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Review article

Photosynthetic terpene hydrocarbon production for fuels and chemicals

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Summary

Photosynthetic hydrocarbon production bypasses the traditional biomass hydrolysis process and represents the most direct conversion of sunlight energy into the next-generation biofuels. As a major class of biologically derived hydrocarbons with diverse structures, terpenes are also valuable in producing a variety of fungible bioproducts in addition to the advanced 'drop-in' biofuels. However, it is highly challenging to achieve the efficient redirection of photosynthetic carbon and reductant into terpene biosynthesis. In this review, we discuss four major scientific and technical barriers for photosynthetic terpene production and recent advances to address these constraints. Collectively, photosynthetic terpene production needs to be optimized in a systematic fashion, in which the photosynthesis improvement, the optimization of terpene biosynthesis pathway, the improvement of key enzymes and the enhancement of sink effect through terpene storage or secretion are all important. New advances in synthetic biology also offer a suite of potential tools to design and engineer photosynthetic terpene platforms. The systemic integration of these solutions may lead to 'disruptive' technologies to enable biofuels and bioproducts with high efficiency, yield and infrastructure compatibility.

Introduction

Central to biological renewable energy production is the efficient harnessing of sunlight energy to transform inorganic carbon into energy-dense fuels and chemicals. Traditionally, biofuel, mainly bioethanol, was produced from corn or sugarcane through microbial fermentation. The on-going development of lignocellulosic biofuel aims to utilize nonfood biomass to resolve the sustainability issue associated with the crop-based biofuel production (Fortman *et al.*, 2008; Hahn-Hagerdal *et al.*, 2006; Stephanopoulos, 2007). In addition, biodiesel can be produced from oil plants or oil-rich microalgae (Chisti, 2008). Despite substantial progresses, these biofuel platforms have several limitations including the energy output per land area, the compatibility with current fuel infrastructures and the insufficient capacity to meet Renewable Fuel Standard (RFS) for petroleum replacement (Chuck and Donnelly, 2014; Yuan *et al.*, 2008b). Metabolic engineering is a powerful tool in advancing biofuel productions. Genetically engineered microbes have the potential to produce a variety of infrastructure-compatible 'drop-in' fuel molecules. Recently, the potential of directly producing advanced hydrocarbon biofuels has been demonstrated in genetically engineered heterotrophic microbes (Choi and Lee, 2013; Peralta-Yahya *et al.*, 2011; Zhang *et al.*, 2011). However, when applying cellulosic biomass as the sugar stock, the yield of fuel molecules is rather low (Bokinsky *et al.*, 2011). The challenges result not only from the low efficiency in hydrolysing biomass, but also from the inherent carbon and energy efficiency in converting sugars to a more reduced hydrocarbon molecule (Dugar and Stephanopoulos, 2011). Heterotrophic microbial systems might succeed in producing high-value chemicals or pharmaceuticals, but they are less attractive in fuel productions due to high costs and the sustainability constraints from sugar consumptions. *In planta* photosynthetic hydrocarbon production represents a viable alternative for hydrocarbon biofuel production and could lead to a sustainable platform in that carbon, ATP and NADPH are directly provided from photosynthesis.

The concept of 'photosynthetic biofuels' is pioneered by Lindberg and Melis, who demonstrated the possibility of directly producing isoprene, a C_5 hydrocarbon, in the engineered cyanobacterium *Synechocystis* (Lindberg *et al.*, 2010). Photosynthetic biofuels are thus, within plants or other photosynthetic platforms, to directly convert sunlight energy to energy-dense fuel molecules that resemble gasoline constituents (C_4–C_{12} hydrocarbon and derivatives) or other transportation fuels (Altin and Eser, 2004; Chuck and Donnelly, 2014; Lindberg *et al.*, 2010). Various biosynthesis pathways are interconnected with photosynthesis and can be employed to produce a wide range of chemical compounds. Photosynthesis-derived fatty acids can be used to synthesize fatty alcohols, fatty acid alkyl esters or directly converted into alkanes/alkenes through decarboxylation (Lu, 2010; Tan *et al.*, 2011; Wang *et al.*, 2013). The downsides of utilizing fatty acid-derived pathways are the intense cell regulations and energy consumptions in fatty acid biosynthesis, and complications in controlling the length of fatty acid chains (Machado and Atsumi, 2012; Peralta-Yahya *et al.*, 2012).

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Terpenoid biosynthesis represents another important route for photosynthetic hydrocarbon production and will be the focus of this review.

