Metabolic Engineering for Carotenoid Production Using Eukaryotic Microalgae and Prokaryotic Cyanobacteria

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Abstract

Eukaryotic microalgae and prokaryotic cyanobacteria are diverse photosynthetic organisms that produce various useful compounds. Due to their rapid growth and efficient biomass production from carbon dioxide and solar energy, microalgae and cyanobacteria are expected to become cost-effective, sustainable bioresources in the future. These organisms also abundantly produce various carotenoids, but further improvement in carotenoid productivity is needed for a successful commercialization. Metabolic engineering via genetic manipulation and mutational breeding is a powerful tool for generating carotenoid-rich strains. This chapter focuses on carotenoid production in microalgae and cyanobacteria, as well as strategies and potential target genes for metabolic engineering. Recent achievements in metabolic engineering that improved carotenoid production in microalgae and cyanobacteria are also reviewed.

Keywords

Microalgae · Cyanobacteria · Metabolic engineering

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10.1 Introduction

Eukaryotic microalgae and prokaryotic cyanobacteria are diverse groups of microscopic photosynthetic organisms that include the green algae *Chlorella* (Liu et al. 2014a), *Dunaliella* (Oren 2014), *Haematococcus* (Shah et al. 2016), the diatom *Phaeodactylum* (Gügi et al. 2015), and the cyanobacteria *Synechocystis* (Yu et al. 2013) and *Synechococcus* (Ruffing et al. 2016). In particular, the green alga *Chlamydomonas reinhardtii* has been extensively studied as a model in genetic researches (Scranton et al. 2015). Microalgae and cyanobacteria produce biomass photosynthetically and grow rapidly compared with terrestrial plants and are therefore promising targets for producing valuable products, such as biodiesel (Taparia et al. 2016; Ho et al. 2017). These organisms can also synthesize high-value natural chemicals that can be used in cosmetics, dietary supplements, and pharmaceuticals (Wang et al. 2015; Chew et al. 2017; Yan et al. 2016). Microalgae and cyanobacteria produce biomass by fixing carbon dioxide using solar energy, which contributes to cost-effective and sustainable production of valuable compounds. In addition, their cultivation in the hydrosphere does not compete with food production on croplands. Some microalgae and cyanobacteria can be grown using seawater, saving limited freshwater resources.

Carotenoids are warm-colored tetraterpenoid pigments primarily synthesized by photosynthetic

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organisms including terrestrial plants, microalgae, and cyanobacteria (Huang et al. 2017). Carotenoids are commonly localized in chloroplasts and chromoplasts, and they function as light-harvesting antennas in photosynthesis as well as scavengers of reactive oxygen species (ROS) to protect cellular components from photooxidative damage (Xiao et al. 2011; Jahns and Holzwarth 2012). Due to their antioxidative properties, carotenoids have begun to attract public attention with respect to both their use as natural coloring agents for foods and their use as dietary supplements (Fiedor and Burda 2014). Microalgae and cyanobacteria produce abundant amounts of a wide variety of valuable carotenoids, such as β-carotene, lutein, zeaxanthin, astaxanthin, and fucoxanthin (Varela et al. 2015; Huang et al. 2017).

In order to meet the increasing worldwide demand for carotenoids, stable and cost-effective carotenoid production technologies using microalgae and cyanobacteria are needed (Lin et al. 2015; Anila et al. 2016). Improved carotenoid productivity is desired for commercialization, which could be realized by breeding valuable strains via metabolic engineering approaches (Gimpel et al. 2015). This chapter focuses on metabolic engineering of microalgae and cyanobacteria for carotenoid production and summarizes recent achievements.

10.2 Technologies for Metabolic Engineering of Microalgae and Cyanobacteria

Genetic engineering enables the overexpression of targeted genes and is therefore a powerful tool for use in metabolic engineering. Carotenoid-rich transgenic organisms have been generated by introducing transgenes related to carotenoid synthesis pathways via plasmid vectors into bacteria (Li et al. 2015; Henke et al. 2016), yeast (Gassel et al. 2014), and plants (Hasunuma et al. 2008a; Zhu et al. 2009). The success of these efforts suggests that genetic engineering is an effective approach for improving carotenoid production. Technologies for genetic engineering of microalgae and cyanobacteria have been developed in model organisms, such as *Chlamydomonas* (Baek et al. 2016a, b; Yamaoka et al. 2016; Wannathong et al. 2016), *Chlorella* (Fan et al. 2015; Yang et al. 2016), *Dunaliella* (Feng et al. 2014; Zhang et al. 2015), *Haematococcus* (Steinbrenner and Sandmann 2006), *Nannochloropsis* (Kilian et al. 2011; Kang et al. 2015), *Phaeodactylum* (Xie et al. 2014; Kadono et al. 2015a), *Synechocystis* (Yu et al. 2013), and *Synechococcus* (Ruffing et al. 2016), as well as in some non-model organisms, such as *Monoraphidium* (Jaeger et al. 2017) and *Scenedesmus* (Chen et al. 2016). In eukaryotic microalgae, transformation of either nuclear or chloroplast would be available for enhancing carotenoid production, because intrinsic carotenoid synthesis occurs primarily in the chloroplasts (Gimpel et al. 2015). In some eukaryotic microalgae, exogenous DNA fragments introduced into cells are randomly integrated into the nuclear genome via non-homologous end joining (NHEJ) pathways (Doron et al. 2016). This makes targeted gene knock-in/out of the nuclear genome via homologous recombination (HR) quite difficult in these eukaryotic microalgal species, including the model microalga *C. reinhardtii*.

