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Journal
Photosynthesis Research, 130(1-3)

ISSN
0166-8595

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Publication Date
2016-12-01

DOI
10.1007/s11120-016-0293-3

Peer reviewed

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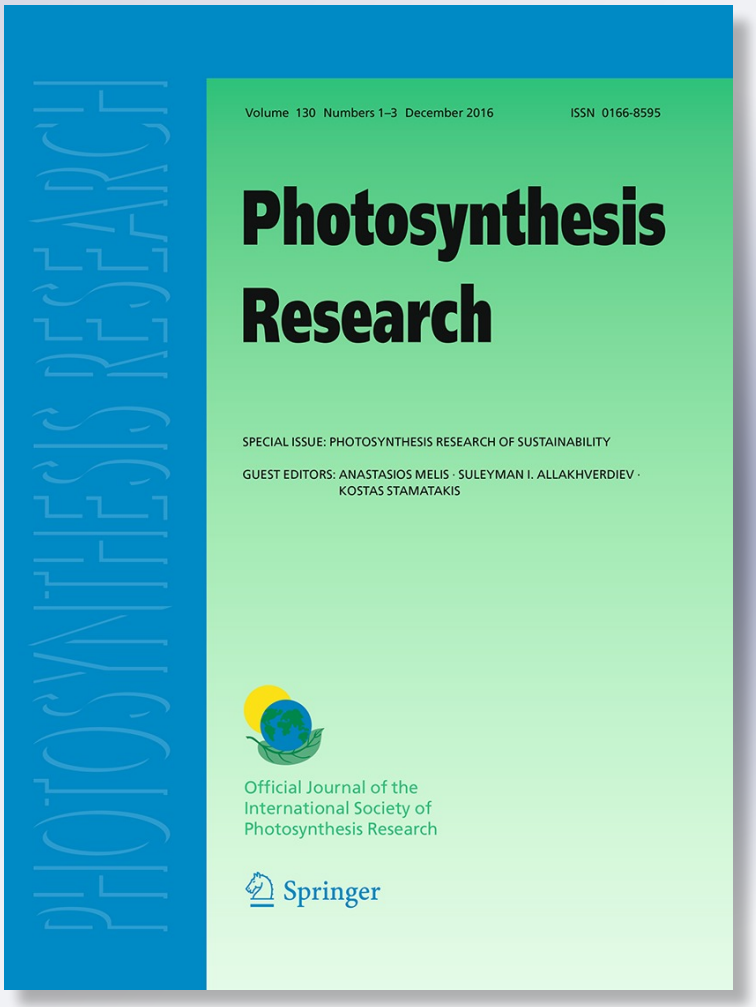
Photosynthesis Research
Official Journal of the International Society of Photosynthesis Research

ISSN 0166-8595
Volume 130
Combined 1-3

Photosynth Res (2016) 130:517-527
DOI 10.1007/s11120-016-0293-3



Springer

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ORIGINAL ARTICLE

Role of isopentenyl-diphosphate isomerase in heterologous cyanobacterial (*Synechocystis*) isoprene production

Julie E. Chaves[1] · Paloma Rueda Romero[1] · Henning Kirst[1] · Anastasios Melis[1]

Received: 27 April 2016 / Accepted: 5 July 2016 / Published online: 13 July 2016

Abstract Heterologous production of isoprene (C_5H_8) hydrocarbons in cyanobacteria, emanating from sunlight, CO_2, and water, is now attracting increasing attention. The concept entails application of an isoprene synthase transgene from terrestrial plants, heterologously expressed in cyanobacteria, aiming to reprogram carbon flux in the terpenoid biosynthetic pathway toward formation and spontaneous release of this volatile chemical from the cell and liquid culture. However, flux manipulations and carbon-partitioning reactions between isoprene (the product) and native terpenoid biosynthesis for cellular needs are not yet optimized for isoprene yield. The primary reactant for isoprene biosynthesis is dimethylallyl diphosphate (DMAPP), whereas both DMAPP and its isopentenyl diphosphate (IPP) isomer are needed for cellular terpenoid biosynthesis. The present work addressed the function of an isopentenyl diphosphate (IPP) isomerase in cyanobacteria and its role in carbon partitioning between IPP and DMAPP, both of which serve, in variable ratios, as reactants for the synthesis of different cellular terpenoids. The work was approached upon the heterologous expression in *Synechocystis* of the "isopentenyl diphosphate isomerase" gene (*FNI*) from *Streptococcus pneumoniae,* using isoprene production as a "reporter process" for substrate partitioning between DMAPP and IPP. It is shown that transgenic expression of the *FNI* gene in *Synechocystis* resulted in a 250 % increase in the "reporter isoprene" rate and yield, suggesting that the FNI isomerase shifted the endogenous DMAPP-IPP steady-state pool size toward DMAPP, thereby enhancing rates and yield of isoprene production. The work provides insight into the significance and functional role of the IPP isomerase in these photosynthetic microorganisms.

Keywords Carbon partitioning · Isopentenyl diphosphate isomerase · Isoprene biosynthesis · Metabolic engineering · *Synechocystis* · Synthetic biology

Abbreviations

DCW	Dry cell weight
MEP	2-C-methyl-D-erythritol 4-phosphate
DMAPP	Dimethylallyl-diphosphate
IPP	Isopentenyl diphosphate
IspS	Isoprene synthase
FNI	Isopentenyl diphosphate isomerase
WT	Wild type
RS	Recipient strain
Cpc	operon encoding the phycocyanin subunits and associated linker polypeptides
Δ*cpc*	*cpc* operon deletion
CmR	Chloramphenicol resistance
HMBPP	Hydroxy-2-methyl-2-butenyl-4-diphosphate
GPP	Geranyl diphosphate

Electronic supplementary material The online version of this article (doi:10.1007/s11120-016-0293-3) contains supplementary material, which is available to authorized users.

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SSpFNI	*Synechocystis* codon-optimized *Streptococcus pneumoniae* isopentenyl diphosphate isomerase (FNI)
SkIspS	*Synechocystis* codon-optimized kudzu isoprene synthase
SkIspS, Δcpc+CmR	*Synechocystis* codon-optimized kudzu isoprene synthase in the *psbA2* site, including replacement of *cpc* operon with chloramphenicol resistance cassette
SkIspS, Δcpc+SSpFNI+CmR	Synechocystis codon-optimized kudzu isoprene synthase in the *psbA2* site, including replacement of *cpc* operon with the *Synechocystis* codon-optimized *Streptococcus pneumoniae* isopentenyl diphosphate isomerase (FNI) followed by the chloramphenicol resistance cassette

Introduction

Isoprenoids, or terpenoids, are the largest class of naturally occurring chemicals. They are functionally important as the basis of photosynthetic pigments comprising the phytol tail of chlorophylls, all carotenoids, quinone prenyl side-chains, growth regulators such as abscisic and gibberellic acid, and structural components of cell walls and membranes such as bactoprenol and steroids. In addition, they are the source of plant defensive compounds against herbivores, and the molecules that confer scents and fragrance to leaves and flowers.

