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REVIEW

Toward a photosynthetic microbial platform for terpenoid engineering

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Abstract Plant terpenoids are among the most diverse group of naturally-occurring organic compounds known, and several are used in contemporary consumer products. Terpene synthase enzymes catalyze complex rearrangements of carbon skeleton precursors to yield thousands of unique chemical structures that range in size from the simplest five carbon isoprene unit to the long polymers of rubber. Such chemical diversity has established plant terpenoids as valuable commodity chemicals with applications in the pharmaceutical, neutraceutical, cosmetic, and food industries. More recently, terpenoids have received attention as a renewable alternative to petroleum-derived fuels and as the building blocks of synthetic biopolymers. However, the current plant- and petrochemical-based supplies of commodity terpenoids have major limitations. Photosynthetic microorganisms provide an opportunity to generate terpenoids in a renewable manner, employing a single consolidated host organism that is able to use solar energy, H_2O and CO_2 as the primary inputs for terpenoid biosynthesis. Advances in synthetic biology have seen important breakthroughs in microbial terpenoid engineering, traditionally via fermentative pathways in yeast and *Escherichia coli*. This review draws on the knowledge obtained from heterotrophic microbial engineering to propose strategies for the development of microbial photosynthetic platforms for industrial terpenoid production. The importance of utilizing the wealth of genetic information provided by nature to unravel the regulatory mechanisms of terpenoid biosynthesis is highlighted.

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Abbreviations

DMAPP	Dimethylallyl pyrophosphate
DXP	1-Deoxy-D-xylulose 5-phosphate
DXR	1-Deoxy-D-xylulose 5-phosphate reductase
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
FPP	Farnesyl pyrophosphate
GAP	Glyceraldehyde 3-phosphate
GGPP	Geranylgeranyl pyrophosphate
GPP	Geranyl pyrophosphate
IDI	Isopentenyl diphosphate isomerase
IPP	Isopentenyl pyrophosphate
LIMS	Limonene synthase
MEP	Methyl-D-erythritol 4-phosphate
MVA	Mevalonate
PTM	Post translational modification
TPS	Terpene synthase
SQS	Squalene synthase

Terpenoids: a natural array of chemical diversity

Terpenoids are a large family of structurally and functionally diverse organic compounds, synthesized by all free-living organisms, but predominantly by plants as secondary metabolites. Over 55,000 different terpenoids have been isolated (Breitmaier 2006), and this number will likely increase over the coming years as bioprospectors mine this valuable chemical resource for novel bioactive compounds. The universal building blocks of all terpenoids

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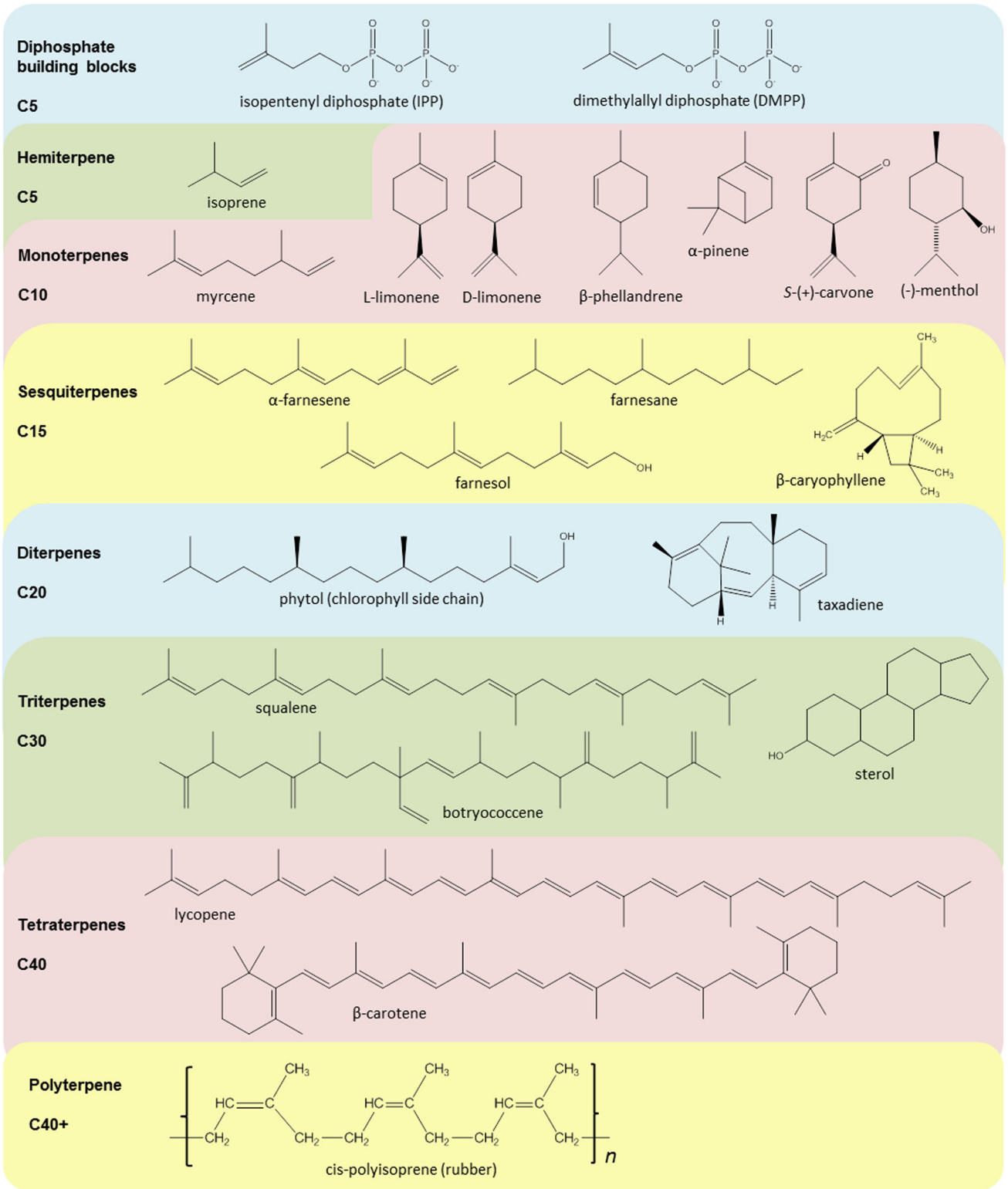

Diphosphate building blocks
C5
isopentenyl diphosphate (IPP)
dimethylallyl diphosphate (DMPP)
Hemiterpene
C5
isoprene
Monoterpenes
C10
myrcene
L-limonene
D-limonene
β-phellandrene
α-pinene
S-(+)-carvone
(-)-menthol
Sesquiterpenes
C15
α-farnesene
farnesane
farnesol
β-caryophyllene
Diterpenes
C20
phytol (chlorophyll side chain)
taxadiene
Triterpenes
C30
squalene
botryococcene
sterol
Tetraterpenes
C40
lycopene
β-carotene
Polyterpene
C40+
cis-polyisoprene (rubber)

◀**Fig. 1** Representative structural diversity of natural plant terpenoids. All terpenoids are derived from IPP and DMAPP building blocks, and classified based on the number of five-carbon isoprene units they contain: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40); polyterpenes (larger chain length). Functional diversity of terpenoids is created by differences in chain length, as well as structural rearrangements that include degree of saturation (compare α-farnesene and.farnesane), isomerization (compare L-limonene and D-limonene), and the addition of functional moieties [hydroxyl group on (−)-menthol, farnesol, phytol, and sterol; ketone group on S-(+)-carvone]

are the five-carbon branched unsaturated pyrophosphate isomers, isopentenyl pyrophosphate (IPP), and dimethylallyl pyrophosphate (DMAPP) (Fig. 1). Isoprene (C_5H_8) is the simplest terpene, and directly synthesized from DMAPP by means of pyrophosphate elimination, whereas longer chained terpenes are derived from the stepwise addition of IPP units to a DMAPP precursor according to the "biogeneic isoprene rule" (Ruzicka 1953). Consequently, terpenes differ in chain length by multiples of five-carbon isoprene units and are classified accordingly: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40), and polyterpenes (larger chain length) (Fig. 1). Rubber (cis-1,4-polyisoprene) is the longest chained terpenoid and may be comprised of over 100,000 isoprene units. Functional diversity of the terpenoid family is not only enabled by variation in chain length, but also by degree of saturation and structural rearrangements of the carbon skeleton that include linearization, cyclization, isomerization, and branching (Thulasiram et al. 2007), as illustrated in Fig. 1. Modification though the addition of functional moieties, such as hydroxyl, carbonyl, ketone, aldehyde, and peroxide groups provides further functional diversity in some instances (Fig. 1). To highlight the correct nomenclature, terpenes are hydrocarbon molecules, while terpenoids (or isoprenoids) are terpenes that have been modified through the addition of functional groups, although these are often used interchangeably in the literature.