Terpenoids, also called isoprenoids due to their isoprene-derived structures, are the largest class of secondary metabolites produced by plants (O'Maille *et al.*, 2008; Wu *et al.*, 2006; Yuan *et al.*, 2009). They can be classified into monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}) and tetraterpenes (C_{40}) according to the number of isoprene structures. Terpenes have diverse industrial applications as chemicals, nutraceuticals, antioxidants and drugs (Ajikumar *et al.*, 2010; Chang and Keasling, 2006; Farhi *et al.*, 2011). For example, the flavour and fragrance industry alone have over $1 billion terpene market (Wu *et al.*, 2006). In addition, the thermochemical and thermophysical properties of some monoterpenes, sesquiterpenes and their derivatives make them ideal candidates as 'drop-in' JP-8, gasoline and diesel fuels. For example, the ring structure of C_{10} limonene enables higher energy density and can serve as 'drop-in' fuel precursors (Chuck and Donnelly, 2014; Filley *et al.*, 2001). C_{15} sesquiterpenes like β-caryophyllene is a major component of *Copaifera* oleoresin that can be directly used as diesel (Chen *et al.*, 2009). Bisabolane, the derivative of the isoprenoid bisabolene, can also serve as a biosynthetic diesel (Peralta-Yahya *et al.*, 2011). However, the natural production of terpenes usually has low yield and makes complex terpene mixtures and thus falls short of the rising demand in the terpene industry. This dilemma enables the role of photosynthetic terpene production as a potential route to produce terpenes instead of harvesting from natural resources.

'MEP' vs. 'MVA' pathway in photosynthetic terpene production

All terpenes are generated from the C_5 precursors isopentenyl pyrophosphate (IPP), and its isomer dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP can be synthesized in two isoprenoid biosynthetic pathways, the MVA (mevalonate) pathway found in eukaryotic cytosol and archaea and the MEP pathway in most prokaryotes, green algae and plants (Eisenreich *et al.*, 2004; Martin *et al.*, 2003). Plants have both cytosolic MVA pathway and chloroplastic MEP pathway for IPP and DMAPP synthesis, while green algae and cyanobacteria predominantly contain the MEP pathway (Hildebrand *et al.*, 2013; Lichtenthaler, 1999; Wu *et al.*, 2006) (Figure 1). Compared to MVA pathway, MEP is considered as an 'energy-deficient' pathway, where additional reducing power is required to produce terpene precursors (Dugar and Stephanopoulos, 2011). From the perspective of fuel production, the pathway's energetic efficiency is a key determinant in product yield. Many pathways produce reducing equivalent such as NADH or highly oxidized by-products such as CO_2 besides the desired molecule. The excess energy and reductant are either balanced into futile pathways or used for cell maintenance, both of which will result in lower yield of the desired product (or carbon efficiency) (Dugar and Stephanopoulos, 2011). Compared to the MVA pathway, the 'energy-deficient' MEP pathway is redox-balanced and more efficient in converting glucose/glycerol to the key terpenoid synthesis precursor IPP (Dugar and Stephanopoulos, 2011). In other words, an 'energy-deficient' pathway produces more desired products rather than leading to energy escape.

Compared to chemoheterotrophic organisms, photosynthetic microbes already have elevated terpene carbon partition due to their extra needs in MEP-derived terpenoids and derivatives such as carotenoids, prenylated plastoquinones and phytol moieties of chlorophylls (Formighieri and Melis, 2014a; Melis, 2013). More importantly, photosynthetic terpenoid production through MEP could intercept glyceraldehyde 3-phosphate (G3P) directly from the photosynthetic carbon reduction cycle within the eukaryotic chloroplast or cyanobacteria (Figure 1). MEP-derived terpenoid biosynthesis can thus serve as a potential photosynthate sink *en route* to fuel productions.

Exploring MEP-derived terpene biosynthesis provides an attractive opportunity for the advanced biofuel production. However, a major extant limitation is the efficient redirection of photosynthates into target compounds. The relatively high terpene titre achieved in heterotrophic systems has not been readily translated into photosynthetic systems. As shown in Table 1, most of the photosynthetic systems yielded much lower terpene titres compared to heterotrophic systems. The substantial efforts invested in engineering heterotrophic organisms such as *Escherichia coli* vs. the fairly recent development in photosynthetic organisms is certainly an important consideration for the dramatic productivity difference in these two platforms. The lower expression levels of heterologous terpene synthase in cyanobacteria due to the chromosomal integration as compared to the plasmid-based higher expression levels in *E. coli* might be another reason for the lower titre (Formighieri and Melis, 2014b). Nevertheless, certain as yet undiscovered metabolic regulations might contribute substantially to the low terpene yield in photosynthetic organisms. After all, the natural accumulation of large amounts of terpenes in the green alga *Botryococcus braunii* (Banerjee *et al.*, 2002), and the precedent success in engineering cyanobacteria to produce high titre of other molecules such as higher alcohols (Atsumi *et al.*, 2009), presented the feasibility of establishing an efficient photosynthetic terpene platform. We hereby discuss four key technical barriers and potential improvement strategies for the current photosynthetic terpene production.