Genome editing is a recently developed genetic engineering tool that involves sequence-specific nucleases. The CRISPR-Cas9 system is now a widely employed technology for genome editing due to its ease of use (Hsu et al. 2014; Mali et al. 2013; Yang 2015). Targeted gene knock-in and knock-out are archived after generated DNA double-strand breaks are repaired by HR and NHEJ pathways, respectively. Targeting knock-out strains generated by genome editing would be valuable because exogenous sequences do not remain in the genome DNA; therefore, these mutants are not regarded as genetically modified organisms (GMOs) (Kanchiswamy et al. 2015). However, genome editing is still uncommon in eukaryotic microalgae, probably because of the random integration characteristic described above. Genome editing using CRISPR-Cas9 is now possible in *Chlamydomonas* (Shin et al. 2016; Kao and Ng

2017), *Nannochloropsis* (Wang et al. 2016a; Ajjawi et al. 2017), and *Phaeodactylum* (Nymark et al. 2016). Knowledge gained through genome editing of model microalgae is expected to lead to improved carotenoid production.

Mutational breeding, which combines random mutagenesis using various mutagens, such as UV, radiation, and chemical agents with screening to identify potentially valuable strains, has been widely utilized as a classical and traditional approach (Bose 2016; Tanaka et al. 2010; Kato et al. 2017; Emmerstorfer-Augustin et al. 2016). Recently, easy and accelerated high-throughput screening techniques have become available due to technological developments. Atmospheric and room temperature plasma (ARTP) has attracted attention as a convenient, safe, and effective tool for random mutagenesis as an alternative to radioactive materials and heavy ion radiation (Fang et al. 2013; Zhang et al. 2014; Cao et al. 2017). Fluorescence-activated cell sorting (FACS) using fluorescent biomarkers accelerates screening processes (Velmurugan et al. 2013; Rumin et al. 2015). In microalgae, oil-rich strains of *Chlamydomonas* (Terashima et al. 2015), *Parachlorella* (Ota et al. 2013), *Desmodesmus* (Hu et al. 2013; Zhang et al. 2016), and *Euglena* (Yamada et al. 2016) have been generated using mutational breeding. This approach is particularly useful in cases in which genomic characteristics of the organism are not fully understood or when other metabolic engineering tools are not available. Knowledge obtained via mutational breeding can be feedbacked into other targeted metabolic engineering approaches. In addition, as strains generated by random mutagenesis are non-GMO, they can be used for outdoor cultivation and food purposes.

10.3 Carotenoid Synthesis Pathways in Microalgae and Cyanobacteria

Basic knowledge regarding carotenoid synthesis pathways is indispensable for determining targets for metabolic engineering. Carotenoids in microalgae are believed to be synthesized via universal pathways in common with plants (Lohr et al. 2005; Cheng 2006; Liang et al. 2006; Wang et al. 2014). The hypothetical and generally accepted pathways in microalgae and cyanobacteria are summarized in Fig. 10.1. The intermediate metabolites for carotenoid synthesis are initially generated in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. In the MEP pathway, pyruvate and glyceraldehyde-3-phosphate (GA3P) are converted to isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) via 1-deoxy-D-xylulose-5-phosphate (DXP), MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME), 4-diphos phocytidyl-2-C-methyl-D-erythritol-2-phosphate (CDP-MEP), 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP), and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) by the sequentially acting enzymes DXP synthase (DXS), DXP reductoisomerase (DXR), MEP cytidylyltransferase (CMS), CDP-ME kinase (CMK), MEcPP synthase (MCS), HMB-PP synthase (HMS), and IPP/DMAPP synthase (IDS). Microalgae and cyanobacteria do not possess the mevalonate pathway, which is also used to synthesize DMAPP and IPP in plants (Lohr et al. 2012; Bentley et al. 2014).