The universal precursors for isoprenoid synthesis in plants, animals, insects, and microorganisms are the 5-carbon metabolites isopentenyl diphosphate (IPP) and its isomeric form dimethylallyl diphosphate (DMAPP) (Arganoff et al. 1959; Lichtenthaler 2007, 2010). In cyanobacteria, microalgae, and plant chloroplasts, IPP and DMAPP are generated through the methylerythritol phosphate (MEP) pathway from glyceraldehyde-3-phosphate (G3P) and pyruvate (Pyr) by the action of a set of seven consecutive enzymatic reactions (Bentley et al. 2014), with the final enzyme, 4-hydroxy-3-methyl-2-(E)-butenyl-diphosphate reductase (*IspH*) generating an IPP/DMAPP ratio of 3:1 (McGarvey and Croteau 1995; Lichtenthaler 2010; Formighieri and Melis 2014), but measured to be 5.6:1 in tobacco (Tritsch et al. 2010) and 6:1 in *E. coli* (Adam et al. 2002). Most isoprenoid synthesis occurs upon head-to-tail additions of one or several IPPs to DMAPP (Lichtenthaler 2010), yielding molecules with 10, 15, 20, or greater than 40 5-carbon structures. Therefore, a high IPP-to-DMAPP steady-state ratio makes sense, as there is a greater requirement for IPP than for DMAPP for the synthesis of the vast majority of isoprenoid products.

It is evident from the preceding that synthesis of natural terpenoids would consume greater stoichiometric amounts of IPP than DMAPP, the IPP/DMAPP ratio required depending on the target molecule. For example, phytol and carotenoids (Lagarde et al. 2000) are synthesized from C-20 diterpenes and would require an IPP/DMAPP ratio of 3:1, whereas the C-45 prenyl tail of the abundant in thylakoids plastoquinone-9 (McCauley and Melis 1986) would require an IPP/DMAPP ratio of 8:1. Other, less abundant terpenoids, e.g., C-10 myrcene and β-phellandrene (Formighieri and Melis 2016), would require an IPP/DMAPP ratio of 2:1. On the contrary, synthesis of C-5 isoprene (C_5H_8) would require only DMAPP, as this is the only substrate serving as its reactant (Zhou et al. 2013).

The IPP/DMAPP ratio may be modulated in vivo depending on the chloroplast or cellular needs by action of the enzyme isopentenyl diphosphate isomerase (*Ipi*), which interconverts IPP and DMAPP (Ramos-Valdivia et al. 1997; Barkley et al. 2004; Okada et al. 2008; Weise et al. 2013). In vitro, the IPP isomerase reportedly shifts the balance toward DMAPP, resulting in an DMAPP/IPP ratio of 2.1:1 of *E. coli* (Zhou et al. 2013) or DMAPP/IPP = 2.2:1 in *Saccharomyces cerevisiae* extracts (Street and Poulter 1990). In vivo, ratios of DMAPP/IPP have been measured to be slightly higher at 2.8:1 in *E. coli* (Zhou et al. 2013). The regulation of this interconversion in cyanobacteria is not well understood, and the in vitro and in vivo DMAPP/IPP ratios produced by the native *Synechocystis* IPP isomerase have not yet been determined. Evidence was presented suggesting a lack of IPP isomerase activity in the cyanobacterium *Synechocystis* sp. PCC 6803 (Ershov et al. 2000), suggesting either inability of *Synechocystis* to modulate the DMAPP/IPP ratio in the cell, or that this enzyme does not play a role in the isoprenoid biosynthesis in this microorganism.

Heterologous isoprene production in cyanobacteria (Lindberg et al. 2010; Bentley and Melis 2012; Pade et al. 2016) can be used as a "reporter process" for the function of the IPP isomerase by monitoring the rate and yield of isoprene production, which depends on the pool of DMAPP in the cell. We hypothesized that shifting the balance of the

IPP/DMAPP toward DMAPP in *Synechocystis* should improve rates and yield of the "reporter process" and result in greater amounts of its product. Accordingly, this work applied heterologous expression of the FNI gene from *Streptococcus pneumoniae*, encoding an IPP isomerase that normally functions in conjunction with the mevalonic acid pathway in these bacteria (Zurbriggen et al. 2012; Bentley et al. 2014) as a tool by which to alter the cellular endogenous DMAPP-to-IPP ratio. Outcome of the transgenic expression of the *FNI* gene in *Synechocystis* was a 250 % increase in the "reporter isoprene" rate and yield, showing the function and significance of the IPP isomerase in these photosynthetic microorganisms.

Materials and methods

Strains and culturing conditions

Synechocystis sp. from the Pasteur Culture Collection strain number 6803 was employed as the experimental strain and is referred to as wild type (WT). The isoprene producing *Synechocystis* strain (*SkIspS*) was previously developed in this lab (Lindberg et al. 2010), expressing a codon-optimized isoprene synthase from *Pueraria montana* (kudzu) in the *psbA2* gene locus. *Synechocystis* strain *SkIspS* was used as the recipient strain in this work.

All strains employed in this work were maintained on 1 % agar-BG11 media supplemented with 10 mM TES-NaOH pH 8.2, and 0.3 % Na-thiosulfate. Kanamycin (25 μg/mL) and chloramphenicol (30 μg/mL) were added into agar plates and used to maintain transformants. Starter cultures were inoculated in 300 mL growth media with cells from an agar plate and allowed to grow initially under illumination at 30 μmol photons m^{-2} s^{-2} until an $OD_{730} = 0.3$ was reached. Illumination was then increased to 50 μmol photons m^{-2} s^{-2} until an $OD_{730} = 0.65$–0.75 was reached. Illumination was further increased to 100 μmol photons m^{-2} s^{-2} until the culture reached a density enough to support dilution in 700 mL growth medium to an $OD_{730} = 0.65$. Liquid cultures were grown in BG11 media buffered with 25 mM NaH_2PO_4 (pH 7.5) at 28 °C, under continuous aeration and illumination at 100 μmol photons m^{-2} s^{-1}.

Synechocystis transformation

Transformations were performed as previously established (Kirst et al. 2014). A DNA construct comprising the chloramphenicol resistance cassette was designed to replace the *cpc* operon in the *SkIspS* strain, generating the control strain *SkIpsS*, Δ*cpc*+*CmR*. A DNA construct comprising the "isopentenyl diphosphate isomerase" gene (*FNI*) from *S. pneumoniae* (Zurbriggen et al. 2012), followed by the chloramphenicol resistance cassette, was designed to replace the *cpc* operon in the *SkIspS* strain, generating the *SkIpsS*, Δ*cpc*+*SSpFNI*+*CmR* transformant strain. The *cpc* locus was chosen for the insertion of transgenes, as it possesses a strong promoter, in addition to affording the option of phycocyanin removal from the cells (Kirst et al. 2014). Transgene constructs were flanked by 500 base pairs of the upstream and downstream sequences of the *cpc* operon for homologous recombination. Complete segregation of the transgenes into all copies of the genome was confirmed by genomic DNA PCR analysis.

