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Paradigm of Monoterpene (β-phellandrene) Hydrocarbons Production via Photosynthesis in Cyanobacteria

Fiona K. Bentley · Jose Gines García-Cerdán · Hsu-Ching Chen · Anastasios Melis

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Abstract A direct "photosynthesis-to-fuels" approach envisions application of a single organism, absorbing sunlight, photosynthesizing, and converting the primary products of photosynthesis into ready-made fuel. The work reported here applied this concept for the photosynthetic generation of monoterpene (β-phellandrene) hydrocarbons in the unicellular cyanobacteria *Synechocystis* sp. PCC 6803. Heterologous expression of a codon-optimized *Lavandula angustifolia* β-phellandrene synthase (*β-PHLS*) gene in *Synechocystis* enabled photosynthetic generation of β-phellandrene in these microorganisms. β-phellandrene accumulation occurred constitutively and in tandem with biomass accumulation, generated from sunlight, CO_2, and H_2O. Results showed that β-phellandrene diffused through the plasma membrane and cell wall of the cyanobacteria and accumulated on the surface of the liquid culture. Spontaneous β-phellandrene separation from the biomass and its removal from the liquid phase alleviated product inhibition of cellular metabolism and enabled a continuous production process. The work showed that oxygenic photosynthesis can be directed to generate monoterpene hydrocarbons, while consuming CO_2, without a prior requirement for the harvesting, dewatering, and processing of the respective biomass.

Keywords Bioenergy · Biofuels · Cyanobacteria · Monoterpenes · β-phellandrene · Photosynthesis · Synechocystis

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Introduction

Terpenoids are the largest and most diverse group of naturally occurring organic compounds [1]. They are classified into groups based on the number of five-carbon isoprene units they comprise; hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40), and polyterpenes (greater than C40). Terpenoids play wide-ranging essential roles in primary growth and development; however, the majority of terpenoids have evolved as secondary metabolites of plant origin, with roles that include defense against herbivory, pollinator attraction, and seed dispersion [2]. This large family of structurally diverse secondary metabolites represents a valuable resource for bioprospectors and the industrial applications for terpenoids range from perfumes to pharmaceuticals to pesticides. Furthermore, there is potential for terpenoids to be developed as hydrocarbon biofuels, to supplement current fuel supplies, and as renewable feedstock for the synthetic chemistry industry [3, 4].

A number of plant species naturally produce the monoterpene β-phellandrene ($C_{10}H_{16}$) as a constituent of their essential oils, including lavender and grand fir. Essential oils are a product of photosynthesis and the associated chloroplast metabolism, and in lavender they accumulate in specialized organs called glandular trichomes, which form on the surface of leaves and flowers and function in chemical defense against herbivores. Harvesting of terpenoids from natural sources is often uneconomical, while so many terpenoids are chemically synthesized at an industrial scale from petrochemical resources. However, chemical synthesis is nonrenewable and often difficult to obtain because of the uniqueness and complexity of the chemical structure of the molecules. Accordingly, there is a need to develop renewable sources of fuels and chemicals, such as

terpenoid hydrocarbons, that will help meet global demands for energy and synthetic chemistry feedstock. The genetic and metabolic engineering of industrially robust photosynthetic microorganisms to drive oxygenic photosynthesis and the associated metabolism toward the synthesis of high impact products, such as β-phellandrene, offers an attractive alternative [5]. Photosynthetic microorganisms, such as cyanobacteria and unicellular microalgae, can simultaneously harvest solar energy and convert it into high-energy molecules. They can also grow to high densities within fully enclosed photo-bioreactors [6–8] and have better solar energy conversion efficiencies in photosynthesis than land plants [9].

The methyl-erythritol-4-phosphate (MEP) pathway, which is of prokaryotic bacterial origin and present in plant plastids, cyanobacteria, and microalgae [10–14] is responsible for the synthesis of most terpenoids. The MEP isoprenoid biosynthetic pathway is closely linked to cellular photosynthetic activity because it uses pyruvate and glyceraldehyde 3-phosphate as substrates and consumes reducing equivalents and cellular energy in the form of NADPH, reduced ferredoxin, CTP, and ATP, all of which are derived from photosynthesis [15]. β-phellandrene is a product of the MEP pathway in plant chloroplasts, e.g., lavender and grand fir and is directly synthesized from geranyl pyrophosphate by the chloroplast-localized β-phellandrene synthase enzyme (Fig. 1). Plant β-phellandrene synthases are encoded by the nuclear *β-PHLS* gene and have been cloned and characterized from lavender (*Lavandular angustifolia*), grand fir (*Abies grandis*), tomato (*Solanum lycopersicum*), and spruce (*Picea sitchensis*) [16–19].

Photosynthetic microorganisms, such as cyanobacteria and microalgae possess the MEP pathway by which to synthesize a wide variety of terpenoid-type molecules that are essential for cell function, such as carotenoids, hormones, phytol for chlorophyll, quinone prenyl tails, sterols, and tocopherols. However, they do not synthesize secondary terpenoid metabolites, such as monoterpenes [20], as they are not endowed with monoterpene synthase genes, e.g., a β-phellandrene synthase gene and enzyme. In this work, proof-of-concept is provided to show that cyanobacteria, e.g. *Synechocystis* sp. PCC 6803 (hereafter termed *Synechocystis*), can be metabolically manipulated to direct flux of the endogenous photosynthetic substrate through the terpenoid biosynthetic pathway toward synthesis and release of β-phellandrene ($C_{10}H_{16}$) hydrocarbons. The β-phellandrene synthase gene from lavender (*L. angustifolia*) was codon optimized and genetically introduced into *Synechocystis* to endow the cyanobacterium with the property of photosynthetic β-phellandrene production. This constitutes the first example of an engineered microorganism producing β-phellandrene monoterpene hydrocarbons, derived entirely via photosynthesis, i.e., from sunlight, carbon dioxide (CO_2), and water (H_2O). The work also presents methods to show how the transformant β-phellandrene-producing cyanobacteria can be grown at scale in photo-bioreactors, under semi-continuous culturing conditions, to renewably provide a supply of hydrocarbons in the form of β-phellandrene that is suitable as fuel or feedstock in chemical synthesis.

