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Journal

ACS Synthetic Biology, 7(3)

ISSN

2161-5063

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Publication Date

2018-03-16

DOI

10.1021/acssynbio.7b00431

Peer reviewed

ACS Synthetic Biology
Research Article
Cite This: ACS Synth. Biol. 2018, 7, 912–921
pubs.acs.org/synthbio

Heterologous Leader Sequences in Fusion Constructs Enhance Expression of Geranyl Diphosphate Synthase and Yield of β-Phellandrene Production in Cyanobacteria (*Synechocystis*)

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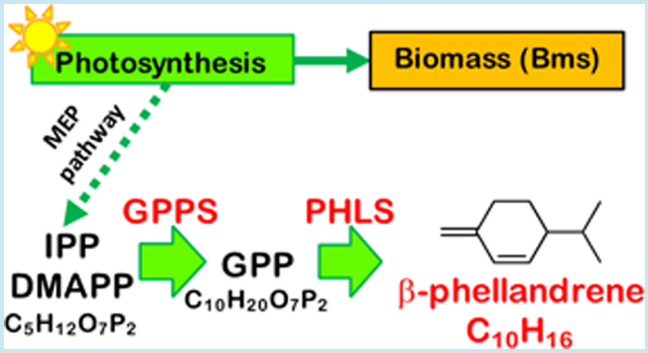
Supporting Information

ABSTRACT: Fusion constructs as protein overexpression vectors proved to be critical in the heterologous expression of terpene synthases in cyanobacteria. The concept was recently applied to the heterologous overexpression of the β-phellandrene synthase (*β-PHLS*) from plants, fused to the highly expressed endogenous *cpcB* gene encoding the β-subunit of phycocyanin. Overexpressed CpcB*PHLS fusion proteins enhanced the heterologous yield of $C_{10}H_{16}$ β-phellandrene hydrocarbons production in *Synechocystis*. This work extended the concept of fusion constructs as protein overexpression vectors by showing that highly expressed heterologous genes could also serve as leader sequences for protein overexpression in cyanobacteria. Examined are the kanamycin *nptI* and chloramphenicol *cmR* resistance cassettes, both of which are overexpressed in *Synechocystis*. Evidence showed a dual purpose of the *nptI* gene, as a leader sequence fused to a heterologous geranyl-diphosphate synthase (*GPPS*), promoting its expression, while at the same time serving as a selectable marker for the screening of transformants. The work further showed that enhanced *GPPS* expression increased the yield of β-phellandrene in *Synechocystis* transformants harboring the *β-PHLS* gene. Moreover, the research evaluated the expression efficacy of a DNA fragment comprising 87 nucleotides from the 5′ end of the *cmR* gene in fusion with the *GPPS* gene. This short fusion construct substantially increased the intracellular geranyl-diphosphate synthase level, suggesting that "short-stretch" *cmR* leader sequences can be used to drive a higher expression level of heterologous biosynthetic genes, while avoiding undesirable internal recombinations, as these sequences are shorter than the threshold of 200 bp, commonly assumed to be the threshold of high efficiency recombinations.



KEYWORDS: *cyanobacteria, fusion protein, GPP synthase, metabolic engineering, β-phellandrene, photosynthesis, terpene synthesis*

Among naturally occurring chemicals, it is the isoprenoid, or terpenoid, biosynthetic pathway that generates the largest class of compounds. Isoprenoids, or terpenoids, are thought as products of the cellular secondary metabolism, meaning their rate and yield is minor compared to that of the primary cellular metabolism. However, isoprenoids are functionally vital for all cells and important to photosynthesis, as the pathway generates required molecules such as the phytol tail of chlorophylls, carotenoids, and quinone prenyl side-chains. In addition, plant hormones such as abscisic and gibberellic acid, and a great variety of molecules that serve to discourage herbivory, as well as compounds that confer scents to leaves and fragrance to flowers in order to attract pollinators, are products of the isoprenoid biosynthetic pathway.[1−4]

The universal precursors for isoprenoid synthesis in all living organisms are the 5-carbon ($C_5H_{12}O_7P_2$) metabolites dimethylallyl diphosphate (DMAPP) and its isomeric form isopentenyl diphosphate (IPP).[1−3] It is of interest that two distinct and separate biosynthetic pathways evolved independently in nature to generate DMAPP and IPP. In general terms, archaea, yeast, fungi, insects, animals, and the eukaryotic plant cytosol operate the mevalonic acid pathway, which begins with acetyl-CoA metabolites and ends with the generation of DMAPP and IPP. Most fermentative aerobic and anaerobic bacteria, anoxygenic photosynthetic bacteria, cyanobacteria, algae (micro and macro), and chloroplasts in all photosynthetic organisms operate the methylerythritol 4-phosphate (MEP) pathway (Figure 1), beginning with glyceraldehyde 3-phosphate and pyruvate metabolites and ending with the generation of DMAPP and IPP.[3,4]

Of interest in this work is the heterologous generation of $C_{10}H_{16}$ monoterpene hydrocarbons, with β-phellandrene, a monocyclic monoterpene, as the case study. Monoterpenes could serve as renewable biogasoline, as well as flavor, fragrance, and synthetic chemistry feedstock in the commercial sector.[5] There is a two-step enzymatic reaction that leads from DMAPP and IPP to the generation of β-phellandrene (Figure 1). The first step entails the covalent linkage of equimolar

Received: November 28, 2017
Published: February 4, 2018

DOI: 10.1021/acssynbio.7b00431
ACS Synth. Biol. 2018, 7, 912–921

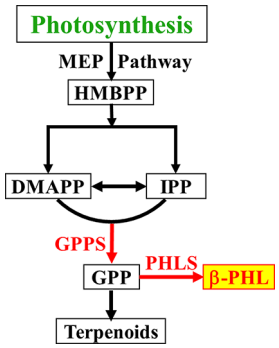


Figure 1. Schematic of substrate flux in the methylerythritol phosphate (MEP) biosynthetic pathway leading to terpenoids and the monoterpene β-phellandrene (PHL) in *Synechocystis*. Glyceraldehyde 3-phosphate and pyruvate metabolites from photosynthesis serve as the primary substrates of the MEP pathway, leading to the synthesis of the 4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) from which the intermediate 5-carbon compounds isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) emanate. The latter serve as the universal precursors to all terpenoids. Head-to-tail condensation of DMAPP and IPP generates the 10-carbon geranyl diphosphate (GPP), catalyzed by the enzyme GPP-synthase (GPPS). GPP is the substrate of the heterologous β-phellandrene synthase (PHLS) from which β-phellandrene hydrocarbons are made.

amounts of the universal terpenoid precursors dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), in a head-to-tail reaction. This step is catalyzed by the enzyme geranyl diphosphate synthase (GPPS) to yield the intermediate isoprenoid metabolite geranyl diphosphate (GPP; $C_{10}H_{20}O_7P_2$) with the concomitant release of pyrophosphate. The second step entails removal of pyrophosphate (PP) from GPP by the enzyme β-phellandrene synthase (PHLS), thereby converting GPP to the $C_{10}H_{16}$ monoterpene β-phellandrene (Figure 1).

β-Phellandrene is naturally generated and stored in the trichomes of many plants (*e.g.*, lavender, tomato, pine, eucalyptus, grand fir, among other) as a secondary metabolite, comprising low amounts relative to the overall plant biomass (low capacity biosynthetic process). Efforts in this lab have sought to use photosynthetic microorganisms (*e.g.*, cyanobacteria) in an attempt to convert the cyanobacteria into modified trichomes, generating and exuding β-phellandrene at high rates and yields. Limiting parameters in this endeavor proved to include the expression level of the PHLS and GPPS enzymes,[6−8] the very low cellular concentration of which was a barrier to achieving high rates and yield of β-phellandrene production.