Key technical barriers and improvement strategies in photosynthetic terpene production

Key barriers from 'source' to 'sink' are discussed to gain insight into improving strategies for photosynthetic terpene production. More specifically, improving photosynthesis efficiency, fine-tuning MEP pathway, optimizing key terpene enzymes and designing proper storage strategies are of imminent importance in improving terpene yield in current photosynthetic systems.

Photosynthesis redesign to improve terpene production—source enhancement

The inferior performance of the engineered terpene-producing apparatus in phototrophic systems indicates its unique metabolic metabolism (Table 1). Importantly, photosynthesis directly competes with the heterologous terpene-producing apparatus for the consumption of terpene precursors (Formighieri and Melis, 2014a). Optimized photosynthesis could be pivotal to direct sufficient carbon and reductant for the 'energy-deficient' MEP pathway. Moreover, a recent genome-scale modelling in the cyanobacterium *Cyanothece* sp. showed that the relative light distribution in photosystem I (PSI) and II (PSII) could substantially impact cell growth and metabolic flux distributions (Vu *et al.*, 2012). The coexistence of the light-induced electron transfer chain (ETC) and respiratory electron transfer in phototrophs thus might be subject to more complex cell regulations

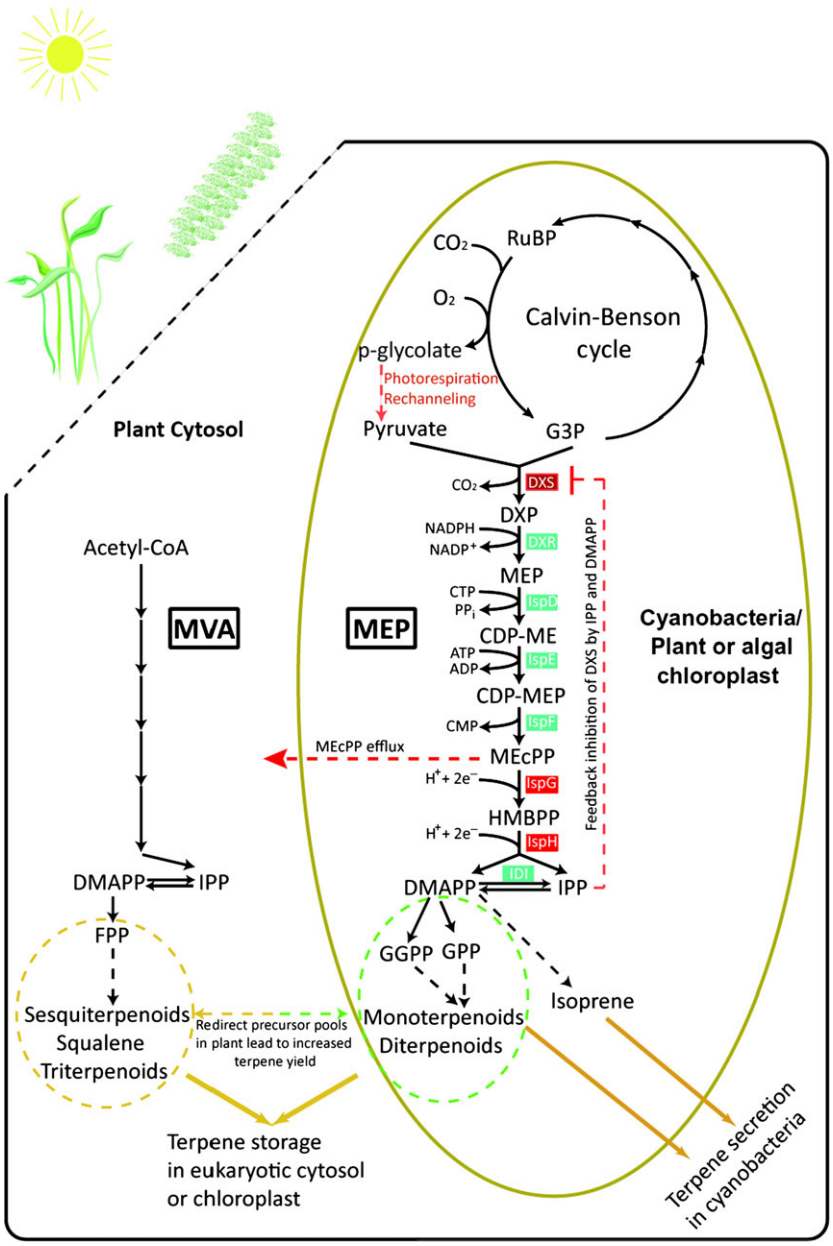

Figure 1 The schematic for photosynthetic terpene productions in cyanobacteria, green algae or plants. The success of the terpene platform is determined by the synergy of photosynthesis, MEP and terpene biosynthesis pathways (PMT), in which photosynthesis redesign (photorespiration rechannelling or other strategies to increase both photosynthesis efficiency and carbon repartition), MEP pathway optimization (DXS, IspG and IspH could be key tuning points to reduce intermediates accumulation and toxicity and enhance the MEP carbon flux), terpene enzymes modification, and terpene molecules storage or secretion need to be integrated to enhance the total terpene yield.

and might limit the reductant availability for MEP-derived terpene biosynthesis.