An incredibly diverse array of plant terpenoids has evolved to perform an equally diverse range of biological functions. Terpenoids have essential roles in primary photosynthetic metabolism, including light harvesting (phytol tail of chlorophyll), electron transfer (plastoquinone), and photoprotection (carotenoids), but also in respiration (ubiquinone), regulation of membrane structure and fluidity (sterols), and developmental regulation (phytohormones). The majority of plant terpenoids, however, are secondary metabolites synthesized in specialized secretory structures with important roles in reproduction, defense, and stress responses (Langenheim 1994). The sweet-smelling volatile terpenoids emitted from floral tissue, and the brightly colored orange/red carotenoid pigmentation of flowers and fruits attract pollinators and seed-dispersing animals. Pine and conifer trees release a toxic mixture of terpenoids that act as a deterrent to attacking pathogens or herbivores, and subsequently harden to a resin that forms a mechanical barrier to protect wound sites. The rubber tree (*Hevea brasiliensis*) adopts a defensive strategy through the secretion of latex, which contains cis-1,4-polyisoprene (Puskas et al. 2006). Other terpenoids are synthesized in response to abiotic stresses. Isoprene is emitted from a variety of herbaceous, deciduous, and conifer plants during episodes of elevated temperatures and is thought to play a role in thermotolerance by enhancing membrane stability or as a mechanism to remove excess cellular carbon (Behnke et al. 2007; Sasaki et al. 2007; Sharkey and Singsaas 1995; Sharkey and Yeh 2001; Singsaas et al. 1997). From such functional diversity, it is clear that plants rely extensively on terpenoid metabolism for a variety of physiological functions.

Harnessing natural terpenoid diversity for industrial applications

The natural chemical diversity of the terpenoid family has led to the identification of many terpenoids suitable for commercial applications. Plant terpenoids are used in diverse markets as flavoring agents, fragrances, disinfectants, agrochemicals, pharmaceuticals, and nutraceuticals (Ajikumar et al. 2008; Bohlmann and Keeling 2008). The monoterpenoid menthol, for example, is used as a flavor enhancer and preservative in the food industry, an analgesic and antibacterial in the pharmaceutical industry, and as a cooling agent in tobacco products (Kamatou et al. 2013). Very slight changes in atomic configurations can result in different physical or chemical characteristics, such as the different odors of limonene enantiomers (D-limonene has a lemon-orange smell, while L-limonene has a fragrance similar to turpentine). Novel functions of plant terpenoids have also been discovered, such as the anticancer properties of taxol, which is isolated from the bark of the Pacific yew (*Taxus brevifolia*), and the antimalarial function of artemisinin, a sesquiterpenoid isolated from sweet wormwood (*Artemisia annua*) (Ajikumar et al. 2008). Most recently, attention has shifted to the potential use of terpenoids as renewable biofuels. Terpene hydrocarbons, in particular, are attractive for fuel applications as they have a greater energy density and lower hygroscopicity than short chain alcohols, and the structural diversity to mimic the alkanes and aromatics of desired chain lengths for gasoline, diesel, and jet fuels. Importantly, this means that terpenes can be blended with petroleum-based fuels,

and are compatible with existing transport fuel infrastructure. Exciting commercial opportunities also exist for harnessing terpenoids as the building blocks of biopolymers (Byrne et al. 2004; Firdaus et al. 2011; Kobayashi et al. 2009; Wilbon et al. 2013) to provide a renewable alternative to petroleum-derived plastics and polymers.

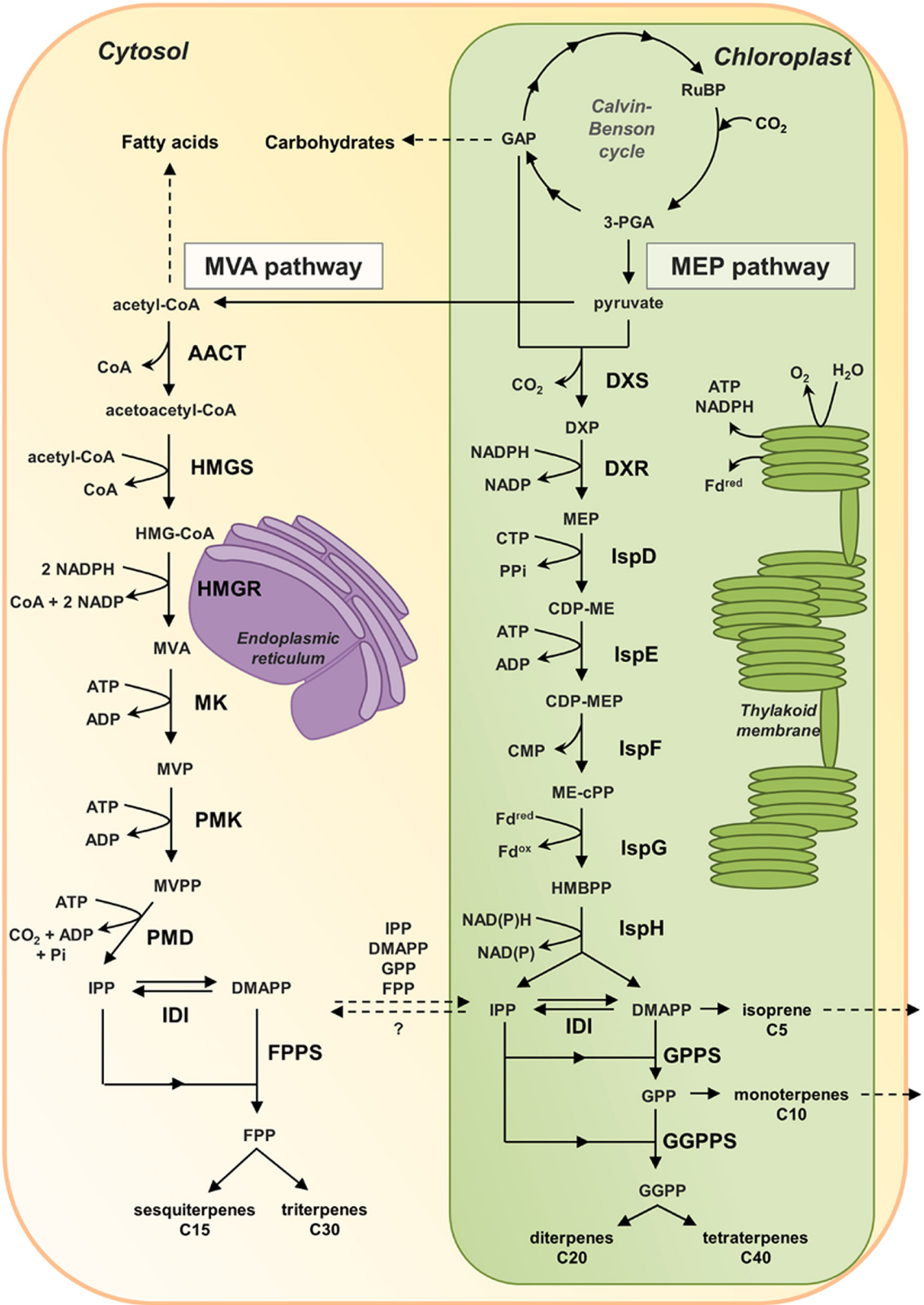
High-value natural terpenoids of plant origin are typically present in very low abundance (Kesselmeier and Staudt 1999). Taxol, for example, when extracted from the bark of the Pacific Yew constitutes only between 0.01 and 0.02 % of bark dry weight (Hezari et al. 1995). Therefore, it is commercially prohibitive to harvest terpenoids from plant tissue due to the large natural resources required to obtain sufficient quantities and the associated ecological and environmental impacts. Some terpenoids are naturally produced in relatively large quantities, including isoprene, which is emitted from terrestrial foliage and accounts for one-third of the annual global emissions of volatile organic compounds from both natural and anthropogenic sources (500–750 Tg isoprene $year^{-1}$) (Guenther et al. 2006, 2012); however, this global emission is dilute and not practical to harvest. Monoterpenes that are currently harvested from plants for commercial use include limonene and pinene, which are extracted from citrus rind oils and gum turpentine, respectively, by energy-intensive distillation procedures. Natural rubber latex from the Para rubber tree (*Hevea brasiliensis*) is another example, providing 40–45 % of the world's rubber supply. However, this resource is under threat by the South American leaf blight and so alternative sources of rubber are being investigated (van Beillen and Poirier 2007), including the Russian dandelion, which was propagated as a source of latex in Germany, Russia, and America during World War II when natural rubber was in short supply. Synthetically derived rubber currently fills the remainder of the world's rubber supply and is generated nonrenewably by polymerization of petroleum-derived monomers. The chemical synthesis of terpenoids is extremely difficult due to their structural complexity and requires a large amount of energy. Properties of synthetically-derived terpenoids can also vary significantly from the natural product, such as the scent of synthetic menthol, which is negatively influenced by reaction contaminants.

Clearly, plant terpenoid products are heavily integrated in our lives, yet the current plant- and petrochemical-based supplies have major limitations. Microbial biosynthesis presents an opportunity for a more reliable, intensive, and energy-efficient method of terpenoid production in a renewable manner. Some photosynthetic microorganisms are fast growing, for example, the marine cyanobacterium *Synechococcus* sp. PCC 7002 has a doubling time of 2–3 h under optimal conditions (Ludwig and Bryant 2012) and, along with many other species of cyanobacteria, is naturally transformable (Eaton-Rye 2011; Frigaard et al. 2004). Marine photosynthetic microbes are particularly suited to industrial-scale culturing due to their growth in salt water, the most abundant water resource on earth. Furthermore, photoautotrophic microorganisms offer the advantage of photosynthetic terpenoid production, using CO_2 as the only carbon source and sunlight for energy. This eliminates the energy-intensive cultivation and administration of an exogenous carbohydrate feedstock, which is required by strictly heterotrophic microorganisms, and provides a more streamlined route for the solar-to-biocommodity generation process. Advances in synthetic biology over the past decade have heightened the reality of using heterotrophic microbial-platforms for the production of commodity chemicals at an industrial scale (Keasling 2012). The challenge now stands to achieve similar advances with a photoautorophic microbial-platform.