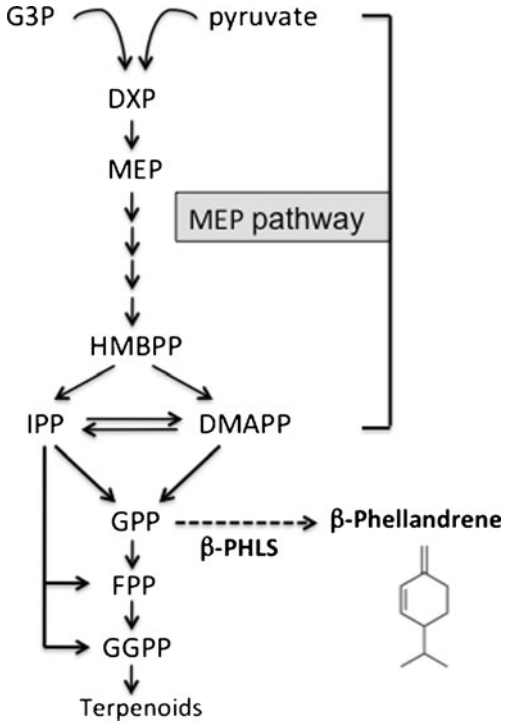

Fig. 1 Terpenoids biosynthesis via the methylerythritol 4-phosphate (*MEP*) pathway in *Synechocystis*. Abbreviations used: *G3P* glyceraldehyde 3-phosphate, *DXP* deoxyxylulose 5-phosphate, *HMBPP* hydroxymethylbutenyl diphosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate, *GGPP* geranylgeranyl diphosphate, *β-PHLS* β-phellandrene synthase. *Solid lines* represent reactions catalyzed by endogenous *Synechocystis* enzymes; whereas, the *dashed line* shows the reaction catalyzed by the heterologously expressed *Syn-β-PHLS* construct

Materials and Methods

Strains and Growth Conditions

The *Escherichia coli* strain DH5α was used for routine subcloning and plasmid propagation and grown in LB media with appropriate antibiotics at 37 °C, according to standard protocols. The glucose-tolerant cyanobacterial strain *Synechocystis* [21] was used as the recipient strain in this study and is referred to as the wild type (WT). WT and transformant strains were maintained on solid BG-11 media supplemented with 10 mM TES-NaOH (pH 8.2), 0.3 % sodium thiosulfate, and 5 mM glucose. Where appropriate, chloramphenicol was used at a concentration of 15 $\mu g mL^{-1}$. Liquid cultures were grown in BG-11 containing 25 mM sodium phosphate buffer, pH 7.5. Liquid cultures for inoculum purposes and for photoautotrophic growth experiments, RNA

isolation, and SDS-PAGE analyses were maintained at 25 °C under a slow stream of constant aeration and illumination at 20 μmol photons $m^{-2}s^{-1}$. Growth conditions employed, when measuring β-phellandrene in *Synechocystis* cultures, are described below in "Growth Conditions for Measuring β-phellandrene".

Codon-Use Optimization of the *β-phellandrene* Synthase Gene for Expression in *Synechocystis*

The nucleotide and translated protein sequences of the β-phellandrene synthase gene from *Lavandula angustifolia* cultivar Lady (accession No. HQ404305) [18] were obtained from the NCBI GenBank database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/nuccore/HQ404305). The protein sequence of the β-phellandrene synthase gene was firstly analyzed by TargetP software (http://www.cbs.dtu.dk/services/TargetP/) for prediction of the subcellular localization of the protein and for the presence of a transit peptide. Based on this analysis, the β-phellandrene synthase from *L. angustifolia* cultivar Lady was predicted to be a chloroplast localized protein with the first 42-amino acids of the protein serving as a chloroplast transit peptide. This analysis indicated that the first 42-amino acids are not part of the mature protein that functions in the chloroplast. Based on this information, we designed a nucleotide sequence from the original sequence of *Lavandula* β-phellandrene synthase (*La-β-PHLS*) gene by replacing the first 42-amino acids with a methionine, suitable for expression in *Synechocystis*. The protein sequence of the β-phellandrene synthase that we employed in this work is composed of 540-amino acids (Fig. 2). To maximize expression of β-phellandrene synthase in *Synechocystis* this protein sequence was back translated and codon-optimized according to the frequency of the codon usage in *Synechocystis*. The codon-use optimization process was performed based on the codon usage table obtained from Kazusa DNA Research Institute, Japan (http://www.kazusa.or.jp/codon/) and using the Gene Designer 2.0 software from DNA 2.0 (https://www.dna20.com/) with a cut-off thread of 15 %. The codon-optimized gene (*Syn-β-PHLS*) was designed with appropriate flanking restriction sites to aid subsequent cloning steps. The nucleotide sequences of the β-phellandrene synthase gene from *L. angustifolia* (*La-β-PHLS*) and the codon-optimized sequence for expression in *Synechocystis* (*Syn-β-PHLS*) are shown in Fig. 2a, b, respectively.