To overcome the enzyme expression barrier,[9,10] recent work from this lab contributed with the use of fusion constructs as protein overexpression vectors. Fusion of the lavender β-phellandrene synthase gene (*PHLS*[11]) to the highly expressed *cpcB* gene of *Synechocystis,* encoding the abundant phycocyanin β-subunit, was shown to substantially enhance the PHLS protein expression level in the cells, to the point where the fusion CpcB*PHLS protein was the most abundant protein in the cyanobacteria, thereby improving the associated photosynthetic yield of β-phellandrene (PHL) production in these cells.[7] However, another important variable in the yield of β-phellandrene biosynthesis is the pool size of geranyl diphosphate (GPP), as this is the reactant from which PHL is generated (Figure 1). As there is no obvious *GPPS* gene in *Synechocystis*, heterologous *GPPS2* from *Picea abies* was recruited and coexpressed along with the *PHLS* gene. In preliminary work, it was observed that coexpression of *PHLS* with the *GPPS2* gene led to a slight increase in PHL production.[8] However, the GPPS2 protein expression level in this initial effort was low and only detectable *via* sensitive Western-blot analysis. The argument was made that low levels of geranyl diphosphate synthase (GPPS) in transformant cyanobacteria do not support high rates and yield of β-phellandrene production. This hypothesis was tested in the present work upon a concerted effort to increase levels of GPPS expression so as to increase flux and the overall production of PHL (Figure 1) in the transformants.

RESULTS AND DISCUSSION

Plasmid Construct and Transgene Organization. The concept of fusion constructs as protein overexpression vectors proved to be critical in the heterologous overexpression of the lavender β-phellandrene synthase (*PHLS*,[7,8]) and of the kudzu isoprene synthase (*ISPS*,[12]) in cyanobacteria. It was concluded that highly expressed DNA in cyanobacteria (*e.g.*, the *cpcB* gene encoding the β-subunit of phycocyanin, an abundant protein), will cause a likewise expression of a fusion protein, when placed in the leader sequence position. In this respect, the CpcB*PHLS and CpcB*ISPS fusion constructs were the most abundant proteins in the respective transformant cyanobacteria, whereas the *PHLS* and *ISPS* genes alone were poorly expressed, regardless of the promoter used.[6,13] Recent work showed that a codon-optimized *nptI* gene, encoding a neomycin phosphotransferase conferring kanamycin resistance, was also highly expressed in *Synechocystis*.[14] Thus, a question was raised as to whether heterologous genes, *e.g.*, the *nptI*, could also serve as leader sequences in fusion constructs to overexpress plant terpenoid biosynthetic pathway proteins in cyanobacteria. This was tested upon the heterologous overexpression of the *GPPS2* gene from *Picea abies*. A fusion construct of the *nptI* and *GPPS2* sequences (*nptI*GPPS*) was made, codon optimized for expression in *Synechocystis*.

The *cpc* operon of *Synechocystis* (Figure 2, wild type) was first transformed with the *cpcB*PHLS* fusion construct, carrying the chloramphenicol resistant marker (Figure 2, Recipient strain).[7,8] Homoplasmic lines of the recipient strain expressing the CpcB*PHLS fusion protein under the control of the strong *cpc* promoter were subsequently transformed by plasmid construct of the *GPPS* gene coupled with a spectinomycin resistance cassette (*smR*) under the control of the P_{TRC} promoter (Figure 2, P_{TRC}-GPPS-SmR). Alternatively, the recipient strain was transformed by plasmid construct of the *GPPS* gene fused to the *nptI* DNA under the control of the P_{TRC} promoter (Figure 2, P_{TRC}-NptI*GPPS). In the latter case, selection was achieved by the kanamycin resistance property, conferred to the cells from the fusion NptI*GPPS protein. The P_{TRC}-GPPS-SmR and P_{TRC}-NptI*GPPS constructs were incorporated into the *Synechocystis* genomic DNA upon double homologous recombination at the *glgA1* (sll0945) locus, replacing this isoform of the endogenous glycogen synthase genes[15] (Figure 2).

Homoplasmy of the Transformants. Attainment of transgenic DNA copy homoplasmy in the above transformants was tested by genomic DNA PCR analysis (Figure 3). Oligonucleotide primers flanking the inset in the *cpc* operon were based on the *cpc* upstream (Figure 2, *cpc*_us, forward) and *cpcA*_Rv (Figure 2, *cpcA*_Rv, reverse) regions. The nucleotide sequence of these primers is listed in Supplemental Table S1. By using primers *cpc*_us (forward) and *cpcA*_Rv (reverse),

DOI: 10.1021/acssynbio.7b00431
ACS Synth. Biol. 2018, 7, 912−921

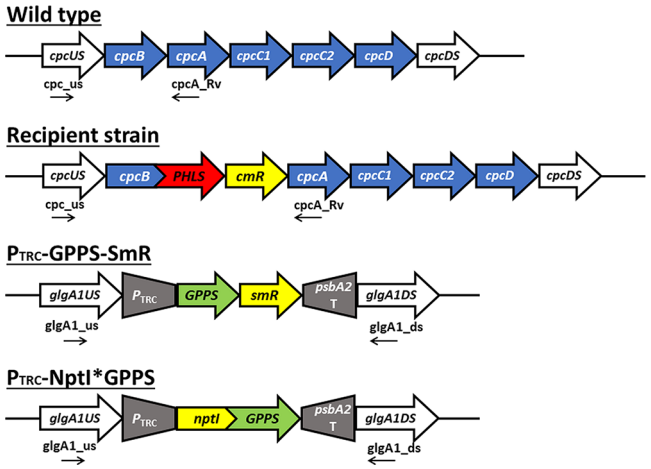


Figure 2. Schematic of the wild type *cpc* operon and recombinant constructs expressed in *Synechocystis* transformants. Thin black-line arrows mark the position of oligonucleotide primers used in the genomic DNA PCR analysis of the transformants. (Wild type) The *cpc* operon locus encoding the abundant *β*- (CpcB) and *α*- (CpcA) subunits of phycocyanin along with the phycocyanin peripheral-rod linker polypeptides (CpcC2, CpcC1, and CpcD). (Recipient strain) The *PHLS* transgene fused to the endogenous *cpcB* gene of *Synechocystis,* with the fusion construct expressed under the control of the *cpc* promoter with the chloramphenicol (*cmR*) resistance cassette for selection.[6−8] The remaining *cpc* operon genes (*cpcA* through *cpcD*) follow the 3′ end of *cmR*. (P_{TRC}-GPPS-SmR) The *GPPS* transgene expressed along with a spectinomycin resistance cassette (*smR*) under the control of the P_{TRC} promoter and the *psbA2* terminator, inserted into the *glgA1* locus upon replacement of the endogenous *glgA1* gene. (P_{TRC}-NptI*GPPS) The *GPPS* transgene fused to the 3′ end of the kanamycin resistance cassette (*nptI*), with the fusion construct expressed under the control of the constitutive P_{TRC} promoter and *psbA2* terminator, inserted into the *glgA1* locus upon replacement of *glgA1* gene.

