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A phycocyanin · phellandrene synthase fusion enhances recombinant protein expression and β-phellandrene (monoterpene) hydrocarbons production in *Synechocystis* (cyanobacteria)

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ABSTRACT

Cyanobacteria can be exploited as photosynthetic platforms for heterologous generation of terpene hydrocarbons with industrial applications. Transformation of *Synechocystis* and heterologous expression of the β-phellandrene synthase (*PHLS*) gene alone is necessary and sufficient to confer to *Synechocystis* the ability to divert intermediate terpenoid metabolites and to generate the monoterpene β-phellandrene during photosynthesis. However, terpene synthases, including the PHLS, have a slow K_{cat} (low V_{max}) necessitating high levels of enzyme concentration to enable meaningful rates and yield of product formation. Here, a novel approach was applied to increase the PHLS protein expression alleviating limitations in the rate and yield of β-phellandrene product generation. Different *PHLS* fusion constructs were generated with the *Synechocystis* endogenous *cpcB* sequence, encoding for the abundant in cyanobacteria phycocyanin β-subunit, expressed under the native *cpc* operon promoter. In one of these constructs, the CpcB · PHLS fusion protein accumulated to levels approaching 20% of the total cellular protein, i.e., substantially higher than expressing the PHLS protein alone under the same endogenous *cpc* promoter. The CpcB · PHLS fusion protein retained the activity of the PHLS enzyme and catalyzed β-phellandrene synthesis, yielding an average of 3.2 mg product g^{-1} dry cell weight (dcw) versus the 0.03 mg g^{-1} dcw measured with low-expressing constructs, i.e., a 100-fold yield improvement. In conclusion, the terpene synthase fusion-protein approach is promising, as, in this case, it substantially increased the amount of the PHLS in cyanobacteria, and commensurately improved rates and yield of β-phellandrene hydrocarbons production in these photosynthetic microorganisms.

1. Introduction

Cyanobacteria are good microbial platforms for the generation of commodity product useful for industrial and domestic consumption (Ducat et al., 2011; Oliver and Atsumi, 2014; Savakis and Hellingwerf, 2015). Compounds synthesized in the cyanobacterial cell and spontaneously separated from the biomass and the extracellular aqueous medium are particularly attractive because product segregation and harvesting are simplified. This is a parameter that weighs heavily on the economics of a microbial production system, as a spontaneous product separation from the biomass alleviates negative effects associated with product accumulation inside the cells. The latter include potential inhibitory or toxic effects of the product molecule on cellular metabolism, and considerably higher costs associated with product extraction from the cell interior, harvesting, and downstream processing (Melis, 2012; Wijffels et al., 2013).

β-Phellandrene ($C_{10}H_{16}$) is a monoterpene with commercial value as a key ingredient in synthetic chemistry, medical, cosmetic and cleaning products, and potentially as a fuel (Bentley et al., 2013). It is a component of plant essential oils, naturally synthesized in plant trichomes from geranyl-diphosphate (GPP) by a nuclear-encoded and plastid localized β-phellandrene synthase (PHLS) enzyme. Heterologous production of β-phellandrene was achieved by genetic engineering of the cyanobacterium *Synechocystis*, showing spontaneous and quantitative separation of the molecule from the biomass and the extracellular aqueous phase. β-Phellandrene efficiently diffused through the plasma membrane and cell wall and, because of its hydrophobicity, accumulated as a floater molecule on the surface of the culture, from where it was

Abbreviations: β-PHL, β-phellandrene; APC, allophycocyanin; Car, carotenoid; Chl, chlorophyll; dcw, dry cell weight; GPP, geranyl-diphosphate; PBS, phycobilisome; Phc, phycocyanin; PHLS, β-phellandrene synthase; PS, photosystem; wt, wild type

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harvested by siphoning (Bentley et al., 2013; Formighieri and Melis, 2014a).

Heterologous expression of the *PHLS* gene *via* genomic DNA chromosome-based transformation is necessary and sufficient for the constitutive photoautotrophic generation of β-phellandrene in *Synechocystis* transformants. More specifically, the codon optimized *PHLS* gene from *Lavandula angustifolia* (lavender) (Demissie et al., 2011) was expressed under the control of the strong endogenous *psbA2* (Bentley et al., 2013) or *cpc* promoter (Formighieri and Melis, 2014a) *via* homologous recombination and replacement of the respective *psbA2* gene or *cpc* operon. In both cases, β-phellandrene was constitutively generated, but the rate and yield of production were low, attributed to the low levels of transgenic protein accumulation. This consideration is important, as high levels of terpene synthase expression are required for enhanced yields in product generation, considering the slow K_{cat} of these enzymes (Demissie et al., 2011; Zurbriggen et al., 2012).

The *cpc* operon (locus 724094–727466 in the *Synechocystis* genome, ⟨http://genome.microbedb.jp/cyanobase⟩) encodes five proteins, i.e., the phycocyanin (Phc) β- and α- subunits (*CpcB* and *CpcA* genes), and their linker polypeptides (encoded by the *CpcC2*, *CpcC1* and *CpcD* genes), all of which assemble to form the peripheral rods of the phycobilisome (PBS) light-harvesting antenna complex. Phycocyanin (Phc) is one of the most abundant proteins in cyanobacteria, suggesting strong expression elements in the promoter and 5′UTR of the *cpcB* gene (Zhou et al., 2014), including aspects of the function of the *cpc* operon transcription and translation processes that, theoretically, could be used to efficiently drive expression of transgenes. Removal/replacement of the *cpc* operon resulted in a Phc-less mutant with a truncated PBS antenna size (Kirst et al., 2014).

Expression of the *PHLS* gene under the control of the *cpc* operon promoter improved accumulation of the PHLS protein over that under the *psbA2* promoter (Bentley et al., 2013) to a point where the transgenic protein was, for the first time, visible in the Coomassie-stained SDS-PAGE of *Synechocystis* total protein extracts (Formighieri and Melis, 2014a). Correspondingly, the yield of β-phellandrene hydrocarbons also increased from about 0.01 to about 0.2 mg of β-phellandrene g^{-1} dry cell weight (Formighieri and Melis, 2014a). It was concluded that limitations in rate and yield of β-phellandrene hydrocarbons production are in part due to the limited concentration of the transgenic enzyme in the transformant cells. However, in spite of this improvement upon use of the *cpc* promoter, levels of PHLS accumulation were nowhere near those of the native phycocyanin β- or α-subunits, and the yield in β-phellandrene was still low, corresponding to a 0.02% β-phellandrene:biomass (w:w) carbon partitioning ratio. This yield accounted for only a small fraction of the carbon flux through the cell's own terpenoid biosynthetic pathway, which was estimated to be 4–5% of all photosynthetically fixed carbon (Lindberg et al., 2010).

It became clear, therefore, that a strong promoter is necessary (Camsund and Lindblad, 2014) but not sufficient to yield high levels of transgenic terpene synthase and product yield. In order to further enhance the level of transgenic enzyme accumulation, as a pre-requisite for greater rates and yield of monoterpene production, we made a fusion of the entire *cpcB* gene with the *PHLS* transgene, expressed under the control of the *cpc* promoter. As a result, we report on the successful generation of a productive *Synechocystis* strain expressing the CpcB · PHLS fusion protein to about 20% of the total cell protein, a feature that allowed us to obtain substantially greater yields of β-phellandrene hydrocarbons from the photosynthesis of the cyanobacteria.