Fig. 2 Subcellular compartmentalization of the MVA and MEP pathways in the plant cell. The MVA pathway generates IPP/DMAPP for the synthesis of sesquiterpenes and triterpenes in the cytosol, whereas the plastidial MEP pathway makes IPP/DMAPP for the synthesis of isoprene, monoterpenes, diterpenes, and tetraterpenes. The photosynthetic machinery in the thylakoid membrane of the chloroplast generates ATP, NADPH, and reduced ferredoxin (Fd^{red}) cofactors that are used by the MEP pathway. Abbreviated MVA pathway enzymes are: *AACT* acetoacetyl-CoA thiolase, *HMGS* HMG-CoA synthase, *HMGR* HMG-CoA reductase, *MK* mevalonate kinase, *PMK* mevalonate 5-phosphate kinase, *PMD* mevalonate 5-pyrophosphate decarboxylase, and *IDI* IPP isomerase. Abbreviated MEP pathway enzymes are: *DXS* 1-deoxy-D-xylulose 5-phosphate synthase, and *DXR* 1-deoxy-D-xylulose 5-phosphate reductase

Terpenoid biosynthetic pathways: origins and mechanisms

Terpenoids are essential to cellular function, and two different enzymatic pathways have evolved to generate DMAPP and IPP, the universal terpenoid precursors. The first pathway to be completely described was the mevalonate (MVA) pathway (McGarvey and Croteau 1995; Lombard and Moreira 2011). For decades, this pathway was thought to be the only terpenoid biosynthetic pathway, until a second pathway was discovered and named the methyl-D-erythritol 4-phosphate (MEP) pathway (Lichtenthaler 1999; Rohmer et al. 1993; Zhao et al. 2013). The MVA pathway is of archael/eukaryotic origin, and the MEP pathway is of prokaryotic bacterial origin, although there is plenty of evidence to support a shuffling of the two pathways between kingdoms. MVA-pathway genes generally cluster into one or two operons (as opposed to the MEP pathway genes that are typically scattered across a genome), which has supported mobility and transfer of the entire MVA pathway to some bacteria. In some cases, the


Cytosol
Chloroplast
Calvin-Benson cycle
RuBP
CO2
GAP
3-PGA
Fatty acids
Carbohydrates
MVA pathway
MEP pathway
acetyl-CoA
pyruvate
CoA
AACT
acetoacetyl-CoA
acetyl-CoA
CoA
HMGS
HMG-CoA
2 NADPH
CoA + 2 NADP
HMGR
Endoplasmic reticulum
MVA
ATP
ADP
MK
MVP
ATP
ADP
PMK
MVPP
ATP
CO2 + ADP + Pi
PMD
IPP
DMAPP
IDI
FPPS
FPP
sesquiterpenes C15
triterpenes C30
CO2
DXS
DXP
NADPH
NADP
DXR
MEP
CTP
PPi
IspD
CDP-ME
ATP
ADP
IspE
CDP-MEP
CMP
IspF
ME-cPP
Fdred
Fdox
IspG
HMBPP
NAD(P)H
NAD(P)
IspH
IPP
DMAPP
GPP
FPP
?
IPP
DMAPP
IDI
isoprene C5
GPPS
GPP
monoterpenes C10
GGPPS
GGPP
diterpenes C20
tetraterpenes C40
ATP
NADPH
Fdred
O2
H2O
Thylakoid membrane

acquired MVA pathway has even displaced the ancestral MEP pathway. Conversely, the bacterial MEP pathway has been acquired by photosynthetic eukaryotic organisms via cyanobacterial endosymbiosis, with the MEP pathway genes subsequently being transferred to the nuclear genome (Lange et al. 2000; Lichtenthaler 1999).

The MVA pathway is the only terpenoid pathway found in archaea, animals, and fungi, and operates exclusively in the cytosol. Plants and algae also utilize the ancestral cytosolic MVA pathway but additionally have the bacterial MEP pathway for terpenoid production in the plastid. Interestingly, many unicellular algae have lost the MVA pathway after acquiring the MEP pathway, and now solely use the MEP pathway to provide isoprenoids for the cytosol and plastid (such as the green alga *Chlamydomonas*, and the stramenoplie *Nannochloropsis* sp.). Bacteria have either or both pathways, as some organisms acquired the MVA pathway and in some instances lost the ancestral MEP pathway. Because a number of human pathogens utilize the MEP pathway, extensive research has focused on drug development to inhibit this pathway (Hale et al. 2012).

Although the two terpenoid pathways synthesize identical end-products (IPP and DMAPP), the primary feedstock molecules are different: the MVA pathway utilizes acetyl-CoA (Miziorko 2011), whereas the MEP pathway requires glyceraldehyde 3-phosphate (GAP) and pyruvate (Lichtenthaler 1999; Rohmer et al. 1996). The enzymatic steps for each pathway are illustrated in Fig. 2. The MVA pathway begins with the condensation of two molecules of acetyl-CoA to produce acetoacetyl-CoA, followed by condensation of a third acetyl-CoA to form 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) and a reduction step to produce MVA. Two rounds of ATP phosphorylation give mevalonate 5-diphosphate, which is phosphorylated a third time and then decarboxylated to form IPP. The interconversion between IPP and DMAPP is catalyzed by an isopentenyl diphosphate isomerase (IDI). In comparison, the MEP pathway begins with the condensation of GAP and pyruvate to form 1-deoxy-D-xylulose 5-phosphate (DXP), which undergoes a reductive isomerization to MEP, and then a coupling with CTP to form methylerythritol cytidyl diphosphate (CDP-ME). Subsequent phosphorylation, cyclization, and reductive dehydration steps generate 4-hydroxy 3-methyl-butenyl 1-phosphate (HMBPP). HMBPP can be converted to either IPP or DMAPP by a further reductive dehydration step, which is why IDI is not essential for the MEP pathway to function, although it is used to balance the IPP:DMAPP ratio. There are two different classes of IDI that are structurally unrelated: IDI-1, which is a Zn^{2+}-dependent metalloprotein (Agranoff et al. 1959, 1960), and IDI-2, which requires reduced flavin mononucleotide (FMN) and Mg^{2+} for activity (Kaneda et al. 2001). Although organisms that are dependent on the MEP pathway do not require IDI activity, both types of IDI have been identified in MEP-dependent bacterial strains (Laupitz et al. 2004).

Downstream terpenoid biosynthesis is the same in all terpenoid-synthesizing organisms, regardless of whether the IPP and DMAPP building blocks were generated from the MVA or the MEP pathways. The process begins with a group of enzymes called prenyltransferases, which catalyze the repetitive addition of active IPP units to a DMAPP precursor by sequential head-to-tail condensation reactions. This process generates linear prenyl pyrophosphate molecules that vary in chain length by 5-carbon units, including C10 geranyl pyrophosphate (GPP), C15 farnesyl pyrophosphate (FPP), and C20 geranylgeranyl pyrophosphate (GGPP). These linear prenyl pyrophosphates of varying chain length are the immediate precursors to terpenes via the action of terpene synthases (GPP is the precursor to monoterpenes, FPP to sesquiterpenes, and GGPP to diterpenes, carotenoids, etc.). Terpene synthase enzymes initiate the structural diversity found among terpenoids by folding the prenyl pyrophosphate substrate within the active site and removing the pyrophosphate group to create highly reactive carbocation intermediates that lead to complex structural rearrangements.

Regulation of terpenoid biosynthesis in natural systems

Unraveling the regulatory mechanisms that control terpenoid biosynthesis via the MVA and MEP pathways is the key to successful bioengineering of microorganisms for terpenoid production. This section describes the current understanding of key regulatory aspects of terpenoid biosynthesis in natural systems, and is followed by a section summarizing efforts to integrate this knowledge into bioengineered microbial systems.

Cellular compartmentalization

Plant cells that contain both the MVA and MEP pathways generate cytosolic and plastidial pools of IPP/DMAPP, and the compartmentalization of terpenoid biosynthesis is thought to create biochemical flexibility. Plants appear to have retained two independent terpenoid biosynthetic pathways to enhance regulatory control of the myriad of terpenoids that must be synthesized in a manner that is tissue specific, developmental stage specific, or in response to environmental cues (Hemmerlin et al. 2012). As a rule of thumb, plastidial IPP/DMAPP from the MEP pathway is used to generate monoterpenoids, diterpenoids, and many of the longer chained products involved with

photosynthetic primary metabolism in the plastid (plastoquinone, carotenoids, and chlorophylls) (Fig. 2). Cytosolic IPP/DMAPP generated from the MVA pathway is used primarily for the synthesis of sesquiterpenoids, as well as diterpenoids and sterols in the cytosol (Fig. 2). The mitochondrian is another subcellular compartment where terpenoids are synthesized using MVA-derived IPP that is imported from the cytosol. There is some debate over the existence of crosstalk between the MEP and MVA pathways in plant cells, which envisions the exchange of intermediate metabolites, such as IPP, DMAPP, GPP, or FPP, between the two pathways across the plastid envelope (Weise et al. 2013).

