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Chapter 1-14 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 rate 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 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

1-14.1 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 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 photo-oxidative damage (Xiao *et al*., 2011, Jahns and Holzwarth, 2012). Due to their antioxidative properties, carotenoids have begun to attract public attention with respect both to their use as natural coloring agents for foods as well as 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.

1-14.2 Technologies for metabolic engineering of microalgae and cyanobacteria

Genetic engineering enables 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*., 2016, 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), *Synechococcus* (Ruffing *et al*., 2016), and also 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 fed back 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.

1-14.3 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 Figure 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-diphosphocytidyl-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 *et al*., 2012). PDS/CrtP is downstream of PSY/CrtB, which catalyzes 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).

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) down-regulation of competitive reactions, and (3) heterogeneous expression of important/absent genes.

1-14.4 Recent achievements through metabolic engineering

1-14.4.1 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

196 increase carotenoid content (Table 1). Overexpression of the *dxs* gene in *Synechocystis*
197 sp. PCC6803 resulted in a 1.5-fold increase in the total carotenoid content (Kudoh *et al*.,
198 2014). In *Phaeodactylum tricornutum*, the fucoxanthin content was increased 2.4-fold
199 compared with the wild type following introduction of the *dxs* gene (Eilers *et al*., 2016).
200 GGPS, which catalyzes the conversion of DMAPP and IPP into GGPP, was also examined
201 as a target of metabolic engineering in microalgae. The *ggps* gene from the thermophilic
202 Archea *Sulfolobus acidocaldarius* was introduced into *C. reinhardtii*, but no significant
203 change was observed in terms of carotenoid content (Fukusaki *et al*., 2003).
204
205
206 **1-14.4.2 Phytoene synthase**
207
208 Phytoene synthase is the gateway enzyme in the carotenoid synthesis pathway and
209 catalyzes the conversion of GGPP into phytoene. Carotenoid deficiency caused by down-
210 regulation of the *psy* gene in some microalgal species has been reported. In *C. reinhardtii*,
211 knockdown of the *psy* gene using artificial miRNA decreased the chlorophyll content,
212 suggesting a deficiency of protective carotenoids that suppress photobleaching (Molnar
213 *et al*., 2009). In *P. tricornutum*, knockdown of the *psy* gene using artificial miRNA
214 resulted in a decrease in total carotenoids (Kaur and Spillane, 2015). These data suggest
215 that phytoene synthase plays an important role in carotenoid synthesis.
216 Metabolic engineering involving the *psy* gene is reportedly an effective way to
217 improve carotenoid content in plants (Lindgren *et al*., 2003). In microalgae, the *psy* gene
218 was also shown to be a key enzyme in carotenoid synthesis in studies mainly involving
219 *C. reinhardtii* and *P. tricornutum* (Table 2). The *psy* gene from *Dunaliella salina* was
220 constitutively overexpressed in *C. reinhardtii* using the promoters of the *rubisco small*
221 *subunit* (*rbcS2*) and *hsp70A* genes. This increased the content of violaxanthin, lutein, β-
222 carotene, and neoxanthin 2.0-, 2.6-, 1.25-, 1.8-fold, respectively, compared with the wild
223 type (Couso *et al*., 2011). In the same way, the *psy* gene from *Chlorella zofingiensis* was
224 overexpressed in *C. reinhardtii* using the *rbcS2* and *hsp70A* promoters. This increased the
225 content of lutein and violaxanthin 2.0- and 2.2-fold, respectively, compared with the wild
226 type (Cordero *et al*., 2011a). Overexpression of the orange protein (OR), which is a DnaJ-
227 like chaperone for PSY, also increased the carotenoid content. Overexpression of OR in
228 *C. reinhardti* 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 compare 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 3-fold by 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).

1-14.4.3 Phytoene desaturase

Phytoene desaturase, downstream of phytoene synthase, catalyzes the conversion of phytoene to ζ-carotene. Silencing of the *pds* gene via RNA interference were 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 down-regulation of the *pds* gene resulted in accumulation of phytoene and decline in 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 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 photo-oxidative damage by ROS generated under high light conditions. Thus, 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 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 was 1.49- 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 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 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.