Protein analysis

Cells were grown in 300 mL volume cultures to an OD_{730} of 2.5, pelleted by centrifugation, and re-suspended in 5–10 mL of 50 mM Tris–HCl (pH 8). The cell suspension was then incubated with lysozyme at room temperature for 30 min, and then washed in fresh 50 mM Tris–HCl (pH 8) three times. Protease inhibitor (1 mM PMSF) was added to samples before lysing of the cells by French press (3 × 1200 psi). Disrupted cell suspensions were centrifuged at 2250 g for 3 min to pellet cell debris and glycogen grains. The supernatant was supplemented with an equal volume of solubilization solution, comprising 250 mM Tris–HCl, pH 6.8, 7 % w/v SDS, 20 % w/v glycerol, 2 M urea, and a few grains of bromophenol blue. Samples were solubilized upon incubation at room temperature for 1–2 h. At the end of the solubilization incubation, samples were supplement with 10 % β-mercaptoethanol. The solubilized total cellular proteins were subjected to SDS-PAGE and Western blot analysis. SDS-PAGE resolved proteins were either stained with Coomassie brilliant blue or transferred to PVDF membrane for immunodetection using rabbit immune serum containing specific polyclonal antibodies against the ISPS (Lindberg et al. 2010) or FNI proteins (Zurbriggen et al. 2012).

Isoprene, photosynthetic pigment, and biomass accumulation

Liquid cultures grown for biomass accumulation and isoprene production were grown photoautotrophically in the absence of antibiotics. Glass bottle bioreactors (1 L volume) were designed in this lab specifically for quantitative biomass and isoprene production measurements, (Bentley and Melis 2012). The 1 L bioreactors were loaded with ~700 mL liquid BG11 growth medium containing 25 mM NaH_2PO_4 (pH 7.5), and then inoculated with *Synechocystis* starter cultures at an $OD_{730} = 0.65$. Unless otherwise indicated, the bioreactors were further loaded with inorganic carbon, delivered to the liquid culture by slowly

bubbling 500 mL of 100 % CO_2 gas through the bottom of the liquid culture to fill the reactor headspace. Bioreactors were then sealed and cultures were stirred slowly and continuously at 28 °C under constant illumination at 100 µmol photons m^{-2} s^{-2}.

Isoprene accumulation in the headspace of the reactor was determined by gas chromatography (Shimadzu 8A GC-FID) analysis of 1 mL gaseous samples from the bioreactor headspace. Isoprene quantification was determined based on a calibration of isoprene standard (Acros Organics, Fair Lawn, NJ, USA), as described (Chaves et al. 2015). Chlorophyll *a* and total carotenoids were extracted in 100 % methanol and measured spectrophotometrically according to Lichtenhaler (1987). Biomass accumulation in the liquid phase of the reactor was determined upon collection of 50 mL aliquots, followed by centrifugation, rinsing with deionized water, and cell re-suspension in 2 mL of deionized water. Samples were then transferred to and dried on aluminum trays for 6 h at 90 °C, and weighed to determine the dry cell weight (DCW). Cell growth was also determined spectrophotometrically by measuring the optical density of live cell cultures at 730 nm with a Shimadzu UV-1800 UV-visible spectrophotometer.

Results

Construction of a chloramphenicol resistant *FNI* overexpressing strain

The *Synechocystis* recipient strain used in this work carried the codon-optimized kudzu isoprene synthase *SkIspS* gene, the latter being in the *psbA2* locus (Fig. 1a). The nucleotide sequence of the *SkIspS* transgene is shown in the supplementary materials. The recipient strain also included an unmodified *cpc* operon (Fig. 1b), which encodes for the phycocyanin β-subunit (cpcB), the α-subunit (cpcA), and the associated linker polypeptides (cpcC1, cpcC2, and cpcD). The *Synechocystis* codon-optimized chloramphenicol resistance *CmR* cassette was introduced via double homologous recombination into the *cpc* operon locus (Fig. 1c) of the recipient strain already carrying the codon-optimized kudzu isoprene synthase *SkIspS* gene. This transformation replaced the *cpc* operon with the chloramphenicol resistance *CmR* cassette, generating the "SkIspS, Δcpc+CmR" strain. Alternatively, the *Synechocystis* codon-optimized *S. pneumoniae* "isopentenyl diphosphate isomerase" gene (*FNI*), followed by the chloramphenicol resistance *CmR* cassette was introduced via double homologous recombination into the *cpc* operon locus (Fig. 1d) of the recipient strain already carrying the codon-optimized kudzu isoprene synthase *SkIspS* gene. This transformation replaced the *cpc* operon with the *S. pneumoniae* "isopentenyl diphosphate isomerase" gene (*FNI*) plus the chloramphenicol resistance *CmR* cassette, generating the "SkIspS, Δcpc+SSpFNI+CmR" strain. Replacement of the native *cpc* operon with either the *CmR* (Fig. 1c) or the *FNI-CmR* construct (Fig. 1d) resulted in phycocyanin-less mutants. These were analyzed molecularly and biochemically for their genetic and protein expression properties.

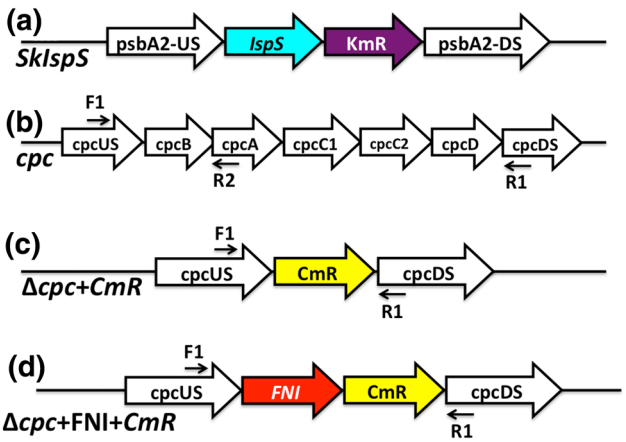

Fig. 1 Schematic overview of the constructs designed to transform the genomic DNA of *Synechocystis*, as used in this work. **a** organization of the DNA in the *psbA2* locus of the *Synechocystis* recipient strain, in which the native *psbA2* gene was replaced by the *IspS-KanR* construct. **b** Organization of the DNA in the *cpc* operon locus of the recipient strain. The CPC-operon includes the *cpcB* gene, encoding the phycocyanin β-subunit; *cpcA*, encoding the phycocyanin α-subunit; *cpcC1* and *cpcC2* encoding the phycocyanin rod linker polypeptides; and the *cpcD* encoding an additional small linker polypeptide. **c** DNA construct for the replacement of the *cpc* operon with a chloramphenicol resistance cassette (*CmR*) designed for double homologous recombination in the *cpc* operon locus of the *Synechocystis* recipient strain. **d** DNA construct for the expression of the "isopentenyl diphosphate isomerase" gene (*FNI*) from *S. pneumoniae* along with a chloramphenicol resistance cassette (*CmR*) designed for double homologous recombination in the *cpc* operon locus of the *Synechocystis* recipient strain. F1 and R1: *forward* (F1) and *reverse* (R1) primers designed to amplify the *cpc* operon DNA region between the *cpc* promoter and terminator. F1 and R2: *forward* (F1) and *reverse* (R2) primers designed to amplify the *cpc* operon DNA region between the *cpc* promoter and *cpcA* gene

Transformation and protein expression profile of the Δcpc+CmR strain

Genomic DNA analysis, the state of homoplasmy, and protein expression profiles were investigated. State of homoplasmy of the *SkIpsS* and *SkIspS, Δcpc+CmR* strains was tested by PCR amplification of genomic DNA using primers flanking the insertion site, as well as primers specific to the recipient strain. In the recipient strain (*SkIpsS*), primers F1 and R1 (Fig. 1b) flanking the *cpc* operon amplified a 3.6 kb product, corresponding to the DNA of the full *cpc* operon (Fig. 2, SkIpsS in left panel).

