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REVIEW

Engineering cyanobacteria for production of terpenoids

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Abstract

Main conclusion **This review summarizes recent advances in cyanobacterial terpenoid production. The challenges and opportunities of improving terpenoid production by cyanobacteria are discussed.**

Terpenoids are a diverse group of natural products with a variety of commercial applications. With recent advances in synthetic biology and metabolic engineering, microbial terpenoid synthesis is being viewed as a feasible approach for industrial production. Among different microbial hosts, cyanobacteria have the potential of sustainable production of terpenoids using light and CO_2. Terpene synthases and the precursor pathways have been expressed in cyanobacteria for enhanced production of various terpene hydrocarbons, including isoprene, limonene, β-phellandrene, and farnesene. However, the productivities need to be further improved for commercial production. Many barriers remain to be overcome in order to efficiently convert CO_2 to terpenoids. In this review, we will summarize recent efforts on photosynthetic production of terpenoids and discuss the challenges and opportunities of engineering cyanobacteria for terpenoid bioproduction.

Keywords Terpenoid · Cyanobacteria · Metabolic engineering · MEP pathway · MVA pathway

Abbreviations

DMAPP	Dimethylallyl pyrophosphate
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
GAP	Glyceraldehyde 3-phosphate
IDI	Isopentenyl diphosphate isomerase
IPP	Isopentenyl pyrophosphate
IspS	Isoprene synthase
MEP	2-C-Methyl-D-erythritol-4-phosphate
MVA	Mevalonate

Introduction

Cyanobacteria are photosynthetic microorganisms that only require sunlight, CO_2, and trace minerals for growth. In recent years, cyanobacteria are emerging hosts for production of chemicals, because these photosynthetic microorganisms directly convert CO_2 into desired compounds. By expressing heterologous pathways in cyanobacteria, CO_2 is converted to many useful products (Sengupta et al. 2018; Knoot et al. 2018), such as ethanol (Dexter and Fu 2009), 2,3-butanediol (Kanno et al. 2017), ethylene (Ungerer et al. 2012), and fatty acids (Liu et al. 2011). Several synthetic biology tools were developed for cyanobacteria in the past decade. For instance, CRISPR/Cas9 (Li et al. 2016; Wendt et al. 2016) and Cpf1 (Ungerer and Pakrasi 2016) techniques enable rapid modification of genome sequences. Development of inducible promoters (Markley et al. 2015; Oliver et al. 2013; Huang and Lindblad 2013) and CRISPR interference (Gordon et al. 2016), and characterization of endogenous promoters (Liu and Pakrasi 2018; Li et al. 2018) allow controllable and tunable expression of heterologous genes. Currently, microbial production of fuels and chemicals is mainly performed by heterotrophic microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, which require sugar feedstocks for growth. However, terrestrial plants that produce sugars have a low energy efficiency to convert solar energy into biomass (< 0.5%) (Brenner et al. 2006), which become a major hurdle for sustainable feedstock production. Cyanobacteria have a higher efficiency (3–9%) than land plants to utilize solar energy (Dismukes et al. 2008). Direct conversion of CO_2 to products is expected to increase the overall efficiency

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of production. Although cyanobacteria have been engineered to produce a variety of useful chemicals, the productivity requires to be further enhanced for commercial application. More research is needed to improve the efficiency of directing the photosynthetic carbon toward desired chemicals.

Terpenoids: functions, applications, and biosynthesis

Terpenoids (or isoprenoids) are the largest group of natural products with extreme diversity. More than 55,000 compounds have been identified (Breitmaier 2006). Some terpenoids are essential in primary metabolism, including photosynthesis (chlorophyll, carotenoids, and plastoquinone), respiration (ubiquinone), developmental regulation in plants (gibberellins and abscisic acid), etc. Most terpenoids are produced by plants as secondary metabolites. Isoprene is a volatile molecule emitted from numerous plant species to protect against heat stress. Isoprene enhances membrane integrity for the purpose of thermotolerance (Siwko et al. 2007), because heat can cause leakiness of thylakoid membrane and affect the efficiency of photosynthesis (Sharkey et al. 2008). Some volatile terpenoids emit floral scents, which can attract pollinators and seed-dispersing animals to facilitate plant reproduction. Moreover, plants release terpenoids with toxicity or strong odor to protect against herbivores and pathogens.

Although terpenoids are extremely diverse compounds, they are all derived from the same building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Fig. 1). One of the simplest types of terpenoids is isoprene, which is a five-carbon compound converted from DMAPP. Terpenoids are categorized into groups based on the number of isoprene units: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40), and polyterpenes (> C40). In terms of nomenclature, terpenes are the hydrocarbon molecules, whereas terpenoids are terpenes modified with additional functional groups or structural rearrangement. However, the two terms are often used interchangeably.