2. Materials and methods

2.1. Synechocystis strains, recombinant constructs, and culturing conditions

Synechocystis sp. PCC 6803 (*Synechocystis*) was used as the recipient strain and referred to as the wild type (wt) in this study (Williams, 1988). The recombinant plasmid for expression of the codon optimized β-phellandrene synthase (PHLS)-encoding gene in the *cpc* genomic locus (Formighieri and Melis, 2014a) was modified in order to fuse the *PHLS* to the C-terminus of the complete *CpcB*-encoding sequence. The upstream 500 bp of the *cpc* operon and the *cpcB* sequence were amplified by PCR from the *Synechocystis* genome, using cpc_us-XhoI as forward primer and cpcB-NdeI as reverse primer (Supplementary material, Table 1S). The resulting PCR product was cloned upstream of *PHLS via* XhoI and NdeI digestion, removing the native stop codon of *cpcB*. Homologous recombination was designed to occur between the 500 bp of the upstream and downstream sequences of the *cpc* operon, leading to replacement of the *cpc* operon by the recombinant CpcB · PHLS construct. Resulting *Synechocystis* transformants in this case are referred to as Δcpc+cpcB · PHLS (Fig. 1b). Homologous recombination was alternatively performed between the upstream sequence of the *cpc* operon and the CpcA-encoding sequence. The latter was amplified by PCR from the *Synechocystis* genome, using cpcA-BamHI and cpcA-SacI as forward and reverse primers, respectively (Supplementary material, Table 1S). The CpcA-encoding DNA was then cloned downstream of the recombinant CpcB · PHLS construct *via* BamHI and SacI digestion, thus replacing the 500 bp of the downstream sequence of the *cpc* operon previously employed. Transformation of *Synechocystis* and homologous recombination allowed substitution of the native *cpcB* sequence by the *cpcB · PHLS* fusion construct while maintaining the other *cpc* genes in the downstream portion of the operon (Fig. 1c, cpcB · PHLS+cpc transformant). In addition, homologous recombination was designed to occur between the upstream sequence of the *cpc*

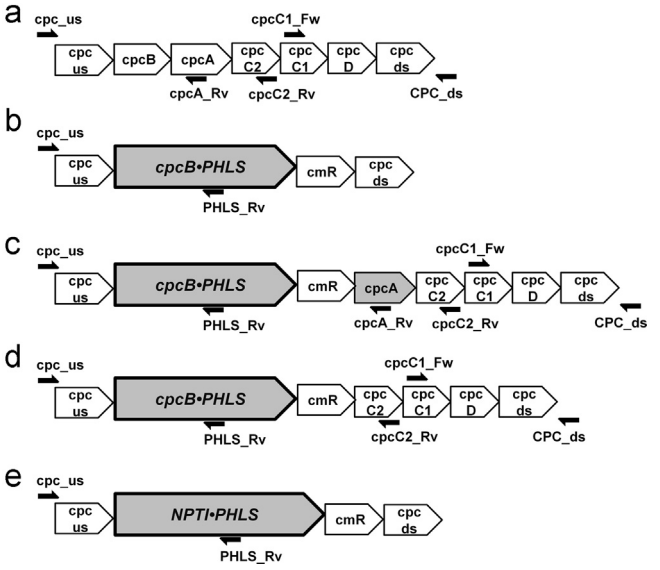

Fig. 1. Schematic overview of DNA constructs designed to transform the genome of *Synechocystis*, as used in this work. (a) The *cpc* operon, as it occurs in wild type cyanobacteria. (b–d) Constructs Δcpc+cpcB · PHLS, cpcB · PHLS+cpc, and cpcB · PHLS+cpc(-cpcA) express a fusion *CpcB · PHLS* and chloramphenicol resistance (*cmR*) genes in the absence of other *cpc* operon genes (b), in the presence of the remainder *cpc* operon genes (c), or in the presence of the remainder *cpc* operon genes, minus the *cpcA* gene (d). (e) Construct Δcpc+NPTI · PHLS was designed to replace the coding sequence of the endogenous *cpc* operon in *Synechocystis* with a kanamycin-resistance phellandrene-synthase fusion (NPTI · PHLS) encoding sequence.

operon and the CpcC2-encoding sequence. The latter was amplified by PCR from the *Synechocystis* genome, using cpcC2-BamHI and cpcC2-SacI as forward and reverse primers, respectively (Supplementary material, Table 1S), and cloned downstream of the recombinant *cpcB·PHLS* construct *via* BamHI and SacI digestion. Transformation of *Synechocystis* and homologous recombination allowed integration of *cpcB·PHLS* upstream of the genes encoding for the CpcC2 linker polypeptide, while deleting the endogenous *cpcB* and *cpcA* sequences (Fig. 1d, cpcB·PHLS+cpc(-cpcA) transformant).

PHLS was alternatively fused to the codon-optimized *NPTI* gene conferring kanamycin resistance. The latter was amplified by PCR using the Δcpc+NPTI plasmid as template (Kirst et al. 2014), and cpc_us-XhoI and NPTI-Rv as forward and reverse primers, respectively (Supplementary material, Table 1S). The PCR product was then cloned upstream of the PHLS sequence *via* XhoI and NdeI digestion and used to replace the *cpc* operon *via* homologous recombination. The resulting *Synechocystis* transformants are referred to Δcpc+NPTI·;PHLS (Fig. 1e).

Synechocystis transformations were made according to established protocols (Eaton-Rye, 2011). Wild type and transformants were maintained on 1% agar BG11 media supplemented with 10 mM TES-NaOH (pH 8.2) and 0.3% sodium thiosulphate. Liquid cultures in BG11 were buffered with 25 mM phosphate (pH 7.5) and incubated under continuous low-stream bubbling with air at 28 °C. Transgenic DNA copy homoplasmy was achieved with cells incubated on agar in the presence of 30 μg/mL chloramphenicol, 5 mM glucose, under illumination of 170 μmol photons m^{-2} s^{-1}.

2.2. PCR analysis of Synechocystis transformants for insert site mapping

Genomic DNA templates were prepared with Chelex®100 Resin (BioRad) as described (Formighieri and Melis, 2014a). The following genomic DNA PCR primers were used to map the insert site of the *Synechocystis* transformants, to look for transgene insertion into the correct genomic locus, and also to test for DNA copy homoplasmy: cpc_us, cpcA_Rv, cpcC2_Rv, cpcC1_Fw, cpc_ds, PHLS_Rv. The location of these primers on the genomic DNA is shown in Fig. 1. The oligonucleotide sequences are given in the Supplementary material, Table 1S.