1-14.4.4 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 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 zeaxanthin content 2.5-fold compared with the wild type (Lagarde *et al*., 2000). Zeaxanthin content can also be increased by down-regulating 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*., 2016). 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 conversion of zeaxanthin to less-valuable carotenoids.

1-14.4.5 β-Carotene ketolase

β-Carotene ketolase, which catalyzes conversion of β-carotene and zeaxanthin to canthaxanthin and astaxanthin, respectively, is an important enzyme for astaxanthin synthesis in microalgae and cyanobacteria. It was designated 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 5. Most microalgae, except for *H. pluvialis*, do not possess the *bkt* gene and are therefore unable to produce astaxanthin (Vila *et al*., 2012). Up-regulation of *bkt* gene in *H. pluvialis* enhanced the astaxanthin content 2- to 3-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 lacking the capability of synthesizing 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, expression of the *crtW* and *crtZ* genes from *Brevundimonas* sp. resulted in 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. Deletion of the *crtO* gene increased the content of myxoxanthophyll and total carotenoids

2.3- and 1.3-fold, respectively. Overexpression of the *crtB* and *crtP* genes combined with *crtO* deletion resulted in 2.6-, 1.6-, and 1.5-fold increases in the content of myxoxanthophyll, zeaxanthin, and total carotenoids, respectively.

1-14.5 Conclusions

Microalgae and cyanobacteria have sufficient potential for economical production of carotenoids due to their rapid growth rate 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 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 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. PCC6803 via dynamic metabolic analyses (Hasunuma *et al*., 2013, Hasunuma *et al*., 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

394 production using microalgae and cyanobacteria. Outdoor cultivation using solar energy
395 is essential for cost-effective production. Therefore, strains for commercialization should
396 be robust in unstable outdoor conditions and in the presence of environmental
397 contaminants. In addition, the resistance of consumers to accept GMOs should be
398 considered, although most microalgae and cyanobacteria are "generally regarded as safe
399 (GRAS)" for food purposes. Strains obtained through mutational breeding could be
400 utilized for the present, as they are non-GMOs. Containment strategies for GMOs, such
401 as the use of auxotrophy, should also be developed.
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Fig. 1. Proposed pathway for carotenoid synthesis in eukaryotic microalgae and cyanobacteria.