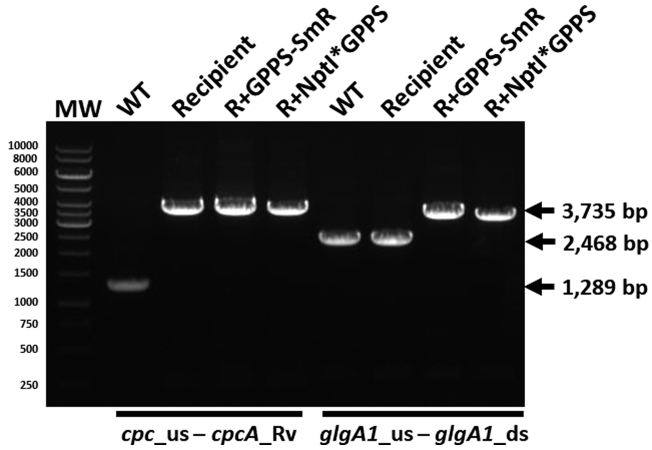


Figure 3. Genomic DNA PCR analysis with selected primers positioned on the genomic DNA of *Synechocystis* wild type (WT) and transformants. Strains expressing the *cpcB*PHLS* fusion construct are referred to as the recipient strains (R). Wild type, the recipient, and transformant lines expressing the *GPPS* along with the *smR* gene (R +GPPS-SmR) or the *nptI*GPPS* fusion construct (R+NptI*GPPS) were tested by genomic DNA PCR for transgene DNA copy homoplasmy. Primers annealed the genomic DNA in positions marked by thin black-line arrows in Figure 2. PCR reactions using primers *cpc*_us and *cpcA*_Rv[6,7] amplified a DNA fragment from the *cpc* promoter to the *cpcA* genomic region. Corresponding PCR reactions performed with the *glgA1*_us and *glgA1*_ds primers amplified the *glgA1* locus.[8]

annealing upstream of the *cpc* operon promoter and within the *cpcA* gene, respectively, the genomic DNA PCR reaction generated the expected 1289 bp product in the wild type, and the expected 3735 bp product in the recipient (R) and *GGPS*-transformant strains (Figure 3). The larger product size generated with these primers in the Recipient and R+GPPS strains is due to the presence of the *cpcB*PHLS* fusion and *cmR* cassette, installed within the *cpc* operon. Transgenic DNA copy homoplasmy was shown by the absence of any wild type amplicon in the transformants containing the *cpcB*PHLS* fusion.[7,8]

The GPPS heterologous constructs were inserted in the *glgA1* (sll0945) locus of *Synechocystis*. Homoplasmy of the transformants was tested by genomic DNA PCR analysis of the transformants with forward primers on the *glgA1* upstream region (Figure 2, *glgA1*_us) and reverse primers on the *glgA1* downstream region (Figure 2, *glgA1*_ds) annealing on the flanking regions of *glgA1* gene. The nucleotide sequence of these primers is also listed in the Supplemental Table S1. In this case, the wild type and recipient strain amplicons showed the expected 2468 bp size (Figure 3, right). Transformants containing the *GPPS-smR* and the *nptI*GPPS* constructs generated amplicon sizes of 3537 bp and 3452 bp, respectively. The larger amplicon size of the two *glgA1* transformants was due to the insertion of the *GPPS* gene sequence with the *smR* resistance cassette, or the presence of the fused sequence *nptI*GPPS*, which replaced the smaller *glgA1* gene. Absence of wild type *glgA1* 2468 bp product in the *GPPS* transformants showed that these reached a state of transgenic DNA copy homoplasmy.

Selection of the GPPS-SmR transformants was attained in the presence of spectinomycin, conferred by the presence of the SmR cassette. In the case of the NptI*GPPS transformant, selection was attained in the presence of kanamycin, conferred by the presence of the NptI protein in the NptI*GPPS fusion. Demonstration of the antibiotic resistance properties of the NptI*GPPS transformant used in this work is given in the results of Figure 4. Shown is the ability, or lack thereof, of *Synechocystis* transformants to grow in the presence of specific antibiotics. The recipient strain was able to grow on BG11-agar in the presence of 30 μg/mL chloramphenicol because of the presence of the *cmR* cassette in the modified *cpc* operon (Figure 2). However, this strain could not grow in the presence of both chloramphenicol and 20 μg/mL kanamycin, as it lacked the KanR protein (Figure 4, Recipient). Conversely, the recipient strain, when also transformed with the *nptI*GPPS* fusion cassette, was able to grow in the presence of chloramphenicol alone, and also in the presence of both chloramphenicol and kanamycin (Figure 4, R+NptI*GPPS). It is concluded that the NptI kanamycin resistance protein retained its functionality, albeit in the presence of the GPPS, when the latter was fused to the NptI C-terminus.

Transformant Protein Analysis. Cellular protein analysis was conducted to evaluate the CpcB*PHLS and GPPS protein expression levels in the various transformants. Since the emphasis of this work is on the expression of the GPPS constructs, three independent transformant lines harboring the *GPPS-smR* or *nptI*GPPS* genes were assayed. The SDS-PAGE Coomassie stain of *Synechocystis* total protein extracts are shown in Figure 5. The wild type (WT) showed dominant bands at ∼56 kDa, attributed to the large subunit of Rubisco

DOI: 10.1021/acssynbio.7b00431
ACS Synth. Biol. 2018, 7, 912−921

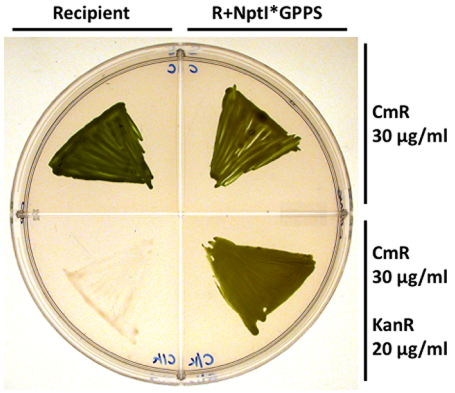


Figure 4. Survival test of recipient (R) strain and R+NptI*GPPS-expressing transformant in the presence or absence of selected antibiotics. Cell cultures were grown in BG11-agar media in a Quad-Plate. In the two upper sections, BG11 was supplemented with chloramphenicol only (30 μg/mL), while in the two lower sections both chloramphenicol and kanamycin (20 μg/mL) were added to the media. Recipient strain, expressing the CpcB*PHLS fusion and carrying the CmR resistance cassette (Figure 2) was grown on the two left-side sections, while the NptI*GPPS fusion-expressing transformant was grown on the two right-side sections of the Quad-Plate.

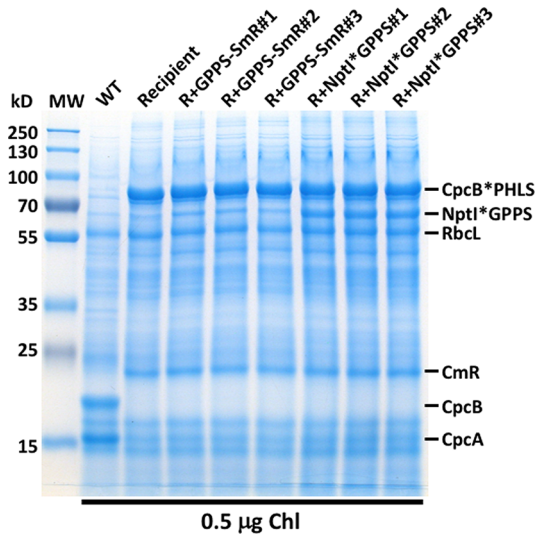


Figure 5. Protein expression analysis of *Synechocystis* wild type and transformants. Total cell proteins were resolved by SDS-PAGE and visualized by Coomassie-stain. Individual native and heterologous proteins of interest are indicated on the right side of the gel. Wild type (WT) *Synechocystis* extracts showed dominant proteins at 56 kDa (RbcL), 19 kDa (CpcB), and 14 kDa (CpcA). The strain expressing the CpcB*PHLS is referred to as the Recipient strain (R). The recipient strain expressed the CpcB*PHLS fusion construct as the most dominant protein in the cells (~75 kDa), while lacking the CpcB and CpcA proteins. Evident in the recipient strain is also the expression of the chloramphenicol resistance protein, migrating to about 23 kDa (CmR). Three independent lines each for the GPPS and SmR (R+GPPS-SmR) and NptI*GPPS (R+NptI*GPPS) expressing transformants were loaded on the gel. Evident in the latter is the expression of the NptI*GPPS fusion protein, migrating to about 62 kDa. Expression of the GPPS could not be discerned in the Coomassie strain of the R+GPPS-SmR extracts. Sample loading corresponded to 0.5 μg of chlorophyll.