Terpenoids have a variety of commercial applications, including pharmaceuticals, nutraceuticals, flavors/fragrances, and industrial chemicals (Ajikumar et al. 2008). Artemisinin is a sesquiterpene produced by the plant *Artemisia annua.* Artemisinin derivatives are the first-line antimalarial drugs recommended by World Health Organization. The price and availability of artemisinin fluctuate wildly due to unstable supply of agricultural materials. With recent improvement in synthetic biology and metabolic engineering, the yeast *S. cerevisiae* was engineered for commercial production of artemisinic acid, the precursor of artemisinin (Paddon and Keasling 2014). Paclitaxol, a complex diterpene isolated from the Pacific yew tree (*Taxus brevifolia*), is a chemotherapy drug for treatment of several types of cancer. However, the low yield of paclitaxol extracted from the bark of the yew tree (< 0.01% of dry weight) is a major barrier for large-scale production (Ciddi et al. 1995). Many terpenoids are used as nutraceuticals due to their health effects to human, such as lutein, lycopene, and astaxanthin. A wide variety of monoterpenes and sesquiterpenes have characteristic fragrances which are used in the flavor and fragrance industry, such as menthol (mint odor), D-limonene (orange peel odor), α-farnesene (green apple odor), and nootkatone (grapefruit odor). Isoprene, the simplest form of terpenoid, is the main component of synthetic and natural rubber. Recently, some terpenoids (limonene, myrcene, and farnesene) or their hydrogenated forms have been determined to be compatible with diesel (Tracy et al. 2009) and aviation fuels (Chuck and Donnelly 2014), demonstrating the potential of using these terpenoids as alternative fuels.

Two distinct metabolic pathways can lead to the synthesis of IPP and DMAPP (Fig. 1). In general, the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (also known as the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway or the non-mevalonate pathway) exists in bacteria, whereas the mevalonate (MVA) pathway is mainly found in archaea and eukaryotes. Plants and algae have both pathways, in which the MVA pathway operates in the cytosol and the MEP pathway functions in the plastids. The MEP pathway consists of seven enzymatic reactions. It begins with glyceraldehyde 3-phosphate (GAP) and pyruvate to form DXP. This step is catalyzed by the rate-limiting enzyme, DXP synthase (DXS). Further, DXP is converted to MEP by DXP reductase (DXR), which requires NADPH as reducing power. Subsequently, MEP is coupled with CTP to form 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME), which further undergoes phosphorylation, cyclization, and reductive dehydration to form 4-hydroxy-3-methyl-butenyl 1-diphosphate (HMB-PP). Finally, the HMB-PP is converted to IPP and DMAPP by HMB-PP reductase (IspH). The enzyme isopentenyl diphosphate isomerase (IDI) interconverts IPP and DMAPP. Although it exists in the MEP pathway, it is not essential because IspH can generate both IPP and DMAPP. The MVA pathway also contains seven reaction steps. It initiates with two condensation steps, in which three molecules of acetyl-CoA are coupled to form 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). Afterward, HMG-CoA is converted to mevalonate, which is then phosphorylated twice to form mevalonate pyrophosphate. IPP is generated by decarboxylation of mevalonate pyrophosphate, and the IDI catalyzes isomerization of IPP to form DMAPP.

Besides their substrate requirements, the MEP and MVA pathways differ in energy utilization and carbon yield. The net reactions of both pathways for synthesizing IPP from GAP are summarized as below:

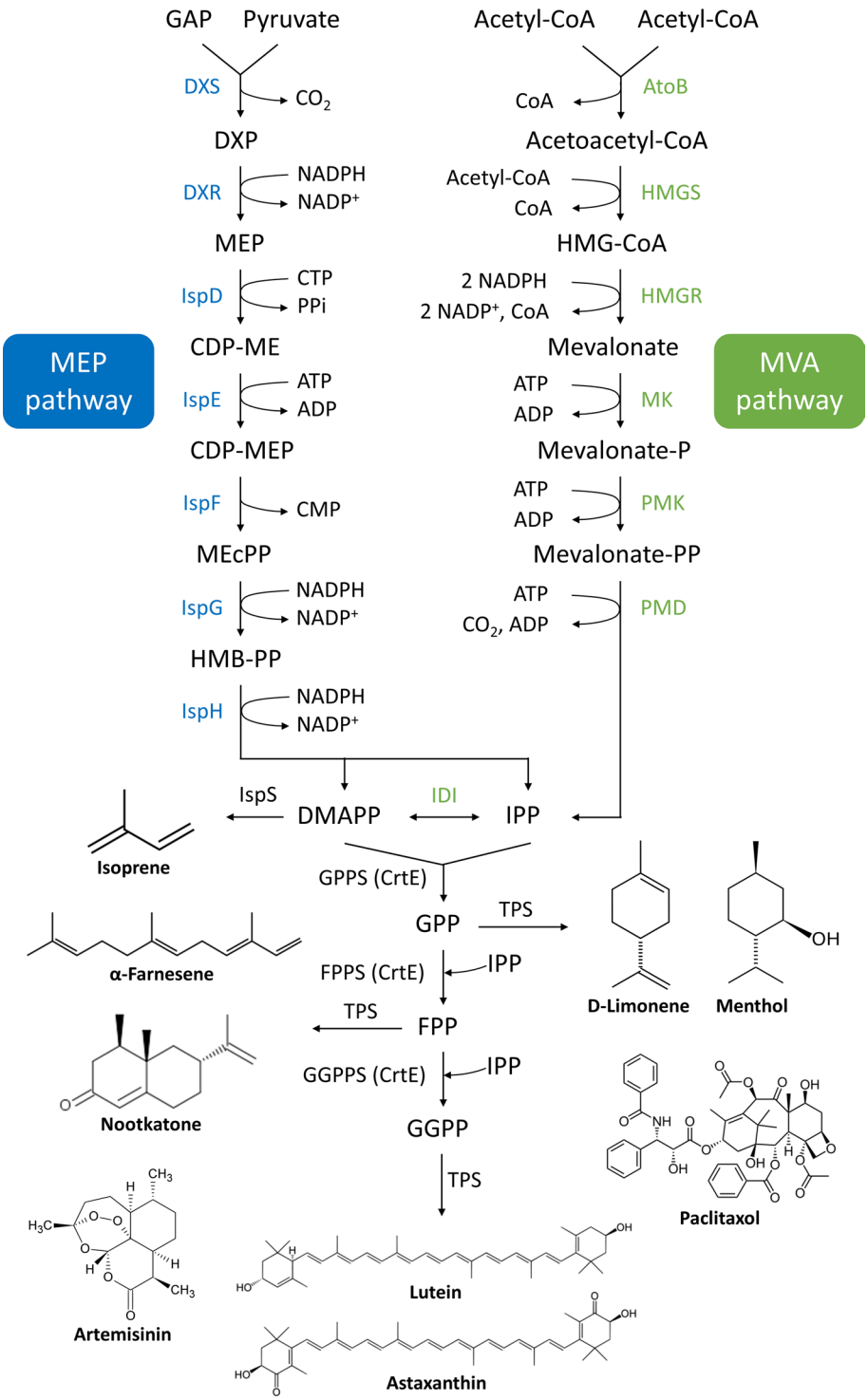

Fig. 1 Metabolic pathway of terpenoid biosynthesis. In cyanobacteria, linear prenyl pyrophosphates (GPP, FPP, and GGPP) are sequentially synthesized by CrtE. Terpenoid production catalyzed by terpene synthases can be from one-step or multiple-step reactions. Some of the commercially used terpenoids are shown here. *GAP* glyceraldehyde 3-phosphate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *DXS* DXP synthase, *MEP* 2-C-methyl-D-erythritol-4-phosphate, *DXR* DXP reductase, *CDP-ME* 4-diphosphocytidyl-2-C-methyl-D-erythritol, *IspD* 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase, *CDP-MEP* 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate, *IspE* 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, *MEcPP* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, *IspF* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *HMP-PP* 4-hydroxy-3-methyl-butenyl 1-diphosphate, *IspG* HMB-PP synthase, *IspH* HMB-PP reductase, *AtoB* acetoacetyl-CoA thiolase, *HMG-CoA* 3-hydroxy-3-methyl-glutaryl-CoA, *HMGS* HMG-CoA synthase, *HMGR* HMG-CoA reductase, *MK* mevalonate kinase, *PMK* mevalonate 5-phosphate kinase, *PMD* mevalonate 5-pyrophosphate decarboxylase, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *IDI* isopentenyl diphosphate isomerase, *IspS* isoprene synthase, *GPP* geranyl pyrophosphate, *GPPS* GPP synthase, *FPP* farnesyl pyrophosphate, *FPPS* FPP synthase, *GGPP* geranylgeranyl pyrophosphate, *GGPPS* GGPP synthase, *TPS* terpene synthase

MEP: $2GAP + ADP + CTP \rightarrow IPP + CO_2 + ATP + CMP$

MVA: $3GAP + 3ADP + 4NAD(P)^+ \rightarrow IPP + 4CO_2 + 3ATP + 4NAD(P)H$.

The MEP pathway utilizes carbon more efficiently, in which 83% (5/6) of the carbon substrate is used for IPP production, whereas the MVA pathway has 56% (5/9) carbon utilization. Conversely, the MVA pathway is more efficient at energy generation, in which three ATP and three NAD(P)H are produced from three molecules of GAP, whereas the MEP pathway generates one ATP from two molecules of GAP.

The downstream process of terpene synthesis begins with sequential head-to-tail condensation of DAMPP with IPP

to generate linear prenyl pyrophosphates, including geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15), geranylgeranyl pyrophosphate (GGPP, C20), etc. (Fig. 1). Further, terpene synthases convert the pyrophosphate intermediates into various terpenes. Notably, the photosynthetic pigments such as carotenoids and the phytol tail on chlorophyll are synthesized from this metabolic pathway. Therefore, the terpene biosynthesis pathway is critically important for cyanobacteria.