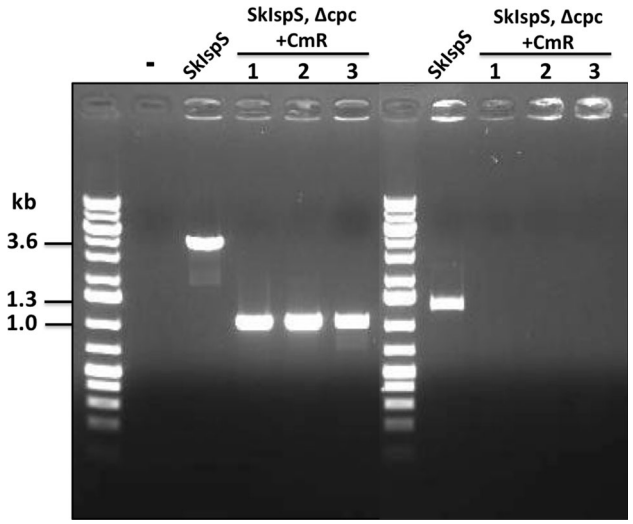

Fig. 2 Genomic DNA PCR analysis with selected *forward* (F) and *reverse* (R) primers positioned on the genomic DNA of *Synechocystis* recipient (*SkIspS*) and *SkIspS*, Δ*cpc*+*CmR* transformants. (*Left panel*) PCR reactions using primers F1 and R1 (primer positions given in Fig. 1) amplifying the DNA region of the *cpc* operon between the *cpc* promoter and terminator. The recipient strain (*SkIspS*) yielded a single 3.6 kb product, corresponding to the full *cpc* operon genes. Three different *SkIspS*, Δ*cpc*+*CmR* lines yielded a single 1 kb product, corresponding to the Δ*cpc*+*CmR* DNA. (*Right panel*) PCR reactions using primers F1 and R2 amplifying the DNA region between the *cpc* promoter and the *cpcA* gene. The recipient strain (*SkIspS*) yielded a single 1.3 kb product. Three different *SkIspS*, Δ*cpc*+*CmR* lines failed to yield any PCR products

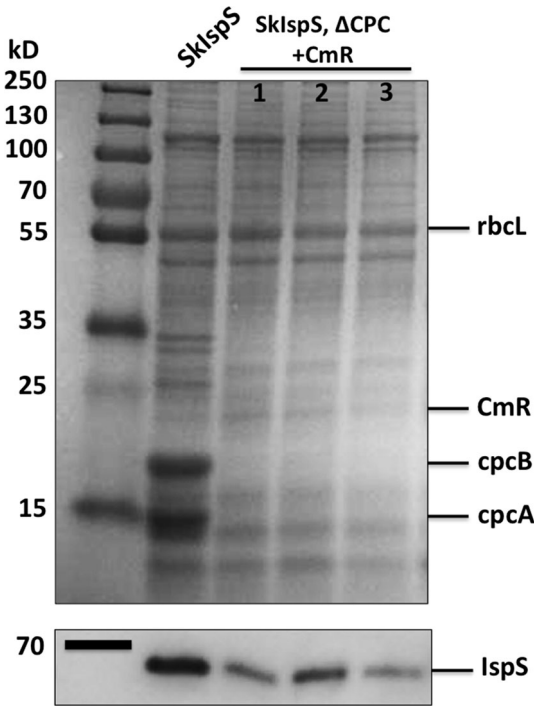

Fig. 3 (*Upper panel*) SDS-PAGE analysis of total protein extracts from *Synechocystis* recipient strain (SkIspS) and three *CmR* transformant lines (*SkIspS*, Δ*cpc*+*CmR*). Pre-stained PageRuler Plus molecular weight markers from ThermoFisher Scientific are indicated in kD. Note the presence of the cpcB and cpcA phycocyanin subunits in the recipient SkIspS strain, migrating in the 15–20 kD region, and the absence of these proteins from the *SkIspS*, Δ*cpc*+*CmR* lines. (*Lower panel*) Western blot analysis of protein extracts from *Synechocystis* recipient strain (*SkIspS*) and three *CmR* transformant lines (*SkIspS*, Δ*cpc*+*CmR*), probed with specific polyclonal antibodies raised against the IspS protein

In the *SkIpsS*, Δ*cpc*+*CmR* transformant, primers F1-R1 amplified a 1.0 kb product, corresponding to the CmR insert (Fig. 2, SkIpsS, Δcpc+CmR in left panel). Primers F1-R2 flanking the cpc upstream region (F1) and the *cpcA* (R2) coding region (Fig. 1b) generated a 1.3 kb product in the *SkIpsS* recipient strain (Fig. 2, SkIpsS in right panel), but failed to generate any product in the *SkIpsS*, Δ*cpc*+*CmR* control strain (Fig. 2, SkIpsS, Δcpc+CmR in right panel). The latter is evidence that the Δ*cpc*+*CmR* transformants have reached a state of DNA copy homoplasmy.

SDS-PAGE and Western blot analysis of total cellular protein extracts was employed to assess expression of the *IspS* and the overall protein expression profile of the recipient strain (SkIspS) and the chloramphenicol expressing transformant (SkIpsS, Δcpc+CmR). The *Synechocystis* recipient strain showed expression of the phycocyanin cpcB (β-subunit) and cpcA (α-subunit) as dominant proteins in the 15–20 kD region (Fig. 3, upper panel). However, the chloramphenicol expressing transformant (SkIpsS, Δcpc+CmR) lacked these major cellular proteins. Instead, it specifically expressed the chloramphenicol resistance conferring protein, seen in Fig. 3, upper panel, as a 24 kD protein. Western blot analysis with isoprene synthase-specific polyclonal antibodies showed the presence of the IspS protein in the three SkIpsS, Δcpc+CmR lines, migrating to about 65 kD in the gel electrophoresis (Fig. 3, lower panel). These results suggest successful replacement of the *cpc* operon by the *CmR* cassette, and show low levels of expression of the CmR protein. The *SkIpsS*, Δ*cpc*+*CmR* transformant served as a control strain in the below measurements.