Lycopene are then synthesized from IPP and DMAPP in the carotenoid synthesis pathway via geranylgeranyl diphosphate (GGPP), phytoene, and ζ-carotene by the sequentially acting enzymes GGPP synthase (GGPS), phytoene synthase (PSY/CrtB), phytoene desaturase (PDS/CrtP), ζ-carotene desaturase (ZDS/CrtQ), and carotenoid isomerase (CRTISO/CrtH), or the bypassed pathway by cyanobacterial phytoene desaturase (CrtI). PSY/CrtB functions at the beginning of this pathway and converts GGPP into phytoene. In plants, PSY is a rate-limiting enzyme in carotenoid synthesis (Ruiz-Sola and Rodríguez-Concepción 2012). PDS/CrtP is downstream of PSY/CrtB, which catalyzes the conversion of phytoene into ζ-carotene. Considerable carotenoid synthesis pathway research in eukaryotic microalgae has focused on PDS due to the availability of specific inhibitors (Simkin et al. 2000).

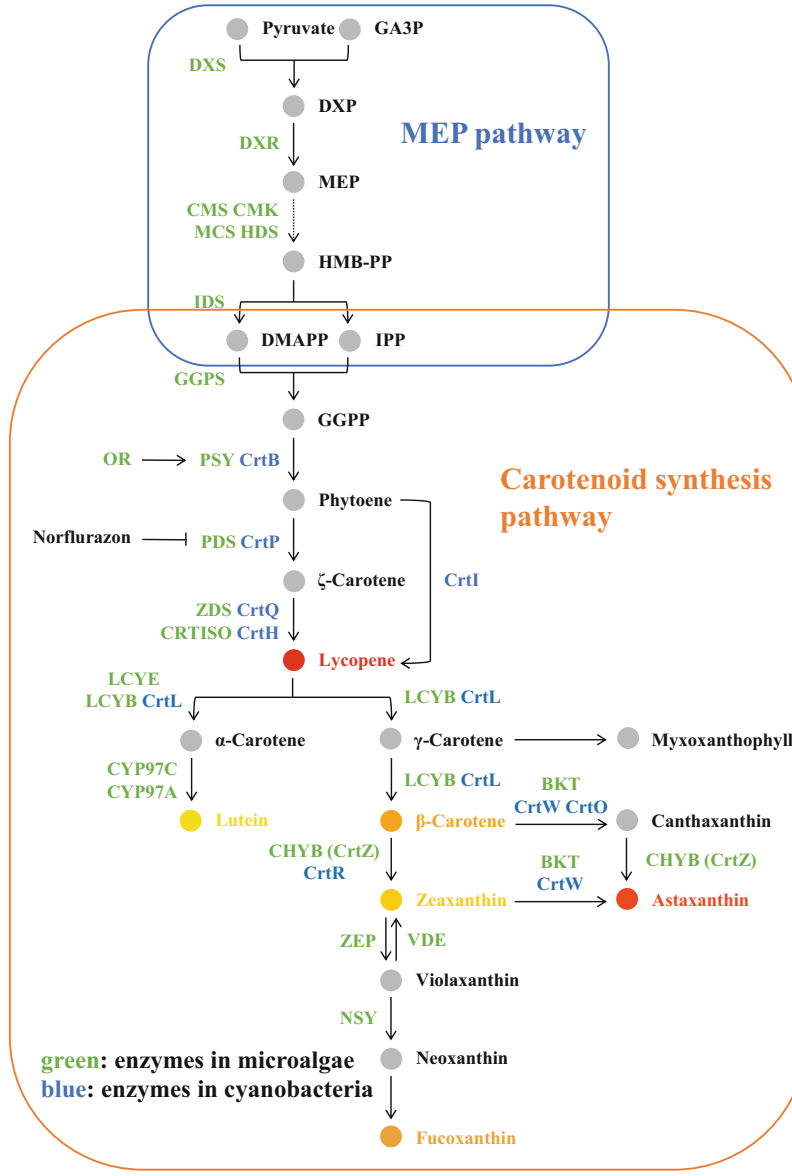

Fig. 10.1 Proposed pathway for carotenoid synthesis in eukaryotic microalgae and cyanobacteria