(Figure 5, RbcL), and also dominant bands migrating to ~19 and 14 kDa, attributed to the phycocyanin CpcB β-subunit and CpcA α-subunit (Figure 5, CpcB and CpcA, respectively). The recipient strain showed dominant bands at ~75 kDa attributed to the CpcB*PHLS fusion protein,[7,8] ~56 kDa RbcL, and ~23 kDa CmR attributed to the expression of the chloramphenicol resistance protein (Figure 5, CmR). Notable was the absence of the CpcB and CpcA phycocyanin subunits, as the phycobilisome antenna is not assembled in strains harboring the CpcB*PHLS fusion construct.[7,8] Transformants with the GPPS and spectinomycin resistance cassette (Figure 5, R+GPPS-SmR #1–3) showed a similar to the recipient strain protein profile. The anticipated 36 kDa GPPS protein, however, could not be discerned in the Coomassie strain of these (R+GPPS-SmR #1–3) transformants. On the contrary, transformants with the NptI*GPPS fusion construct (Figure 5, R+NptI*GPPS #1–3) showed, in addition to all the pronounced protein bands seen in the recipient strain, clear presence of a ~62 kDa band, attributed to expression of the NptI*GPPS fusion construct.

To further explore the *GPPS* transgene expression levels and the assignment of the GPPS protein bands made in the results of Figure 5, Western-blot analysis with specific polyclonal antibodies against the *Picea abies* GPPS2 protein was undertaken (Figure 6). GPPS expression was evaluated in the

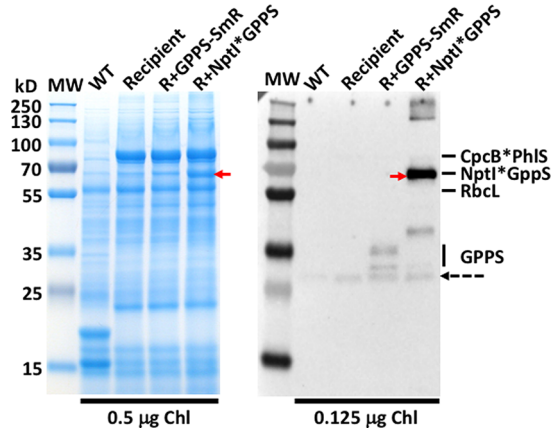


Figure 6. Protein expression analysis of *Synechocystis* wild type (WT) and transformants. Total cell proteins were resolved by SDS-PAGE and Coomassie-stained (left panel), or transferred to nitrocellulose and probed with specific α-GPPS2 polyclonal antibodies (right panel). Individual native and heterologous proteins of interest are indicated on the right side of the figure. The strain expressing the CpcB*PHLS fusion is referred to as the recipient strain (R). Transformant lines expressing GPPS along with SmR (R+GPPS-SmR) or NptI*GPPS only (R+NptI*GPPS) were loaded on the gel. Sample loading corresponds to 0.5 μg of chlorophyll for the Coomassie-stained gel and 0.125 μg of chlorophyll for the Western blot analysis. Red arrows indicate presence of the NptI*GPPS fusion protein.

two different transformants, compared to that of the wild type and the recipient strain. A faint cross-reaction was detected between the polyclonal *Picea abies* GPPS2 antibodies and a protein band migrating to about 32 kDa in the wild type, recipient, and GPPS transformant strains (Figure 6, dashed line arrow). This may reflect the presence of a low-level expression of an endogenous GPPS enzyme, or GPPS-like catalytic domain in *Synechocystis*. A native *GPPS* gene is still unknown in *Synechocystis*. However, bioinformatic analysis using as a reference the *Mycoplasma tuberculosis GPPS*, GenBank accession number AF082325.1[16] allowed identification of slr0611, an ORF encoding a putative prenyltransferase of 32 kDa. The faint cross-reactions at ~32 kDa observed in wild

DOI: 10.1021/acssynbio.7b00431
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type and transformants could thus be attributed to this protein. Cross-reactions were also detected between the polyclonal *Picea abies* GPPS2 antibodies and protein bands migrating to about 36 kDa in the GPPS-SmR transformant, showing that the P_{TRC}-GPPS-SmR construct was expressed at the protein level, albeit at very low levels (Figure 6, GPPS). A considerably stronger specific cross-reaction was detected between the polyclonal *Picea abies* GPPS2 antibodies and a protein band migrating to 62 kDa in the NptI*GPPS2 fusion transformant, showing that the P_{TRC}-NptI*GPPS construct was truly overexpressed at the protein level in *Synechocystis* (Figure 6, NptI*GPPS, marked with red arrow). This Western blot analysis corroborated the SDS-PAGE Coomassie stain findings on protein expression level shown in Figure 5 and Figure 6 (left panel).

Partial *cmR* and *nptI* Sequences as Leader Nucleotides in Fusion Constructs. In the preceding analysis, the full length *nptI* DNA was successfully used as a leader sequence in the *nptI*GPPS* fusion construct. We tested whether use of a truncated version comprising the 5′ end of the *nptI* gene could be sufficient to confer the strong expression of the *GPPS2* gene. Moreover, we noted that the chloramphenicol *cmR* resistance gene showed a high expression level in *Synechocystis* (Figure 5 and Figure 6), hence *cmR* could be used as a leader sequence to enhance the level of expression of *GPPS*. The full-length *cmR* sequence could not be used in this test because it was already present in the recipient strain as the selectable marker of the *cpcB*PHLS* transgenic DNA. On the basis of these observations, we tested to see if N-terminal truncated or "short stretch" leader sequences of the *nptI* and *cmR* genes could confer high level of expression of the subsequent GPPS fusion protein.

Truncated 5′ end leader sequences were chosen for *nptI* comprising 135 nucleotides (45 amino acids) and 309 nucleotides (103 amino acids). These are referred to as NptI45 and NptI103, respectively. Similarly, truncated 5′ end leader sequences were chosen for *cmR* comprising 87 nucleotides (29 amino acids) and 207 nucleotides (69 amino acids). These are referred to as CmR29 and CmR69, respectively. These leader sequences were selected on the basis of bioinformatic analysis, conducted on the NptI (http://www.rcsb.org/pdb/protein/P00551), and CmR (http://www.rcsb.org/pdb/protein/P62577) protein structures so as to avoid interrupting identifiable secondary α-helixes or β-sheets in the N-terminal domain of these proteins. The 3′ end of these partial sequences was fused to the 5′ end of the *GPPS2* DNA and the fusion constructs were then assembled in vectors containing the spectinomycin resistance (*smR*) cassette and the associated flanking regions for homologous recombination of the constructs in the *glgA1 Synechocystis* genomic DNA locus (for details, please see Materials and Methods and Supporting Information sections).