2.3. Protein analysis

Cells were harvested by centrifugation and resuspended in a buffer containing 50 mM Tris–HCl pH 8, 50 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$. The cell suspension was treated first with lysozyme (Thermo Scientific) then with bovine pancreas DNaseI (Sigma) for 30 min each at room temperature. Cell disruption was achieved by passing the suspension through a French press cell at 20,000 psi in the presence of protease inhibitors (1 mM PMSF, 2 mM aminocaproic acid, and 1 mM benzamidine). The sample was then treated with 1% v/v Triton X-100 for 20 min and centrifuged at 21,000*g* for 20 min to separate the cleared lysate from the pellet. The supernatant was solubilized upon incubation at room temperature with 62 mM Tris–HCl pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol. The pellet was solubilized upon incubation with 62 mM Tris–HCl pH 6.8, 3.5% SDS, 1 M urea, 5% β-mercaptoethanol, and 10% glycerol. Unsolubilized material was removed upon centrifugation at 21,000*g* for 5 min and the supernatant was loaded on a SDS-PAGE (Bio-Rad, USA). The SDS-PAGE resolved proteins were stained with Coomassie Brilliant Blue G-250 and densitometric analysis of the protein bands was performed in each lane upon application of the GelPro Analyzer software. For Western blot analysis, resolved proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane and probed with CpcA polyclonal antibodies (Abbiotec, Cat. no. 250488).

2.4. Measurement of photoautotrophic growth and β-phellandrene production

Photoautotrophic growth kinetics of wild type and transformants were measured from the optical density of the cultures at 730 nm. β-Phellandrene production and separation from *Synechocystis* cultures were performed as described (Bentley et al., 2013; Formighieri and Melis, 2014a). Briefly, 500 mL liquid cultures of *Synechocystis*, with an optical density OD_{730}=0.5, were placed in a 1 L gaseous/aqueous two-phase reactor (Bentley and Melis, 2012), supplemented with 100% CO_2 gas to fill the ~500 mL gaseous headspace, then sealed for 48 h and incubated under 50 μmol photons m^{-2} s^{-1} of incident light intensity. At the end of the incubation period, β-phellandrene was collected as a floater molecule from the surface of the liquid culture. This was achieved upon dilution of the floater β-phellandrene fraction with hexane, while gently stirring for 2 h. The amount of β-phellandrene present was measured in the hexane extract by absorbance spectroscopy and sensitive gas chromatography (GC-FID), according to Formighieri and Melis (2014a, 2014b), and normalized per g of dried biomass collected at the end of the 48 h incubation period.

The *in vitro* assay for β-phellandrene synthase activity and β-phellandrene hydrocarbons synthesis was performed according to Demissie et al. (2011), with measurements performed either with total cell extracts, following cell disruption by French Press, or pellet fractions, after centrifugation at 21,000*g* for 5 min. Samples were suspended in 50 mM Tris–HCl pH 6.8, 5% glycerol, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mg/mL BSA, 1 mM DTT, 50 μM geranyl-pyrophosphate (GPP, by Echelon Biosciences), and incubated for 1 h at 30 °C with a half volume of hexane as reaction mix overlayer. The latter was eventually collected and measured by absorbance spectroscopy and GC-FID analysis.

3. Results

3.1. Design of constructs for heterologous transgene expression in Synechocystis

In the *Synechocystis* genome, the *cpc* operon includes the *cpcB* and *cpcA* genes, encoding the phycocyanin β- and α-subunits, respectively. These, together with the products of the *cpcC2*, *cpcC1* and *cpcD* genes, encoding associated linker polypeptides (Fig. 1a), assemble into the peripheral rods of the light-harvesting phycobilisomes. The *cpcB* and *cpcA* genes are highly expressed in cyanobacteria, providing for the abundant phycocyanin β- and α-subunits in the phycobilisome light-harvesting antenna of these microorganisms. In the present work, the *PHLS* gene was fused to the C-terminus of the entire CpcB-encoding sequence, investigating whether the strong expression of the CpcB phycocyanin β-subunit might also be manifested in the CpcB·PHLS fusion protein synthesis. In this respect, three alternative strategies of homologous recombination were employed for transgene integration of the *CpcB·PHLS* fusion in the *cpc* operon locus: (i) the *cpc* operon was deleted and replaced by the *cpcB·PHLS* fusion sequence, denoted as Δcpc+cpcB·PHLS (Fig. 1b); (ii) the *cpcB·PHLS* fusion construct replaced the native *cpcB* sequence only, inserted upstream of the *cpcA*, *cpcC2*, *cpcC1* and *cpcD* genes, denoted as cpcB·PHLS+cpc (Fig. 1c); (iii) the *cpcB·PHLS* fusion construct replaced the native *cpcB* and *cpcA* sequences, and was integrated upstream of the *cpcC2*, *cpcC1* and *cpcD* genes, denoted as cpcB·PHLS+cpc(-cpcA) (Fig. 1d). This strategy allowed us to evaluate the specific contribution of the Cpc subunits in the

accumulation of the CpcB · PHLS fusion protein. The gene conferring resistance to chloramphenicol (cmR) was cloned in operon configuration immediately downstream of the *PHLS* gene. Positive transformants from the various constructs were selected on chloramphenicol-supplemented media.

In addition, *PHLS* was fused downstream of the *Synechocystis* codon-optimized *NPTI* sequence conferring resistance to kanamycin. This kanamycin resistance cassette was highly expressed under the control of the *cpc* operon promoter in *Synechocystis* transformants (Kirst et al., 2014). NPTI was used in this respect as an upstream moiety of a *NPTI · PHLS* heterologous fusion, with the recombinant fusion-protein expressed under the *cpc* promoter upon replacement of the entire *cpc* operon, denoted as Δcpc+NPTI · PHLS (Fig. 1e).

3.2. Analysis of Synechocystis transformants and evidence of homoplasmy

Genomic DNA PCR analysis was undertaken to test for insert integration and DNA copy homoplasmy in transformants with each of the above-mentioned constructs. Results from this analysis are shown in Fig. 2. In lanes a–e, location of the PCR primers is shown in Fig. 1, a–e, respectively. By using primers cpc_us and cpcA_Rv, annealing upstream of the *cpc* operon promoter and within the *cpcA* gene, respectively, the PCR reaction generated a 1289bp product in the wild type (Fig. 2A, lane a) and a 3735bp product in the cpcB · PHLS+cpc transformant (Fig. 2A, lane c). The larger product size in the latter is due to the CpcB · PHLS fusion and cmR insert. The other transformants (Fig. 2A, lanes b, d, e) did not yield a PCR product with these primers, consistent with the absence of the *cpcA* gene. The specific absence of wild type 1289 bp product in Fig. 2A, lanes b–e, is evidence of having attained transgenic DNA copy homoplasmy in the transformants.

Genomic DNA PCR analysis using primers cpc_us and cpcC2_Rv, annealing upstream of the *cpc* operon and within the *cpcC2* gene, respectively, showed a single 2681bp product in the wild type (Fig. 2B, lane a). In the cpcB · PHLS+cpc and cpcB · PHLS+cpc (-cpcA) transformants, insertion of the CpcB · PHLS fusion encoding sequence increased the size of the PCR product to 5135 bp and 4535 bp, respectively (Fig. 2B, lanes c and d). The larger size of the PCR products is due to the insertion of the CpcB · PHLS and cmR cassette. In the other transformants in which the *cpcC2* gene was deleted upon insertion of the fusion construct (Fig. 2B, lanes b, e), no PCR products could be detected with the aforementioned primers. The specific absence of wild type 2681bp product in Fig. 2B, lanes b–e, is evidence of transgenic DNA copy homoplasmy in the transformants.