The synthetic pathway upstream of lycopene is common in many microalgae and cyanobacteria, but pathways and synthesized carotenoids downstream of lycopene diverge across species (Takaichi 2011). Lutein, mainly found in eukaryotic Chlorophyta, is synthesized from lycopene via α-carotene by lycopene ε-cyclase (LCYE), lycopene β-cyclase (LCYB/CrtL), and two cytochrome P450 enzymes (CYP97C and CYP97A). Zeaxanthin, mainly found in the Rhodophyta and Cyanophyta, is synthesized by LCYB/CrtL and carotene β-hydroxylase (CHYB/CrtZ/CrtR) using lycopene and β-carotene as the substrates. Heterokontophyta and Haptophyta cells contain abundant amounts of fucoxanthin, which is synthesized from zeaxanthin via violaxanthin and neoxanthin by zeaxanthin epoxidase (ZEP) and neoxanthin synthase (NSY). The xanthophyll cycle, which consists of reversible reactions catalyzed by ZEP and violaxanthin deepoxidase

(VDE), is an important photoprotection mechanism in plants and microalgae (Goss and Jakob 2010). Astaxanthin, found in a limited number of eukaryotic microalgae (such as *Haematococcus pluvialis*) and cyanobacteria, is synthesized from β-carotene via zeaxanthin and canthaxanthin by carotene β-ketolase (BKT/CrtW/CrtO) and CHYB/CrtZ/CrtR (Shah et al. 2016). Although most eukaryotic microalgae do not possess the *bkt* gene, genetic engineering enables the synthesis of astaxanthin from β-carotene, as described below (Vila et al. 2012).

Myxoxanthophyll, a cyanobacteria-specific carotenoid, is synthesized from lycopene via γ-carotene (Graham and Bryant 2009). To increase the carotenoid content in microalgae and cyanobacteria, previous metabolic engineering studies generally employed one of three main approaches: (1) overexpression of genes encoding rate-limiting enzymes, (2) downregulation of competitive reactions, and (3) heterogeneous expression of important/absent genes.

10.4 Recent Achievements Through Metabolic Engineering

10.4.1 Enzymes in the MEP Pathway

Challenges associated with metabolic engineering of microalgae and cyanobacteria and their consequences are summarized in this section. The MEP pathway is upstream of the carotenoid synthesis pathway that synthesizes IPP and DMAPP from pyruvate and GA3P. DXS is the gateway enzyme in the MEP pathway and catalyzes the conversion of pyruvate and GA3P into DXP. In plants, this step is rate limiting in carotenoid synthesis (Estévez et al. 2001; Rodríguez-Concepción 2006); therefore, the *dxs* gene is a potential target for metabolic engineering to improve carotenoid production (Hasunuma et al. 2008b). Unfortunately, limited data are available regarding metabolic engineering of the MEP pathway in microalgae and cyanobacteria. Some studies reported that metabolic engineering of microalgae and cyanobacteria targeting the *dxs* gene led to increased carotenoid content (Table 10.1). Overexpression of the *dxs* gene in *Synechocystis* sp. PCC6803 resulted in a 1.5-fold increase in the total carotenoid content (Kudoh et al. 2014). In *Phaeodactylum tricornutum*, the fucoxanthin content was increased 2.4-fold compared with the wild type following the introduction of the *dxs* gene (Eilers et al. 2016). GGPS, which catalyzes the conversion of DMAPP and IPP into GGPP, was also examined as a target of metabolic engineering in microalgae. The *ggps* gene from the thermophilic Archaea *Sulfolobus acidocaldarius* was introduced into *C. reinhardtii*, but no significant change was observed in terms of carotenoid content (Fukusaki et al. 2003).

10.4.2 Phytoene Synthase

Phytoene synthase is the gateway enzyme in the carotenoid synthesis pathway and catalyzes the conversion of GGPP into phytoene. Carotenoid deficiency caused by the downregulation of the *psy* gene in some microalgal species has been reported. In *C. reinhardtii*, knockdown of the *psy* gene using artificial miRNA decreased the chlorophyll content, suggesting a deficiency of protective carotenoids that suppress photobleaching (Molnar et al. 2009). In *P. tricornutum*, knockdown of the *psy* gene using artificial miRNA resulted in a decrease in total carotenoids (Kaur and Spillane 2015). These data suggest that phytoene synthase plays an important role in carotenoid synthesis.