Transformation and protein expression profile of the SkIpsS, Δcpc+SSpFNI+CmR strain

The *Synechocystis* codon-optimized *S. pneumoniae* "isopentenyl diphosphate isomerase" gene (*FNI*), followed by the chloramphenicol resistance *CmR* cassette (Fig. 1d), was introduced via double homologous recombination into the *cpc* operon locus (Fig. 1b) of a strain already carrying the codon-optimized kudzu isoprene synthase *SkIspS* gene, the latter being in the *psbA2* locus (Fig. 1a). Replacement of the native *cpc* operon with the *FNI-CmR* construct

generated an isoprene producing, *FNI* overexpressing strain termed SkIpsS, Δcpc+SSpFNI+CmR. State of homoplasmy of three independent lines of the SkIpsS, Δcpc+SSpFNI+CmR strains was tested by PCR amplification of genomic DNA using primers flanking the insertion site, as well as primers specific to the recipient strain. In the recipient strain (SkIspS), primers F1 and R1 (Fig. 1b) flanking the *cpc* operon amplified a 3.6 kb product, corresponding to the DNA of the full *cpc* operon (Fig. 3a). In the Δcpc+SSpFNI+CmR transformants, the same F1-R1 primers (Fig. 1d) amplified only a 2 kb product, corresponding to the FNI-CmR insert, and failed to amplify a product corresponding to the genes of the full *cpc* operon. Conversely, primers F1 and R2 (Fig. 1b) flanking the *cpc* upstream region (F1) and the *cpcA* gene (R2), amplified a 1.3 kb product in the recipient strain only (Fig. 4b) but failed to amplify any products in the Δcpc+SSpFNI+CmR strains. This genomic DNA PCR analysis provided evidence that the FNI-CmR transgene properly integrated into the recipient *Synechocystis* genomic DNA and segregation of transgenic DNA copies (homoplasmy) has occurred in the three transgenic lines under our selection conditions.

Synechocystis wild type and the recipient (SkIspS) strains showed expression of the phycocyanin cpcB (β-subunit) and cpcA (α-subunit) as dominant proteins in the 15–20 kD region (Fig. 5a, Wild type and SkIspS). Importantly, the Δcpc+SSpFNI+CmR transgenic lines lacked the phycocyanin proteins but showed the presence of distinct FNI and CmR proteins migrating to about 37 and 24 kD, respectively

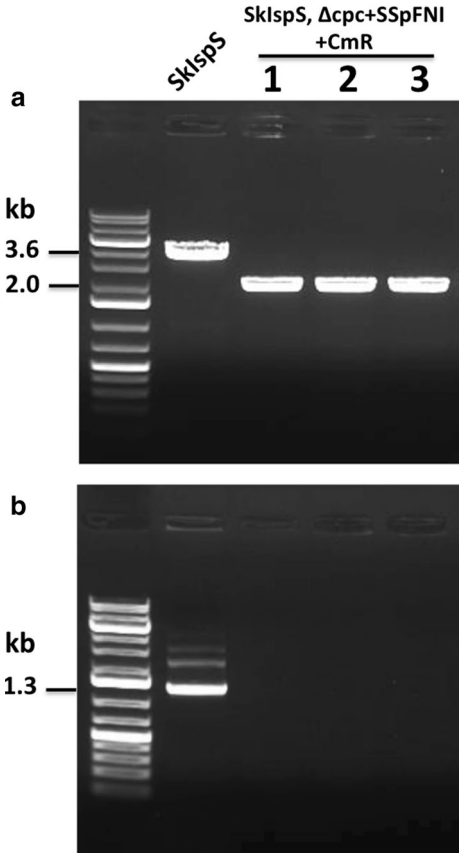

Fig. 4 Genomic DNA PCR analysis with selected *forward* (F) and *reverse* (R) primers positioned on the genomic DNA of *Synechocystis* recipient (*SkIspS*) strain and three *FNI-CmR* transformant lines (*SkIspS*, Δ*cpc+SSpFNI+CmR*). **a** PCR reactions using primers F1 and R1 (primer positions given in Fig. 1) amplifying the DNA region of the *cpc* operon between the *cpc* promoter and terminator. The recipient strain (*SkIspS*) yielded a single 3.6 kb product, corresponding to the full *cpc* operon genes. Three different *FNI-CmR* lines yielded a single 2 kb product, corresponding to the *FNI-CmR* DNA. **b** PCR reactions using primers F1 and R2 amplifying the DNA region between the *cpc* promoter and the *cpcA* gene. The recipient strain (*FNI-CmR*) yielded a single 1.3 kb product. Three different *FNI-CmR* lines failed to yield any PCR products

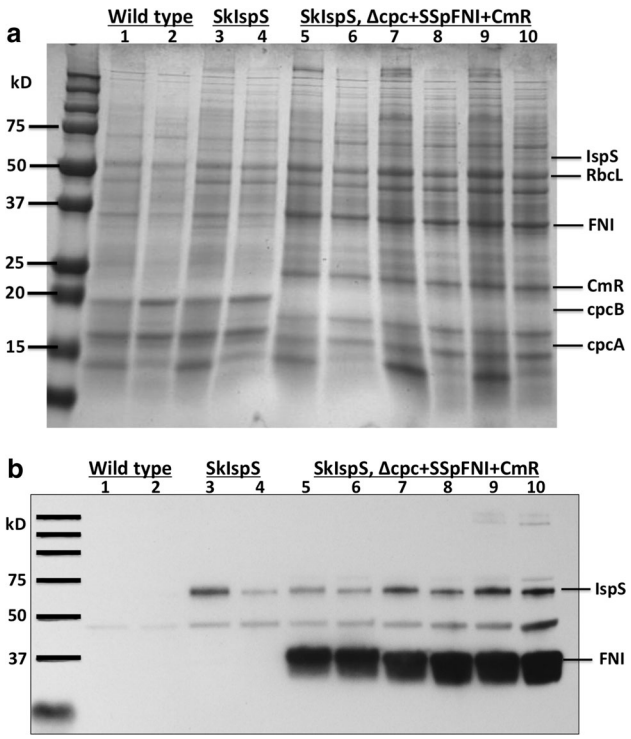

Fig. 5 **a** SDS-PAGE analysis of total protein extracts from *Synechocystis* wild type, the recipient strain (*SkIspS*) and three *FNI-CmR* transformant lines (*SkIspS*, Δ*cpc+SSpFNI+CmR*). Pre-stained Precision Plus protein standard molecular weight markers from BioRad are indicated in kD. Note the presence of the cpcB and cpcA phycocyanin subunits in the wild type and recipient strain (*SkIspS*), migrating to about 18 and 17 kD, respectively, and the absence of these proteins from the *FNI-CmR* transformant lines. Also note the pronounced expression of the FNI and CmR proteins in the *FNI-CmR* lines, and the low expression level of the IspS protein in both the recipient strain (*SkIspS*) and three *FNI-CmR* transformant lines. **b** Western blot analysis of total protein extracts from *Synechocystis* wild type, the recipient strain (*SkIspS*) and three *FNI-CmR* transformant lines (*SkIspS*, Δ*cpc+SSpFNI+CmR*) probed with specific polyclonal antibodies raised against the IspS and FNI proteins

(Fig. 5a, SkIpsS, Δcpc+SSpFNI+CmR lanes). Smaller amounts of the IspS can also be seen in SDS-PAGE Coomassie stain of the recipient strain (SkIpsS) and Δcpc+SSpFNI+CmR lines, migrating to about 65 kD (Fig. 5a, IspS). Expression of the FNI and IspS proteins was confirmed by Western blot analysis (Fig. 5b), showing specific cross-reactions with 37 and ~65 kD proteins, respectively. This Western blot analysis with the IspS immune serum also showed a minor cross-reaction with a protein migrating to about 45 kD, possibly a partial proteolysis product of the IspS protein. It is evident from both the Coomassie stain in the SDS-PAGE (Fig. 5a) and the Western blot analysis (Fig. 5b) that FNI is expressed at higher levels than the IspS protein.