Plasmid Construction and Generation of *Synechocystis* Transformants with Heterologous Expression of the *Syn-β-PHLS* Gene

A plasmid construct was generated to allow the heterologous expression of the β-phellandrene synthase gene in *Synechocystis* through the replacement of the endogenous

A

La-β-PHLS: *L. angustifolia β-PHLS* cDNA nucleotide sequence

```
ATGTCTACCATTATTGCGATACAAGTGTTGCTTCCTATTCCAACTACTAAAACATACCCTAGTCA
TGACTTGGAGAAGTCCTCTTCGCGGTGTCGCTCCTCCTCCACTCCTCGCCCTAGACTGTGTTGCT
CGTTGCAGGTGAGTGATCCGATCCCAACGGGCCGGCGATCCGGAGGCTACCCGCCCGCCCTATGG
GATTTCGACACTATTCAATCGCTCAACACCGAGTATAAGGGAGAGAGGCACATGAGAAGGGAAGA
AGACCTAATTGGGCAAGTTAGAGAGATGCTGGTGCATGAAGTAGAGGATCCCACTCCACAGCTGG
AGTTCATTGATGATTTGCATAAGCTTGGCATATCTTGCCATTTTGAGAATGAAATCCTCCAAATC
TTGAAATCCATATATCTTAATCAAAACTACAAAAGGGATTTGTACTCAACATCTCTAGCATTCAG
ACTCCTCAGACAATATGGCTTCATCCTTCCACAAGAAGTATTTGATTGTTTCAAGAATGAGGAGG
GTACGGATTTCAAGCCAAGCTTCGGCCGTGATATCAAAGGCTTGTTACAATTGTATGAAGCTTCT
TTCCTATCAAGAAAAGGAGAAGAAACTTTACAACTAGCAAGAGAGTTTGCAACAAAGATTCTGCA
AAAAGAAGTTGATGAGAGAGAGTTTGCAACCAAGATGGAGTTCCCTTCTCATTGGACGGTTCAAA
TGCCGAATGCAAGACCTTTCATCGATGCTTACCGTAGGAGGCCGGATATGAATCCAGTTGTGCTC
GAGCTAGCCATACTTGATACAAATATAGTTCAAGCACAATTTCAAGAAGAACTCAAAGAGACCTC
AAGGTGGTGGGAGAGTACAGGCATTGTCCAAGAGCTTCCATTTGTGAGGGATAGGATTGTGGAAG
GCTACTTTTGGACGATTGGAGTGACTCAGAGACGCGAGCATGGATACGAAAGAATCATGACCGCA
AAGGTTATTGCCTTAGTAACATGTTTAGACGACATATACGATGTTTATGGCACGATAGAAGAGCT
TCAACTTTCACAAGCACAATCCAAAGATGGGATTTGGAATCAATGAAGCAACTCCCTACCTACA
TGCAAGTAAGCTTTCTTGCACTACACAACTTTGTAACCGAGGTGGCTTACGATACTCTCAAGAAA
AAGGGCTACAACTCCACACCATATTTAAGAAAAACGTGGGTGGATCTTGTTGAATCATATATCAA
AGAGGCAACTTGGTACTACAACGGTTATAAACCTAGTATGCAAGAATACCTTAACAATGCATGGA
TATCAGTCGGAAGTATGGCTATACTCAACCACCTCTTCTTCCGGTTCACAAACGAGAGAATGCAT
AAATACCGCGATATGAACCGTGTCTCGTCCAACATTGTGAGGCTTGCTGATGATATGGGAACATC
ATTGGCTGAGGTGGAGAGAGGGGACGTGCCGAAAGCAATTCAATGCTACATGAATGAGACGAATG
CTTCTGAAGAAGAAGCAAGAGAATATGTAAGAAGAGTCATACAGGAAGAATGGGAAAAGTTGAAC
ACAGAATTGATGCGGGATGATGATGATGATGATTTACACTATCCAAATATTACTGTGAGGT
GGTTGCTAATCTTACAAGAATGGCACAGTTTATATACCAAGATGGATCGGATGGCTTCGGCATGA
AAGATTCCAAGGTTAATAGACTGCTAAAAGAGACGTTGATCGAGCGCTACGAATAA
```