PCR reactions using primers cpcC1_Fw and cpc_ds, annealing within the *cpcC1* gene and downstream of the *cpc* operon, respectively, gave a single 1270bp PCR product in the wild type (Fig. 2C, lane a), cpcB · PHLS+cpc (Fig. 2C, lane c), and cpcB · PHLS+cpc(-cpcA) (Fig. 2C, lane d) transformants. This result is evidence that genes encoding for the PBS linker polypeptides are present in the genome of these transformants. In contrast, no PCR product could be obtained with the aforementioned primers in the remaining strains (Fig. 2C, lanes b, e) because of deletion of the *cpc* operon upon homologous recombination for the insertion of the *PHLS*-containing cassette. Absence of a PCR product in the latter is evidence of transgenic DNA copy homoplasmy.

Finally, genomic DNA PCR analysis using primers cpc_us and PHLS_Rv, annealing upstream of the cpc operon and within the *PHLS* sequence, respectively, assessed integration of the *PHLS* construct in the *cpc* locus. Products of different sizes, depending on the transgenic construct, were obtained in the transformants including a 1927bp for Δcpc+cpcB · PHLS (Fig. 2D, lane b), cpcB · PHLS+cpc (Fig. 2D, lane c), and cpcB · PHLS+cpc(-cpcA) (Fig. 2D, lane d), and 2224bp for Δcpc+NPTI · PHLS (Fig. 2D, lane e). With the above-mentioned primers, wild type strains generated no PCR product (Fig. 2D, lane a).

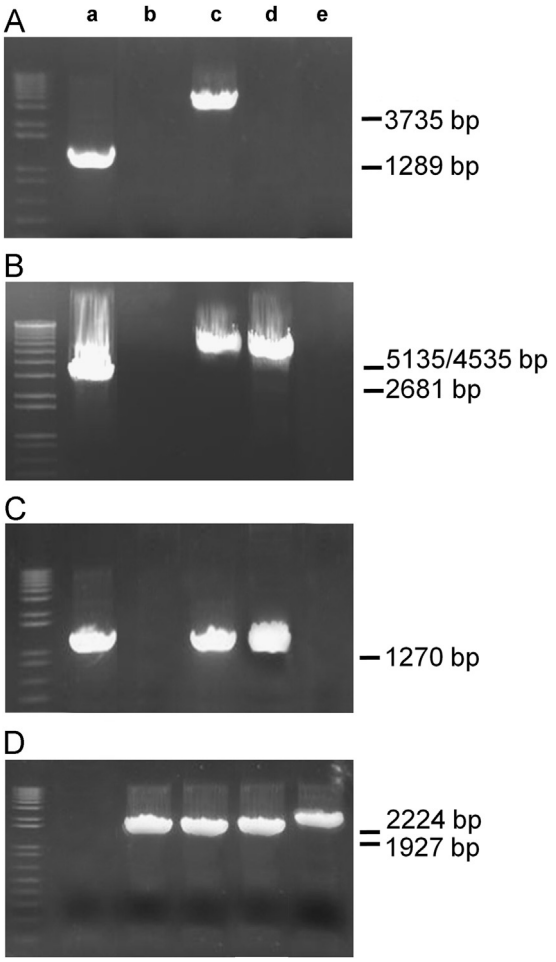

Fig. 2. Genomic DNA PCR analysis with selected forward (us) and reverse (ds) primers positioned on the genomic DNA of *Synechocystis* wild type and *PHLS* transformants. Strains **a**–**e** were generated by constructs **a**–**e**, as shown in Fig. 1. Fig. 1 (arrows) also shows the position of primers for this analysis. (A) PCR reactions using primers cpc_us and cpcA_Rv, amplifying the *cpc* promoter-to-*cpcA* genomic region. (B) PCR reactions using primers cpc_us and cpcC2_Rv, amplifying the *cpc* promoter-to-*cpcC2* genomic region. (C) PCR reactions using primers cpcC1_Fw and cpc_ds, amplifying the *cpcC*1-to-3′end downstream region of the *cpc* operon, where genes encoding for the linker polypeptides are localized. (D) PCR reaction using primers cpc_us and PHLS_Rv, amplifying the *cpc* promoter-to-*PHLS* transgene genomic region, and designed to test integration of *PHLS* in the transformants.

3.3. Transgenic protein accumulation

Synechocystis wild type and transformants cells were broken by French press treatment. Supernatant and pellet fractions were separated to improve resolution of the cell constituent proteins. Supernatant (Fig. 3A) and pellet (Fig. 3B) protein extracts were resolved by SDS-PAGE. In the Δcpc+cpcB · PHLS (Fig. 3, lanes b) and cpcB · PHLS+cpc(-cpcA) transformants (Fig. 3, lanes d), the CpcB · PHLS fusion protein (expected molecular weight of 82 kD) could barely be detected upon Coomassie staining of the gels, suggesting low transgenic protein expression. In contrast, the CpcB · PHLS fusion protein accumulated to high levels in the cpcB · PHLS+cpc transformant, where a protein band migrating to about 75 kD was detected, both in the supernatant and pellet fractions (Fig. 3, lanes c, CpcB · PHLS marked with asterisks). This protein band was absent from the wild type (Fig. 3, lane a). The

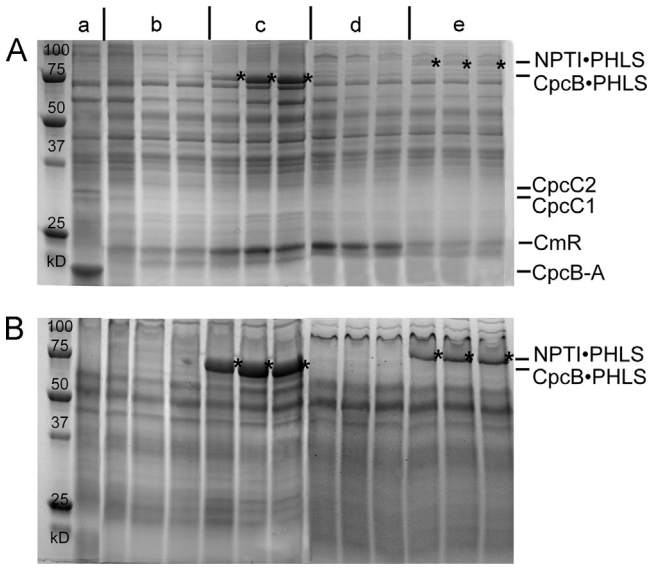

Fig. 3. SDS-PAGE analysis of total protein extracts from *Synechocystis* wild type and *PHLS* transformants. Strains **a**–**e** were generated by constructs **a**–**e**, as shown in Fig. 1. Three independent transformant lines were considered for the analysis of each genotype. Molecular weight markers are indicated in kD. Proteins of interest are labeled and marked with asterisks. (A) Coomassie-stained SDS-PAGE profile of proteins of cell lysate supernatant fractions. (B) Coomassie-stained SDS-PAGE profile of proteins of cell lysate pellet fractions.