Recent advances in cyanobacterial terpenoid production

Engineering the terpene synthases

To engineer cyanobacteria for terpenoid production, the choice of terpene synthase is critical. An ideal enzyme should exhibit superior catalytic rate and good solubility in the host cells. The abundance of a soluble enzyme and its activity are correlated with the production titer (Gao et al. 2016). For isoprene production, the commonly used *isoprene synthases* (*ispS*) are derived from *Populus alba* and *Pueraria montana* (also known as kudzu). Recently, the *ispS* genes from various high isoprene emission plant species were expressed in *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus* 7942). The IspS from *Eucalyptus globulus* produced the highest amount of isoprene, over 5- and 25-fold higher than that from *P. alba* and *P. montana*, respectively (Gao et al. 2016). Similarly, a recent work reported that IspS from *E. globulus* and *Ipomoea batatas* increased isoprene production more than 20 fold compared to the *P. montana* IspS (Englund et al. 2018). The monoterpene limonene is widely found in plants. The limonene synthase (Lims) from *Mentha spicata* is the commonly used enzyme for limonene production. It exhibits the highest production level than that from other plant species (Table 1).

Another feature of terpene synthases that needs to be taken into account is enzyme promiscuity. Many terpene synthases produce multiple products. For instance, limonene synthase from *Mentha spicata* primarily produces S-limonene with trace amounts of α-pinene, β-pinene, and myrcene (Colby et al. 1993). Pinene synthase from *Citrus limon* mainly generates β-pinene but also α-pinene, sabinene, and limonene (Lucker et al. 2002). Expressing a terpene synthase with a higher product specificity prevents the formation of unwanted products (Lin et al. 2017).

To enhance expression of terpene synthase, strong promoters such as P_{cpcB} and P_{trc} promoters are widely used to drive gene expression in cyanobacteria (Lin et al. 2017; Wang et al. 2016; Kiyota et al. 2014; Davies et al. 2014). A recent work attempted stepwise metabolic engineering of *Synechococcus* 7942 for limonene production, while obtaining a marginal productivity increase (Wang et al. 2016). Surprisingly, by replacing the P_{trc} promoter by the pea P_{psbA} promoter for Lims expression, the production titer increased over 100-fold (Wang et al. 2016). Proteomic analysis confirmed the increased expression of Lims by the P_{psbA} promoter in the host strain (Wang et al. 2016). In addition, protein fusion is a strategy to increase protein expression. Using the highly expressed c-phycocyanin beta subunit (CpcB) protein as a leader sequence, the CpcB–IspS fusion constructs led to a significant improvement in protein abundance and isoprene productivity (Chaves et al. 2017). Although the IspS activity was attenuated, the increased expression level compensated the reduced activity of IspS, resulting in a 27-fold increase in isoprene production (Chaves et al. 2017).

Unlike plants, cyanobacteria do not have specific prenyltransferases to generate GPP and FPP, substrates for monoterpene and sesquiterpene synthases, respectively. These diphosphate intermediates are synthesized sequentially by the enzyme CrtE, which is a GGPP synthase (Fig. 1). A common strategy for enhancing monoterpene and sesquiterpene production is to express specific GPP and FPP synthases, respectively. Such strategy increases the amounts of GPP and FPP for terpenoid production (Lin et al. 2017; Wang et al. 2016; Choi et al. 2016; Englund et al. 2015). However, expression of these prenyltransferases reduces the carbon flux toward pigment synthesis, lowering the amount of photosynthetic pigments in cyanobacteria (Lin et al. 2017; Choi et al. 2016; Englund et al. 2015).

Engineering the precursor pathways

To enhance production of terpenoids, it is critical to increase the amount of the precursors (IPP and DMAPP), which can be achieved by engineering the MEP or MVA pathways. In *E. coli*, it is widely known that the DXS and IDI catalyze the bottleneck reactions in the MEP pathway. Overexpression of genes encoding the DXS and IDI leads to significant increase in terpene production, and this has been summarized in a recent review (Niu et al. 2017). Moreover, a previous study extensively engineered the MEP pathway in *E. coli* to produce taxadiene, the precursor of the potent anticancer drug paclitaxol. By overexpressing and optimizing four bottleneck genes (*dxs*, *ispD*, *ispF*, and *idi*) in the MEP pathway, the optimized strain increased taxadiene production 15,000-fold, leading to 1 g L^{-1} yield in fed-batch bioreactors (Ajikumar et al. 2010).