Metabolic engineering involving the *psy* gene is reportedly an effective way to improve carotenoid content in plants (Lindgren et al. 2003). In microalgae, the *psy* gene was also shown to be a key enzyme in carotenoid synthesis in studies mainly involving *C. reinhardtii* and *P. tricornutum* (Table 10.2). The *psy* gene from *Dunaliella salina* was constitutively overexpressed in *C. reinhardtii* using the promoters of the *rubisco small subunit* (*rbcS2*) and *hsp70A* genes. This increased the content of violaxanthin, lutein, β-carotene, and neoxanthin 2.0-, 2.6-, 1.25-, and 1.8-fold, respectively,

Table 10.1 Genetic engineering of the MEP pathway

Gene	Microalga	Strategy	Effect on carotenoid production	References
dxs	*Synechocystis* sp.	Genetic engineering	1.5-fold increase in total carotenoid content	Kudoh et al. (2014)
dxs	*Phaeodactylum tricornutum*	Genetic engineering	2.4-fold increase in fucoxanthin content	Eilers et al. (2016)
ggps (*Sulfolobus acidocaldarius*)	*Chlamydomonas reinhardtii*	Genetic engineering	No significant change	Fukusaki et al. (2003)

compared with the wild type (Couso et al. 2011). In the same way, the *psy* gene from *Chlorella zofingiensis* was overexpressed in *C. reinhardtii* using the *rbcS2* and *hsp70A* promoters. This increased the content of lutein and violaxanthin 2.0- and 2.2-fold, respectively, compared with the wild type (Cordero et al. 2011a). Overexpression of the orange protein (OR), which is a DnaJ-like chaperone for PSY, also increased the carotenoid content. Overexpression of OR in *C. reinhardtii* increased the content of lutein and β-carotene 1.9- and 1.7-fold, respectively, compared with the wild type (Morikawa et al. 2017). The *psy* gene was also overexpressed in *P. tricornutum*. Expression of the intrinsic *psy* gene using the *fcpA* (fucoxanthin chlorophyll *a*/*c*-binding protein) promoter increased the fucoxanthin content 1.45-fold compared with the wild type. By contrast, the level of β-carotene, which is an intermediate metabolite for fucoxanthin in the carotenoid synthesis pathway, was not affected (Kadono et al. 2015b). Another study reported that expression of the *psy* gene in *P. tricornutum* increased the fucoxanthin content 1.8-fold compared with the wild type (Eilers et al. 2016). A unique study reported increased carotenoid content in a green alga, *Scenedesmus* sp., via *psy* gene expression. In *Scenedesmus* sp., the β-carotene content increased approximately threefold by the expression of a synthetic *psy* gene encoding consensus amino acid sequences from the *C. reinhardtii*, *D. salina*, and *Mariella zofingiensis* proteins (Chen et al. 2017). In *Synechocystis* sp. PCC6803, overexpression of *crtB*, which encodes phytoene synthase in cyanobacteria, increased the content of both zeaxanthin and myxoxanthophyll 1.5-fold compared with the wild type (Lagarde et al. 2000).

Table 10.2 Genetic engineering related to phytoene synthase

Gene	Microalga	Strategy	Effect on carotenoid production	References
psy (*Dunaliella salina*)	*Chlamydomonas reinhardtii*	Genetic engineering	2.0-, 2.6-, 1.25-, and 1.8-fold increases in violaxanthin, lutein, β-carotene, and neoxanthin content	Couso et al. (2011)
psy (*Chlorella zofingiensis*)	*Chlamydomonas reinhardtii*	Genetic engineering	2.0- and 2.2-fold increases in lutein and violaxanthin content	Cordero et al. (2011a, b)
or	*Chlamydomonas reinhardtii*	Genetic engineering	1.9- and 1.7-fold increases in lutein and β-carotene content	Morikawa et al. (2017)
psy	*Phaeodactylum tricornutum*	Genetic engineering	1.45-fold increase in fucoxanthin content	Kadono et al. (2015a, b)
psy	*Phaeodactylum tricornutum*	Genetic engineering	1.8-fold increase in fucoxanthin content	Eilers et al. (2016)
Synthetic *psy*	*Scenedesmus* sp.	Genetic engineering	3.0-fold increase in β-carotene content	Chen et al. (2017)
crtB	*Synechocystis* sp.	Genetic engineering	1.5-fold increase in zeaxanthin and myxoxanthophyll content	Lagarde et al. (2000)

10.4.3 Phytoene Desaturase

Phytoene desaturase, downstream of phytoene synthase, catalyzes the conversion of phytoene to ζ-carotene. Silencing of the *pds* gene via RNA interference was conducted in *C. reinhardtii* and *D. salina*, but no effect on carotenoid content was reported (Vila et al. 2008; Sun et al. 2008). Another study reported that the downregulation of the *pds* gene resulted in the accumulation of phytoene and decline in the levels of lycopene, β-carotene, and lutein in *D. salina* (Srinivasan et al. 2017).