B

Syn-β-PHLS: *Synechocystis codon-optimized version of La-β-PHLS*

```
   PacI     NdeI
TTAATTAACATATGTGTAGTTTGCAAGTTTCTGATCCTATTCCTACCGGACGCCGTTCCGGTGGT
TATCCCCCGGCCTTATGGGATTTCGATACTATTCAATCCCTGAATACCGAATATAAGGGCGAACG
TCACATGCGTCGGGAAGAAGACTTAATTGGTCAAGTTCGGGAAATGTTGGTGCACGAAGTAGAAG
ATCCCACTCCCCAGTTGGAATTCATTGACGATCTGCATAAATTGGGCATTTCCTGCCATTTTGAA
AACGAGATTCTGCAAATTCTCAAATCCATTTATCTCAACCAAAACTATAAACGGGACCTCTATTC
TACCAGTTTAGCCTTCCGTCTCTTGCGTCAATACGGGTTTATCTTGCCGCAGGAAGTTTTTGACT
GCTTTAAAAACGAAGAAGGTACGGATTTTAAACCCAGCTTCGGCCGGGATATTAAGGGTCTGTTA
CAGTTGTACGAAGCCTCCTTTTTGTCCCGGAAGGGGGAAGAAACTTTACAACTCGCCCGCGAATT
TGCTACCAAAATCTTGCAAAAGGAAGTCGATGAACGGGAATTTGCTACTAAAATGGAATTTCCCA
GTCACTGGACCGTACAAATGCCTAACGCTCGGCCTTTTATCGATGCCTATCGTCGGCGTCCCGAC
ATGAACCCCGTGGTTCTGGAACTCGCCATTCTCGATACCAATATCGTGCAAGCTCAGTTTCAAGA
AGAATTGAAGGAGACCTCCCGTTGGTGGGAAAGCACGGGGATTGTTCAAGAACTGCCGTTTGTTC
GGGACCGGATTGTGGAAGGTTATTTTTGGACCATTGGTGTTACTCAACGCCGTGAACACGGTTAC
GAACGTATTATGACGGCCAAAGTCATCGCTTTGGTGACCTGTTTGGATGATATTTATGACGTATA
TGGCACTATTGAAGAATTGCAACTCTTCACCTCTACGATTCAGCGTTGGGATTTGGAGTCTATGA
AGCAGTTACCGACTTATATGCAGGTAAGCTTCCTGGCCTTGCACAATTTTGTAACCGAAGTGGCC
TATGATACGCTGAAGAAAAAGGGCTACAACTCTACCCCCTATTTGCGGAAGACTTGGGTGGATTT
GGTCGAAAGTTACATTAAGGAAGCCACTTGGTACTATAATGGGTACAAACCCTCTATGCAGGAAT
ACCTCAACAACGCCTGGATCTCTGTGGGCAGCATGGCTATTTTGAATCATTTGTTTTTTCGCTTT
ACTAATGAACGCATGCATAAGTACCGGGACATGAATCGTGTATCCTCTAATATTGTGCGGTTAGC
CGACGATATGGGAACCTCTTTGGCCGAAGTTGAACGCGGTGACGTGCCCAAAGCTATCCAATGTT
ACATGAATGAAACGAACGCCTCTGAGGAGGAGGCCCGCGAATATGTGCGGCGCGTTATCCAGGAA
GAATGGGAAAAACTGAACACTGAACTGATGCGCGACGACGACGATGACGATGATTTCACCTTAAG
TAAATACTACTGCGAAGTCGTTGCTAACCTGACCCGGATGGCTCAGTTCATTTACCAAGATGGTT
CCGATGGGTTTGGGATGAAAGATTCCAAAGTAAATCGTTTACTGAAAGAAACGCTGATTGAGCGC
TATGAGTGAAGATCTGCGGCCGC
                                  BglII     NotI
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C

Mature Syn-β-PHLS amino acid sequence