To increase the terpene precursors in cyanobacteria, most studies focused on optimizing the native MEP pathway. A recent study engineered *Synechococcus* 7942 for isoprene production. Overexpressing the native *dxs* gene increased 20% of the isoprene yield (Gao et al. 2016). Similarly,

Table 1 Selected recent literature on cyanobacterial terpenoid production

Product	Strain[a]	Titer	Time (days)	Origin of terpene synthase (TPS)	Engineering strategies	Conditions	References
Isoprene	7942	1.26 g L^{-1}	21	*Eucalyptus globulus*	P_{trc}-idi-ispS-dxs P_{tac}-ispG	100 µmol photons m^{-2} s^{-1}, 5% CO_2, 50 mM $NaHCO_3$, 37 °C	Gao et al. (2016)
Isoprene	6803	2.5 mg L^{-1}	4	*Pueraria montana*	CpcB-IspS fusion protein	100 µmol photons m^{-2} s^{-1}, 25 °C	Chaves et al. (2017)
Isoprene	6803	250 µg g^{-1} DCW	8.2	*Pueraria montana*	MVA pathway	150 µmol photons m^{-2} s^{-1}, 100% CO_2[b], 35 °C	Bentley et al. (2014)
Isoprene	6803	2.8 mg g^{-1} DCW	1	*Eucalyptus globulus*	P_{trc}-ispS P_{trc}-idi-dxs	50 µmol photons m^{-2} s^{-1}, 50 mM $NaHCO_3$, 30 °C	Englund et al. (2018)
Limonene	6803	6.7 mg L^{-1}	7	*Mentha spicata*	P_{trc}-lims, P_{psbA2}-gpps, P_{rbcL}-rpi-rpe	130 µmol photons m^{-2} s^{-1}, 30 °C	Lin et al. (2017)
Limonene	7002	4 mg L^{-1}	4	*Mentha spicata*	P_{cpcB}-lims	250 µmol photons m^{-2} s^{-1}, 1% CO_2, 37 °C	Davies et al. (2014)
Limonene	7942	2.5 mg L^{-1}	4	*Mentha spicata*	P_{psbA}-lims	100 µmol photons m^{-2} s^{-1}, 5% CO_2, 30 °C	Wang et al. (2016)
Limonene	6803	1 mg L^{-1}	30	*Schizonepeta tenuifolia*	P_{trc}-lims, P_{trc}-dxs-idi-crtE	100 µmol photons m^{-2} s^{-1}, 1% CO_2, 30 °C	Kiyota et al. (2014)
Limonene	7120	0.52 mg L^{-1}	12	*Sitka spruce*	P_{nir}-P_{psbA1}-lims-dxs-idi-gpps	150 µmol photons m^{-2} s^{-1}, 30 °C	Halfmann et al. (2014b)
Phellandrene	6803	3.2 mg g^{-1} DCW	2	*Lavandula angustifolia*	CpcB-PHLS fusion protein	50 µmol photons m^{-2} s^{-1}, 100% CO_2[b]	Formighieri and Melis (2015)
Phellandrene	6803	5.95 mg g^{-1} DCW	2	*Lavandula angustifolia*	CpcB-PHLS, NptI-GPPS fusion proteins	100 µmol photons m^{-2} s^{-1}, 100% CO_2[b]	Betterle and Melis (2018)
Phellandrene	6803	10 mg g^{-1} DCW	2	*Lavandula angustifolia*	MVA pathway	100% CO_2[b], light intensity not specified	Formighieri and Melis (2016)
Amorphadiene	7942	19.8 mg L^{-1}	10	*Artemisia annua*	P_{trc}-dxs-idi-ispA P_{trc}-ads	100 µmol photons m^{-2} s^{-1}, 5% CO_2, 30 °C	Choi et al. (2016)
Farnesene	7120	0.3 mg L^{-1}	15	*Picea abies*	P_{nir}-P_{psbA1}-fas	50 µmol photons m^{-2} s^{-1}, 1% CO_2, 30 °C	Halfmann et al. (2014a)
Manoyl oxide	6803	0.45 mg g^{-1} DCW	4	*Coleus forskohlii*	P_{psbA2}-CfTPS-dxs	100 µmol photons m^{-2} s^{-1}	Englund et al. (2015)

lims limonene synthase, *rpi* ribose 5-phosphate isomerase, *rpe* ribulose 5-phosphate 3-epimerase, *crtE* GGPP synthase, *PHLS* phellandrene synthase, *NptI* neomycin phosphotransferase I, *ispA* FPP synthase, *ads* amorphadiene synthase, *fas* farnesene synthase, *CfTPS* terpene synthase from *C. forskohlii*

[a]7942, *Synechococcus elongatus* PCC 7942; 6803, *Synechocystis* sp. PCC 6803; 7002, *Synechococcus* sp. PCC 7002; 7120, *Anabaena* sp. PCC 7120