Gene duplication

Gene duplication is another mechanism known to provide metabolic plasticity through the functional diversification of specific enzymes. Multigene families are commonly associated with genes involved in secondary metabolism. The increased gene copy number may allow greater flux though a pathway, or increase the potential for regulation through differential expression of isoforms among tissues and organelles or in response to environmental stimuli. A multigene family hints that the enzyme may be a key regulatory gatekeeper within a metabolic pathway. The enzyme that catalyzes the first step of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate sythase (DXS), is one such example in the terpenoid biosynthesis family. Land plants have evolved multiple functionally specialized isoforms of DXS. For example, the black cottonwood tree (*Populus trichocarpa*), from the poplar family that emits 2–5 % of assimilated carbon as isoprene (Schnitzler et al. 2010), has five isoforms annotated as DXS in its genome sequence (Tuskan et al. 2006). The DXS isoforms have evolved as three phylogenetically distinct clades (Cordoba et al. 2011) and evidence suggests that DXS1 synthesizes essential terpenoids involved with chloroplast primary metabolism, while DXS2 and DXS3 are required for secondary metabolism (Phillips et al. 2007; Cordoba et al. 2011; Walter et al. 2002). Most species of bacteria, cyanobacteria, and unicellular microalgae have only one copy of DXS in their genome, however, a recent study identified three distinct DXS isoforms in the green microalga *Botryococcus braunii* (race B) (Matsushima et al. 2012). The *Botryococcus* isoforms do not cluster with any of the three land plant DXS clades. However, it is unlikely to be coincidental that the only known green microalga to possess multiple DXS isoforms is also distinguished from other algal strains by an ability to produce the triterpenoid botryococcene, which can comprise 30–40 % of dry cell weight (Metzger et al. 1985; Okada et al. 1995). Clearly, there is selective pressure for organisms that are heavily invested in terpenoid production to duplicate key biosynthetic genes, such as DXS, which may be differentially expressed for superior regulatory control of terpenoid biosynthesis, or participate in the formation of discrete biosynthetic enzyme complexes for dedicated product synthesis. It is now widely accepted that DXS is a key regulatory enzyme in the MEP pathway and that it constitutes a pathway "bottleneck" that will likely need to be manipulated for pathway bioengineering.

Bifunctional fusion proteins

An interesting genetic observation associated with terpenoid metabolism is the presence of a bifunctional IpsDF fusion protein in some bacteria that catalyzes two non-consecutive steps in the MEP pathway (Testa et al. 2006; Gabrielsen et al. 2004; Perez-Gil et al. 2010). In vitro experiments have shown a physical association of the fusion protein with IspE, the enzyme catalyzing the linking reaction between IspD and IspF (Fig. 2) although no substrate channeling between the proteins was observed (Lherbet et al. 2006). The other reported fusion protein is an acetoacetyl-CoA thiolase/HMG-CoA reductase from *Enterococcus faecalis*, which catalyzes non-consecutive steps of the MVA pathway (Hedl et al. 2002).

We recently discovered a third example of a fusion protein associated with terpenoid metabolism; a predicted bifunctional IDI/squalene synthase (IDI/SQS) in the *Nannochloropsis gaditana* nuclear genome (Radakovits et al. 2012; Jinkerson et al. 2013). The IDI/SQS fusion appears to be conserved among photosynthetic heterokonts (stramenopiles) including diatoms, brown algae, and *Aureococcus anophagefferens*, and is also found in the haptophyte *Emiliania huxleyi* and in several dinoflagellates (Fig. 3). Squalene synthase catalyzes the reductive dimerization of two FPP molecules in a head-to-head orientation to form the triterpene squalene, which is an essential precursor of all sterols (Fig. 3). Plants synthesize squalene in the cytosol via the MVA pathway, and the absence of a chloroplast transit peptide sequence associated with the IDI/SQS fusion protein is suggestive of a cytosolic localization. *Nannochloropsis* and several other microalgae utilize the plastidial MEP pathway exclusively for terpenoid biosynthesis, suggesting that IPP and/or DMAPP building blocks are transported across the chloroplast envelope to the cytosol for squalene/sterol biosynthesis. The SQS enzyme associates with the endoplasmic reticulum membrane, where it is anchored by a short C-terminal membrane-spanning domain. It is possible that FPPS physically interacts with the IDI/SQS bifunctional protein to complete the series of reactions necessary for squalene synthesis (Fig. 3). The IDI encoded by the fusion protein appears to function exclusively as a cytosolic IPP

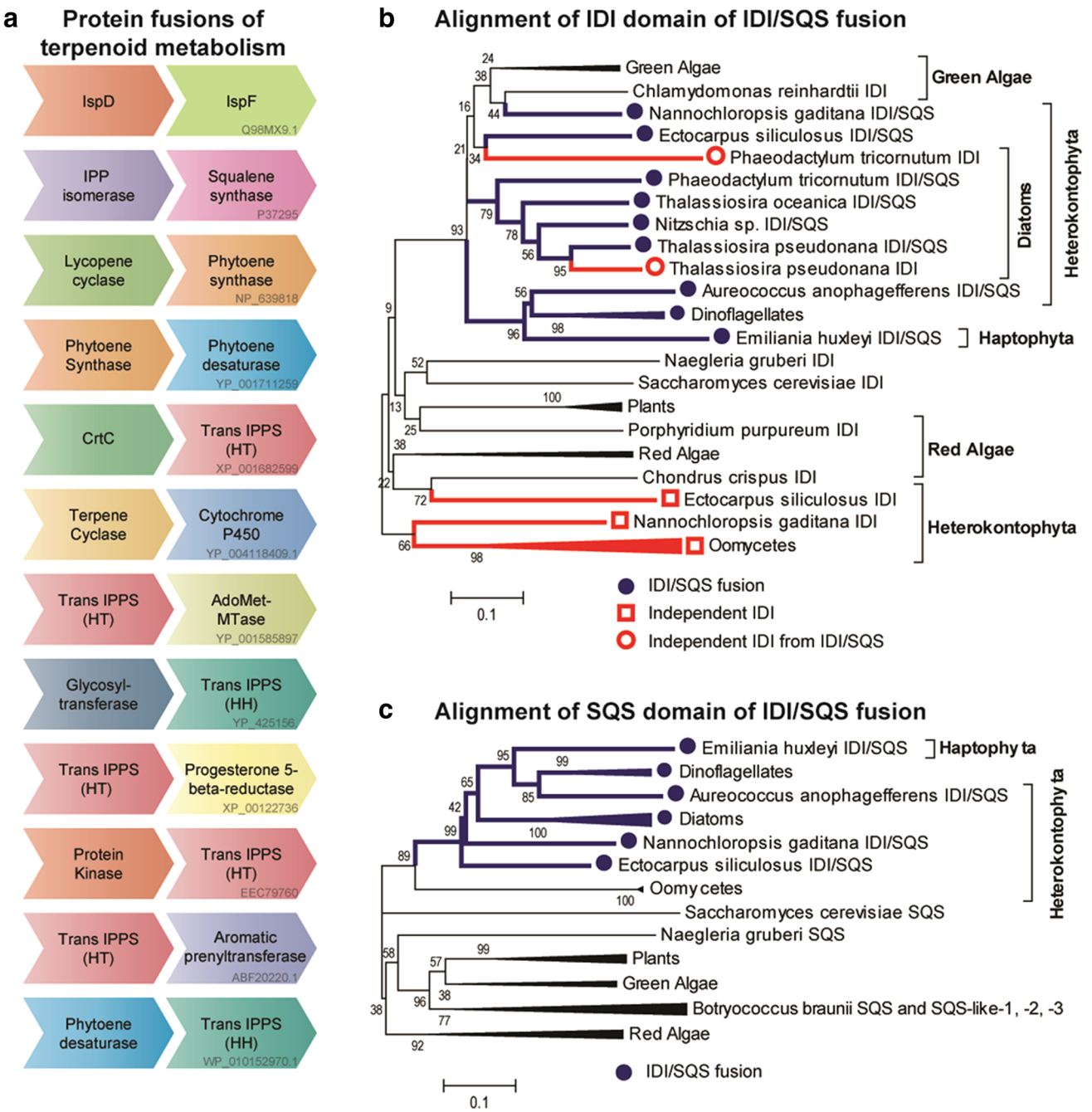


Fig. 3 **a** A variety of fusion proteins involved in terpenoid biosynthesis. The NCBI Accession number of a representative fusion protein is given. Fusion proteins were identified using the Conserved Domain Architecture Retrieval Tool (Geer et al. 2002). **b** Evolutionary relationships of IDI across algal taxa. Only the IDI domain from independent IDI or fusion IDI/SQS was used. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 6.86792575 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 31 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 143 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). Blue entries are IDI from the fused IDI/SQS, while red entries are from independent IDI found in heterokonts. **c** Evolutionary relationships of SQS across algal taxa. Only the SQS domain from independent SQS or fusion IDI/SQS were used. The method used was the same as above except that the analysis involved 26 amino acid sequences and there were a total of 270 positions in the final dataset. The optimal tree with the sum of branch length = 6.26047703 is shown. *Blue* entries are SQS from the fused IDI/SQS

isomerase, because a second IDI with a predicted chloroplast transit peptide is also annotated in the *Nannochloropsis gaditana* draft genome (Radakovits et al. 2012), and is predicted to function in plastidial terpene biosynthesis. Both of the predicted IDI genes encode a Type-1 IDI. Interestingly, the fused IDI proteins are more closely related to IDI enzymes found in green algae, while the independent IDIs found in *N. gaditana*, *Ectocarpus siliceous*, and the nonphotosynthetic oomycetes are more closely related to those in red algae (Fig. 3b). The independent IDI was likely found in early heterokonts but was subsequently lost in some lineages, including several diatoms, *Aureococcus anophagefferens*, and the dinoflagellates *Alexandrium tamarense* and *Lingulodinium polyhedron*, which have only one IDI that is fused to SQS. Interestingly, independent IDI isoforms have reemerged in some diatoms (e.g., *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*) that are paralogs of their respective IDI/SQS enzymes, and are likely the result of independent IDI domain duplication events. SQS domains in Heterokontophyta are all monophyletic (Fig. 3c).