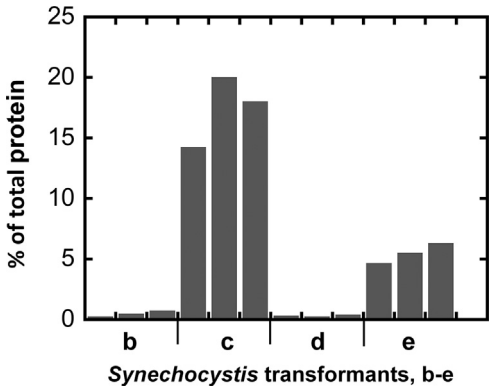

Fig. 4. Densitometric analysis of recombinant protein bands shown in Fig. 3. The Coomassie-stained band intensity of recombinant PHLS proteins was normalized to the total protein load per lane and expressed as a percentage of the total. The analysis was performed with GelPro Analyzer software. Bars **b**–**e** measure the recombinant protein expression level in *Synechocystis* transformants derived from constructs **b**–**e**, as shown in Fig. 1.

results suggested that expression of PHLS as a fusion with the CpcB protein can result in substantial recombinant protein accumulation, when the rest of the *cpc* operon, and in particular the *cpcA*, is in place.

The premise of a fusion construct in the amplification of transgene expression was examined further in detail, upon replacing the *cpcB* gene with a highly expressing kanamycin resistance sequence (Kirst et al., 2014). The rationale behind this design was to test if highly expressed genes, other than the *cpcB* gene, could act as lead fusion sequences for amplification of expression of the *PHLS* transgene. The NPTI · PHLS fusion protein with an expected molecular weight of 95 kD, was clearly visible, especially so in the Coomassie-stained gel of the pellet fractions (Fig. 3, lanes e). It is concluded that a highly expressed endogenous (*cpcB)* or heterologous (*NPTI*) gene, when placed as the lead sequence in a fusion construct, will cause amplification in the expression of the trailing terpene synthase transgene.

Quantification of transgenic protein accumulation, as a function of total cell protein, is provided in Fig. 4. Relative amounts of the recombinant proteins are based on Coomassie staining and corroborate the results, as qualitatively shown in Fig. 3. The highest recombinant protein accumulation was observed in the cells transformed with the cpcB · PHLS+cpc construct, reaching up to 20% of total cell protein (Fig. 4, lanes c). The fusion construct with NPTI as leader sequence also produced noticeable amounts of the transgenic protein, about 5% of the total cellular protein (Fig. 4, lanes e). In contrast, the Δcpc+cpcB · PHLS (Fig. 4, lanes b) and cpcB · PHLS+cpc(-cpcA) constructs (Fig. 4, lanes d) accumulated the cpcB · PHLS fusion to levels less than 1% of the total cell protein.

3.4. Modulation of CpcB · PHLS expression by CpcA

Of interest is the observation that inclusion of the *cpcA* gene downstream of the *cpcB · PHLS* fusion and cmR construct was required to enhance accumulation of the CpcB · PHLS fusion protein. To gain a better understanding of this property in the transgenic systems, SDS-PAGE and Western blot analysis with anti-CpcA polyclonal antibodies (Abbiotec) was employed to test for the relative level of expression of the β- and α-phycocyanin subunits (these polyclonal antibodies cross-react with both the β- and α-phycocyanin protein subunits.) Wild type *Synechocystis* protein extracts showed an abundance of CpcB and CpcA proteins in the supernatant fraction, with these protein bands being clearly visible in equimolar quantities both in the Coomassie-stained SDS-PAGE profile (Fig. 5A, lane a, supernatant) and in the corresponding Western blot analysis (Fig. 5B, lane a, supernatant). The *Synechocystis* CpcB · PHLS+cpc protein extracts showed an abundance of the CpcB · PHLS fusion protein in both the supernatant and pellet fractions, with the protein band migrating to about 75 kD in the Coomassie-stained SDS-PAGE (Fig. 5A, lanes c) and in the Western blot analysis (Fig. 5B, lanes c). In the latter, the anti-CpcA polyclonal antibodies recognized the CpcB protein in its configuration as a CpcB · PHLS fusion.

Low levels of the CpcA protein were detected by Western blot analysis in the CpcB · PHLS+cpc supernatant fraction (Fig. 5B, lane c, supernatant). Traces of CpcA were in far lower quantity than the CpcB · PHLS fusion protein, and also lower than the CpcA protein measured in the wild type. The Δcpc+cpcB · PHLS transformant showed no detectable amounts of the CpcA subunit by Western blot with anti-CpcA polyclonal antibodies (Fig. 5B, lane b, supernatant).

3.5. Growth properties of Synechocystis wild type and PHLS transformants

Photoautotrophic growth was measured with the wild type (wt) and the CpcB · PHLS transformants. At 50 μmol photons m^{-2} s^{-1}, the Δcpc+cpcB · PHLS and cpcB · PHLS+cpc transformants grew with only about 30% of the rate measured with the wild type (Fig. 6A). Reason for this quantitative difference at low irradiance is that Δcpc+cpcB · PHLS and cpcB · PHLS+cpc transformants failed to assemble functional Phc peripheral rods. Such disruption of the PBS antenna in the CpcB · PHLS strains has led to a phenotype consistent with that of ΔCpc mutants (Kirst et al., 2014; Formighieri and Melis, 2014a). Truncated light-harvesting antenna mutants have a diminished light-harvesting capacity, a disadvantage under limiting light conditions that translated into lower photosynthetic productivity. However, this phenotype was alleviated under higher growth irradiance conditions. When grown at 170 μmol photons m^{-2} s^{-1}, rate of growth accelerated for the wild type by about 35%, whereas rate of growth for the Δcpc+cpcB · PHLS and cpcB · PHLS+cpc transformants accelerated by 280% (Fig. 6B). Comparable rates of cell growth

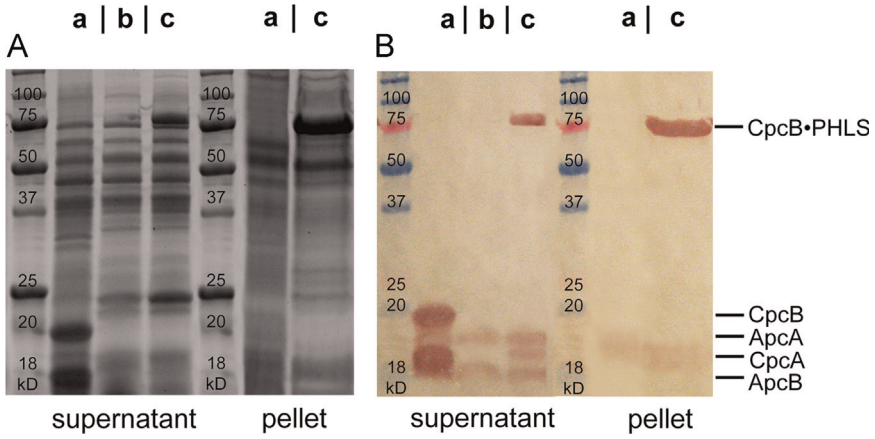

Fig. 5. Analysis of CpcB and CpcA protein expression in *Synechocystis* wild type and *PHLS* transformants. Strains **a**, **b**, and **c** were generated by constructs **a**, **b**, and **c**, as shown in Fig. 1. (A) Total protein of *Synechocystis* cell lysate pellet and supernatant fractions, as indicated, were resolved by SDS-PAGE and visualized by Coomassie staining. (B) Western blot analysis of the SDS-PAGE-resolved proteins shown in (A), obtained upon incubation with CpcA polyclonal antibodies (Abbiotec). The endogenous Cpc and Apc subunits, and CpcB protein from the CpcB•PHLS fusion cross-reacted with the CpcA polyclonal antibodies and are accordingly labeled.