Transgenic DNA copy homoplasmy was tested by using the *glgA1*_us (forward) and *glgA1*_ds (reverse) oligonucleotide primers shown in Figure 2 and Table S1. Genomic DNA PCR analysis with these primers on wild type and transformants yielded products of expected sizes, *i.e.*, 2468 bp for the wild type, 3627 bp for CmR29; 3747 bp for CmR69; 3678 bp for NptI45; and 3852 bp for NptI103 (Figure 7, upper panel). No amplification of wild type sequence could be observed in the transformants, providing evidence of having reached transgenic DNA copy homoplasmy in the latter. The different heterologous proteins had an expected MW between 37 kDa and 43 kDa, but no protein overexpression was evident in the Coomassie strain of the SDS-PAGE analysis of any of the transformants with the truncated leader sequences (not shown). A subsequent Western-blot analysis using GPPS-specific polyclonal antibodies showed that heterologous fusion CmR29*GPPS and CmR69*GPPS proteins were expressed in the transformants, albeit to lower-than-expected levels. Levels of expression were better for the CmR29*GPPS than the CmR69*GPPS construct (Figure 7, lower panel). Surprisingly, no transgenic proteins could be detected with either the NptI45*GPPS or the NptI103*GPPS constructs.

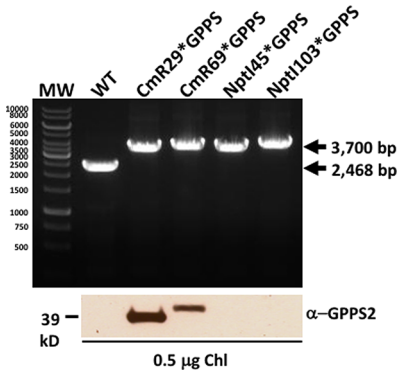

Figure 7. (Upper panel) Genomic DNA PCR analysis with selected primers positioned on the genomic DNA of *Synechocystis* wild type (WT) and transformants. PCR reactions were performed using *glgA1*_us and *glgA1*_ds primers, which amplified the *glgA1* locus. Primers annealed the genomic DNA in the positions marked by thin black arrows (Figure 2). Leader sequences fused at the N-terminus of GPPS were named according to their origin (CmR or NptI sequences) and their amino acid lengths are indicated. Wild type *Synechocystis* (WT) generated a 2468 bp product. Fusion constructs *cmR29*GPPS*, *cmR69*GPPS*, *nptI45*GPPS*, and *nptI103*GPPS* generated products in the 3700 bp region. (Lower panel) Western blot analysis of *Synechocystis* wild type and transformants. Total cell proteins were transferred to nitrocellulose and probed with specific α-GPPS2 polyclonal antibodies. Lanes were loaded with 0.5 μg for the Western blot analysis.

On the basis of the better expression of the CmR29*GPPS construct, we transformed the recipient strain of Figure 2 with the CmR29*GPPS-SmR construct, as described above for the results of Figure 2 and Figure 3. Upon attainment of transgenic DNA copy homoplasmy, SDS-PAGE and Western-blot analyses were conducted, comparing the GPPS expression level in *Synechocystis* recipient (R = CpcB*PHLS) strain, and transformants R+GPPS-SmR, R+NptI*GPPS, and R+CmR29*GPPS-SmR. The SDS-PAGE profile of total cell protein extracts from *Synechocystis* wild type, the recipient strain and these three transformants is shown in Figure 8, left panel. The SDS-PAGE Coomassie stain profile of the wild type proteins is dominated by the phycocyanin β- and α-subunits migrating to ~19 and 14 kDa, respectively (Figure 8, left panel, WT). The profile of the recipient strain proteins is dominated by the ~75 kDa CpcB*PHLS fusion protein, while the phycocyanin β- and α-subunits are missing (Figure 8, left panel, Recipient). The profile of the R+GPPS-SmR proteins is similar to that of the recipient strain, and it offers no evidence of overaccumulation of the GPPS protein (Figure 8, left panel, R+GPPS-SmR). The corresponding Western blot (Figure 8, right panel, R+GPPS-SmR, marked by red arrow) showed the presence of low levels of the GPPS transgenic protein. The profile of the R+NptI*GPPS proteins showed, in addition to

DOI: 10.1021/acssynbio.7b00431
ACS Synth. Biol. 2018, 7, 912–921

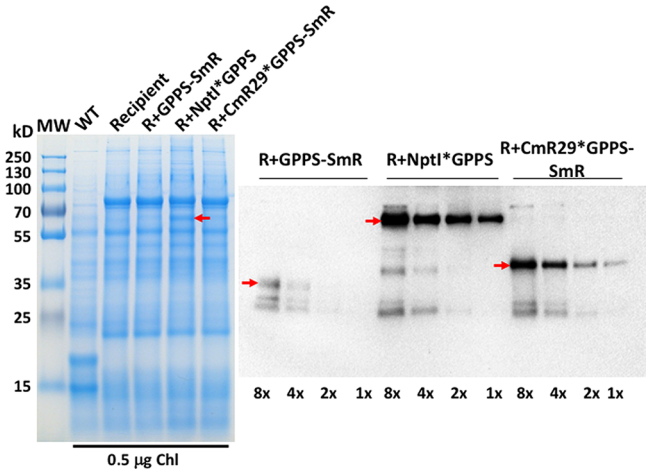

Figure 8. Protein expression analysis of *Synechocystis* wild type and transformants. Total cell proteins were resolved by SDS-PAGE and Coomassie-stained (left panel), or transferred to nitrocellulose and probed with specific α-GPPS2 polyclonal antibodies (right panel). The strain expressing the CpcB*PHLS fusion construct is referred to as the Recipient strain (R). Wild type *Synechocystis* (WT) and transformant lines expressing the GPPS along with SmR (R+GPPS-SmR), NptI*GPPS (R+NptI*GPPS) or CmR29 "short stretch" leader sequence fused to GPPS (R+CmR29*GPPS) were loaded on the gel. Sample loading corresponded to 0.5 μg of chlorophyll for Coomassie-stained SDS-PAGE. Samples corresponding to 0.250 (8×), 0.125 (4×), 0.0625 (2×), and 0.03125 (1×) μg of chlorophyll were loaded for the Western blot analysis. Red arrows indicate the electrophoretic migration position of the GPPS, NptI*GPPS and CmR29*GPPS proteins.

the overaccumulation of the CpcB*PHLS fusion, a notable accumulation of the 62 kDa NptI*GPPS fusion protein (Figure 8, left panel, R+NptI*GPPS, marked by red arrow). The identity of this band was confirmed by the corresponding Western blot analysis (Figure 8, right panel, R+NptI*GPPS), which showed strong cross-reactions between the polyclonal GPPS2 antibodies and a protein band migrating to ~62 kDa. Lastly, the profile of the R+CmR29*GPPS-SmR proteins offered no clear evidence of overaccumulation of the GPPS protein in the SDS-PAGE Coomassie stain (Figure 8, left panel, R+CmR29*GPPS-SmR). However, the corresponding Western blot analysis (Figure 8, right panel, R+Cm29*GPPS-SmR, marked by red arrow) clearly showed the presence of the transgenic GPPS fusion protein as a ~39 kDa band, albeit at quantities not sufficient to be visualized in the SDS-PAGE Coomassie stain analysis. These results showed that greater amounts of transgenic GPPS fusion protein accumulate, when the full-length DNA of a highly expressed leader sequence is used, compared to using only their respective truncated 5′ end.