The wide-spread distribution of terpenoid biosynthetic gene fusions (Fig. 3a) across a variety of organisms suggests advantages to these gene arrangements, which may include the control of ratios of terpenoid precursors (IPP, DMAP, FPP, etc.), the streamlined import of terpenoid biosynthetic proteins into eukaryotic organelles that in some cases need to pass through four membranes, and/or a mechanism to allow simultaneous expression of genes within a given metabolic pathway. However, it also brings to attention the concept of metabolically-related enzymes forming multiprotein complexes as a method for enhancing biosynthetic efficiency through close physical interactions that allow substrate channeling. Understanding the functional roles and the evolutionary origins of terpenoid biosynthetic protein fusions may give key insights into the way nature organizes these pathways, which could potentially be exploited to enhance terpenoid production.

Transcriptional control

There are numerous pieces of evidence detailing the transcriptional control of terpenoid biosynthetic genes, and this represents an important level at which terpenoid production is regulated. In the essential oil-rich leaves of *Melaleuca alternifolia* (tea tree), for example, the expression of MEP pathway genes accounts for 87 % of the variation of monoterpene concentrations (Webb et al. 2013). However, the importance of other levels of regulation in terpenoid metabolism is becoming more obvious, especially at the post-translational level. Post-translational modifications usually promote conformational changes that alter enzymatic activity, such as phosphorylation, glycosylation, or ubiquitination. The current understanding of these alternative levels of regulation in terpenoid metabolism is limited; but summarized nicely in a recent review (Hemmerlin 2013). One example in the MEP pathway is the differential phosphorylation of the two DXS isoforms in *Arabidopsis thaliana* (Reiland et al. 2009). The DXS1 isoform has a phosphorylated serine, which is not conserved in the DXS2 isoform, suggesting a mechanism by which the synthesis of terpenoids associated with primary or secondary metabolism may be separated. Posttranslational regulation of DXS was recently described in *Arabidopsis thaliana*, where a J20 protein was found to associate with DXS (Pulido et al. 2013). This interaction is hypothesized to target damaged DXS to the heat shock protein (Hsp70) to induce degradation under stress and down regulate terpenoid biosynthesis, or facilitate proper folding under normal conditions for maintenance of terpenoid metabolism. DXS is considered the gatekeeper of the MEP pathway, and HMG-CoA reductase (HMGR) is the regulatory counterpart in the MVA pathway. The HMGR enzyme, which catalyzes the formation of mevalonate, is the most highly regulated enzyme of the MVA pathway. It is the only known enzyme of both terpenoid biosynthetic pathways that is subject to all levels of regulation: transcriptional, post-transcriptional, translational, and post-translational (Hemmerlin 2013).

Redox regulation

Redox regulation is a form of post-translational regulation that is characteristic of the MEP pathway. The IspG and IspH enzymes contain iron sulfur clusters that must be reduced for enzymatic activity. Both of these enzymes can be reduced by thioredoxin (Balmer et al. 2003; Lemaire et al. 2004), which in turn is reduced by the photosynthetic electron transport chain. Ferredoxin, the final electron acceptor of the photosynthetic electron transport chain, can also donate electrons to IspG, as has been documented in *Arabidopsis thaliana* (Seemann et al. 2006) and cyanobacteria (Okada and Hase 2005). Flavodoxin is thought to be the reduction system used by *Escherichia coli* (Puan et al. 2005). The MEP pathway in photosynthetic organisms is, therefore, strongly linked to photosynthesis. Not only is photosynthesis the source of reducing electrons, but it also provides the carbon-based precursors to the MEP pathway (GAP and pyruvate). Supporting the concept of photosynthetic regulation are studies that show the light-activated emission of isoprene (Sanadze 1969), as well as the incorporation of ^{13}C-labeled CO_2 into isoprene (Sanadze et al. 1972; Ghirardo et al. 2011; Mgalobilishvili et al. 1978). Considering that, a number of terpenoid products from the MEP pathway are essential for photosynthesis (chlorophyll, carotenoids, plastoquinone), it makes sense

that photosynthetic activity, as communicated by the redox status of the plastid, is a key regulatory mechanism.

Bioengineering to increase the IPP/DMAPP terpenoid precursor pool: lessons from the heterotrophic platform

Most efforts in terpenoid bioengineering have employed bacterial or yeast host systems because of their high fermentation rates and ability to utilize sugar feedstocks, which ultimately drives high growth rates and increases terpenoid yields. The production of carotenoids, such as lycopene and β-carotene, has been a major focus because they are precursors to the drugs artemisinin and taxol, and have a bright orange/red color that allows high-throughput screening of colonies to identify those with enhanced terpenoid metabolic flux. These strains can subsequently be used as a platform for the production of other terpenoids. In many cases, the transformation of bacteria or yeast with a single heterologous TPS gene has enabled the use of endogenous IPP/DMAPP pools for the production of non-native terpenoids, including taxadiene (Huang et al. 2001), amorphadiene (Martin et al. 2003; Ro et al. 2006), and limonene (Carter et al. 2003). Other terpenoid products require an ensemble of heterologously-expressed enzymes to catalyze multistep reactions, as required for zeaxanthin, β-carotene, lycopene, and astaxanthin synthesis (Misawa et al. 1990; Miura et al. 1998; Wang et al. 1999). The first successful terpenoid engineering experiments were reported in *E. coli* cells by heterologously expressing a plasmid-based carotenoid biosynthetic pathway, and yielded zeaxanthin, β-carotene, and lycopene at $\sim$2 mg g^{-1} dry cell weight (Misawa et al. 1990). Subsequently, many milestones have been achieved toward increasing terpenoid yield in bacterial and yeast systems and these have been extensively reviewed (Ajikumar et al. 2008; Kirby and Keasling 2009; Immethun et al. 2013). The goal of many terpenoid pathway engineering studies has been to increase the cellular IPP/DMAPP pool size. In *E. coli*, some important advances have included the overexpression of *dxs*, encoding the "gatekeeper" enzyme of the native MEP pathway, and *idi*, the IPP isomerase that regulates cellular IPP/DMAPP ratios (Harker and Bramley 1999; Kajiwara et al. 1997). Similarly, metabolic flux has been improved in the native yeast MVA pathway though overexpression of a truncated form of the highly regulated HMGR, as well as down regulation of the competing sterol biosynthetic pathway through the repression of the native squalene synthase gene (Shimada et al. 1998). An important breakthrough came with the introduction of the entire *Saccharomyces cerevisiae* MVA pathway to *E. coli*, which resulted in a 36-fold yield improvement of artemisinin over the native MEP pathway (Martin et al. 2003). Many studies have subsequently employed this strategy to boost terpenoid yields in *E. coli*, including Genencor, who in partnership with Goodyear Tire and Rubber Company, expressed an engineered MVA pathway in concert with the *Populus alba* isoprene synthase gene to produce isoprene at rates of 2 g L^{-1} h^{-1} in glucose fed-batch reactors (Whited et al. 2010). Engineering the heterologous MVA pathway has proven more successful than the native MEP pathway in *E. coli*, as it likely provides a bypass in the flux to terpenoid biosynthesis that avoids the native regulatory mechanisms associated with the MEP pathway (Martin et al. 2003; Morrone et al. 2010; Zurbriggen et al. 2012). However, important advances continue to be made toward improving metabolic flux though the MEP pathway, including a combinatorial approach to optimally balance the pathway through modular gene expression (Ajikumar et al. 2010), and the use of metabolite profiling to identify pathway bottlenecks (Zhou et al. 2012).

Despite significant improvements in terpenoid yield though engineering of the MEP and MVA pathways, it has become apparent that the abundance of the immediate precursors to the MEP and MVA pathways (GAP/pyruvate and acetyl-CoA, respectively) are the major limitations to further yield increases. Subsequently, a recent shift in focus for terpenoid engineering has been toward understanding the regulation of central metabolism, and how central carbon metabolites may be pushed, pulled, or diverted toward terpenoid pathways. The concept of engineering global cellular metabolism envisions the ability to control partitioning of assimilated carbon between major metabolic pathways and sinks in a way that does not severely impact cell viability. An elegant regulatory circuit was designed that sensed the accumulation of acetyl phosphate as an indicator of excess glycolytic flux (glucose availability), and stimulated expression of phosphoenolpyruvate synthase (Pps), the gluconeogenic enzyme that balances the ratio of GAP and pyruvate (Farmer and Liao 2000). Equimolar amounts of GAP and pyruvate are required for MEP pathway terpenoid biosynthesis, and it appears that GAP is the limiting precursor because the channeling of flux back to GAP from pyruvate enhanced lycopene production in *E. coli* (Farmer and Liao 2001). This was achieved by the overexpression of Pps and PEP carboxykinase (Pck), or the inactivation of pyruvate kinase genes (*pykF* and *pykA*). Channeling metabolic flux toward pyruvate, by the overexpression of PEP carboxylase (Ppc), resulted in a decrease in lycopene yield (Farmer and Liao 2001). Increasing the pool of PEP by deletion of genes encoding the carbohydrate phosphotransferase system, a PEP-consuming pathway, also provided a significant boost to lycopene production in *E. coli* via the MEP pathway to give a yield of 20 mg g^{-1} dry cell weight (Zhang et al.