```
MCSLQVSDPIPTGRRSGGYPPALWDFDTIQSLNTEYKGERHMRREEDLIGQVREMLVHEVEDPTP
QLEFIDDLHKLGISCHFENEILQILKSIYLNQNYKRDLYSTSLAFRLLRQYGFILPQEVFDCFKN
EEGTDFKPSFGRDIKGLLQLYEASFLSRKGEETLQLAREFATKILQKEVDEREFATKMEFPSHWT
VQMPNARPFIDAYRRRPDMNPVVLELAILDTNIVQAQFQEELKETSRWWESTGIVQELPFVRDRI
VEGYFWTIGVTQRREHGYERIMTAKVIALVTCLDDIYDVYGTIEELQLFTSTIQRWDLESMKQLP
TYMQVSFLALHNFVTEVAYDTLKKKGYNSTPYLRKTWVDLVESYIKEATWYYNGYKPSMQEYLNN
AWISVGSMAILNHLFFRFTNERMHKYRDMNRVSSNIVRLADDMGTSLAEVERGDVPKAIQCYMNE
TNASEEEAREYVRRVIQEEWEKLNTELMRDDDDDDDFTLSKYYCEVVANLTRMAQFIYQDGSDGF
GMKDSKVNRLLKETLIERYE*
```

Fig. 2 The β-phellandrene synthase nucleotide and protein sequences employed in this study. **a** The *L. angustifolia β-PHLS* (*La-β-PHLS*) cDNA sequence (accession No. HQ404305) [18]. The chloroplast transit peptide is indicated in *bold lettering*, and start and stop codons are *highlighted*. **b** Codon-optimized version of *La-β-PHLS* cDNA sequence minus the chloroplast transit peptide for expression in *Synechocystis* and *E. coli*. We have termed this codon-optimized sequence *Syn-β-PHLS*. Start and stop codons are indicated. Restriction sites incorporated into the synthesized sequence for cloning purposes are *underlined*; *Pac*I and *Nde*I sites at the start of the sequence, and *Bgl*II and *Not*I sites after the stop codon. **c** The mature protein sequence of *Syn-β*-PHLS for expression in *Synechocystis*

psbA2 gene with the *Syn-β-PHLS* transgene via double homologous recombination. The synthesized *Syn-β-PHLS* transgene was PCR amplified using the following primers: PHLS_F, 5′-CCTGGGCGGTTCTGATAACG-3′, and PHLS_*Bam*HI_R, 5′-CGCGGATCCTTTTGACGGCGGC CGCAGAT-3′. A *Bam*HI site was incorporated into the PHLS_*Bam*HI_R primer to allow the cloning of *Syn-β-PHLS* PCR product into the *Nde*I and *Bam*HI sites of the plasmid pBA2A2, which contains 500 bp of the upstream and downstream sequences of the *psbA2* gene [22], generating plasmid pSyn-β-PHLS-ΔA2. Finally, a chloramphenicol-resistance cassette from plasmid pACYC184 was PCR amplified using primers with strategically incorporated restriction sites: CamR_NotI_F, 5′-AAGGAAAAAAGCGGCCGCTTGATG CGGCACGTAAGAGGTTC-3′ and CamR_*Bam*HI_R, 5′-CGCGGATCCCCAGGCGTTTAAGGGCACCAATAAC-3′ and cloned into the *Not*I and *Bam*HI sites of plasmid pSyn-β-PHLS-ΔA2, to generate plasmid pSyn-β-PHLS-CmR-ΔA2. This plasmid was used to transform WT *Synechocystis* according to established procedures [21, 23]. Chloramphenicol was used for selection and maintenance of transformant strains on agar plates. The heterologous transformed cyanobacteria are referred to as *Syn-β-PHLS* transformants. Successful transgene incorporation and complete DNA cyanobacterial copy segregation for the *Syn-β-PHLS* gene was tested by genomic DNA PCR, using primers designed to genomic DNA regions just outside of the upstream and downstream regions of the *psbA2* gene that were used for homologous recombination: A2us_F, 5′-TATCAGAATCCTG TGCCCAGATG-3′ and A2ds_R, 5′-GGTAGAGTTGCGG AGGGCAAT-3′.

Reverse-Transcription PCR

Cells were harvested in the late-exponential growth phase for RNA isolation. Trizol reagent (Life Technologies) was used to extract total RNA from 25 mL of the liquid culture according to the manufacturer's instructions. Contaminating DNA was removed by treating the isolated RNA with DNaseI (Life Technologies). cDNA was generated from 1 μg of RNA by reverse transcription using SuperScript III (Life Technologies). This was used as the template for PCR analysis, where gene-specific primers were used to amplify the *Syn-β-PHLS* transgene, in addition to the Rubisco large subunit (*RbcL*) gene as a positive control for transcription. Primers designed for *Syn-β-PHLS* amplification were: RT_Syn-β-PHLS_F, 5′-TTGGTGACCTGGTT TGGATGA-3′ and RT_Syn-β-PHLS_R, 5′-CCAGGCGTTGTTGAGGTATT-3′; and for RbcL amplification: RT_RbcL_F, 5′-GTATCACCATGGGCTT CGTT-3′, and RT_RbcL_R, 5′-CACAAGCTTCCAA AGCAACA-3′.

Antibody Generation and Western Blot Analysis

For expression in *E. coli*, the *Synechocystis* codon-optimized *β-PHLS* gene (*Syn-β-PHLS*) was PCR amplified using the forward primer 5′-GGAATTCCATATGTG TAGTTTGCAAGTTTCTGAT-3′ and reverse primer 5′-ACAGGATCCTCACTCATAGCGCTCAATCAGCGT-3′ and subcloned into the pET28a(+) vector (Novagen). Expression of the *Syn-β-PHLS* construct was induced by IPTG in *E. coli* BL21 (DE3) cells (Novagen), and the 6xHis-tagged Syn-β-*PHLS* protein was purified under native conditions through a nickel-nitrilotriacetic acid agarose column (Qiagen) according to the manufacturer's instructions. Specific polyclonal antibodies were generated in rabbit against the whole β-phellandrene synthase recombinant protein as the antigen following the instructions of ProSci Inc, USA.