A considerable amount of attention has been paid to photosynthesis efficiency improvement (Blankenship *et al.*, 2011; Evans, 2013; Zhu *et al.*, 2010). Significant progresses have been made in identifying key bottlenecks in photosynthesis from both carbon fixation and light capture perspectives (Carraretto *et al.*, 2013; Peers *et al.*, 2009). Classical breeding and emergence of systems biology and synthetic biology are providing new opportunities to develop a more photosynthetically productive germplasm (Ort *et al.*, 2011). For example, improving leaf display in crop canopies can avoid light saturation, and further integrating photorespiratory bypass has already improved the productivity of model plant species (Kebeish *et al.*, 2007; Ort *et al.*, 2011; Zhu *et al.*, 2010). In the longer term, various strategies are being explored in improving photosynthesis efficiency. Some examples include engineering plant carboxylases that are better adapted to current and forthcoming CO_2 concentrations, the conversion of species from C_3 to C_4 pathways and molecular optimization of resource investment among the components of the photosynthetic apparatus (von Caemmerer and Evans, 2010; Evans, 2013). Other promising approaches include introducing bicarbonate transporters to improve carbon concentrating mechanisms (Price *et al.*, 2011), increasing carbon assimilation by synthetic carbon fixation pathways (Bar-Even *et al.*, 2010) and decreasing feedback regulation on photosynthesis via end product storage or secretion. Many of these strategies are applicable to the photosynthetic terpene production. For example, in higher plants, C_4 perennial grasses have higher yield potential than nearly all C_3 plants. However, most of the model high-terpene plants (e.g. peppermint, *Copaifera langsdorffii*, and *Euphorbia*) are C_3 plants (Croteau *et al.*, 1971; Forgo *et al.*, 2011; Groeneveld, 1987; Lange and Croteau, 1999a,b; Piazza and Holzwarth, 1989; Rodrigues and Machado, 2009). Calvin cycle is mainly localized in

Table 1 Recent development in terpene productions from both heterotrophic and photosynthetic organisms

Terpenoids	Pathway applied	Titre* (mg/L)	Organism	Reference
Taxadiene	MEP	~1000	*E. coli*	Ajikumar *et al.* (2010)
Bisabolene	Exogenous MVA	>900	*E. coli*	Peralta-Yahya *et al.* (2011)
Limonene	Exogenous MVA	400	*E. coli*	Alonso-Gutierrez *et al.* (2013)
Pinene	Exogenous MVA	32	*E. coli*	Sarria *et al.* (2014)
Bisabolene	Exogenous MVA	>900	*Saccharomyces cerevisiae*	Peralta-Yahya *et al.* (2011)
Isoprene	Exogenous MVA	0.25 (mg/g DCW)	*Synechocystis* sp. PCC 6803	Bentley *et al.* (2014)
Squalene	mutant (Δ*shc*)	0.67 (mg/L/OD_{750})	*Synechocystis* sp. PCC 6803	Englund *et al.* (2014)
Limonene	MEP	4	*Synechococcus* sp. PCC 7002	Davies *et al.* (2014)
β-Phellandrene	MEP	~0.25 (mg/g DCW)	*Synechocystis* sp.	Formighieri and Melis (2014b)
Bisabolene	MEP	0.6	*Synechococcus* sp. PCC 7002	Davies *et al.* (2014)
Limonene	MEP	<0.01	*Chlamydomonas reinhardtii*	Our unpublished data
Squalene	MEP	~1.76 (mg/g FW)	Tobacco	Wu *et al.* (2012)
Limonene	MEP	~500 ng/g FW	Tobacco	Wu *et al.* (2006)
	MVA	>300 ng/g FW		
Isoprene	MEP	~12–25 nmol/m^2/s	Tobacco	Vickers *et al.* (2011)
Taxadiene	MEP	~400 μg/g DW	Tomato	Kovacs *et al.* (2007)

*The highest titres from the engineered microbes/plants were provided.

bundle sheath cells of C_4 plants, whereas the mesophyll cells contain phosphoenolpyruvate carboxylase, the primary carboxylase of the C_4 pathway and the initial step leading to CO_2 concentrate at the site of Rubisco in bundle sheath cells. C_4 metabolism with its unique Kranz anatomy minimizes the oxygenation reaction of Rubisco and thus photorespiration, whereas in C_3 plants, metabolically expensive photorespiration lowers the rate and efficiency of photosynthesis. The rechannelling of photorespiration products (Kebeish *et al.*, 2007) to pyruvate for terpene biosynthesis has the potential to improve the partition of photosynthates into terpene fuel molecules (our unpublished work).

