidi et al. 2014) or the thylakoid-embedded chlorophyll-carotenoid-protein complexes that form the photosystems and the light-harvesting complexes (Lohr et al. 2005). The coordinated regulation of these processes has to be understood for the successful engineering of the pathway, avoiding undesirable collateral effects or low efficiencies of the expressed proteins.

Recent efforts towards a comprehensive analysis of algal genomes have generated a large amount of data. Over

the last few years, about 20 microalgal genome sequences have been completed (http://www.genomesonline.org). High-throughput sequencing is becoming an essential technology, not only for genome sequencing, but also for transcriptome analysis. Massively parallel cDNA sequencing has been used to determine the changes in transcript abundance under different conditions. This approach seems to overcome some of the shortcomings of microarray analysis (González-Ballester et al. 2010). There are interesting examples of the use of this technique to study the synthesis of TAGs under nitrogen deprivation (Miller et al. 2010; Msanne et al. 2012), and it will undoubtedly help clarify the regulation of other metabolic processes, such as carotenoid biosynthesis. High-throughput sequencing has even been proposed as an alternative to insertional mutagenesis since it provides a fast and efficient method to pinpoint the mutations generated by radiation and chemical mutagenesis.

Advances in genetic transformation techniques for microalgae will allow the development of strains with novel, more efficient phenotypes, including higher carotenoid contents. Synthetic biology employing engineering principles, such as modulation and assembly, for the genetic manipulation of microorganisms has been successfully applied to the manipulation of isoprenoids pathways in bacteria (Chang and Keasling 2006), including cyanobacteria (Berla et al. 2013), and they will, most probably, provide interesting results when applied to microalgae.

The synthesis of carotenoids takes place in the chloroplast and is catalyzed by nuclear-encoded enzymes. Direct expression of exogenous genes in the plastome (i.e. chloroplast genome) is other interesting approach for improving carotenoid productivity in microalgae. In this case, DNA integration takes place by homologous recombination, as in bacteria and yeast. Expression of transgenes in the chloroplast is not submitted to transcriptional or post-transcriptional silencing phenomena, so usual in the nuclear expression of transgenes in eukaryotes (Purton 2007), and could thus allow the expression of complete mini-pathways encoded by multigene operons, as has been described for higher plants (Quesada-Vargas et al. 2005; Giuliano 2014).

Taken together, all these perspectives announce exciting times for those interested in the biosynthesis and production of microalgal carotenoids in order to fulfil the growing demand for these molecules with such a wide array of biotechnological applications.

Acknowledgments This study was supported by the Andalusian government (Project P09-CVI-5053 and BIO214) and the Foundation for Science and Technology (FCT), Portugal, via the XTREMEBIO project (PTDC/MAR-EST/4346/2012). This is a publication (No. 94) from CEIMAR Publication Series.

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