2013). Another study aimed at manipulating glycolytic flux demonstrated that knocking out glucose-6-phosphate dehydrogenase increased lycopene production by 130 % in *E. coli*, and coincided with an increase in transcript abundance of *dxs* and *idi*, which may have contributed to the yield increase (Zhou et al. 2013b). The blocking of competing pathways has also proven to be an effective strategy for increasing terpenoid yield, for example, the inactivation of acetate-forming pathways at the pyruvate and acetyl-CoA nodes in *E. coli* led to a 45 % improvement in lycopene production (Vadali et al. 2005).

Photosynthetic platform for terpenoid bioengineering

As discussed in the introductory section, photosynthetic microorganisms are an attractive host platform for terpenoid production because they streamline the solar-to-biofuel generation process. The pioneering work of terpenoid bioengineering in photosynthetic microorganisms has utilized a cynanobacterial host system. Most studies have used the model cyanobacterium, *Synechocystis* sp. PCC 6803, and introduced a single TPS gene to the native *psbA2* locus of the chromosome via double homologous recombination. Expression of the transgenes was driven by the native *psbA2* promoter to promote high expression in a light-dependent manner (Lindberg et al. 2010). The *psbA2* locus is well established as a suitable "neutral" site for transgene integration in cyanobacteria because the deletion of *psbA2* is compensated by a strong up-regulation of the homologous *psbA3* gene (Mohamed et al. 1993). The transformation of a *Synechocystis* codon-optimized isoprene synthase (IspS) gene from *Pueraria lobata* (kudzu) enabled photosynthetic isoprene production at a rate of 4 μg isoprene L^{-1} h^{-1} with almost 0.1 % of assimilated CO_2 partitioning as isoprene (Lindberg et al. 2010; Bentley and Melis 2012). The importance of incorporating codon-usage information into the design strategy for heterologous gene expression was highlighted here, where a tenfold increase in IspS protein expression was observed upon codon-optimization (Lindberg et al. 2010). Similarly, *Synechocystis* was successfully transformed with the β-caryophyllene synthase from *Aretemisia annua* (Reinsvold et al. 2011) and a codon-optimized version of the β-phellandrene synthase from *Lavandular angustifolia* (Bentley et al. 2013), which allowed accumulation of the sesquiterpene β-caryophyllene at rates of 0.3 μg L^{-1} h^{-1}, or the monoterpene β-phellandrene at 1.0 μg L^{-1} h^{-1}, respectively. Cyanobacteria have multiple copies of their chromosomal DNA, and importantly, all cyanobacterial transformants in these studies reached homoplasmy for the introduced TPS transgene. This is a situation where all chromosomal DNA copies contain the transgene, and the cells may be cultured in the absence of antibiotic selection, which is a requirement for any robust industrial strain.

Increasing carbon flux through the terpenoid pathway to enhance IPP/DMAPP pools

A wealth of information has been obtained from the studies conducted in yeast and *E. coli* to enhance carbon flux to the IPP/DMAPP precursors of the terpenoids (discussed in the previous section). This valuable resource must be drawn upon as we move to the next stages of cyanobacterial terpenoid engineering. Bentley et al. (2014) have made the first step aimed at enhancing intracellular IPP/DMAPP pools in cyanobacteria through the heterologous expression of the MVA pathway in *Synechocystis* sp. PCC 6803, which led to an increase in isoprene production by ~2.5-fold. The entire MVA pathway was integrated to the chromosomal DNA as two separate operons. The first operon contained the upper MVA pathway genes encoding HMG synthase (HMGS) and HMGR from the bacterium *E. faecalis*, as well as *atoB* from *E. coli* to provide extra thiolase activity and pull more acetyl-CoA to the MVA pathway. The second operon contained the lower MVA pathway genes encoding mevalonate kinase (MVK), mevalonate 5-phosphate kinase (PMK), mevalonate 5-pyrophosphate decarboxylase (PMD), and an IPP isomerase from *S. pneumonia*. A much larger increase in isoprene yield was measured upon the introduction of the identical MVA pathway to *E. coli* (Zurbriggen et al. 2012), which highlights a number of issues surrounding the differences in feedstock between photosynthetic microorganisms and the more traditional host platforms (yeast and *E. coli*), as well as differences in methods of heterologous gene expression. Supplementation of yeast and *E. coli* with fixed carbon feedstocks, such as glucose, promotes rapid growth rates, and high-density cultures to produce higher terpenoid yields than cyanobacteria, which are limited by carbon-fixation reactions and become light-limited at higher cell densities. However, when calculating the true carbon and energy costs of each system, the photosynthetic cost of the plant-derived glucose feedstock must be included to make a valid comparison between photoautotrophic and heterotrophic terpenoid production.

An additional reason for the higher rates of terpenoid production observed in *E. coli* is likely due to the higher rates of heterologous protein expression achieved by a plasmid-based expression system. Plasmids that contain genes for heterologous expression are maintained within *E. coli* cells through antibiotic selection and replicate to produce multiple copies for high levels of protein expression. For this reason, most gene-overexpression studies in *E coli* utilize plasmid-based expression systems, but the plasmids are not stable without antibiotic selection and this

is an undesirable trait for industrial strains. A number of cyanobacterial strains naturally contain endogenous plasmids in addition to the chromorosmal DNA, such as *Synechococcus* sp. PCC 7002, which has six endogenous plasmids that range in copy number from ~6 to 50 (Xu et al. 2011). The functions of these plasmids are largely unknown, but they have been employed successfully for heterologous gene expression and represent a valuable tool for expression-based studies. The smallest plasmid, pAQ1, has the highest copy number (~50); however, it is difficult to achieve homoplasmy for introduced transgenes with this plasmid (D. Bryant, pers. comm.). Therefore, the stable integration of transgenes to the chromosomal DNA is a more appropriate choice when the aim is to create an industrially robust cyanobacterial strain.

Bypassing the native regulation of terpenoid biosynthesis

The presence of two unique terpenoid biosynthetic pathways raises an important bioengineering question—which is better suited for terpenoid production in photosynthetic microorganisms? The ability to heterologously express a functional MVA pathway in a cyanobacterium has been demonstrated (Bentley et al. 2014), with the rationale being that the MVA pathway provides a bypass to the highly regulated native MEP pathway. This strategy requires careful validation that every enzyme in the pathway is expressed and active, and will need to be followed by the tuning of protein production to maximize product titers. A similar approach to avoiding native regulatory systems could be employed for the MEP pathway via replacement of key regulatory genes with those from different species that may have evolved different regulatory mechanisms. One example is the DXR-like enzyme (DXR-II) that is found in many pathogenic bacteria, which, despite having no sequence homology with DXR, appears to have functionally replaced this enzyme in the MEP pathway (Carretero-Paulet et al. 2013; Sangari et al. 2010). Accordingly, replacement of the cyanobacterial DXR with DXR-II may allow unregulated enzymatic activity. Other considerations to be made when comparing the two terpenoid pathways are the requirements for energy, carbon, and reducing equivalents. The MVA pathway is more energy efficient as it generates ATP and has a net gain in NAD(P)H reducing equivalents. In contrast, the MEP pathway is more carbon efficient, with only two GAP molecules required for IPP synthesis (loss of one CO_2), compared with three GAP molecules required by the MVA pathway (loss of four CO_2). Engineering these pathways will alter the cellular requirements for energy, carbon, and reducing equivalents, so attention must be paid to ensure that these are met. The added energy demand from protein synthesis upon gene overexpression, especially when under the transcriptional control of strong promoters, is often overlooked and will have a large influence on the overall energy cost to produce IPP/DMAPP from any metabolically-engineered pathway.

Relieving terpenoid pathway bottlenecks

Gene overexpression studies of the native cyanobacterial MEP pathway, particularly key regulatory genes such as *dxs* and *ipi*, will likely release a certain amount of regulatory control and increase titers. It is important to make use of nature's biological toolbox and learn from plants that are geared toward terpenoid production, such as poplar trees that produce large amounts of isoprene, tomatoes that produce lycopene, and citrus trees that accumulate essential oils in glandular trichomes. Cloning genes from such plant species that have high levels of terpenoid metabolite flux may prove most successful for expression in cyanobacterial systems. The limitation here will be access to genetic sequence information for less well-studied species. Overexpression of key regulatory genes to relieve known pathway bottlenecks will likely have the effect of introducing new bottlenecks as downstream enzymes become limiting. A good example is the overexpression of *dxs* or *dxr* in *E. coli*, where an increase in terpenoid yield was additive upon overexpression of *idi* (Albrecht et al. 1999). Although *dxs* or *dxr* overexpression relieved the initial bottlenecks of the MEP pathway (and likely increased IPP pools), the isomerase overexpression was required to translate this to greater terpenoid production by converting the excess IPP to DMAPP and balancing the skewed IPP:DMAPP ratio.