Cyanobacteria combat Rubisco oxygenation with a different form of carbon concentrating mechanisms (CCM) than C_4 metabolism of higher plants, in which Rubisco is sequestered within the unique carboxysome structure where CO_2 is concentrated by active transport of bicarbonate and its rapid dehydration to CO_2 by carbonic anhydrase (Rae *et al.*, 2013). Efforts have been taken to introduce cyanobacterial carboxysome components and bicarbonate transporters to higher plants to improve photosynthesis (Lin *et al.*, 2014a,b; Price *et al.*, 2013). Better understanding of cyanobacterial CCM may help guide the optimization of plant photosynthesis as well as lead to the development of terpene production in the photosynthetic prokaryotic system. For example, a recent study pointed out the importance of a thylakoid potassium channel in achieving efficient photosynthesis in cyanobacteria. The study indicated its possible involvement in regulating electron components in building proton motive force for generating ATP (Checchetto *et al.*, 2012). In cyanobacteria, to achieve the optimal photosynthetic performance, ATP/NADPH ratio represents a key fine-tuning parameter in light energy conversion. The ATP/NADPH produced by the photosynthetic linear electron flow (LEF) yields an output ratio of 1.28, yet the ATP/NADPH sink such as the Calvin cycle requires an input ratio of 1.5 (Kramer and Evans, 2011; Nogales *et al.*, 2012). It is therefore important to fine-tune the ATP/NADPH (energy vs. reductant) ratio to drive the MEP and downstream terpene productions.

It is also worth pointing out that the improvement in photosynthesis efficiency does not necessarily translate into more carbon partition in terpene biosynthesis. The low baseline carbon partition towards secondary metabolisms like terpene biosynthesis requires metabolic designs to efficiently channel photosynthates towards terpene biosynthesis precursors. In particular, the ratio of G3P and pyruvate was found to be essential for an increased terpene yield (Liu *et al.*, 2013). The redesign of photosynthesis pathways to generate an appropriately balanced G3P and pyruvate ratio thus may lead to more carbon partition into terpene biosynthesis.

Tuning MEP pathway to increase terpene precursors—enhancing the carbon flux to the sink

Since the discovery of MEP pathway, the enzymes leading to IPP and DMAPP have been gradually identified (Eisenreich *et al.*, 2001). As shown in Figure 1, the MEP pathway starts with condensation of pyruvate and G3P to 1-deoxy-D-xylulose 5-phosphate (DXP) by the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS). DXP is then isomerized to the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by DXP reductoisomerase (DXR or IspC) (Takahashi *et al.*, 1998). MEP is converted by IspD to 4-diphosphocytidyl-2-*C*-methylerythritol (CDP-ME), followed by phosphorylation by IspE to form 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate (CDP-MEP), which is then cyclized by IspF to form 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP). Following reductive MEcPP ring opening by IspG to produce 4-hydroxy-3-methyl-butenyl 1-diphosphate (HMBPP), IspH catalyses the formation of IPP and DMAPP. Unlike eukaryotic MVA pathway, where IPP is isomerized to DMAPP by IPP:DMAPP isomerase (IDI), IDI is not essential for many organisms with MEP pathway (Chang *et al.*, 2013).

With recent advances in understanding MEP pathway regulations, it is clear that several steps of MEP pathway are subject to different degrees of regulations (Banerjee and Sharkey, 2014; Cordoba *et al.*, 2009). Some regulatory steps are of particular interests in guiding metabolic engineering efforts (Figure 1). Accumulating evidence indicates that DXS controls fluxes towards

MEP pathway (Estevez *et al.*, 2001; Ghirardo *et al.*, 2014; Wright *et al.*, 2014) and is negatively regulated by IPP and DMAPP (Banerjee *et al.*, 2013). The direct feedback regulation prevents the accumulation of the IPP and DMAPP at high levels and requires an efficient downstream pathway to increase MEP flux. In addition, MEcPP was found to serve as a stress signal to modulate the expression of nuclear-encoded stress response genes targeted for chloroplasts in plants (Xiao *et al.*, 2012). MEcPP efflux thus could potentially limit terpene yield (Zhou *et al.*, 2012). These regulatory steps reveal the flux imbalance among the MEP and downstream terpene pathways, which could potentially be optimized through controlling gene expressions.