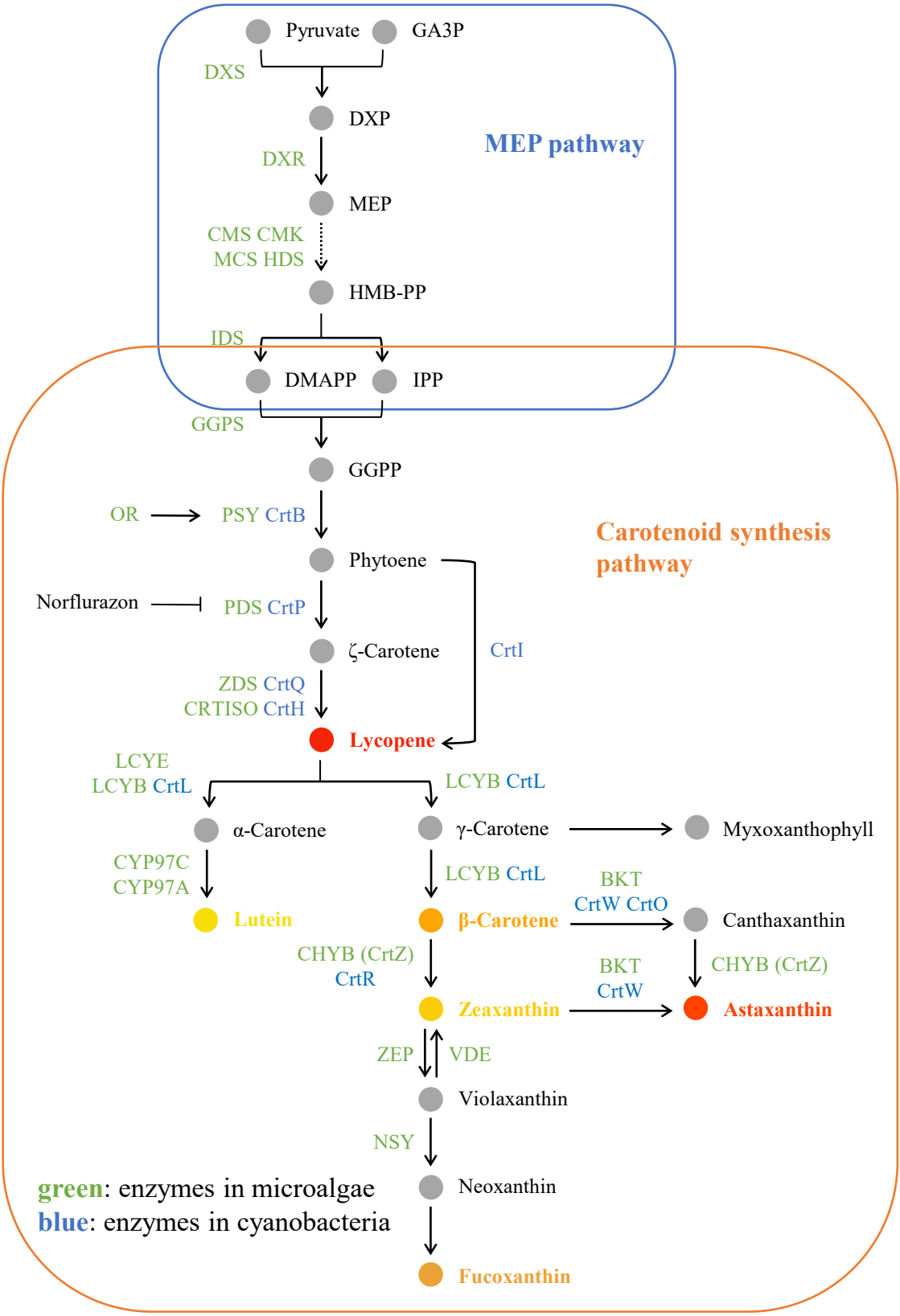

Table 1. Genetic engineering of the MEP pathway.

Gene	Microalga	Strategy	Effect on carotenoid production	Reference
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

Table 2. Genetic engineering related to phytoene synthase.

Gene	Microalga	Strategy	Effect on carotenoid production	Reference
psy (*Dunaliella salina*)	*Chlamydomonas reinhardtii*	Genetic engineering	2.0-, 2.6-, 1.25-, 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-, 2.2-fold increases in lutein and violaxanthin content	Cordero, *et al*. 2011
or	*Chlamydomonas reinhardtii*	Genetic engineering	1.9-, 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*. 2015
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

Table 3. Genetic engineering of phytoene desaturase.

Gene	Microalga	Strategy	Effect on carotenoid production	Reference
pds	*Chlorella sorokiniana*	Mutational breeding	1.55-fold increase in lutein content	Cordero, *et al*. 2011
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, *et al*. 2006
pds	*Chlorella zofingiensis*	Mutational breeding	1.44-fold increase in astaxanthin content	Liu, *et al*. 2010
pds	*Chlorella zofingiensis*	Mutational breeding	1.54-, 1.32-fold increases in astaxanthin and total carotenoid content	Liu, *et al*. 2014
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

Table 4. Genetic engineering of β-carotene hydroxylase and zeaxanthin epoxidase.

Gene	Microalga	Strategy	Effect on carotenoid production	Reference
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*. 2016
Δzep?	*Dunaliella salina*	Mutational breeding	Constitutive zeaxanthin accumulation; no neoxanthin, violaxanthin, or antheraxanthin accumulation	Jin, *et al*. 2003

Table 5. Genetic engineering of β-carotene ketolase.

Gene	Microalga	Strategy	Effect on carotenoid production	Reference
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*. 2016
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, *et al*. 1997
crtW, *crtZ* (*Brevundimonas* sp.)	*Synechococcus* sp.	Genetic engineering	Production of astaxanthin	Hasunuma, *et al*. 2019
ΔcrtO	*Synechocystis* sp.	Genetic engineering	2.3-, 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-, 1.5-fold increases in myxoxanthophyll, zeaxanthin, and total carotenoid content	Lagarde, *et al*. 2000