Biomass Accumulation and β-Phellandrene Productivity. Wild type and transformants (R = CpcB*PHLS, R +GPPS-SmR, R+NptI*GPPS, R+CmR29*GPPS-SmR) were grown in the lab under a combination of continuous cool-white and warm-white illumination providing an incident visible light intensity of ~100 μmol photons m^{-2} s^{-1}. The rate of photoautotrophic cell growth and biomass accumulation was measured from the density of the cultures at 730 nm, OD_{730} (Figure 9). It was also measured from the specific rate of cell growth (μ), shown in Table 1. At this limiting growth irradiance, all transformants had a slower rate of cell growth and biomass accumulation compared to the wild type. Such

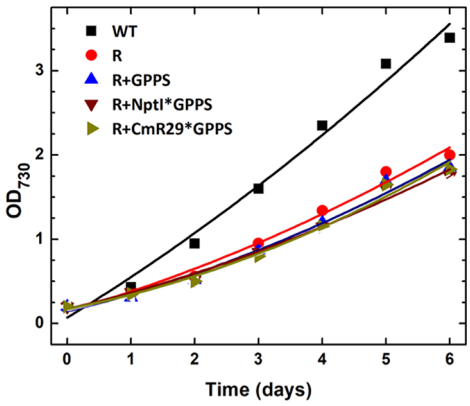

Figure 9. Biomass accumulation curves of *Synechocystis* wild type and transformants, as measured from the optical density of the cultures at 730 nm (OD_{730}). The strain expressing the CpcB*PHLS is referred to as the Recipient strain (R). Wild type *Synechocystis* (WT) and transformant lines expressing the GPPS along with SmR (R+GPPS-SmR), NptI*GPPS (R+NptI*GPPS) or CmR29 "short stretch" leader sequence fused to GPPS (R+CmR29*GPPS) were also analyzed. Cells were grown under 100 μmol photons m^{-2} s^{-1} of incident visible light intensity. Cultures were inoculated to an OD_{730} of about 0.2, as the initial cell concentration in the biomass accumulation experiment. Such kinetic measurements were repeated yielding similar results between different runs.

Table 1. Cell Growth and Photosynthetic Carbon Partitioning between β-Phellandrene and Cellular Biomass in *Synechocystis* in Relation to the Level of GPPS Expression[a]

genotype	growth rate, μ day^{-1}	β-phellandrene yield, mg g^{-1} dcw	amount of transgenic GPPS protein, rel. units
wild type	2.10 ± 0.26	0	native
recipient (cpcB*PHLS)	0.90 ± 0.19	1.42 ± 0.66	native
R+GPPS-smR	0.90 ± 0.14	1.89 ± 0.53	1.0
R+cmR29*GPPS-smR	0.76 ± 0.13	2.42 ± 0.86	9 ± 5
R+nptI*GPPS	0.81 ± 0.16	5.95 ± 1.31	60 ± 15

[a]Rates of *Synechocystis* cell growth were measured during the initial exponential growth phase from the increase in cell number as a function of time. The growth rate (μ) was calculated from the equation $\mu = \ln(N_2 / N_1)/(t_2 - t_1)$.[14] Yields are expressed as mg of β-phellandrene relative to the increment in biomass observed during a 48-h culture incubation period.[7,8] Amount of transgenic GPPS protein was measured in relative units, normalized to the level of GPPS expression in the R+GPPS-SmR transformant, which was set equal to 1.0. Averages and standard deviations listed are the result of three independent measurements. All transformant strains have a phenotype deficient in phycobilisome peripheral rod assembly.

differences in the rate of cell growth and biomass accumulation were expected because of the smaller light-harvesting antenna size in the transformants, minimizing the rate of light absorption in the latter.[7,14] The slope of the biomass accumulation curves in Figure 9 showed that, under these experimental conditions, the transformants accumulated biomass with only about 55% of the rate displayed by the wild type.

The above-mentioned wild type and transformant lines were tested to evaluate β-phellandrene (PHL) production. In this experiment, the various cell lines were inoculated in the gaseous/aqueous two-phase reactor, designed in this lab.[13] The

DOI: 10.1021/acssynbio.7b00431
ACS Synth. Biol. 2018, 7, 912−921

gaseous phase was loaded with 100% CO_2 and incubated in the light for 48 h. At the end of this incubation period, a known volume of organic solvent (heptane) was added to the surface of the culture to help dilute and collect the floating hydrocarbon products. The biomass content of the culture was assayed by OD_{730} and by gravimetric approaches,[12] whereas PHL productivity was assayed by UV-absorbance spectrophotometry and gas chromatography with flame ionization detector (GC-FID) analyses. β-Phellandrene has a well-defined absorbance spectrum with a primary absorbance peak at 232.4 nm in hexane/heptane. The concentration of this monoterpene was calculated from its specific absorbance at this wavelength (ε [232.4 nm] = 15.7 mM^{-1} cm^{-1})[6] and by application of Beer's law. Total absorbance in the UV region for the various samples was then normalized to the amount of biomass generated during the 48-h incubation in the light (Figure 10), as previously described.[8] The results showed that

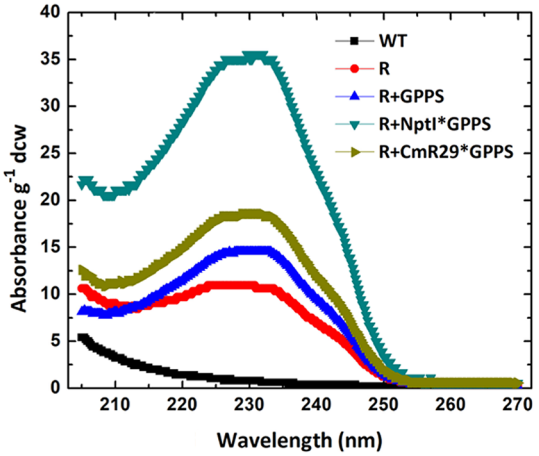

Figure 10. Detection of β-phellandrene hydrocarbons production by *Synechocystis* transformants. β-Phellandrene was siphoned off the top of the culture medium after applying a known volume of heptane solvent overlayer. Absorbance spectra of the heptane extracts were normalized on a per g dry cell weight (dcw) of the photosynthesizing biomass. Analyzed cultures were the same as in Figure 9. In contrast to *Synechocystis* transformants, no β-phellandrene was detected in wild type cultures. This experiment was repeated yielding similar results between different runs.

wild type cells did not produced any β-phellandrene, as evidenced by the zero absorbance of the heptane extracts from these cultures (Figure 10, black line). Among the transformants, the lowest amount of PHL (equivalent to 10 $A_{232.4}$ units g^{-1} dcw) was generated by the recipient R = CpcB*PHLS fusion strain (Figure 10, red line), or about 1.42 mg PHL per g biomass (Table 1). The presence of the GPPS protein in the unfused R+GPPS-SmR configuration caused an increase in the yield of PHL (equivalent to 14 $A_{232.4}$ units g^{-1} dcw, Figure 10, blue line), or about 1.89 mg PHL per g biomass (Table 1). Presence of the GPPS protein as a fusion protein with 29 amino acids of the N-terminal domain of the chloramphenicol resistance protein in the R+CmR29*GPPS-SmR configuration, caused a further increase in the yield of PHL (equivalent to 17 $A_{232.4}$ units g^{-1} dcw, Figure 10, olive-green line), or about 2.42 mg PHL per g biomass (Table 1). Best outcome was obtained with the GPPS protein as a fusion protein with the entire sequence of the kanamycin resistance cassette in the R+NptI*GPPS configuration, which resulted in a yield increase of PHL (equivalent to 35 $A_{232.4}$ units g^{-1} dcw, Figure 10, blue-green line), or about 5.95 mg PHL per g biomass (Table 1). The results suggested a strong correlation between the yield of PHL production in the CpcB*PHLS fusion recipient strain (Figure 10 and Table 1) and the amount of the transgenic GPPS protein expressed in the transformants (Figure 8 and Table 1).