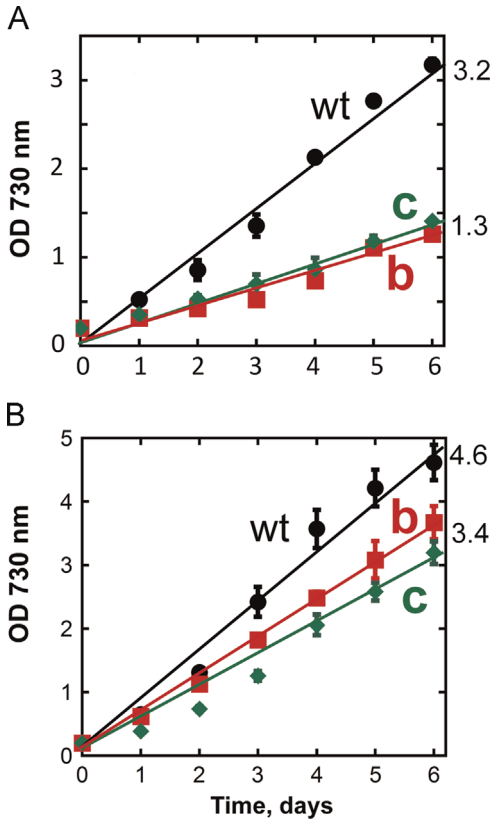

Fig. 6. Growth curves of *Synechocystis* wild type and PHLS transformants, as measured from the optical density (OD) of the cultures at 730 nm. Strains **b** and **c** refer to *Synechocystis* transformed with constructs **b** and **c**, as shown in Fig. 1. (A) Cells were grown under 50 μmol photons $m^{-2} s^{-1}$ of incident light intensity. (B) Cells were grown under 170 μmol photons $m^{-2} s^{-1}$ of incident light intensity. Averages and standard deviations were calculated from three independent biological replicates for each genotype. Cultures were inoculated to an OD at 730 nm of about 0.2, as the initial cell concentration in the growth experiment. Best fit of the points from the cell-density measurements were straight lines, reflecting a deviation from exponential growth due to increasing cell density and shading, gradually limiting the effective light intensity through the cultures.

for the wild type, Δcpc+cpcB·PHLS, and cpcB·PHLS+cpc transformants under saturating irradiance conditions suggested that substantial accumulation of the CpcB·PHLS protein up to 20% of total cellular protein in the latter does not exert a negative impact on cell fitness and biomass accumulation.

3.6. β-Phellandrene hydrocarbons production in PHLS transformants

The productivity of *Synechocystis* strains expressing the CpcB·PHLS or NPTI·PHLS fusion protein was then investigated in terms of β-phellandrene hydrocarbon yields from the photosynthesizing cyanobacteria. β-Phellandrene was collected as a non-miscible compound floating on top of the aqueous medium of transformant cultures. The floating β-phellandrene product was diluted upon addition of hexane, siphoned off the culture and quantified by absorbance spectroscopy, where the compound is distinguished by a specific absorbance in the UV region of the spectrum, showing a primary peak at 232.4 nm in hexane (Formighieri and Melis, 2014a, 2014b). The absorbance spectra of such hexane extracts from the wild type and *PHLS* transformants are shown in Fig. 7A, normalized on a per g of dry cell weight (dcw) of the biomass at the end of a 48 h cultivation period. It is evident that cpcB·PHLS+cpc (Fig. 7A, c) and NPTI·PHLS (Fig. 7A, e) produced substantially greater amounts of β-phellandrene, compared to the Δcpc+cpcB·PHLS (Fig. 7A, b), whereas the wild type produced none (Fig. 7A, wt).

Quantification of the yields of β-phellandrene as mg β-PHL g^{-1} dcw are reported in Table 1 and referred to measurements from three independent transformant lines for each genotype. The Δcpc+cpcB·PHLS transformant yielded low β-phellandrene amounts, an average of 0.03 mg β-PHL g^{-1} dcw. In contrast, the cpcB·PHLS+cpc transformant yielded an average of 3.2 mg β-PHL g^{-1} dcw. This constitutes a 100-fold yield increase over that of the Δcpc+cpcB·PHLS strain, a difference that is directly attributed to the corresponding amounts of the CpcB·PHLS protein accumulation, which is low in the Δcpc+cpcB·PHLS strain and high in the cpcB·PHLS+cpc strain (Fig. 4, lanes b, c). The NPTI·PHLS transformant generated an intermediate yield of 0.64 mg β-PHL g^{-1} dcw (Table 1), reflecting the intermediate level of PHLS fusion protein accumulation in this case (Fig. 4, lanes e).

A question was asked as to whether β-phellandrene synthase found in the pellet of *Synechocystis* extracts (Fig. 3B) is active in product generation or whether it constitutes insoluble and inactive protein aggregates. The activity of the β-phellandrene synthase was thus assessed *in vitro* with total cell extracts, after cell disruption, and with the pellet fraction following centrifugation. Absorbance spectra of hexane extracts from the *in vitro* reactions are shown in Fig. 7B, comparing the results obtained with the wild type and the CpcB·PHLS+Cpc transformant. Wild type extracts gave a featureless flat absorption spectrum in the 230 nm region. However, both total cell extract and pellet fractions from the CpcB·PHLS+Cpc transformant yielded measurable amounts of β-