[b]Cells were grown in a gaseous–aqueous two-phase reactor (Bentley and Melis 2012)

production of limonene was enhanced by overexpressing the *dxs* gene together with other genes involved in the upstream pathway. A previous work overexpressed genes encoding the DXS (from *E. coli*), IDI (from *Haematococcus pluvialis*), and GPPS (from *Mycoplasma tuberculosis*) in *Anabaena* sp. PCC 7120, resulting in a 6.8-fold increase

in limonene production (0.52 mg L^{-1} in 12 days) (Halfmann et al. 2014b). By expressing the same set of genes (*dxs*, *idi*, and *gpps* from *Synechocystis* sp. PCC 6803), the limonene yield in *Synechocystis* 6803 increased 40% to 1 mg L^{-1} in 30 days (Kiyota et al. 2014). The productivity of limonene in *Synechococcus* 7942 was increased 2.3-fold to 76.3 µg L^{-1} OD_{730}^{-1} day^{-1} by overexpressing the *dxs* gene from *Botryococcus braunii* and the *gpps* gene from *Abies grandis* (Wang et al. 2016). Moreover, increased production of the plant diterpenoid manoyl oxide was achieved by expressing the *dxs* gene from the plant *Coleus forskohlii*, which resulted in a 4.2-fold improvement in low light condition (20 µmol photons m^{-2} s^{-1}) (Englund et al. 2015). However, the *dxs*-overexpressing strain showed no improvement in production under high light condition (100 µmol photons m^{-2} s^{-1}) (Englund et al. 2015).

Compared to DXS, IDI seems to be a more important target for enhancing isoprene production in cyanobacteria. *Synechococcus* 7942 has a low ratio of DMAPP/IPP concentration (i.e., 0.03 in an isoprene-producing strain) (Gao et al. 2016). Such low ratio may affect the substrate availability for isoprene production, since DMAPP is the substrate for isoprene synthase. In addition, the isoprene-emitting kudzu leaves have a high DMAPP/IPP ratio (i.e., > 2) (Zhou et al. 2013), indicating that isoprene production is related to DMAPP availability. To increase the DMAPP/IPP ratio, the *idi* gene from *S. cerevisiae* was overexpressed in an isoprene-producing *Synechococcus* 7942, which increased the DMAPP/IPP ratio to 2.25 and resulted in a twofold improvement in isoprene production (Gao et al. 2016). However, co-expression of *dxs* and *idi* genes produced similar amount of isoprene compared to the *idi*-expressing strain (Gao et al. 2016), suggesting that the IDI has a more critical role than DXS for enhancing isoprene production in cyanobacteria.

Besides DXS and IDI catalyzing the critical reactions for terpene synthesis, IspD and IspG were recently identified as bottleneck enzymes of the MEP pathway in cyanobacteria. Systematic overexpression of each enzyme in the MEP pathway identified IspD as the third enzyme for enhanced isoprene production in *Synechocystis* 6803 (Englund et al. 2018). Kinetic flux profiling showed that the flux through the reaction catalyzed by IspG was very low in *Synechococcus* 7942 (Gao et al. 2016). Overexpressing the *ispG* gene from *Thermosynechococcus elongatus* increased isoprene production 60% (Gao et al. 2016). The final strain in the study, with expression of *ispS*, *dxs*, *idi*, and *ispG*, produced 1.26 g L^{-1} isoprene in a photobioreactor. The productivity was 102 mg L^{-1} day^{-1} in the first 9 days, which is over two orders of magnitude higher than all the reported literature on cyanobacterial terpenoid production (Table 1).

Another strategy to increase terpene precursors is to express the heterologous MVA pathway, which is widely used for terpene production in *E. coli*. Introduction of this pathway increases the carbon flux toward the terpene precursors, which further enhances productivity of various terpenoids such as isoprene, lycopene, limonene, and farnesene (Niu et al. 2017). With cyanobacteria, the Melis group successfully introduced the heterologous MVA pathway into *Synechocystis* 6803 for enhanced production of isoprene (Bentley et al. 2014) and β-phellandrene (Formighieri and Melis 2016). Unlike previous work in *E. coli* which expressed the MVA pathway from *S. cerevisiae*, the upper MVA pathway in their studies was derived from *Enterococcus faecalis* (HMGS, HMGR) and *E. coli* (AtoB), whereas the lower MVA pathway was obtained from *Streptococcus pneumoniae* (MK, PMK, PMD, and IDI) (Formighieri and Melis 2016; Bentley et al. 2014). Both pathways were successfully introduced into the genome of *Synechocystis* 6803, and all enzymes were actively expressed (Bentley et al. 2014). Expressing the MVA pathway increased terpene production over twofold (Formighieri and Melis 2016; Bentley et al. 2014), suggesting that the heterologous pathway indeed directs an extra carbon flux toward terpene synthesis. However, such improvement was less impactful than previous efforts in *E. coli*. For instance, expression of the *S. cerevisiae* MVA pathway increased artemisinic acid production 36 fold (Martin et al. 2003). Also, limonene production improved nearly 90 fold by optimizing the MVA pathway from *Staphylococcus aureus* and *S. cerevisiae* (Alonso-Gutierrez et al. 2013). The heterologous MVA pathway used in *E. coli* has been extensively studied and perhaps this optimized pathway can be expressed in cyanobacteria to see if terpene production could be further improved.