Physiology of recipient, Δ*cpc+CmR*, and Δ*cpc+SSpFNI+CmR Synechocystis* transformants

A number of physiological parameters were measured to assess functional properties of the transformants used in this work. Pigment content in relation to biomass accumulation was measured after 96 h of cultivation. Chl/OD_{730} and Chl/DCW were lowered to between 59 and 68 % in the Δ*cpc+CmR* relative to the recipient *SkIpsS* strain (Table 1). Similarly, Chl/OD_{730} and Chl/DCW were lowered to between 57 and 64 % in the Δ*cpc+SSpFNI+CmR Synechocystis* transformant relative to the recipient *SkIpsS* strain. These results are consistent with previous pigment measurements in Δ*cpc* (*cpc* operon deletion) strains (Kirst et al. 2014), attributed to a decrease in the PSI/PSII stoichiometry as a result of cell acclimation to the removal of the phycocyanin rods (Kirst et al. 2014; Collins et al. 2012; Ajlani and Vernotte 1998). Removal of phycocyanin from the phycobilisomes substantially attenuates the light-harvesting capacity of PSII resulting in a potential over-excitation of PSI. Down-regulation of PSI accumulation by the cells rebalances the excitation energy distribution between the two photosystems (Table 1). As PSI contains more chlorophyll *a* molecules than PSII, this PSI/PSII ratio adjustment results in lowered chlorophyll *a* per cell, manifested as lower chlorophyll *a* per biomass. Total carotenoid accumulation in the above transformants appeared to be slightly elevated to 108–124 %, relative to the recipient *SkIpsS* strain. This was not further investigated in this work.

Isoprene and biomass accumulation in control and *FNI* expressing transformants

Rates of *Synechocystis* growth were determined in sealed gaseous-aqueous two-phase photobioreactors developed by Bentley and Melis (2012). Strains overexpressing the *FNI* protein (*SkIpsS*, Δ*cpc+SSpFNI+CmR*; Fig. 6, red squares) grew with the same rate as the recipient (*SkIspS*; Fig. 6, black circles) and control (*SkIpsS*, Δ*cpc+CmR*; Fig. 6, blue triangles) under a light intensity of 100 μmol photons m^{-2} s^{-1}. Although the OD_{730} increase appeared to be slightly slower in the Δ*cpc* strains relative to the recipient strain (Fig. 6a), dry cell weight (DCW) biomass accumulation (Fig. 6b) was the same in all strains. The discrepancy indicates that the optical properties of cell scattering may be altered upon deletion of phycocyanin and the ensuing lowering of the chlorophyll content in the Δ*cpc* strains. Explanation as to why strains with or without phycocyanin grew at about the same rate is provided upon consideration of the high level inoculation of the cultures at $OD_{730} = 0.65$ (Fig. 6a) or DCW = 0.2 g L^{-1} (Fig. 6b). This high inoculum in combination with the use of photobioreactors with a ~9.5 cm internal optical pathlength was necessary and sufficient to ensure absorption of all incoming irradiance by the cultures, regardless of the antenna configuration of the strains employed. The linear increase of the biomass as a function of incubation time, with an average doubling of the initial biomass every about 24 h (Fig. 6, see also Bentley and Melis 2012), suggested an overall light-limitation in cell growth, defined by the actual quantitative absorption of incident irradiance by the culture.

Table 1 Pigment content, PSI/PSII photosystem reaction center ratio, and light-saturated rates of *Synechocystis* photosynthesis *SkIspS* recipient strains and Δcpc transformants grown photoautotrophically in the laboratory

Parameter measured	SkIspS	SkIspS, Δcpc+CmR	SkIspS, Δcpc+SSpFNI+CmR
Chl/OD_{730} (μg)	4.78 ± 0.24 (100 %)	3.24 ± 0.22 (68 %)	3.05 ± 0.25 (64 %)
Chl/DCW (μg mg^{-1})	17.29 ± 1.27 (100 %)	10.21 ± 0.82 (59 %)	9.94 ± 0.72 (57 %)
Car/OD_{730} (μg)	1.64 ± 0.18 (100 %)	2.03 ± 0.06 (124 %)	1.96 ± 0.13 (120 %)
Car/DCW (μg mg^{-1})	5.89 ± 0.47 (100 %)	6.29 ± 0.48 (108 %)	6.29 ± 0.14 (108 %)
PSI/PSII (mol:mol)	2.5 ± 0.08*	1.8 ± 0.13*	1.5 ± 0.15
P_{max}, mmol O_2 (mol Chl)$^{-1}$ s^{-1}	150*	165*	155

Photosystem reaction center ratios were measured spectrophotometrically (Melis 1989). n ≥ 3; mean ± SD

* Kirst et al. (2014)

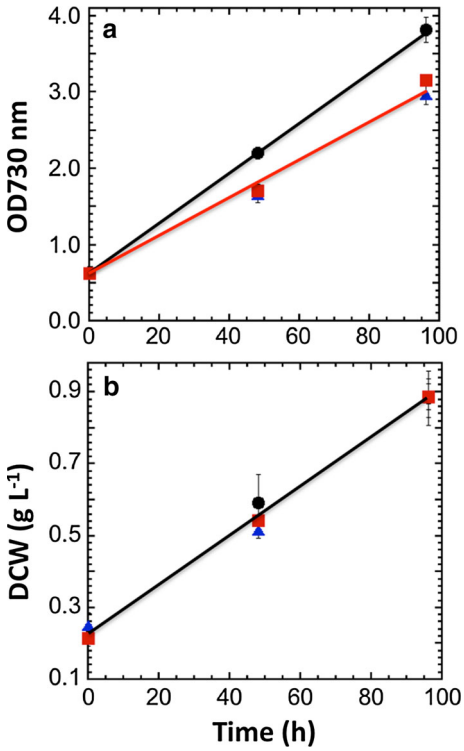

Fig. 6 Photoautotrophic growth of the recipient *SkIspS* strain (*black circles*), the *CmR* transformant (*SkIspS*, Δ*cpc*+*CmR*) (*blue triangles*), and FNI-CmR expressing strain *SkIspS*, Δ*cpc*+*SSpFNI*+*CmR* strain (*red squares*) measured from the increment in the optical density of the cultures at 730 nm (**a**) or from the accumulating dry cell weight (DCW) of the respective biomass (**b**). Cells were cultivated in a sealed gaseous-aqueous two-phase photobioreactor system (Bentley and Melis 2012) in which the liquid phase comprised 700 mL of BG11 growth medium and the gaseous phase comprised 500 mL of 100 % CO_2