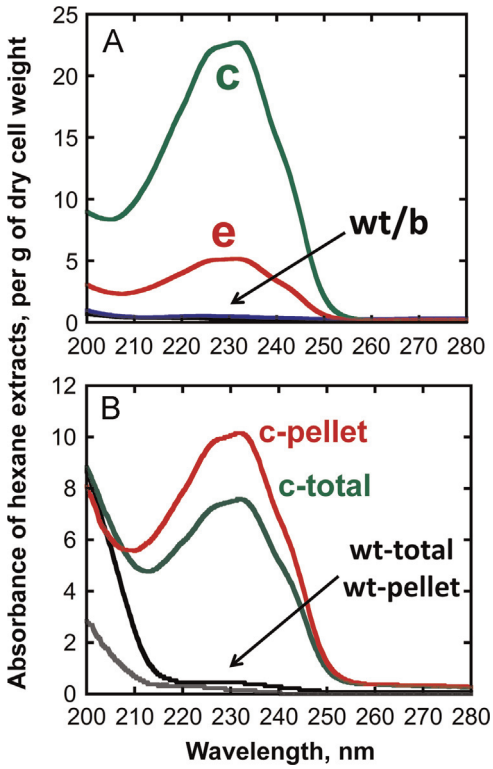

Fig. 7. β-Phellandrene hydrocarbons production by *Synechocystis* transformants. Strains **b**, **c** and **e** refer to *Synechocystis* transformed with constructs **b**, **c** and **e**, as shown in Fig. 1, and they are compared with the wild type. (A) β-Phellandrene hydrocarbons were collected as a non-miscible compound floating on top of the aqueous phase of live and actively photosynthesizing *Synechocystis* cultures. β-Phellandrene was diluted upon addition of 15 mL hexane to the surface of the culture and siphoned off the top of the growth medium. Absorbance spectra were normalized on per g of dry cell weight (dcw). Averages were calculated from three independent biological replicates for each genotype. Calculated β-phellandrene yields are reported in Table 1. (B) β-Phellandrene hydrocarbons synthesis was assayed *in vitro* after *Synechocystis* cell disruption. Assays were performed with total cell extracts or with the pellet fraction of cell lysates following centrifugation. Reaction mixtures were incubated in the presence of 50 μM of extraneously added GPP for 1 h at 30 °C. A 1 mL of hexane over-layer was applied at the surface of the reaction mixture from the beginning of the reaction for product sequestration. Absorbance spectra of the hexane extracts were normalized on a per g of dry cell weight (dcw) basis. Averages were calculated from three independent biological replicates, error bars are within 20% of the presented results.

Table 1
β-Phellandrene hydrocarbons production measurements over a 48 h photoautotrophic cultivation of *Synechocystis* transformants. Yields are expressed as mg of β-phellandrene per g of dry cell weight (dcw). Three independent transformant lines were tested for each genotype, with corresponding averages and standard deviations of the mean.

Genotype	β-phellandrene, mg g^{-1} dcw Independent transformant lines		
	a	b	c
ΔCpc+CpcB · PHLS	0.05 ± 0.02	0.02 ± 0.01	0.03 ± 0.02
CpcB · PHLS+Cpc	3.70 ± 0.48	2.57 ± 0.50	3.28 ± 0.90
ΔCpc+NPTI · PHLS	0.72 ± 0.10	0.62 ± 0.2	0.56 ± 0.14

phellandrene, detected as a clear UV absorbance band at 232.4 nm. Yields were 72 μg g^{-1} dcw h^{-1} (total extract) and 78 μg g^{-1} dcw h^{-1} (pellet fraction) (standard deviation within ±20%). These *in vitro* experiments are evidence that the abundant CpcB · PHLS fusion protein from the pellet fraction (Figs. 3 and 5, lanes c) was active in catalyzing β-phellandrene synthesis.

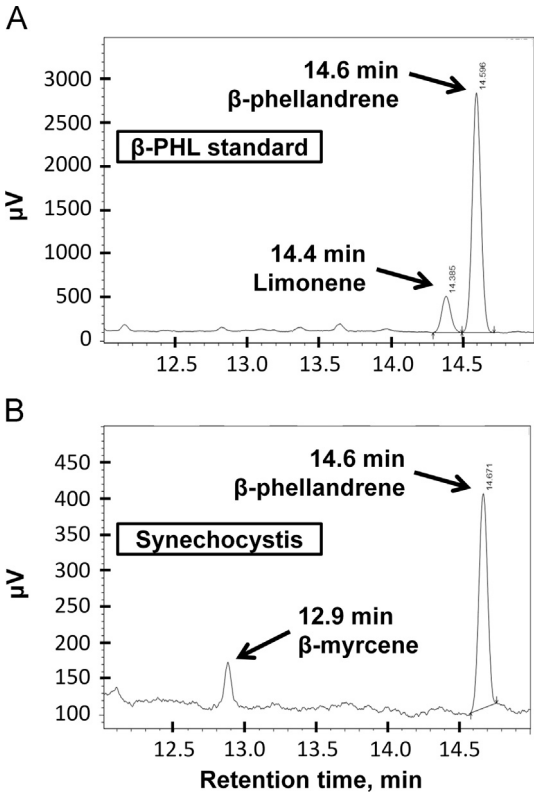

Fig. 8. GC-FID sensitive analysis of hexane extracts from *PHLS* transformant *Synechocystis* cultures. (A) GC-FID analysis of a β-phellandrene standard (Chemos GmbH) showing a retention time of 14.6 min under these conditions. The standard contained, in addition to β-phellandrene (retention time of 14.6 min), other monoterpenes, with limonene as the main impurity. (B) GC-FID analysis of hexane extracts from cpcB · PHLS+cpc transformant lines, showing the presence of β-phellandrene (retention time of 14.6 min) as the major product, confirmed by GC–MS, and of β-myrcene as a minor byproduct (retention time of 12.9 min). GC-FID analysis of hexane extracts from wild type cultures, measured under the same conditions, displayed a flat profile, showing no discernible peaks in the 5–20 min retention time region (not shown).

Fig. 8 shows the GC-FID profile of the hexane extract from the cpcB · PHLS+cpc transformants, as compared to that of a β-phellandrene standard. The results showed the presence of β-phellandrene as the major product with a retention time of 14.6 min. A small amount of limonene was detected in the β-phellandrene standard, and a small amount of β-myrcene was detected as the byproduct of the recombinant PHLS enzymatic activity, consistent with independent results from this lab (Formighieri and Melis, 2014b).

4. Discussion

Aquatic organisms, both multicellular and unicellular, including cyanobacteria, do not have the ability to generate essential oils such as terpene hydrocarbons, as these organisms lack the terpene synthase genes required for their synthesis (Van Wagoner et al., 2007). Terpene synthesis and accumulation in specialized organs, the trichomes, is typically a trait of terrestrial plants. Nevertheless, cyanobacteria express the methyl-erythritol-4-phosphate (MEP) pathway (Lichtenthaler, 2000), *via* which they synthesize a wide variety of terpenoid molecules essential for cell growth and function. These include carotenoids, phytol moieties of chlorophyll, and prenyl tails of plastoquinone molecules, which constitute the vast majority of isoprenoids serving the photosynthetic apparatus (Formighieri and Melis, 2014b). Carbon flux through the cell's terpenoid biosynthetic pathway was estimated to be 4–5% of

all photosynthetically fixed carbon (Lindberg et al., 2010). This endogenous MEP pathway in cyanobacteria can sustain heterologous synthesis of terpenes, and expression of a transgenic terpene synthase alone was necessary and sufficient to endow cyanobacteria with heterologous terpene hydrocarbon biosynthesis (Lindberg et al., 2010; Bentley et al., 2013; Davies et al., 2014; Halfmann et al., 2014). However, rate and yield of product generation were limited by the low amounts of the recombinant enzyme in the transgenic cells (Formighieri and Melis, 2014a). It became evident that high expression levels of the heterologous terpene synthase are needed to enhance carbon flux toward the desired product. The present work reports on the production of the monoterpene β-phellandrene in *Synechocystis* transformants heterologously expressing the β-phellandrene synthase (PHLS) as a fusion protein with highly expressed endogenous CpcB or heterologous NPTI proteins. Over-expression of fusion proteins is a successful part of the effort to increase the amount of the transgenic enzyme that in turn benefits rate and yield in product generation.