Many studies successfully increased carotenoid production using metabolic engineering approaches targeting phytoene desaturase (Table 10.3). Data resulting from metabolic engineering of phytoene desaturase are now available for microalgae and cyanobacteria. Many of these studies used PDS/CrtP inhibitors, such as the herbicide norflurazon (Chamovitz et al. 1991). In a wide range of microalgae and cyanobacteria, norflurazon inhibits PDS/CrtP, which has an adverse effect on carotenoid production. For example, norflurazon caused a decrease in the carotenoid content in *Dunaliella bardawil* by inhibiting the conversion of phytoene to ε-carotene (Salguero et al. 2003; León et al. 2005). As they are antioxidants, carotenoids suppress photooxidative damage by ROS generated under high light conditions. Thus, the inhibition of the carotenoid synthesis pathway using PDS/CrtP inhibitors negatively affects cell viability under high light conditions. In other words, resistance to PDS/CrtP inhibitors could be obtained by increasing the levels of PDS/CrtP; therefore, resistance to PDS/CrtP inhibitors has been used as the indicator of high carotenoid production in mutational breeding. A wide range of metabolic engineering studies have succeeded in increasing carotenoid content using PDS/CrtP inhibitors. Carotenoid content was increased in *Chlorella sorokiniana* using a mutational breeding approach with norflurazon and nicotine. By selective breeding using nicotine and norflurazon, the lutein content in the resulting mutants was1.49- and 1.55-fold higher, respectively, than that of the wild type (Cordero et al. 2011b). Mutational breeding was also conducted in *H. pluvialis* using nicotine, diphenylamine, fluridone, and norflurazon. The nicotine-resistant mutant produced 2.08-fold more astaxanthin than the wild type (Chen et al. 2003).

An important and widely conserved motif was identified that controls the activity of phytoene desaturase. Point mutations in this motif were studied in *Synechococcus* sp., *H. pluvialis*, *C. zofingiensis*, and *C. reinhardtii*. Changes in phytoene desaturase activity resulting from changes in the amino acid sequence of the motif affected carotenoid content and resistances to norflurazon (Liu et al. 2014b). In *H. pluvialis*, expression of the *pds* gene modified by site-directed mutagenesis increased the astaxanthin content 1.33-fold compared with the wild type. Thus, conversion of phytoene to ζ-carotene catalyzed by PDS is thought to be the rate-limiting step in astaxanthin synthesis (Steinbrenner and Sandmann 2006). A *C. zofingiensis* mutant with a single amino acid substitution in PDS was generated using a chemical mutagen. Compared with the wild type, the mutant showed 31-fold greater resistance to norflurazon and 1.44-fold higher astaxanthin content under high light conditions (Liu et al. 2010). A subsequent study reported that by inducing point mutation in the *pds* gene in *C. zofingiensis*, the transformant acquired norflurazon resistance, and the astaxanthin and total carotenoid content increased by 1.54- and 1.32-fold, respectively, compared with the wild type (Liu et al. 2014b). Expression of modified PDS protein also increased carotenoid content in *C. reinhardtii*. A single amino acid substitution mutant of PDS was designed based on the above studies. The transformant expressing this modified PDS showed 27.7-fold greater resistance to norflurazon than the wild type and significantly higher content of carotenoids, such as lutein, β-carotene, and violaxanthin (Liu et al. 2013).

Norflurazon-resistant *Synechococcus* sp. PCC7942 mutants have also been analyzed. Three mutants with point mutations in the *crtP* gene accumulated phytoene but exhibited

Table 10.3 Genetic engineering of phytoene desaturase

Gene	Microalga	Strategy	Effect on carotenoid production	References
pds	*Chlorella sorokiniana*	Mutational breeding	1.55-fold increase in lutein content	Cordero et al. (2011a, b)
pds	*Haematococcus pluvialis*	Mutational breeding	2.08-fold increase in astaxanthin content	Chen et al. (2003)
Modified *pds*	*Haematococcus pluvialis*	Genetic engineering	1.33-fold increase in astaxanthin content	Steinbrenner and Sandmann (2006)
pds	*Chlorella zofingiensis*	Mutational breeding	1.44-fold increase in astaxanthin content	Liu et al. (2010)
pds	*Chlorella zofingiensis*	Mutational breeding	1.54- and 1.32-fold increases in astaxanthin and total carotenoid content	Liu et al. (2014a, b)
Modified *pds*	*Chlamydomonas reinhardtii*	Genetic engineering	Increase in lutein, β-carotene, and violaxanthin content	Liu et al. (2013)
pds promoter	*Synechococcus* sp.	Mutational breeding	Increase in carotenoid content	Chamovitz et al. (1993)

decreased carotenoid content. Another mutant had a deletion in the *crtP* promoter and overexpressed the *crtP* gene. Like eukaryotic microalgae with *pds* point mutations, this mutant accumulated high level of carotenoids compared with the wild type and was highly resistant to norflurazon. Thus, phytoene desaturase is also thought to be the rate-limiting enzyme in carotenoid production in cyanobacteria (Chamovitz et al. 1993). A positive correlation between carotenoid content and norflurazon resistance has been reported for many microalgae and cyanobacteria. For increasing carotenoid content, modification of the *pds*/*crtP* gene by selective breeding using PDS/CrtP inhibitors is now possible for a wide variety of microalgae and cyanobacteria.