Recently, the pentose phosphate (PP) pathway in cyanobacteria was engineered for enhanced production of limonene. Lin et al. (2017) used the Optforce computational algorithm to identify two genes in the PP pathway, *ribose 5-phosphate isomerase* (*rpi*) and *ribulose 5-phosphate 3-epimerase* (*rpe*) that are important for limonene synthesis. The limonene titer increased 2.3 fold to 6.7 mg L^{-1} by expressing the *rpi* and *rpe* genes from *E. coli* and the *gpps* gene from *Abies grandis*.

Challenges in enhancing terpenoid production in cyanobacteria

Redirection of photosynthetic carbon

One of the major challenges to enhance terpenoid production by cyanobacteria is carbon partitioning in photosynthesis. Most of the fixed carbon is directed toward sugar phosphates for biomass instead of the products. The sugar biosynthetic pathways in *Synechocystis* 6803 consumes more than 80% of the fixed carbon for biomass accumulation, whereas approximately 5% of the photosynthetic carbon is allocated to terpene biosynthesis, including carotenoids, phytol groups of chlorophyll, and prenyl tails of quinone (Melis 2013). The

low carbon flux to the terpene metabolic pathway results in the low yield of heterologous terpenoid production.

A common strategy for redirecting the carbon flux is to remove the competing pathway(s) of desired products. In cyanobacteria, glycogen serves as a major carbon sink for energy storage under nutrient starvation conditions, in which the carbon flux is diverted to glycogen accumulation instead of cellular growth (Hickman et al. 2013; Yoo et al. 2007). Several studies attempted to delete the glycogen biosynthetic pathway for the purpose of redirecting the fixed carbon toward desired products. A recent study reported a two-fold increase in lactic acid production using a glycogen deficient *Synechocystis* 6803 mutant (van der Woude et al. 2014). Furthermore, overexpressing a heterologous isobutanol pathway served as an alternative carbon sink that rescued the growth phenotype of a *Synechococcus* 7942 glycogen mutant, and increased the carbon flux to isobutanol synthesis (Li et al. 2014). However, inactivation of glycogen sinks leads to metabolic imbalance under nitrogen-deplete conditions, in which organic acids such as pyruvate and α-ketoglutarate are excreted from the cells (Davies et al. 2014; Hickman et al. 2013; Carrieri et al. 2012). Unfortunately, removing the glycogen synthesis pathway did not lead to improved terpenoid production in cyanobacteria. Davies et al. (2014) constructed a glycogen-deficient mutant for limonene and bisabolene production. However, the production titer was extremely low due to the absence of growth under nitrogen starvation.

Compared to other compounds produced by engineered cyanobacteria, the productivities of terpenes are relatively low, even though many efforts have been done to increase terpene production. Presumably, the MEP pathway in cyanobacteria is highly regulated so that limited amount of the fixed carbon is directed to terpene synthesis. The terpene precursors IPP and DMAPP are distant from the central carbon metabolism. Quantitative analysis reveals that the concentrations of IPP, DMAPP, and other intermediates (MEP and HMB-PP) in the MEP pathway are three orders of magnitude lower than pyruvate in cyanobacteria (Dempo et al. 2014). This indicates that the sparse amount of precursor pools may be a limiting factor for enhancing terpene synthesis. A previous study using cell extracts of *Synechocystis* 6803 demonstrated that terpene production was increased by compounds in the PP pathway but not by substrates (pyruvate and GAP) of the MEP pathway (Ershov et al. 2002). In addition, expression of the *rpi* and *rpe* genes in the PP pathway led to increased production of limonene (Lin et al. 2017). These evidences indicate a connection between the PP pathway and terpene synthesis in cyanobacteria. Moreover, a recent study determined that the *E. coli* enzymes YajO and RibB (G108S) are able to convert ribulose 5-phosphate to DXP, the first intermediate in the MEP pathway (Kirby et al. 2015). Since cyanobacteria have a relatively high carbon flux in the PP pathway (Abernathy et al. 2017; Young et al. 2011), this novel route can be investigated in order to avoid feedback regulation in the MEP pathway (Banerjee et al. 2013) and direct the carbon flux from the PP pathway to terpene production.

MEP vs. MVA pathways: which is better for cyanobacterial terpenoid production?