Metabolite analysis is an effective measure to identify pathway bottlenecks due to the accumulation and/or secretion of metabolites immediately upstream of the limiting enzyme. There have been a number of recent technological advances for metabolite quantification, including a nanospray desorption electrospray ionization (nano-DESI) method coupled with MS/MS analysis for the detection of metabolites from living *Synechococcus* sp. PCC 7002 colonies, which eliminates the need for any special pretreatment (Lanekoff et al. 2013). A solid phase extraction method coupled with ultra-performance liquid chromatography mass spectrometry (SPE UPLC-MS) has also been developed to selectively enrich phosphorylated metabolites to allow the simultaneous quantification of MEP pathway intermediates (Zhou et al. 2012). As discussed above, the IPP:DMAPP ratio is crucial to optimize downstream metabolic flux. The concentrations and ratios of IPP/DMAPP in natural systems are not well characterized due to analytical challenges. However, improved methods for measuring IPP and DMAPP have recently been developed, and were used to measure an isomerase

equilibrium ratio of DMAPP/IPP in light-adapted kudzu leaves of 2.04:1 (Zhou et al. 2013a). A much lower DMAPP/IPP ratio of 15:85 has been reported in nonisoprene emitting tobacco cells (Tritsch et al. 2010), which highlights the importance of engineering a balance of DMAPP/IPP that is optimized for the synthesis of specific terpenoid chain lengths. Isoprene is synthesized directly from DMAPP; therefore, a higher proportion of DMAPP would be beneficial for isoprene production. In contrast, longer-chained terpenoids require a single initiating DMAPP molecule coupled with chain-elongating IPP building blocks (the longer the chain length, the more IPP units required). The ability to accurately measure IPP/DMAPP pools in engineered cyanobacteria and tailor the ratio for specific product chain lengths will be crucial to optimize terpenoid yields.

Understanding the role of post-translational regulation of terpenoid metabolism

As discussed earlier, the importance of post-translational modifications (PTMs) in regulating terpenoid metabolism is becoming increasingly clear, and it is apparent that in many cases there is little correlation between transcript and protein abundance. A recent genome-wide study in *Synechocystis* sp. PCC 6803 revealed that some proteins (including some photosynthesis-related) increased in abundance upon nitrogen starvation despite a down regulation of the corresponding mRNAs (Huang et al. 2013). It makes sense to regulate enzymatic activity at the post-translational level by switching enzymes on or off, rather than via the more energetically inefficient process of protein degradation and re-synthesis. However, the identification of PTMs remains a challenging task. Many valuable resources are emerging from global proteomic studies that map redox-regulated protein thiols (Go and Jones 2013; Sadler et al. 2013) and phosphorylated proteins (Yang et al. 2013b; Macek et al. 2008). The phosphoproteome of *Synechococcus* sp. PCC 7002 was recently published and is the largest phosphoproteome described in a single growth condition to date (Yang et al. 2013b). The authors found a high number of protein kinases and phosphatases with specificity on serine, threonine, and tyrosine residues, suggesting that reversible protein phosphorylation is an important PTM mechanism in cyanobacteria. Of the 280 phosphopeptides identified in this study, two were involved in terpenoid biosynthesis (IspH and IDI), which could be potential targets for protein engineering, as a method to switch enzymes to a permanently activated state.

The concept that enzymes of metabolic pathways form multiprotein complexes for allosteric regulation or to increase efficiency through substrate channeling, is intriguing and yet to be fully explored in terpenoid metabolic engineering. The identification of protein–protein interactions between terpenoid biosynthetic enzymes will provide insights into additional modes of PTM regulation in cyanobacteria. The use of synthetic fusion proteins, for example, a fusion of the yeast FPP synthase with a plant-derived sesquiterpenes synthase (patchoulol synthase) have proven successful in enhancing terpenoid yields in yeast (Albertsen et al. 2011), although incorrect protein folding may be an issue in some cases. Fusion proteins play important roles by not only reducing the transit time for pathway metabolites, but also by reducing the accumulation of toxic intermediates. Dueber et al. (2009) took this concept a step further to create a synthetic protein scaffold in *E. coli* that physically linked the first three enzymes of a heterologously-expressed MVA pathway (AtoB, HMGS, and HMGR), to induce a 77-fold increase in mevalonate concentration. The use of a DNA scaffold has also proven successful through the fusion of MVA-pathway enzymes with zinc finger DNA-binding domains to increase mevalonate titers by twofold to threefold (Conrado et al. 2012).

Funneling photosynthate from competing metabolic pathways

A crucial consideration for terpenoid engineering in cyanobacteria will be to optimally balance the ratio of photosynthetically derived GAP and pyruvate, as the immediate precursors to the native MEP pathway, and ensure that GAP concentrations are not limiting. Drawing carbon from competing metabolic pathways to increase intracellular pools of GAP and pyruvate will also be a key challenge to increase terpenoid titers. Recent studies investigating the effects of blocking glycogen biosynthesis in cyanobacteria, through inactivation of ADP-glucose pyrophosphorylase (*glgC*), observed secretion of large amounts of organic acids, including pyruvate and α-ketoglutarate when cells were nitrogen-stressed (Carrieri et al. 2012; Grundel et al. 2012; Hickman et al. 2013). It appears that photosynthetically fixed carbon, that would normally be stored as a glycogen carbon sink under these stress conditions, were instead redirected toward central metabolism and secreted as overflow metabolites in the form of organic acids. This is an example where inhibition of a competing carbon sink in cyanobacteria resulted in the accumulation of an immediate terpenoid pathway precursor (pyruvate), and a promising metabolic profile in which to increase terpenoid metabolism. Unfortunately, photosynthetic metabolism is not maintained under nitrogen-deprivation, which ultimately inhibits cell growth. Investigations should, therefore, be made in the cyanobacterial Δ*glgC* background using stress conditions that would normally promote glycogen biosynthesis, but also

allow cell growth, such as nitrogen-*limitation* or hypersaline stress. Ducat et al. (2012) have demonstrated the concept of "sink regulation", where the expansion of carbon sinks (in this case through the engineering of sucrose efflux) enhanced photosynthetic activity in the cyanobacterium *Synechococcus elongatus*. This may have important implications for cyanobacterial terpenoid production, where engineered terpenoid efflux may trigger a similar feedback response to enhance photosynthesis to maintain high growth rates and product yield.

The possibility that other metabolic pathways feed into the terpenoid biosynthetic pathways should not be ignored. For example, alternative routes of entry have been suggested via pentose phosphate cycle substrates derived from photosynthesis from in vivo experiments using cell extracts of the cyanobacterium *Synechocystis* sp. PCC 6803 (Ershov et al. 2002; Poliquin et al. 2004). Evidence to suggest channeling of the polyamine biosynthesis dead-end product, 5′-methylthiodenosine, toward terpenoid metabolism has been presented in the bacterium *Rhodospirillum rubrum* (Erb et al. 2012). Further, a study in *E. coli* demonstrated that a mutation in the gene encoding the E1 subunit of the pyruvate dehydrogenase subunit could rescue Δ*dxs* mutants in vivo, suggesting that the mutation allows the synthesis of DXP or an alternative substrate to DXS (Sauret-Gueto et al. 2006). Pyruvate dehydrogenase normally catalyzes the conversion of pyruvate to acetyl-CoA; however, this mutation may function to divert carbon away from competing pathways (such as acetyl-CoA-derived fatty acid biosynthesis) and instead funnel it toward terpenoid biosynthesis, an interesting prospect to pursue in a cyanobacterial host.

Product and terpene synthase enzyme selection: implications for photoautotrophic platform scale-up

Terpene synthase (TPS) enzymes have the capacity to produce a huge natural diversity of terpenoids. In that respect, it is important to assess the suitability of a terpenoid for a given industrial application, as well as compile information on TPS enzymatic activity to determine suitability for expression in a photosynthetic host. For the purpose of understanding the regulation of terpenoid biosynthesis via central metabolism or the MVA/MEP pathways, selection of an easy-to-screen terpenoid reporter product will enable high-throughput experimentation. This method has worked well in yeast and *E. coli* using lycopene and β-carotene products that are easily quantified by means of colorimetric assays. However, as a broader range of terpenoid products are developed for diverse markets, it is crucial to advance high-throughput screening methods that encompass a greater suite of terpenoids, such as a fluorescent dye-based screening method developed by the renewable products company, Amyris, for the rapid detection of farnesene in high-yielding strains of yeast and *E. coli* (Frenz and Ubersax 2012).

The drive to develop renewable energy sources has led to the identification of a number of terpenoid products with physical and chemical properties similar to petroleum-derived fuel. Farnesene is a promising fuel candidate and can be chemically hydrogenated to fully saturated farnesane, which has superior fuel qualities (Kung et al. 2012). Engineered strains of *E. coli* expressing the *Artemisia annua* farnesene synthase gene produced farnesene at yields of 380 mg L^{-1} (Wang et al. 2011). The sesquiterpene bisabolene, and its reduced equivalent bisabolane, have similar properties to diesel; the bisabolene synthase gene from *Abies grandis* has been expressed in *E. coli* to produce bisabolene at titers greater than 900 mg L^{-1} via the MVA pathway (Peralta-Yahya et al. 2011). The monoterpenes, pinene, and limonene are suited for use as jet fuel and have been produced in *E. coli* strains at yields of 0.97 g L^{-1} (Yang et al. 2013a) and 0.4 g L^{-1} (Alonso-Gutierrez et al. 2013), respectively. Combustion and emission characteristics should also be considered when selecting terpenoid products as fuel molecules (Hellier et al. 2013).