The identity of the products with absorbance in the ultraviolet (Figure 10) was further assessed by sensitive GC-FID analysis. Such analysis with a β-phellandrene standard in heptane showed a single main peak with a retention time of 13.3 min (Figure 11, β-phellandrene standard). Heptane extracts from the wild type did not show any features in the

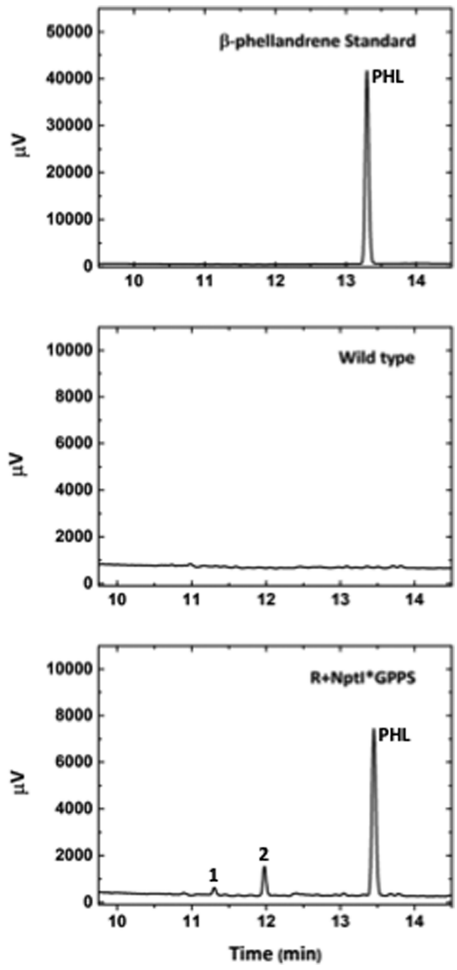

Figure 11. Gas chromatography with flame ionization detector (GC-FID) analysis of heptane extracts from *Synechocystis* wild type and transformant cultures. (Upper panel) GC-FID analysis of a β-phellandrene standard (Santa Cruz Biotechnology), showing a retention time of 13.3 min under these experimental conditions. (Middle panel) GC-FID analysis of heptane extracts from a *Synechocystis* wild type culture. Heptane extracts from wild type cultures displayed a flat profile, without any discernible peaks. (Lower panel) GC-FID analysis of heptane extracts from a *Synechocystis* transformant culture heterologously coexpressing the CpcB*PHLS and NptI*GPPS fusion constructs. β-Phellandrene was the major terpene hydrocarbon product detected, showing a retention time of 13.3 min under these experimental conditions. Smaller amounts of α-pinene (peak #1) and β-myrcene (peak #2) with retention times of 11.3 and 12 min, respectively, were also detected as secondary products of the PHLS catalysis in the transformants.

DOI: 10.1021/acssynbio.7b00431
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10 to 15 min retention time (Figure 11, wild type). Heptane extracts from the transformant with the R+NptI*GPPS configuration clearly showed a peak with the 13.3 min retention time, attributed to β-phellandrene (Figure 11, R +NptI*GPPS). This transformant also showed minor peaks with shorter retention times (Figure 11, R+NptI*GPPS, peaks marked 1 and 2). On the basis of analysis with a number of monoterpene standards (not shown), these were attributed to pinene (peak #1), and myrcene (peak #2). The appearance of other monoterpenes as a byproduct of the β-phellandrene synthase in cyanobacteria was noted earlier.[7]

CONCLUSIONS

Photosynthetic carbon partitioning to the terpenoid biosynthetic pathway in cyanobacteria and green microalgae was estimated to be 4−5% of total fixed carbon.[17] Thus, the slow carbon flux and low level of expression of the terpenoid biosynthetic pathway-associated genes and proteins is consistent with the fact that terpenoids comprise a small fraction of the total cellular biomass. This may underline a general principle whereby genes of cellular secondary metabolism are inherently expressed at low levels under physiological conditions.[18] In the past, strong promoters were used, in combination with codon use optimization, in an effort to obtain high levels of heterologous terpene synthase expression in *Synechocystis*. Terpene synthases under the control of promoters such as the *psbA2* (encoding the abundant D1/32 kDa photosystem-II reaction center protein), the *cpc* operon (encoding the abundant phycocyanin proteins), the *glgA1* (encoding the abundant glycogen synthase protein), or the P_{TRC} strong constitutive promoter, all yielded rather low protein expression levels.[6,13,17,19−21] A tentative explanation for this observation postulated that elements in the coding region of secondary metabolism genes are designed to slow down translation (Shota Atsumi, personal communication). This limitation in the expression of terpene synthases in cyanobacteria apparently includes the *GPPS* gene, endogenous and heterologous levels of which are only detectable upon sensitive Western blot analysis (Figure 6).

Earlier work from this lab showed that the PHLS protein expression barrier was overcome upon fusion of the *PHLS* gene to the endogenous *cpcB* gene, encoding the highly expressed phycocyanin β-subunit in *Synechocystis*.[7,8] In this case, the fusion protein accounted for a major portion of the total cell protein. This work showed that enhancement in the level of *cpcB*PHLS* construct expression is not unique to the *cpcB,* as a leader, and the *PHLS,* as a fusion gene. Similar results were obtained with the kanamycin resistance *nptI* and chloramphenicol resistance *cmR* genes, as leader sequences in a fusion configuration with the *GPPS* gene. Both leader sequences helped to overcome the translation slow-down in *GPPS* gene expression, and significantly greater amounts of the GPPS fusion protein accumulated in such transformants (Figures 5, 6, and 8), compared to expression levels of the GPPS by itself.[8,22] It is concluded that both homologous (*cpcB*) and heterologous (*nptI* and *cmR*) highly expressed genes can be used in cyanobacteria as leader sequences leading to the overexpression of a fusion construct. This may be viewed as a universal method by which to overexpress an otherwise difficult, in terms of low level of expression, protein.

Whereas terpene synthases could not be overexpressed in cyanobacteria immediately under the control of a number of strong promoters, bacterial-origin genes appear not to have this problem. Notable in this respect is the overexpression of bacterial-origin trans-enoyl-CoA reductase (*ter*),[23] D-lactate dehydrogenase (*dldhE*),[23] *nptI*,[15] *cmR*,[6−8] and isopentenyl diphosphate isomerase (*fni*)[24] genes in *Synechocystis* directly or indirectly under the control of the *cpc* promoter. Thus, there appears to be a dichotomy in the expression of plant-origin terpene synthesis *versus* bacterial-origin genes under the control of the *cpc* operon. Our work showed that the difficulty-to-express terpene synthesis genes is alleviated, when the latter are expressed as fusion constructs with an endogenous or heterologous highly expressed gene in *Synechocystis*, when the latter is placed in the leader sequence position.

The use of 5′ end truncated short leader sequences derived from NptI failed to enhance GPPS expression (Figure 7). CmR is another highly expressed heterologous protein in *Synechocystis* (Figure 5), but its use as a full leader sequence was not possible in this work because of its contemporary presence in the *cpc* transgene construct (Figure 2). Use of the full *cmR* gene, as a leader sequence in the overexpression of the *GPPS*, would have been subject to undesirable internal recombination with the existing *cmR* cassette. Such undesirable internal DNA recombinations can be prevented by using DNA fragments shorter than 200 nucleotides.[23] The finding that a "short stretch" CmR29 leader sequence was able to substantially increase GPPS levels is of great importance in this respect. Indeed, application of "short stretch" CmR leader sequences can then be used to drive a higher expression level of heterologous genes comprising an operon, in which constituents are usually poorly expressed, *e.g.*, the upper and lower portions of the heterologous MVA pathway in *Synechocystis*.[8,22,25] In this context, the "short stretch" CmR29 leader sequence could be added to the 5′ end of each gene in the MVA operon, possibly leading to a higher level of expression for each of these genes.