Samples for SDS-PAGE analyses were prepared from *Synechocystis* cells resuspended in phosphate buffer at pH 7.4 at a concentration of 0.12 mgml^{-1} chlorophyll. The suspension was supplemented with 0.05 % (*w*/*v*) lysozyme (Thermo Scientific) and incubated with shaking at 37 °C for 45 min. Cells were then precipitated upon centrifugation at 4,000×*g*, washed twice with fresh phosphate buffer, and disrupted with a French Press chamber (AMINCO, USA) at 1,200 psi in the presence of 1 mM PMSF. Soluble proteins were separated from the crude cell extract by centrifugation at 21,000×*g* and removed as the supernatant fraction. Samples for SDS-PAGE analysis were solubilized with denaturing cell extraction buffer (0.2 M Tris at pH 6.8, 4 % SDS, 2 M urea, 1 mM EDTA, and 20 % glycerol). Samples were supplemented with β-mercaptoethanol to 5 % (*v*/*v*) prior to gel loading. For Western blot analyses, Any kDTM (BIO-RAD) precast SDS-PAGE gels were used for protein separation, prior to transfer and immobilization on PVDF membrane (Immobilon-FL 0.45 μm, Millipore, USA) for immunodetection using the rabbit immune serum containing specific polyclonal antibodies against the Syn-β-PHLS protein. Cross-reactions were visualized by Supersignal West Pico Chemiluminiscent substrate detection system (Thermo Scientific, USA).

Chlorophyll Determination, Photosynthetic Productivity, and Biomass Quantitation

Chlorophyll *a* concentrations in cultures were determined spectrophotometrically in 90 % methanol extracts of the cells according to Meeks and Castenholz [24]. Photosynthetic productivity of the cultures was tested polarographically with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England). Cells were harvested at mid-exponential growth phase and maintained at 25 °C in BG-11 containing 25 mM HEPES–NaOH, pH 7.5, at a chlorophyll *a* concentration of 10 μgmL^{-1}. Oxygen evolution was

measured at 25 °C in the electrode upon yellow actinic illumination, which was defined by a CS 3-69-long wavelength pass cutoff filter (Corning, NY). Photosynthetic activity of a 5-mL aliquot of culture was measured at varying actinic light intensities in the presence of 15 mM $NaHCO_3$ pH 7.4, to generate the light saturation curve of photosynthesis. Culture biomass accumulation was measured gravimetrically as dry cell weight, where 5 mL samples of culture were filtered through 0.22-μm Millipore filters and the immobilized cells dried at 90 °C for 6 h prior to weighing the dry cell weight.

Growth Conditions for Measuring β-phellandrene

Synechocystis cultures for β-phellandrene production assays were grown photo-autotrophically in 1 L gaseous/aqueous two-phase photobioreactors, which are described in detail in [25]. Bioreactors were seeded with a 700-mL culture of *Synechocystis* cells at an OD_{730} of 0.05 in BG-11 medium containing 25 mM sodium phosphate buffer, pH 7.5, and grown under continuous illumination at 75 μmol photons $m^{-2}s^{-1}$, and continuous bubbling with air, until an OD_{730} of approximately 0.3±0.1 was reached. Inorganic carbon was delivered to the culture in the form of 500 mL aliquots of 100 % CO_2 gas, which was slowly bubbled though the bottom of the liquid culture to fill the bioreactor headspace. Once atmospheric gases in the headspace were replaced with 100 % CO_2, the headspace of the reactor was sealed and the culture was incubated under continuous illumination of 150 μmol photons $m^{-2}s^{-1}$ at 35 °C for 48 h. Slow continuous mechanical mixing was employed to keep cells in suspension and to promote balanced cell illumination and gaseous CO_2 diffusion into the liquid culture to support growth. Uptake and assimilation of headspace CO_2 by cells was concomitantly exchanged for O_2 during photoautotrophic growth. The sealed bioreactor headspace allowed for the trapping, accumulation and concentration of photosynthetically produced β-phellandrene as either a volatile product in the headspace, or in liquid form associated with the aqueous phase.

For the semi-continuous culturing experiment, cells were diluted following the 48 h of CO_2-supported photoautorophic growth and the cycle was repeated. More specifically, an aliquot of cells was taken to inoculate a new bioreactor to an OD_{730} of about 0.3±0.1, which was then subject to the CO_2 treatment and subsequent 48 h of CO_2-supported biomass accumulation. β-Phellandrene was measured in the original culture after the inoculation of the new bioreactor so as to avoid heptane contamination of the newly inoculated culture. This process was repeated up to four cycles.

β-phellandrene Identification and Quantification Assays

Gas from the headspace of sealed bioreactors was sampled and analysed by gas chromatography–mass spectrometry (GC-MS) to detect volatilized, photosynthetically produced β-phellandrene. Comparison of retention time and mass spectrum with a vaporized α-phellandrene standard (containing β-phellandrene and other impurities; MP Biomedicals) allowed for positive identification of β-phellandrene in the headspace. Photosynthetically produced non-miscible β-phellandrene in liquid form was trapped by overlaying 20 mL heptane on top of the liquid culture in the bioreactor and incubating for 2 h with gentle stirring at room temperature. The heptane layer was subsequently removed and 1 μL was analysed by GC-MS. A 1:500 dilution of a liquid α-phellandrene standard (MP Biomedicals) in heptane was used as a positive control due to the presence of small amounts of β-phellandrene impurities. GC-MS analyses were performed with an Agilent 6890GC/5973 MSD equipped with a DB-XLB column (0.25 mm i.d.×0.25 μm× 30 m; J&W Scientific). Oven temperature was initially maintained at 50 °C for 2 min, followed by a temperature increase of 10 °C/min to 150 °C, and a carrier gas (helium) flow rate of 1.2 mL/min.