To minimize the regulations and increase MEP carbon flux, the first step is to identify and overcome key metabolic bottlenecks in the MEP pathway. Earlier studies of MEP-derived terpenes mainly focused on carotenoids production in the heterotrophic system such as *E. coli* (Barkovich and Liao, 2001). Microarray work on *E. coli* showed that most of MEP genes with the exception of *IspG* are expressed at low levels, and *dxs*, *ispD*, *ispF* and *ispE* genes were found to be rate limiting (Wei *et al.*, 2001). Through chromosomal promoter replacement of these key bottleneck genes, high titres of β-carotene (6 mg/g dry cell weight) was achieved in *E. coli* (Yuan *et al.*, 2006). Coupling an optimized MEP pathway with different terpene synthases, various terpenoids can be produced in *E. coli* or yeast system in reasonable amounts. These efforts have been extensively reviewed (Kirby and Keasling, 2009). While most of these earlier studies sought to relieve these pathway bottlenecks through gene overexpression, the studies neglected the effect of pathway imbalance and toxic intermediate accumulation. A recent study on MEP-based taxadiene production in *E. coli* identified the production of indole as a by-product along with taxadiene. Although the interaction between indole and MEP is still obscure, the indole accumulation is toxic to cells. A multivariate pathway optimization through coarse adjustment of expression levels between MEP and downstream taxadiene pathway generated a strain that can mitigate the indole toxicity and accumulate taxadiene at 1 g/L (Ajikumar *et al.*, 2010).

Most of pathway engineering efforts were carried out in heterotrophic micro-organisms, and the translation into photosynthetic systems is much more challenging to achieve a comparable yield. DXS but not DXR was suggested to be the rate-limiting step for terpenoid biosynthesis in the cyanobacterium *Synechococcus leopoliensis* (Miller *et al.*, 2000). Recently, limonene production was demonstrated in the cyanobacterium *Anabaena* sp. PCC 7120 by overexpressing the Sitka spruce limonene synthase (LS) along with three potential bottleneck genes (*dxs-ipphp-gpps*). Under optimal conditions, the maximum achievable yield was 172.7 ± 16.9 μg limonene/L 48 h (Halfmann *et al.*, 2014). Similarly, another monoterpene β-phellandrene was produced under various conditions in the cyanobacterium *Synechocystis* with the highest titre of ~250 μg/g gcw 48 h (Formighieri and Melis, 2014b). Metabolic engineering in eukaryotic microalgae to produce terpenes did not turn out be fruitful either. Production of carotenoids in the green alga *Chlamydomonas reinhardtii* has been accomplished by engineering two phytoene synthase genes (*psy*) from *Dunaliella salina* and *Haemotococcus pluvialis* leading to a 2.6- and 2.2-fold increase of carotenoids (Couso *et al.*, 2011; Gimpel *et al.*, 2013). Our work in engineering *C. reinhardtii* by expressing a rice limonene synthase yielded <10 μg of limonene (R.D.S., X.W., Y.K., H.C., S.Y.D, and J.S.Y.). The low terpene yield in photosynthetic organisms could potentially attribute to a more complex cell regulatory mechanism related to terpene pathways. It is essential to further understand MEP pathway regulation to optimize the design.

To minimize the influence of the MEP pathway regulation, an alternative strategy is to introduce a heterologous pathway for terpene production. For example, most bacteria only contain MEP but not the MVA pathway. A yeast MVA pathway was introduced into *E. coli*, leading to a significant increase in amorphadiene, the sesquiterpene olefin precursor to the antimalarial drug artemisinin (Martin *et al.*, 2003). The approach laid down the foundation for other terpene molecule productions in *E. coli* including producing isoprene at the rate of 2 g/L/h in glucose fed-batch reactors using an engineered strain (Whited *et al.*, 2010). Although an exogenous MVA pathway in *E. coli* was proved to be successful, the terpene yield depends heavily on the nature of terpene molecules and their terpene synthase (TPS) activities. The highest levels for pinene synthesis achieved 32 mg/L in an engineered *E. coli*, where three pinene synthases (PS) and three geranyl diphosphate synthases (GPS) genes were combinatorially introduced (Sarria *et al.*, 2014). Limonene was also produced in *E. coli* through an exogenous MVA pathway, where a titre of over 400 mg/L was achieved (Alonso-Gutierrez *et al.*, 2013).

Similar strategies have also been applied to photosynthetic organisms to increase terpene yield. However, it is proved to be far more challenging to achieve similar performance as those in heterotrophic systems (Table 1). An exogenous MVA pathway was introduced into the cyanobacterium *Synechocystis* PCC 6803, coupled with the heterologous expression of an isoprene synthase, in which the isoprene yield only reached 250 μg/g gcw (Bentley *et al.*, 2014). A possible reason for the low terpene yield through the MVA pathway is that acetyl-CoA pool in photosynthetic organisms is low under photosynthetic conditions (Lan and Liao, 2012). Compared to cyanobacteria, the plant and algae MEP pathway enzymes are compartmentalized in chloroplasts. The compartmentalization might supply additional opportunities to integrate photosynthesis with MEP to achieve higher terpene yield. One such example is the green microalgae *Botryococcus braunii,* which can accumulate hydrocarbon up to 75% of its dry weight (Banerjee *et al.*, 2002). The further exploration of the pathway regulation and photosynthesis integration might help guide the design of an efficient photosynthetic terpene platform.