The work further showed a dual-purpose use of the *nptI* gene as a leader sequence in the *nptI*GPPS* fusion construct, *i.e.*, promoting the expression level of the heterologous *GPPS* gene, while at the same time serving as a selectable marker for the screening of the transformants (Figure 4). Moreover, the work clearly showed that expression levels of the *GPPS* gene, albeit as a fusion construct with either the *nptI* and *cmR* leader sequences, invariably enhanced the yield of β-phellandrene production catalyzed by the CpcB*PHLS fusion protein in *Synechocystis*. In this respect, a nonparallel correlation was noted between the GPPS level of expression and the yield of PHL production (Table 1), whereby a disproportional increase in the concentration of GPPS (by up to 60-fold) translated into a mere 4-fold increase in β-phellandrene accumulation. This quantitative discrepancy is not clearly understood at present, it may reflect limitations in cellular substrate flux to DMAPP and IPP, imbalance in the stoichiometric ratio between these two precursor molecules,[24] or hindrances in the catalytic activity of the GPPS enzyme.[12] In spite of the need for optimization of the two-step β-phellandrene synthesis reaction, however, our results support the notion that the activity of the GPPS enzyme and pool levels of the GPP metabolite are determinants in the rate and yield of monoterpene generation in cyanobacteria.

MATERIALS AND METHODS

***Synechocystis* Strains, Recombinant Constructs, and Culturing Conditions.** The cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*) was used as the experimental strain

DOI: 10.1021/acssynbio.7b00431
ACS Synth. Biol. 2018, 7, 912−921

in this work and referred to as the wild type (WT). β-Phellandrene (PHL) production by *Synechocystis* transformants was promoted by the expression of the codon optimized β-phellandrene synthase gene (*PHLS*) from *Lavandula angustifolia* (lavender), as described in recent work from this lab.[6−8] *Synechocystis* codon-optimized genes coding for kanamycin resistance (NptI), chloramphenicol resistance (CmR) and the geranyl diphosphate synthase (GPPS2) from grand fir were synthesized with the original sequences taken from the literature.[6−8,14] A fused *nptI*GPPS2* gene sequence was obtained upon DNA synthesis (Biomatik), followed by cloning in a vector containing 500 bp of upstream and downstream *glgA1* sequences for homologous recombination. The fusion *nptI*GPPS2* construct was placed under the control of the heterologous P_{TRC} promoter in a P_{TRC}-*nptI*GPPS2* configuration to ensure constitutive transgene expression.[6] *GPPS2*, *smR* and *smR/nptI* leader sequences were amplified by PCR using plasmids already available in the lab as templates.[8] The *GPPS2* amplicon was fused to the 3′ end of *cmR* or *nptI* derived-sequences (Phusion polymerase, NEB) and then assembled (Gibson Assembly, NEB) together with the selective cassette *smR* into the same vector used for the *nptI*GPPS2* sequence. Nucleotide sequences of constructs used in this work are reported in the Supporting Information.

Synechocystis transformations were made according to established protocol.[26,27] Wild type and transformants were maintained on 1% agar BG11 media supplemented with 10 mM TES-NaOH (pH 8.2) and 0.3% sodium thiosulfate. Liquid cultures in BG11 were buffered with both 25 mM sodium bicarbonate pH 8.2 and 25 mM dipotassium hydrogen phosphate pH 9 and incubated under continuous bubbling with air at 26 °C. Transgenic DNA copy homoplasmy in the cells was achieved upon transformant incubation on agar in the presence of the antibiotic selectable marker (30 μg/mL CmR, 25 μg/mL KmR, or 25 μg/mL SmR). Cells were grown using a balanced combination of white LED bulbs supplemented by incandescent light to yield a final visible light intensity of ∼100 μmol photons m^{-2} s^{-1}.

Genomic DNA PCR Analysis of *Synechocystis* Transformants. Genomic DNA templates were prepared with a Chelex100 Resin (BioRad), as described.[6] Transgenic DNA copy homoplasmy in *Synechocystis* was tested using suitable primers already available in literature.[8] The genomic DNA location of these primers is indicated in the figures with the appropriate DNA constructs.

Protein Analysis. Cells were harvested by centrifugation at 4000*g* × 10 min. The pellet was resuspended in a solution buffered with 25 mM Tris-HCl, pH 8.2, containing a cOmplete Mini protein inhibitor cocktail (one 50 mg Roche tablet added per 50 mL suspension). Cells were disrupted by passing the suspension through a French press cell at 1500 psi. For protein electrophoretic analysis, cell suspensions were solubilized upon incubation for 2 h at room temperature in the presence of 125 mM Tris-HCl, pH 6.8, 3.5% SDS, 10% glycerol, 2 M urea, and 5% β-mercaptoethanol. SDS-PAGE was performed using Mini-PROTEAN TGX precast gels (BIORAD). Western blot analysis entailed a transfer of the SDS-resolved proteins from the polyacrylamide gel to a nitrocellulose membrane, followed by probing with rabbit-raised GPPS2 specific polyclonal antibodies, as previously described.[28,29]

Quantification of β-Phellandrene Synthesis by *Synechocystis* Transformants. β-Phellandrene production and separation from the *Synechocystis* cultures were performed according to previous literature.[8,13] OD_{730} values of dense cultures were taken and, after centrifugation at 4000*g* for 10 min, cell pellets were resuspended in fresh BG11 media to reach an OD_{730} of about 0.5 in a total volume of 550 mL. Refreshed cultures were incubated in the aqueous/gaseous two-phase bioreactor system.[13] Aliquots of 50 mL volume were harvested from each culture to determine the biomass content (g) at time zero. Cultures were slowly bubbled for 5 s per day with 100% CO_2 and stirred continuously while under illumination. After 48 h of incubation, an aliquot of 50 mL was again harvested to determine the final dry cell weight of the biomass content. Fifteen ml of heptane were then added to the surface of the culture to help collect the floating terpene hydrocarbon products. The presence of the monoterpene was quantified by absorbance spectrophotometry in the ultraviolet and sensitive flame ionization detector gas chromatography (GC-FID), as recently described.[6,29] The amount of β-phellandrene generated by the culture was normalized to the dried biomass (OD:w) that was produced during the 48 h incubation period in the light, as described.[8]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00431.

Sequences of oligonucleotide primers used in this work; Heterologous gene sequences employed in the present work for expression in *Synechocystis* (PDF)

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Author Contributions

NB and AM designed the project. NB conducted the experimental work. NB and AM wrote the manuscript.

Notes

The authors declare the following competing financial interest(s): Some of the authors are inventors of intellectual property in the area of the publication.

■ ACKNOWLEDGMENTS

The work was supported by University of California Berkeley fund 1-45033-13618-44-ME1AM to Anastasios Melis.

■ ABBREVIATIONS

Bms, Biomass; CmR, Chloramphenicol resistance; *cpc*, Operon encoding the phycocyanin subunits and associated linker polypeptides; *cpcB*, First gene in the *cpc* operon encoding the β-phycocyanin subunit; dcw, Dry cell weight; DMAPP, Dimethylallyl-diphosphate; GPP, geranyl-diphosphate; GC-FID, Gas-chromatography Flame-ionization detector; GPPS, geranyl-diphosphate synthase; IPP, Isopentenyl diphosphate; MEP, 2-C-Methyl-D-erythritol 4-phosphate; NptI, Neomycin phosphotransferase conferring kanamycin resistance; PHL, β-Phellandrene; PHLS, β-Phellandrene synthase; WT, Wild type

DOI: 10.1021/acssynbio.7b00431
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DOI: 10.1021/acssynbio.7b00431
ACS Synth. Biol. 2018, 7, 912−921