Accumulation of β-phellandrene was quantified spectrophotometrically according to known absorption maximum at 231 nm and extinction coefficients $\varepsilon_{231}=9.12\ mM^{-1}cm^{-1}$ of β-phellandrene [26, 27]. The majority of photosynthetically produced β-phellandrene accumulated on the surface of the liquid phase of the bioreactor, therefore, the non-miscible, heptane-extracted β-phellandrene (described above) was used to generate the absorption spectra of β-phellandrene in heptane for quantification purposes.

Results

Codon-Use Optimization of the β-phellandrene Synthase Gene

A de novo synthesized version of the native *L. angustifolia* β-phellandrene synthase gene was first designed with the preferred codon usage for *Synechocystis*. Codons that are rarely used by *Synechocystis* were also modified to more frequently used codons; in this case we eliminated codons used less frequently than 15 % in *Synechocystis*. The *de novo Synechocystis* codon-optimized version of the β-phellandrene synthase gene was termed *Syn-β-PHLS*. The β-phellandrene synthase sequences used in this study are presented in Fig. 2. The native *L. angustifolia β-PHLS* cDNA sequence, including the predicted chloroplast transit peptide (accession number HQ404305) is shown in Fig. 2a. The *L. angustifolia β-PHLS* cDNA sequence (*La-β-PHLS*) minus the chloroplast transit peptide, with codon usage optimized for *Synechocystis* (*Syn-β-PHLS*) is shown in Fig. 2b. The β-PHLS protein sequence for expression in *Synechocystis* and *E. coli* (*Syn-β-PHLS*) is shown in Fig. 2c.

Syn-β-PHLS Plasmid Construction and *Synechocystis* Transformation

The *Synechocystis* codon-optimized β-phellandrene synthase (*Syn-β-PHLS*) gene was cloned into a plasmid construct that was designed to enable double homologous recombination at the *psbA2* site of the *Synechocystis* genome [22]. The *Syn-β-PHLS* gene, together with an antibiotic resistance cassette, was inserted in-between two 500 bp sequences that immediately flank the *psbA2* gene (Fig. 3). The resulting plasmid, which we termed p*Syn-β-PHLS*-CmR-ΔA2, was used to transform *Synechocystis*. A number of transformant lines were obtained in which the *psbA2* gene was replaced with the *Syn-β-PHLS* construct via homologous recombination (Fig. 4). Complete segregation of all *Syn-β-PHLS* DNA copies in the cyanobacterial genome (homoplasmy) was achieved upon cultivation of the transformants for several generations under strong antibiotic selective pressure, which promoted deletion of WT copies of the *Synechocystis* DNA and replacement with the *Syn-β-PHLS* transgene-containing copy (Fig. 4a). Genomic DNA PCR analysis, using primers that flank the upstream and downstream regions of the *psbA2* gene (Fig. 4a) was used to provide evidence of homoplasmy. A larger product (3.6 kb) was amplified in the transformant lines compared with the WT DNA (2.3 kb), due to the replacement of the endogenous 1,083 bp *psbA2* gene with the larger 1,632 bp *Syn-β-PHLS* gene construct fused to an 810-bp chloramphenicol-resistance cassette (Fig. 4b). The smaller 2.3-kb product was clearly amplified only in the untransformed WT (Fig. 4b), compared with the larger 3.6 kb product that was amplified in twelve independent *Syn-β-PHLS* transformant lines. Absence of the WT 2.3-kb product from the transformant lines is evidence for complete segregation of the *Syn-β-PHLS* DNA copies, and elimination of all original WT DNA copies.

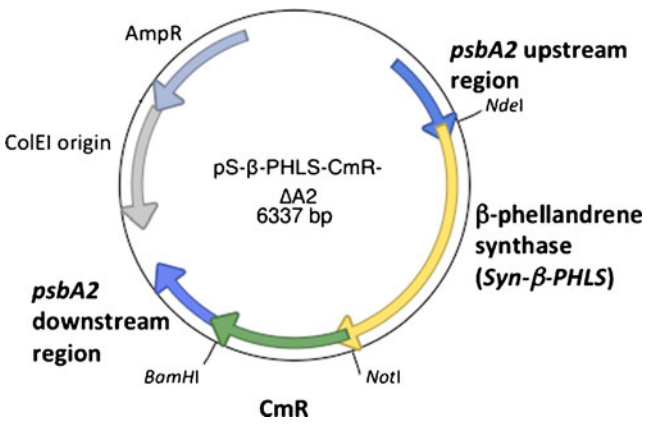

Fig. 3 Plasmid construct for expression of *Syn-β-PHLS* in *Synechocystis*. The *Synechocystis* codon-optimized β-phellandrene synthase gene (*Syn-β-PHLS*) and a chloramphenicol-resistance cassette (*CmR*), were cloned into a vector containing upstream and downstream regions of the *Synechocystis psbA2* gene. Restriction sites used for cloning purposes are indicated. This plasmid was used for the transformation of WT *Synechocystis* cells and facilitated the integration of the *Syn-β-PHLS*-CmR cassette within the *Synechocystis* genome at the *psbA2* locus via double homologous recombination

DNA sequencing of the *Syn-β-PHLS* PCR products was carried out to test whether (1) the correct nucleotide sequence of the *Syn-β-PHLS* construct had been incorporated into the *Synechocystis* genome, (2) was correctly inserted in frame with the endogenous *psbA2* promoter, and (3) no errors had been introduced within the regions of homologous recombination at the nucleotide level. All analyzed *Syn-β-PHLS* transformant lines were found to be stably homoplasmic for the introduced *Syn-β-PHLS* transgene over an indefinite number of generations.

Expression of the *Syn-β-PHLS* Transgene

Successful transcription of the introduced *Syn-β-PHLS* transgene in transformant lines was shown by RT-PCR (Fig. 5). The expected 312 bp *Syn-β-PHLS* amplicon was present in four representative transformant lines, but absent from the WT, confirming the expression of *Syn-β-PHLS* mRNA in the transformant lines (Fig. 5, left panel). The *RbcL* was amplified as a positive control and the expected 264-bp amplicon was present in all lines, including WT (Fig. 5, middle panel). No *RbcL* products were amplified in control samples that were prepared in the absence of the Superscript RNA transcriptase (Fig. 5, right panel), showing absence of genomic DNA contamination in all cDNA samples.