10.4.4 β-Carotene Hydroxylase and Zeaxanthin Epoxidase

To enhance zeaxanthin accumulation in microalgae and cyanobacteria, metabolic engineering of β-carotene hydroxylase and zeaxanthin epoxidase is effective (Table 10.4). CrtR, the β-carotene hydroxylase in cyanobacteria, is important for zeaxanthin synthesis (Masamoto et al. 1998). In *Synechocystis* sp. PCC6803, overexpression of the *crtR* gene increased the zeaxanthin content 2.5-fold compared with the wild type (Lagarde et al. 2000). Zeaxanthin content can also be increased by downregulating ZEP, which is a competitive enzyme that converts zeaxanthin to violaxanthin. *C. reinhardtii ZEP* mutants accumulate zeaxanthin constitutively (Niyogi et al. 1997). This result was confirmed by a more recent study using genome-editing technology. A strain that constitutively produces zeaxanthin was generated by the knock-out of the *zep* gene in *C. reinhardtii* using CRISPR-Cas9 (Baek et al. 2016a, b). The ZEP protein in *C. zofingiensis* was also shown to be functional by expressing the *zep* gene from *C. zofingiensis* in the *zep* mutant of *C. reinhardtii* (Couso et al. 2012). Mutational breeding with visual screening of pale-green coloration was conducted in *D. salina*, and a mutant lacking neoxanthin, violaxanthin, and antheraxanthin but that constitutively accumulates zeaxanthin was isolated. These data strongly suggested that ZEP in this mutant is functionally defective (Jin et al. 2003). Thus, commercially valuable strains that accumulate high levels of useful zeaxanthin have been generated via both enhancing zeaxanthin synthesis and blocking the conversion of zeaxanthin to less-valuable carotenoids.

10.4.5 β-Carotene Ketolase

β-Carotene ketolase, which catalyzes the conversion of β-carotene and zeaxanthin to canthaxanthin and astaxanthin, respectively, is an important enzyme for astaxanthin synthesis in microalgae

Table 10.4 Genetic engineering of β-carotene hydroxylase and zeaxanthin epoxidase

Gene	Microalga	Strategy	Effect on carotenoid production	References
crtR	*Synechocystis* sp.	Genetic engineering	2.5-fold increase in zeaxanthin content	Lagarde et al. (2000)
Δzep	*Chlamydomonas reinhardtii*	Mutational breeding	Constitutive zeaxanthin accumulation	Niyogi et al. (1997)
Δzep	*Chlamydomonas reinhardtii*	Genetic engineering	Constitutive zeaxanthin accumulation	Baek et al. (2016a, b)
Δzep?	*Dunaliella salina*	Mutational breeding	Constitutive zeaxanthin accumulation; no neoxanthin, violaxanthin, or antheraxanthin accumulation	Jin et al. (2003)

and cyanobacteria. It was designated as BKT in microalgae and CrtW (the BKT ortholog) in bacteria (Kajiwara et al. 1995). Cyanobacteria also possess CrtO, which has a limited catalytic function, e.g., not to convert zeaxanthin to astaxanthin (Choi et al. 2007). Studies involving metabolic engineering of β-carotene ketolase in microalgae and cyanobacteria are summarized in Table 10.5. Most microalgae, except *H. pluvialis*, do not possess the *bkt* gene and are therefore unable to produce astaxanthin (Vila et al. 2012). Upregulation of the *bkt* gene in *H. pluvialis* enhanced the astaxanthin content two- to three-fold compared with non-transformed cells (Kathiresan et al. 2015). Mutational breeding was also conducted in *H. pluvialis* using the astaxanthin synthesis inhibitor diphenylamine, generating a mutant with 1.7-fold higher astaxanthin content than the wild type (Wang et al. 2016b). Metabolic engineering was also employed to produce astaxanthin using microalgae and cyanobacteria that originally lack the capability to synthesize astaxanthin. The *bkt* gene from *H. pluvialis* was expressed in *C. reinhardtii* using a constitutive *rbcS2*promoter. The transformant cells accumulated a ketocarotenoid, but interestingly, it was neither astaxanthin nor canthaxanthin (León et al. 2007). The *bkt* gene derived from *H. pluvialis* was also introduced in *Synechococcus* sp. PCC7942. The transformed cells produced various carotenoids (including astaxanthin) not normally synthesized by this species (Harker and Hirschberg 1997). In *Synechococcus* sp. PCC7002, the expression of the *crtW* and *crtZ* genes from *Brevundimonas* sp. resulted in the production of astaxanthin at the cost of β-carotene and zeaxanthin accumulation (Hasunuma et al. 2019).