In one configuration, the *PHLS* transgene was expressed as a fusion with the *cpcB* gene under the control of the *cpc* endogenous promoter. We found that the CpcB · PHLS fusion protein accumulated to about 20% of the total cell protein in the cpcB · PHLS + cpc transformant, becoming the most abundant protein in the transformant extracts (Figs. 3–5, lanes c). Moreover, highly expressed genes other than *cpcB* could also act as lead fusion sequences in the amplification of transgene expression. In this work, a heterologous *NPTI · PHLS* fusion construct was made that allowed for accumulation of the NPTI · PHLS protein to about 5% of the total cell protein (Figs. 3 and 4, lanes e). Enhancements in the amount of the transgenic PHLS, albeit as a fusion protein, were a pre-requisite for greater β-phellandrene yields, as evidenced from the analysis of β-PHL hydrocarbons generation (Table 1). The high level of CpcB · PHLS protein expression was required to support synthesis of an average of 3.2 mg of β-PHL g^{-1} dcw (Table 1), a yield that was 100-fold greater than that measured with a transformant expressing low levels of PHLS. Similarly, the enhanced NPTI · PHLS protein expression led to a product yield of 0.64 mg of β-PHL g^{-1} dcw, consistent with the intermediate level of transgenic protein accumulation.

It is of interest to observe in this and earlier work from this lab (Formighieri and Melis, 2014a) that rate and yield of product (β-phellandrene) accumulation increased in excess of the percent increase in the amount of the β-phellandrene synthase (PHLS). This non-stoichiometric improvement in product accumulation, relative to the amount of the PHLS, can be viewed upon consideration of additive beneficial effects that are manifested with the greater enzyme concentration. (i) Enhanced amounts of PHLS help to compensate for the slow catalytic turnover of this terpene synthase. (ii) As shown in the schematic of Fig. 9, enhanced amounts of PHLS also help in the competition for GPP substrate in the synthesis of β-phellandrene versus that of endogenous terpenoids. (iii) Enhanced PHLS amounts may help in the homodimerization of the enzyme, with the resulting quaternary structure of the enzyme improving rates of catalysis. Such homodimerization was observed in other terpene synthases, such as limonene synthase (Hyatt et al., 2007) and isoprene synthase (Köksal et al., 2010). (iv) Enhancement in a possible dimer formation could be facilitated by the CpcB moiety of the CpcB-phellandrene synthase fusion protein, as dimerization, albeit with a homologous CpcA subunit, is the natural tendency of the CpcB subunits in the assembly of phycocyanin disks (Glazer, 1982). The latter scenario is likely, given that the CpcB moiety of the fusion protein was found to covalently bind a phycocyanobilin chromophore (results not shown). It could be argued that such allosteric arrangements help enhance the ability of the fusion-

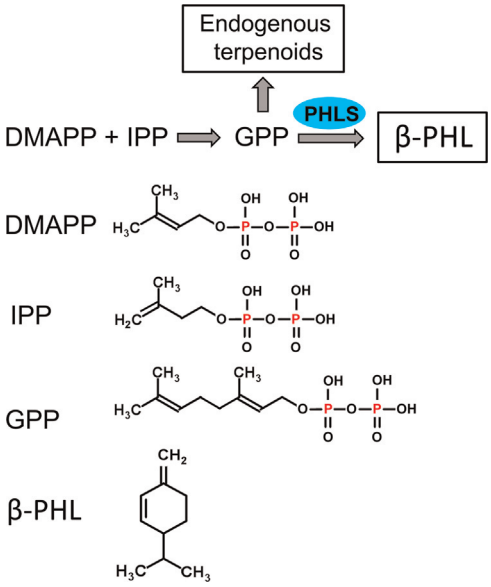

Fig. 9. Schematic depiction of the metabolic reaction for synthesis of geranyl-diphosphate (GPP), and the competition between the endogenous (terpenoids) and heterologous (β-phellandrene) pathways for GPP substrate. Enhancements in PHLS expression lead to disproportionate increase in β-phellandrene accumulation. Also shown are the chemical structures of the compounds involved in this pathway (adapted from ChemSpider ⟨http://www.chemspider.com⟩.)

synthase construct to attract and bind the GPP reactant (Fig. 9), disproportionately shifting the rate and yield of the reaction toward β-phellandrene synthesis.

We observed that CpcB protein fused to PHLS is found both in the supernatant and in the pellet of cell lysates, instead of being exclusively in the supernatant, as the case is for the native CpcB subunits (Figs. 3 and 5). This may be due to the PHLS protein and the properties that it confers. PHLS has no predicted transmembrane domains, however terpene synthases are found both in the plastid stroma of higher plants and thylakoid-membrane pellets (Wildermuth and Fall, 1998), so that recombinant PHLS may be tethered to membranes and thereby moves to the pellet with the heavy-fraction of *Synechocystis* transformants. Nevertheless, PHLS found in the pellet of lysed cells was active in β-phellandrene hydrocarbons production (Fig. 7B), showing also the applicability of the system for *in vitro* β-phellandrene generation.

In the past, heterologous expression in cyanobacteria of terpene synthases from higher plants did not yield high levels of recombinant protein, even under the control of strong promoters such as the *psbA2* (encoding the D1/32 kD photosystem-II reaction center protein) or the *cpc* operon promoter, and following the necessary codon-use optimization (Lindberg et al., 2010; Bentley et al., 2013; Davies et al., 2014; Formighieri and Melis, 2014a; Halfmann et al., 2014). This limitation in the expression of terpene synthases in cyanobacteria negatively impacts product yield, thus undermining commercial exploitation of these photosynthetic microorganisms in the generation of plant-based terpene products. This barrier in the expression of the PHLS protein was overcome in this work upon fusion of the PHLS to the highly expressed phycocyanin β-subunit or to the highly expressed heterologous NPTI in cyanobacteria, which was necessary and sufficient to substantially increase the yield of the β-phellandrene produced.

In summary, successful application of cyanobacteria as platforms for the generation of commodity products requires reprogramming of photosynthesis and attainment of high endogenous carbon partitioning ratios toward the desired product. However, catalysis of heterologous terpene synthesis in cyanobacteria was in

the past compromised by low levels of transgenic terpene synthase expression. In this work, we showed that fusion of the transgene to a highly expressed gene in cyanobacteria could be a means by which to substantially enhance transgene translation and recombinant protein accumulation. In the field of heterologous terpene biosynthesis, high levels of recombinant enzyme are an absolute prerequisite, needed to increase flux of endogenous substrate toward the desirable biosynthetic pathway.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2015.09.010.

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