A current restraint for terpenoid engineering is the lack of genetic sequence information available for TPS enzymes, especially from nonmodel plant species that heavily invest in terpenoid metabolism. Of the thousands of isolated terpenoids, relatively few synthases have been cloned and had functional activity demonstrated upon heterologous expression. Data on the catalytic activities of TPS enzymes is also relatively scarce, but is crucial for designing successful engineering strategies. Plant TPSs typically have low turnover rates; for example the limonene synthase from mint (*Mentha x piperita* and *Mentha spicata*) has a k_{cat} of 0.3 s^{-1} (Rajaonarivony et al. 1992; Alonso et al. 1992). The K_m values for plant TPS are often in the μM range; however some have a high K_m, such as isoprene synthase, which has been measured in the mM range up to 9 mM (Sasaki et al. 2005; Schnitzler et al. 2005; Silver and Fall 1995; Zurbriggen et al. 2012). This means a very high intracellular concentration of DMAPP is required to have a significant effect on the rate of isoprene synthesis, which may in part explain the differences in isoprene yield observed upon heterologous expression of the MVA pathway between *E coli* and cyanobacterial hosts (Bentley et al. 2014; Zurbriggen et al. 2012). The plasmid-based expression system used in *E. coli* likely generated a greater pool of IPP/DMAPP than the chromosomally integrated MVA pathway of the cyanobacterium because of the associated differences in gene copy number, which may have translated to greater isoprene synthase activity.

In situations where the catalytic information for TPS is unavailable, natural composition profiles of terpenoids may provide some guidance. For example, lemon peel oil has a terpenoid composition of 75 % limonene, 11 % γ-terpinene, 4 % β-pinene, 2 % *p*-cymene, 1 % α-pinene, and 1 % myrcene (Lucker et al. 2002), which may be indicative of greater limonene synthase (LIMS) activity over other native monoterpene synthases. Transcriptional and/or translational regulation is likely to be a factor, but it is tempting to speculate that lemon LIMS may have evolved superior catalytic activity over those from species with low limonene content. The low k_{cat} and high K_m values associated with TPS enzymes present a significant challenge to obtaining high terpenoid yields in an engineered strain. However, there are opportunities to enhance catalytic activity via directed protein evolution or random mutagenesis strategies, as well as the use of TPS crystal structures (Hyatt et al. 2007; Koksal et al. 2010) to provide insights into the catalytic mechanism to assist targeted protein engineering.

Plant TPS enzymes are known for their promiscuity, often catalyzing the formation of different terpenoids from the same prenyl-pyrophosphate precursor (Colby et al. 1993; Wagschal et al. 1991). This is a result of the complex nature of the carbocation rearrangement reactions catalyzed by the TPS, which can invariably yield side products with altered chemical structures. As a result, plant TPS heterologous expression may not yield a single terpenoid product, as was observed upon expression of the lavender β-phellandrene synthase in *Synechocystis* sp. PCC 6803, where β-myrcene and limonene also accumulated as minor products in addition to β-phellandrene (Bentley et al. 2013). Likewise, there is no guarantee that the terpenoid profile produced from a TPS *in planta* will be identical to that produced by the heterologously-expressed enzyme. However, as the generation of a pure terpenoid product is likely the goal, it may be beneficial to select an enzyme that has a higher specificity for a single product, such as the LIMS from *Mentha spicata*, which catalyzed in vitro a terpenoid profile comprising 94 % limonene, 2.0 % β-pinene, 1.9 % myrcene, and 1.8 % α-pinene from GPP (Colby et al. 1993). Importantly, genetic sequence information is usually not enough to predict the major terpenoid product generated by a given TPS. In plants, different TPS enzymes within a species often have greater identity than TPS that produce identical terpenoid products from different species (Chen et al. 2011). For the successful identification of a superior plant TPS for heterologous expression, that has high activity and specificity, a large in vitro screening effort for such qualities among a suite of cloned TPS enzymes is a prerequisite.

Because plant TPS enzymes are nuclear-encoded they must be targeted to the plastid via an N-terminal chloroplast transit peptide, which is cleaved in the plastid to yield the mature protein. This transit peptide must be excluded from the TPS when expressed in a prokaryotic host as it can cause protein insolubility leading to protein aggregation or the formation of inclusion bodies. Hence, certainty over the amino acid residues that constitute the transit peptide is important so as to not remove functional residues of the mature protein. An example of the effect of transit peptide truncations on the catalytic activity of spearmint LIMS was presented by Williams et al. (1998). The chloroplast transit peptide prediction software, ChloroP, predicted a transit peptide of 48 AA for the spearmint LIMS, which preceded a tandem pair of arginine residues (R58R59) that are highly conserved among monoterpenes synthases. Truncation of the transit peptide at Q53 resulted in a K_m of 8.6 μM and a k_{cat} of 0.036 s^{-1} for LIMS, which was an improvement over the pre-protein (with full-length transit peptide) activity, indicating that the transit peptide negatively influenced protein activity. However, truncation of the transit peptide upstream of the conserved tandem arginine residues (R58R59) yielded <1 % of native activity. When choosing a plant TPS for heterologous expression in a prokaryotic host, it is also wise to identify enzymes that require eukaryotic-specific post-translational modifications to enable correct folding, protein activity and expression rates, and base enzyme selection of those that have greater compatibility with a prokaryotic system.

Harvesting at industrial scale

The efficient harvesting of terpenoid products at industrial scale is a major challenge. Commercial-scale cultures of *Chlorella* (microalga) and *Anthrospira* (cyanobacteria) have successfully supplied markets for β-carotene and astaxanthin for many decades. However, the harvesting and drying of cells grown in aqueous media requires a large input of energy, which is the major limitation for commercial terpenoid production in photosynthetic microorganisms. The beauty of small, hydrophobic terpenoids, such as isoprene, is that they are volatile at atmospheric conditions and able to diffuse though cellular membranes to naturally separate from the biomass (Sharkey et al. 2008; Sharkey and Yeh 2001). The volatile hydrocarbons may be harvested as a condensate, as has been demonstrated at scale by Algenol for ethanol recovery from cyanobacteria (Legere et al. 2008), or via more elaborate carbon-trapping methods. A major issue with the cultivation of photosynthetic microorganisms is the ability to maintain photoautotrophic growth conditions in an enclosed bioreactor that is necessary to allow the accumulation and concentration of the volatile terpenoid product prior to harvesting. Flushing the aqueous culture and reactor headspace with 100 % CO_2

has proven successful at lab-scale for maintaining photoautotrophic growth and isoprene production over prolonged time periods (Bentley and Melis 2012), and has the potential to be applied at scale using waste industrial flue gases as the source of concentrated CO_2. The cellular localization of other terpenoids that have been engineered in cyanobacteria, including the sesquiterpenes β-caryophyllene and β-phellandrene (Reinsvold et al. 2011; Bentley et al. 2013), are not well understood. As longer-chain hydrocarbons, they are less volatile and less likely to diffuse though cellular membranes, and may accumulate within the cell (possibly within membranes because of their hydrophobic nature), or be secreted from the cell and accumulate as a nonmiscible product on the surface of the aqueous culture. Heterologously-expressed efflux pumps may help with product/biomass separation, as has been demonstrated in *E. coli* for sesquiterpenes (Dunlop et al. 2011) and longer-chained terpenoids such as lycopene and β-carotene (Doshi et al. 2013). Extraction from cyanobacterial cultures with organic solvents has proven successful at lab-scale (Bentley et al. 2013); however, this is not commercially feasible on a larger scale. When yields are improved to levels that suggest commercial viability, terpenoids should accumulate in a relatively pure form at volumes conducive to physical separation from the aqueous culture. Many hydrocarbons are toxic to microorganisms, and care must be taken to ensure that growth of the engineered host organism is not adversely affected by terpenoid accumulation. Efflux pumps are commonly used by microorganisms to expel natural toxic products, and heterologous expression of novel efflux pumps alleviated limonene toxicity in engineered strains of *E. coli* (Dunlop et al. 2011). Such a strategy could be employed in photosynthetic microorganisms to reduce toxicity while enabling the separation of product from biomass.

Future perspectives for photoautotrophic terpenoid engineering

Photosynthetic microorganisms are a platform from which terpenoid products may be synthesized in a manner that has distinct advantages over current plant- and petroleum-based supplies. Cyanobacteria, in particular, offer the benefits of high growth rates and easy genetic manipulation. Significant challenges do exist toward improving photosynthetic efficiency and the cyanobacterial metabolic engineering toolbox (Huang et al. 2010; Huang and Lindblad 2013; Work et al. 2012); however, a major challenge is the redirection of carbon flux in a highly-regulated photosynthetic cell (Melis 2013). Regulation of carbon partitioning during the growth phase is particularly stringent due to the high demand for metabolic intermediates across multiple pathways. Accordingly, the full potential for terpenoid biosynthesis will not be realized until an open pipeline is enabled that allows the highly efficient flow of photosynthate to IPP/DMAPP terpenoid precursors. Nature has provided a wealth of information regarding terpenoid biosynthetic regulation that should be exploited for this purpose. Overcoming pathway "bottlenecks" through gene overexpression or by engineering increased enzyme activity though the inhibition of post-translational events, such as phosphorylation or dithiol bond formation, are promising strategies for MEP pathway deregulation in photosynthetic microorganisms. It is important that photosynthesis, as the source of terpenoid feedstock (GAP and pyruvate), is not compromised as a result of metabolic manipulation. Finally, once an unregulated terpenoid pathway capable of high metabolic flux is established, and the pipeline for photosynthetic carbon fixation remains open, a systematic and step-wise reduction of competing metabolic pathways will provide the necessary steps toward the ultimate goal of creating a photoautotrophic cellular factory for terpenoid biosynthesis.

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