Enzyme manipulations in improving terpene production —increasing pathway efficiency

Enzyme catalysis is the basis for pathway efficiency, in particular, for overcoming metabolic bottlenecks. Several approaches have been used to improve terpene production including the selection of high-performance enzymes, enzyme improvement by engineering, construction of synthetic enzyme complexes to achieve substrate channelling and compartmentation of enzymes and pathways.

Whereas the mechanisms of most enzymes in MEP pathway have been illustrated and extensively summarized in a few recent reviews (Chang *et al.*, 2013; Zhao *et al.*, 2013), the mechanistic models of IspG and IspH are still unclear. IspG and IspH are believed to be iron–sulphur proteins (Wolff *et al.*, 2003; Zhao *et al.*, 2013). As these two steps catalysed by IspG and IspH in MEP pathway are reductive reactions, an efficient reduction system usually also leads to better enzyme activity (Xiao *et al.*, 2009). Importantly, HMBPP was reductively dehydrated by IspH to

IPP and DMAPP in a ratio of 5 : 1 (Rohdich *et al.*, 2002). The engineering of heterologous IDI thus presents a unique chance for balancing the ratio of IPP and DMAPP. Indeed, many studies have shown the positive effect of IDI engineering in improving final terpene yield (Sun *et al.*, 1998). Besides IDI, the choice of other enzymes for both MEP and downstream pathways is also important. Evolutionally speaking, terpene biosynthesis is heavily involved in plant defence against insects and pathogen and thus evolved rapidly with diverse product profile for enzymes (Yuan *et al.*, 2008a, 2009). It is therefore important to choose the enzyme with the right product and higher efficiency for engineering photosynthetic terpene production. In particular, previous researches have established several enzymes including limonene synthase and bisabolene synthase to be widely used for engineering microbes to produce terpenes (Alonso-Gutierrez *et al.*, 2013; Davies *et al.*, 2014; Hyatt *et al.*, 2007).

In addition to the enzyme selection, enzyme engineering is another approach to generate biocatalysts for improving terpene yield. Many downstream terpene synthases (TPS) are known to be of low efficiency, and extensive efforts have been invested in enzyme engineering through both rational design and directed evolution. Regardless, engineering of TPS and MEP enzymes has been highly challenging. Through rational design, TPS and penyltransferases have been engineered in terms of reaction pathway, thermostability, product and substrate specificity (Kampranis *et al.*, 2007; Kang *et al.*, 2014; Yoshikuni *et al.*, 2006a,b). However, most of the studies were focused on understanding structure–function relationship, perhaps explaining why there has been little progress towards increased enzyme activity and end product yield (Gao *et al.*, 2012). Directed evolution has been hampered by the lack of high-throughput product assays. A recent development in the high-throughput assay for terpene synthase has enabled broader applications in directed evolution of terpene biosynthesis (Furubayashi *et al.*, 2014; Lauchli *et al.*, 2013). In their system, various terpenes can be indirectly measured through the decrease of carotenoid pigments, in that these targeted terpene molecules will compete with carotenoid for precursor consumptions. The enhanced terpene synthase can thus be identified by their capacity to compete with carotenoid pathways. In addition, an evolutionarily based algorithm has been developed to redesign TPS and MVA pathway enzymes based on the so-called plasticity residues (Yoshikuni *et al.*, 2006a). The approach has led to enzymes with better specificity and activities, eventually to approximate 1000-fold increase in productivity (Yoshikuni *et al.*, 2008). Enzyme engineering of TPS and key MEP enzymes for higher catalysis represents a key challenge for the future engineering of photosynthetic terpene production.

An alternative approach to increase carbon flux towards terpene is the synthetic design of enzyme complexes to enable the substrate channelling. The protein scaffoldin presents the opportunity to design enzyme complexes based on interacting protein domains. For example, the cellulosome scaffoldin can tether multiple enzymes of the same pathway (You and Zhang, 2013). The designed protein complex may facilitate substrate channelling to improve reaction pathway efficiency and carbon flux. The proof of concept for a synthetic enzyme complex to improve pathway efficiency has been established for the MVA pathway (Dueber *et al.*, 2009). A recent study applied a similar approach to fuse pinene synthase and GPPS into a protein complex to achieve better yield of pinene, a biofuel precursor (Sarria *et al.*, 2014). The approach can be further adapted to the design of synthetic enzyme complexes for MEP pathway and downstream terpene biosynthesis to further increase photosynthetic terpene production.