Overexpression of the heterologous MVA pathway is a common strategy to improve terpenoid production in heterotrophic microorganisms such as *E. coli* and *S. cerevisiae*. Since the native MEP pathway is highly regulated (Banerjee and Sharkey 2014), the non-native MVA pathway is able to avoid transcriptional regulation in the hosts and enable a bypass of the carbon flux to terpene synthesis. Previous studies on cyanobacterial terpenoid production were mainly focused on the MEP pathway. A few studies expressed the heterologous MVA pathway for enhanced terpene synthesis (Table 1). This may indicate the difficulty of introducing the MVA pathway into photosynthetic microorganisms. The MVA pathway contains seven genes, about nine kilobases in length (Bentley et al. 2014). Introducing the entire pathway into cyanobacterial genome may be challenging. Although the heterologous MVA pathway has been successfully expressed in *Synechocystis* 6803 (Formighieri and Melis 2016; Bentley et al. 2014), the production titer is similar to other studies engineering the MEP pathway (Table 1). The MEP and MVA pathways use different substrates. The former uses GAP and pyruvate, whereas the later requires acetyl-CoA. Isotopically nonstationary ^{13}C flux analyses showed that cyanobacteria have a relatively low flux toward acetyl-CoA compared to the sugar phosphate pathways (Abernathy et al. 2017; Young et al. 2011). Moreover, a recent study compared the precursor pools of the MEP and MVA pathways between *Synechococcus* 7942 and *E. coli* BL-21, and found that the concentrations of GAP and pyruvate in *Synechococcus* 7942 were 5- and 21-fold higher than that in *E. coli*, whereas the acetyl-CoA pool was 20-fold lower (Gao et al. 2016). These data indicate that expressing the MVA pathway in cyanobacteria may be less effective than in *E. coli* for enhanced terpene production. Hence, the native MEP pathway is presumably a more attractive target for metabolic engineering, even though further research is needed to elucidate the regulation of the MEP pathway in cyanobacteria. It is known that overexpressing the enzymes in the MEP pathway can reduce terpenoid production in *E. coli* (Ajikumar et al. 2010; Kim and Keasling 2001). The expression level needs to be optimized in order to maximize the productivity. Despite many efforts to engineer the MEP pathway in cyanobacteria, optimization of the pathway is yet to be attempted. This can be achieved by modifying the ribosome binding site (RBS) (Wang et al. 2018; Oliver et al.

2014) or using inducible promoters (Markley et al. 2015; Oliver et al. 2013; Huang and Lindblad 2013) to modulate gene expression.

Balancing pigment and product synthesis

The photosynthetic pigments such as chlorophyll and carotenoids are essential for cyanobacteria. Production of terpenes will necessarily compete with pigment synthesis because the same precursor pathway is used. Although the expression of prenyltransferases such as GPP and FPP synthases is critical in enhancing terpenoid production (Lin et al. 2017; Choi et al. 2016), the carbon flux for photosynthetic pigments is diverted toward the terpene product. Previous studies reported that photosynthetic pigment levels were lowered in terpene-producing cyanobacteria (Lin et al. 2017; Choi et al. 2016; Englund et al. 2015). Decreased amount of pigments may affect the efficiency of light harvesting and photoprotection and alter the structural stability of photosynthetic complexes (Toth et al. 2015), thus leading to impaired cellular growth and terpene production. To further increase terpenoid production in cyanobacteria, the carbon fluxes between the product and pigment synthesis should be balanced in addition to increasing the terpene precursors. Modulating the expression of prenyltransferases could presumably optimize carbon fluxes and improve terpene synthesis. A recent study reported that the expression level of GPP synthase is critically important for enhancing monoterpene production in *E. coli*. Optimization of GPP synthase expression by engineering the RBS sequence led to a sixfold improvement in geraniol production (Zhou et al. 2015).

Future perspectives

Cyanobacterial production of terpenoids demonstrates the potential of sustainable bioproduction using light and CO_2, although further research is needed to enhance the productivity. Challenges including the regulation of MEP pathway, carbon partitioning, and balancing pigment and limonene synthesis should be addressed collectively in order to further increase the productivity of terpenoids. With many obstacles required to be overcome, recent developments on the genetic tools for cyanobacteria (Sengupta et al. 2018) can facilitate strain construction and accelerate the design-build-test cycle, which will benefit systematic engineering of cyanobacteria. The advantage of using cyanobacteria as a production platform is direct conversion of CO_2 to desired compounds without the use of sugar feedstocks. However, the relatively slow rates of growth and CO_2 fixation of standard laboratory strains presumably lead to low productivity of chemicals. Particularly, the small fraction of fixed carbon allocated to terpene metabolism makes terpenoid productivity even lower than other compounds. To utilize cyanobacteria for commercial bioproduction, a better chassis is needed to achieve higher production titer and productivity. *Synechococcus elongatus* UTEX 2973 is a fast-growing cyanobacterium with the fastest growth rate reported to date (Yu et al. 2015). The doubling time of this strain can be as fast as 1.5 h under high light and CO_2 conditions (Ungerer et al. 2018), which is comparable to the heterotrophic growth rate of the yeast *S. cerevisiae*. The fast growth of *Synechococcus* 2973 reflects the rapid accumulation of biomass, at a rate of over 1 g L^{-1} day^{-1} (Ungerer et al. 2018). Moreover, this strain synthesizes significant amount of glycogen as a way to reserve excess fixed carbon, with over 35% of dry cell weight after 3 days of growth (Ungerer et al. 2018). Recently, *Synechococcus* 2973 was engineered for sugar production. The productivity of sucrose reaches 0.85 g L^{-1} day^{-1} (the highest amount from photoautotrophic production), demonstrating the potential of using this strain for biotechnology applications (Song et al. 2016). With recent advances in metabolic engineering of terpene biosynthetic pathway, this fast-growing cyanobacterium offers an opportunity to improve the productivity of terpenoids.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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