Deletion of the cyanobacterial β-carotene monoketolase gene *crtO* combined with overexpression of the *crtB* and *crtP* genes was investigated in *Synechocystis* sp. PCC6803 (Lagarde et al. 2000). The *crtP* gene encodes phytoene desaturase in cyanobacteria. The deletion of the *crtO* gene increased the content of myxoxanthophyll and total carotenoids 2.3- and 1.3-fold, respectively. The overexpression of the *crtB* and *crtP* genes combined with the deletion of *crtO* resulted in2.6-, 1.6-, and 1.5-fold increases in the content of myxoxanthophyll, zeaxanthin, and total carotenoids, respectively.

10.5 Conclusions

Microalgae and cyanobacteria have sufficient potential for economical production of carotenoids due to their rapid growth and high carotenoid content. The use of terrestrial plants is currently more profitable; therefore, further improvements in carotenoid production are required to commercialize carotenoid production by microalgae and cyanobacteria. As the technology progresses, metabolic engineering has become less time-consuming and more effective and thus could be further utilized to generate valuable strains. Selecting suitable host strains and genes for targeting to increase carotenoid production as well as the best production strategy are important for the most efficient utilization of metabolic engineering. More basic data regarding carotenoid synthesis and metabolic engineering in microalgae and cyanobacteria are needed for each

Table 10.5 Genetic engineering of β-carotene ketolase

Gene	Microalga	Strategy	Effect on carotenoid production	References
bkt	*Haematococcus pluvialis*	Genetic engineering	2- to 3-fold increase in astaxanthin content	Kathiresan, et al. (2015)
Not identified	*Haematococcus pluvialis*	Mutational breeding	1.7-fold increase in astaxanthin content	Wang et al. (2016a, b)
bkt (*Haematococcus pluvialis*)	*Chlamydomonas reinhardtii*	Genetic engineering	Accumulation of a ketocarotenoid	León et al. (2007)
bkt (*Haematococcus pluvialis*)	*Synechococcus* sp.	Genetic engineering	Production of various carotenoids, including astaxanthin	Harker and Hirschberg (1997)
crtW, *crtZ* (*Brevundimonas* sp.)	*Synechococcus* sp.	Genetic engineering	Production of astaxanthin	Hasunuma et al. (2019)
ΔcrtO	*Synechocystis* sp.	Genetic engineering	2.3- and 1.3-fold increases in myxoxanthophyll and total carotenoid content	Lagarde et al. (2000)
crtB, *crtP*, *ΔcrtO*	*Synechocystis* sp.	Genetic engineering	2.6-, 1.6-, and 1.5-fold increases in myxoxanthophyll, zeaxanthin, and total carotenoid content	Lagarde et al. (2000)

species. Regarding mutational breeding, screening tools to identify high carotenoid producers are still lacking. High-throughput methods for measuring carotenoid content in living cells should be developed.

Metabolomics studies can provide a comprehensive understanding of cellular metabolites in organisms, including microalgae and cyanobacteria. For example, dynamic metabolic profiling using *in vivo* ^{13}C-labeling combined with transcription analysis revealed the details of starch-to-lipid biosynthesis switching in *Chlamydomonas* sp. and identified the metabolic rate-limiting step, thus highlighting a potential target for metabolic engineering to improve lipid accumulation (Ho et al. 2017). Similarly, the metabolic flux of glycogen biosynthesis was determined in the cyanobacterium *Arthrospira platensis*, and enhanced carbon dioxide incorporation was revealed in a transgenic strain of *Synechocystis* sp. PCC6803via dynamic metabolic analyses (Hasunuma et al. 2013, 2014). There is no doubt that metabolomics will also play an important role in increasing carotenoid production through the study of wild type strains and mutants obtained via metabolic engineering.

Other challenges must be overcome to successfully commercialize carotenoid production using microalgae and cyanobacteria. Outdoor cultivation using solar energy is essential for cost-effective production. Therefore, strains for commercialization should be robust in unstable outdoor conditions and in the presence of environmental contaminants. In addition, the resistance of consumers to accept GMOs should be considered, although most microalgae and cyanobacteria are "generally regarded as safe (GRAS)" for food purposes. Strains obtained through mutational breeding could be utilized for the present, as they are non-GMOs. Containment strategies for GMOs, such as the use of auxotrophy, should also be developed.

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