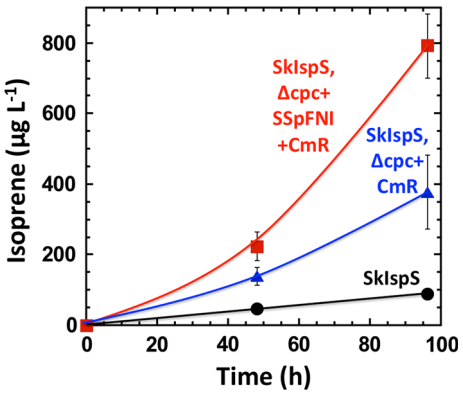

Fig. 7 A sealed gaseous-aqueous two-phase 1.2 L reactor (Bentley and Melis (2012) was used for *Synechocystis* isoprene production measurements. The liquid culture comprised 700 mL of BG11 growth medium, whereas the gaseous phase was filled with 100 % CO_2. Upon assembly and culture inoculation, the reactor was sealed and incubated under continuous illumination of 100 µmol photons m^{-2} s^{-1}. Every 48 h, sampling of the reactor for biomass and isoprene content was followed by flushing the gaseous products and by refilling the gaseous phase with 100 % CO_2 to sustain growth and productivity. Note the much steeper rate of isoprene production by the control strain *SkIspS*, Δ*cpc*+*CmR* (*blue triangles*) compared to the recipient *SkIspS* strain (*black circles*), and the even greater rate of isoprene production by the FNI-CmR strain *SkIpsS*, Δ*cpc*+*SSpFNI*+*CmR* (*red squares*)

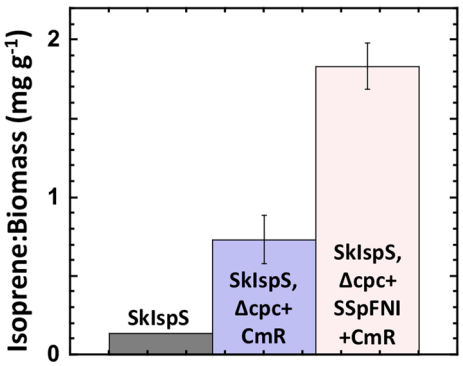

Fig. 8 Calculated isoprene-to-biomass (w:w) ratios for the recipient (*SkIspS*), CmR control (*SkIpsS*, Δ*cpc*+*CmR),* and FNI-CmR overexpression strain (*SkIpsS*, Δ*cpc*+*SSpFNI*+*CmR)*. A fivefold increase (from 0.13 to 0.73 mg g^{-1}) was observed in the isoprene-to-biomass ratio upon the deletion of the cpc operon in the (*SkIspS*, Δ*cpc*+*CmR)* compared to the recipient strain (*SkIspS*). An additional 2.5-fold increase (from 0.73 to 1.8 mg g^{-1}) in the isoprene-to-biomass carbon-partitioning ratio was noted upon expression of the isopentenyl diphosphate isomerase (*FNI*) gene in the *SkIpsS*, Δ*cpc*+*SSpFNI*+*CmR* strain

Rates of isoprene production were also linear as a function of incubation time, but differed substantially between the recipient strain, the control strain, and the FNI transformants. The recipient strain (*SkIspS*) generated about 1.1 µg isoprene L^{-1} h^{-1}, whereas the control strain (*SkIpsS*, Δ*cpc*+*CmR)* generated about 5.1 µg isoprene L^{-1} h^{-1} a fivefold improvement. We hypothesize that such substantial increase in the yield of isoprene may be attributed to the deletion of the *cpc* operon in the *SkIpsS*, Δ*cpc*+*CmR* transformant, resulting in the accumulation of endogenous metabolites, as these transformants no longer consume photosynthetic carbon for the synthesis of phycocyanin. The extra metabolic resources are then available to alternative sinks, e.g., isoprene synthesis. This hypothesis was not further investigated in this work.

The FNI transformants (*SkIpsS*, Δ*cpc*+*SSpFNI*+*CmR*) produced 12.8 µg isoprene L^{-1} h^{-1}, an additional 2.5-fold improvement (Fig. 7). This differential rate of isoprene production translated into an altered isoprene-to-biomass carbon-partitioning ratio from 0.13 mg g^{-1} in the recipient (*SkIspS*) strain, to 0.73 mg g^{-1} in the control (*SkIpsS*, Δ*cpc*+*CmR)* strain, and 1.8 mg g^{-1} in the (*SkIpsS*, Δ*cpc*+*SSpFNI*+*CmR*) FNI over-expressing strain (Fig. 8).

Discussion

Isoprenoids are useful compounds mediating the ecological interactions of plants with both herbivores and pollinators. Isoprenoids with toxic properties (pinene, linalool, camphor, ocimene) bolster plant defenses against herbivores and infectious microbes, while others may serve to attract pollinators (bees, pollen wasps, ants, hoverflies, butterflies and moths, and flower beetles) serving the cross-pollination process in both wild land and crop plants. Isoprenoids have also gained attention for their chemical properties that make them valuable commercial products. In 2002 the worldwide sales of terpene-based pharmaceuticals was $12 billion (Khosla and Keasling 2003), most notably including taxol from the pacific yew tree as an anti-cancer agent, and artemisinin from sweet wormwood used to treat malaria. Food additives contain many plant-based terpenoids such as myrcene, one of the chemicals from hops which flavors beer, and limonene from citrus to provide flavoring in desserts. The perfume industry also employs isoprenoids for their signature scents and fragrances such as terpineol to produce lilac scent, and nerolidol to produce orange blossom scent.

One of the most commonly used in commercial applications and the building block of all terpenoids is isoprene (C_5H_8). In the year 2000, 360 billion pounds of isoprene was used in the United States, mainly in the form of poly (cis1,4-isoprene) as a tough elastic rubber in vehicle tires. Isoprene was also co-polymerized with styrene to form thermal elastomers and pressure-sensitive adhesives (Lee et al. 2007). The utility of isoprene as a fuel and synthetic chemistry feedstock is important, and thus a study of its renewable biological synthesis is valuable.

The work provided evidence that a heterologous IPP isomerase in cyanobacteria can play a role in modulating the DMAPP/IPP steady-state ratio of the cells, as evidenced by the FNI effect on the isoprene "reporter process," i.e., rate and yield of photosynthetic isoprene production. The effect is schematically depicted in the reaction pathway of Fig. 9. The intermediate metabolite HMBPP is preferentially partitioned toward IPP, generating an IPP:DMAPP ratio of 3:1 in the cell (McGarvey and Croteau 1995; Lichtenthaler 2010; Formighieri and Melis 2014), but measured to be 5.6:1 in tobacco (Tritsch et al. 2010). The greater relative amounts of IPP are needed for the synthesis of longer chain terpenoids in the cell (phytol, carotenoids, quinones). The heterologous expression of FNI in this case shifted the steady-state between IPP and DMAPP toward DMAPP, effectively enhancing the concentration of DMAPP in the cell. As DMAPP is the substrate of the IspS enzyme and reactant for isoprene synthesis (Fig. 9), this metabolic steady-state shift resulted in greater rates and yield of isoprene production by the SkIspS, Δcpc+SSpFNI+CmR strain.