Lastly, terpene biosynthesis enzymes and pathways can also be compartmentalized into organelles as a strategy to improve product yield by increasing local enzyme concentrations, mitigating cell regulations and potentially eliminating downstream competing pathways in its original compartment. One of the advantages in direct plant engineering for terpene production is the existence of two independent terpene production pathways compartmented into different cellular locations. MVA and MEP pathways in plants are compartmentalized to produce specific sets of terpene molecules. The cytosolic MVA pathway predominantly produces C_{15}- and C_{30}-derived terpenes such as sterols, whereas C_{10}-derived terpenes (monoterpene, diterpene) are produced from plastidial MEP pathway (Lichtenthaler, 1999; Wu *et al.*, 2006). Through redirecting C_5 intermediates IPP and DMAPP to exogenous terpenes pools by introducing specific TPS, plants were able to accumulate high level of terpene molecules (Wu *et al.*, 2006). The success in terpene accumulation lies in the division of labour between MEP and MVA, their relative independency (Bouvier *et al.*, 2005; Lange *et al.*, 1998) and the potential absence of downstream pathways for the introduced terpene molecules in ectopic compartments. Overall, various strategies can be integrated to overcome the metabolic bottlenecks and improve the overall terpene pathway efficiency.

Storage and secretion of end products—enhancing sink capacity

Besides the enzyme and pathway optimization, another important consideration for engineering photosynthetic terpene production is the compartmentation and storage of targeted biofuel molecules. It is well known that insufficient sink capacity can result in the feedback down-regulation of photosynthetic capacity (Evans, 2013; Melis, 2012). Installing an efficient storage or excretion strategy thus may help mitigate the challenges presented above. In plant system, terpenes can be stored in special plant structures including glandular trichomes, sheath cells and vascular tissues (Aziz *et al.*, 2005; Besser *et al.*, 2009; Xie *et al.*, 2008). Cyanobacteria readily secrete various fuel molecules. To facilitate fuel secretion, membrane-embedded ABC transporters could also be used to secrete triterpene and tetraterpene in cyanobacteria (Doshi *et al.*, 2013). In other studies, improving triacylglycerol (TAG) storage by engineering lipid droplets has synergistically increased both lipid production and carbon assimilation (Vanhercke *et al.*, 2013; Winichayakul *et al.*, 2013). Enhancing terpene storage or secretion thus will be particularly important to further increase yield, when metabolic engineering strategies achieve their limit.

Concluding remarks and perspectives

The MEP-derived photosynthetic terpene biosynthesis is achieved through a multimodule photosynthesis–MEP–terpene synthesis (PMT) pathway. When overcoming the aforementioned technical barriers, it is pivotal to integrate these different modules and induce a balanced gene expression in order to reduce cell toxicity and produce terpenes more efficiently. With the emergence of various synthetic biology tools, a systematically optimized PMT pathway and downstream storage could be engineered to achieve high levels of terpenes in photosynthetic systems. Multiplex automated genome engineering (MAGE) is an approach based on accelerated evolution and thus can screen

for potential mutants with optimized phenotypes (Wang *et al.*, 2009). In their study, 24 genes related to lycopene production were simultaneously optimized to maximize lycopene production. High lycopene-producing strain can be screened based on colour density of colonies in the mutant library. However, a high-throughput method would be necessary for screening other terpene-producing strains. Recently, a microfluidic high-throughput system was applied in screening potential high xylose-consuming yeast cells, an important trait in lignocellulosic biomass consumption (Wang *et al.*, 2014). In their system, single mutant cells were encapsulated into an oil droplet and cultured for a certain period of time. After coalescence with another oil droplet containing fluorescent enzymatic assay reagents, the droplet fluorescence was measured. With this system, a mutant cell with high xylose-consuming ability was isolated in a matter of hours. Provided with a terpene detection method, it is feasible to develop a microfluidic high-throughput platform to screen high terpene-producing cells or strains.

With advances in both technique development and pathway understanding, we could achieve a significant improvement in photosynthetic terpene yields. It is also important to simultaneously explore various photosynthetic systems (plant, algae and cyanobacteria) for terpene productions. In the meantime, an alternative metabolic design involving network biology theory should be considered for future manipulation in terpene and other pathways. The discovery of the power-law flux distribution showed that cells are dominated by certain 'high-flux backbone (HFB)' reactions (Almaas *et al.*, 2004; Jeong *et al.*, 2000). To produce high titre of desired chemicals, the utilization of high-flux metabolic pathways are particularly desired. A few examples have demonstrated the theory unintentionally. An synthetic pathway was engineered into both *E. coli* and *Synechococcus elongatus* PCC 7942 to produce high titre of higher alcohols, in which highly active amino acid pathways were utilized to achieve high yield (Atsumi *et al.*, 2008, 2009). Recently, an ethylene-forming enzyme was engineered into *Synechocystis* sp. PCC 6803. Through utilizing the active TCA pathway, a record ethylene yield was achieved (Ungerer *et al.*, 2012). However, these high-flux pathways are usually primary metabolic pathways leading to cell biomass accumulation. With an improved knowledge on terpene biosynthesis regulations, photosynthate partition and model high terpene-producing systems like *B. braunii*, it is possible to either redesign the PMT pathway to achieve high fluxes or connect the dots between primary and secondary metabolic pathways to enhance terpene production for fuels and chemicals.

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Conflict of interest

The authors declare no conflict of interests.

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