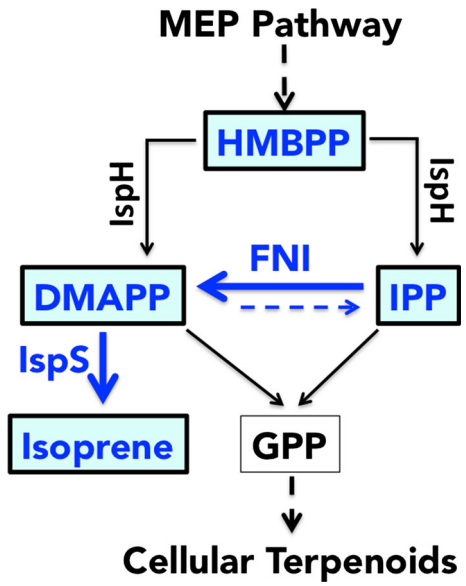

Fig. 9 Schematic depicting the regulation of carbon flow through the native MEP pathway for the generation of the universal terpenoid precursors dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). DMAPP and IPP are isomers derived from the same precursor metabolite, the hydroxy-2-methyl-2-butenyl-4-diphosphate (HMBPP), upon the action of the IspH enzyme. The native steady-state ratio between DMAPP and IPP in the cell is shifted toward DMAPP by a heterologous isopentenyl diphosphate isomerase (FNI). As DMAPP is the only substrate for the isoprene synthase (IspS), which produces the hemiterpene isoprene, the FNI-induced shift in the IPP-DMAPP steady-state ratio favors isoprene production. Native terpenoid biosynthesis requires DMAPP and IPP condensation to produce geranyl diphosphate (GPP), the precursor for all other cellular terpenoids

Expression of the *FNI* gene, a *S. pneumoniae* IPP isomerase, in a *Synechocystis* recipient strain that harbored the isoprene synthase (*IspS*) gene, resulted in a 250 % increase in the yield of isoprene production by these cyanobacteria (Fig. 8). This finding is qualitatively similar to that recently reported in work by Gao et al. (2016). They employed a different cyanobacterial strain (*Synechococcus elongatus* PCC 7942), and a different isopentenyl diphosphate isomerase cloned from yeast, to show enhancements in phototrophic isoprene production. Interestingly, Gao et al. (2016) reported conditions that improved yield, from the native 4–5 % MEP pathway carbon fux, up to 40 % of photosynthetic carbon partitioning toward isoprene production, achieved upon improvement in carbon flux through the MEP pathway by overexpressing the *dxs* and *IspG* genes. However, these important findings cannot be independently tested, as Gao et al. (2016) did not provide the DNA sequences of the constructs used for any of their transformants. Provision of these details is important, as

work by other investigators (Leonard et al. 2010; Xiao et al. 2012; Zurbriggen et al. 2012; Li and Sharkey 2013) has indicated that overexpression of endogenous MEP pathway genes may not substantially enhance the highly regulated endogenous flux of metabolites through the MEP pathway.

Expression of the *FNI* and *IspS* genes in *Synechocystis* in this work was assessed by SDS-PAGE Coomassie stain (Fig. 5a) and Western blot analysis (Fig. 5b) of total cyanobacterial protein extracts. Both of these protein chemistry analytical methods showed sufficient expression of the 37 kD protein FNI protein, with excellent visibility of this transgenic protein in the Coomassie stain of the SkIspS, Δcpc+SSpFNI+CmR SDS-PAGE gels and Western blot analysis. Expression levels of the 65-kD IspS protein, however, were less pronounced in the Coomassie stained SDS-PAGE gels and Western blot analysis, showing a faint band at 65 kD, present in the recipient SkIspS and SkIspS, Δcpc+SSpFNI+CmR strains but not in the wild type (Fig. 5a, b). Under these conditions, it is likely that limiting amounts of the IspS protein caused a limitation in the yield of product synthesis.

In *Synechocystis* PCC 6803, a stable knock out line of the sll1556 gene encoding the IPP isomerase was previously made by Poliquin et al. (2004), and examined by scanning electron microscopy. It was reported that thylakoid membrane development was significantly altered by the absence of the IPP isomerase, though these developmental changes did not alter growth rates under a variety of low light intensity conditions. The outer wall of the mutant cells lacking the sll1556 gene contained more fibrous extensions, and there were fewer thylakoids per central cell section in the mutant than in the wild type, indicating deficient isoprenoid biosynthesis in the absence of the IPP isomerase. However, the specific isoprenoids affected by this mutation were not identified in this study.

The IPP isomerase was also reported to be a rate-limiting enzyme in the terpenoid biosynthesis of green algae (Sun et al. 1998), plants (Albrecht and Sandmann 1994), and multiple other organisms (Chen et al. 2012; Kajiwara et al. 1997). Three different IPP isomerase enzymes from *Haematococcus pluvialis*, *Phaffia rhodozyma*, and *Saccaromyces cerevisiae* were overexpressed independently in *E. coli,* along with the carotenoid biosynthesis gene cluster from *Erwinia* (Kajiwara et al. 1997). The presence of an overexpressed IPP isomerase contributed to 1.7- to 4.5-fold increase in carotenoid production, indicating that the IPP isomerase plays a role in substrate partitioning for heterologous carotenoid biosynthesis in these organisms. Several other groups have also overexpressed the IPP isomerase to enhance heterologous carotenoid synthesis in *E. coli* (Lemuth et al. 2011; Wang et al. 2009; Gallagher et al. 2003; Albrecht and Sandmann 1994). Overexpression of the native IPP isomerase in *Eucommia ulmoides* resulted in a 3- to 4-fold increase in the production of the most widely used isoprenoid polymer, trans-polyisoprene rubber (Chen et al. 2012).

Isomerase enzymes of other biosynthetic pathways were also reported to enhance product formation. Overexpression of the chalcone isomerase from the flavonol biosynthetic pathway up-regulated end product synthesis by up to a 66-fold in tomato (Muir et al. 2001), and 12-fold increase in *Salvia Involucrata* (Li et al. 2006). Integration of multiple copies of the *Piromyces* xylose isomerase (*XYLA*) gene, resulting in xylose isomerase overexpression, was employed to enhance by about ninefold xylose assimilation for ethanol production in *Saccharomyces cerevisiae* (Zhou et al. 2012). Thus, isomerase enzymes have proven to play critical roles in multiple biosynthetic pathways.

Acknowledgments The work was conducted in partial satisfaction of the requirements for the degree of Doctor of Philosophy by JEC, supported by a graduate student fellowship from the NSF Sage IGERT program.

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