Specific polyclonal antibodies were raised against the *E. coli*-expressed recombinant *Syn-β-PHLS* protein, and used to test for translation and presence of the *Syn-β-PHLS* protein in transformant *Synechocystis* cells. The *Syn-β-PHLS* protein has a predicted molecular weight of 63.7 kDa, and the polyclonal *Syn-β-PHLS* antibodies had a strong and specific cross-reaction to a protein of this size in both the total cell extract (*Syn-β-PHLS*, TCE) and supernatant fractions (*Syn-β-PHLS*, SP) of the *Syn-β-PHLS* transformants (Fig. 6). The absence of a similar cross-reaction in the WT provides evidence for the successful expression of the recombinant Syn-β-PHLS protein in the transformant lines. Detection of the Syn-β-PHLS protein in the soluble fraction further indicates that it accumulates as a soluble protein in the cell.

β-phellandrene Production in *Synechocystis Syn-β-PHLS* Transformants

The above results clearly show that *Synechocystis* is amenable to stable incorporation and expression of the *Syn-β-PHLS* gene, as it leads to accumulation of the soluble Syn-β-PHLS recombinant protein. To determine whether the expressed Syn-β-PHLS protein is functionally active, WT and *Syn-β-PHLS* transformant lines were cultivated under the conditions of the gaseous/aqueous two-phase

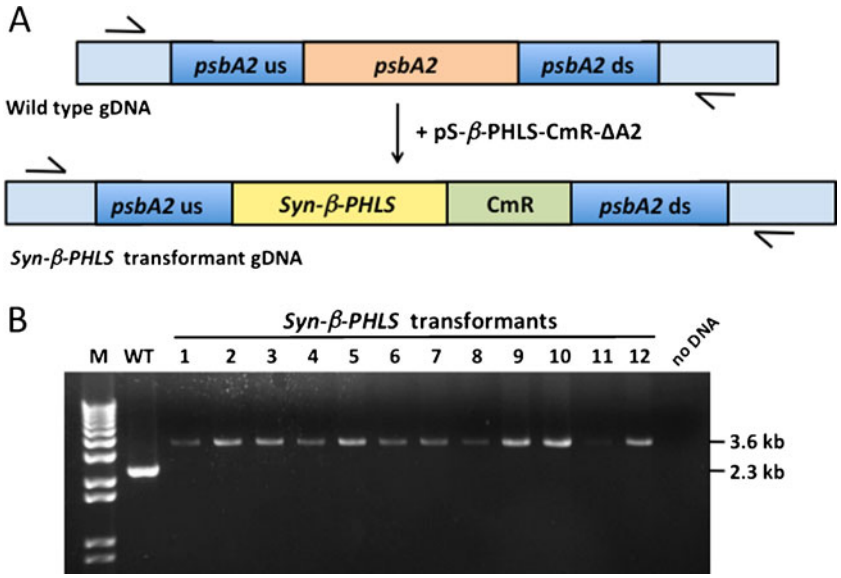

Fig. 4 Double homologous recombination and *Synechocystis* DNA copy segregation. **a** Maps of the *psbA2* gene locus in wild-type (*WT*) S*ynechocystis* and in the *Syn-β-PHLS* transformants upon integration of the *Syn-β-PHLS*-CmR gene construct into the *Synechocystis* genome via double homologous recombination upon transformation with plasmid pS-*β-PHLS*-CmR-ΔA2. Genomic PCR primers (*arrows*) were designed to flanking regions of the upstream and downstream regions of the *psbA2* gene (*psbA2* us and *psbA2* ds) that were used for homologous recombination and amplify a 3.6-kb product in the *Syn-β-PHLS* transformant compared with a 2.3-kb product in the WT. **b** Complete DNA copy segregation in the transformants following the replacement of *psbA2* with the heterologous *Syn-β-PHLS* transgene construct using the above-mentioned primers. A PCR product of ~2.3 kb was amplified in the WT containing the endogenous *psbA2*; whereas, larger products of ~3.6 kb were amplified in 12 different *Syn-β-PHLS* transformant lines (*1–12*). Absence of the 2.3-kb product from the latter indicates homoplasmy for the introduced transgene. *M*, 1 kb plus marker

bioreactor [25], where 100 % CO_2 gas slowly filled the reactor headspace, before sealing the bioreactor to allow autotrophic biomass accumulation for 48 h.

β-phellandrene has a relatively high boiling point (171–172 °C) and is non-miscible in aqueous solution; therefore, we anticipated that if photosynthetically generated *β*-phellandrene is released from the cell, it would accumulate as a non-miscible product on the surface of the liquid culture. Accordingly, a small volume of heptane was layered on top of the liquid culture to trap non-miscible liquid hydrocarbons floating on the surface. Samples of the heptane extract were subjected to GC-MS analysis. A supply of commercially available *β*-phellandrene standard was difficult to source. Therefore, we opted instead to use an *α*-phellandrene standard for testing and control purposes. Upon GC-MS analysis of the *α*-phellandrene standard, a major peak with retention time of 6.85 min was identified as *α*-phellandrene (Fig. 7a). However, this chemical standard contained a number of impurities, mainly of monoterpene nature, including *β*-myrcene, 2-carene, benzene, eucalyptol and *β*-phellandrene (Fig. 7a). Accordingly, we were able to use the impurity of the *β*-phellandrene and its 7.3-min

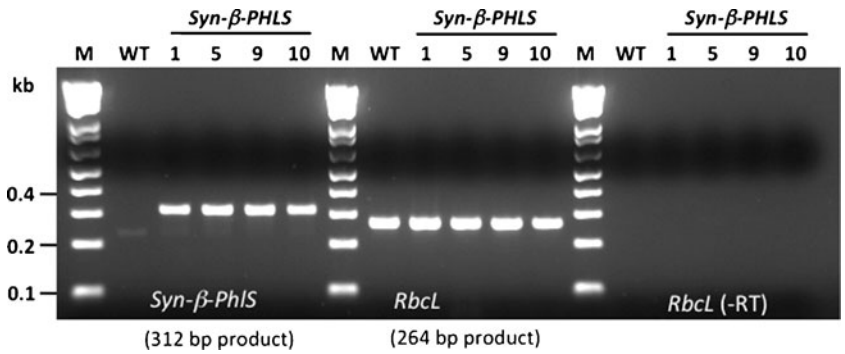

Fig. 5 Expression of the recombinant *Syn-β-PHLS* gene as shown by RT-PCR. Primers designed to the *Syn-β-PHLS* coding region amplify a PCR product from the cDNA samples of four different *Syn-β-PHLS* transformant lines (numbered 1, 5, 9, and 10), which is absent in the WT cDNA. As a positive control, primers designed to the *RbcL* gene amplify a product in all lines, including WT. cDNA samples prepared in the absence of the reverse transcriptase (*RT*) enzyme do not yield products with the RbcL primers, indicating the absence of DNA contamination in all samples

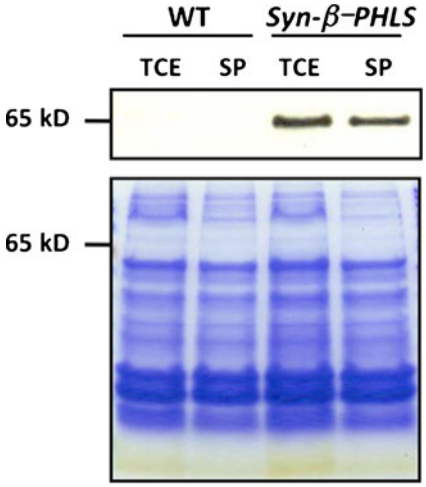

Fig. 6 Immunodetection of the *Syn-β*-PHLS protein in transformant *Synechocystis* cells. (*Upper*) Western blot analysis of proteins from wild-type (*WT*) and *Syn-β*-PHLS transformant cells probed with specific polyclonal antibodies. Lanes were loaded with a total cell extract (*TCE*) sample, or the soluble fraction of *Synechocystis* cells (*SP*), as obtained by collection of the supernatant following cell disruption and centrifugation to pellet insoluble material. (*Lower*) Coomassie-stained SDS-PAGE lanes corresponding to the protein profile of the Western blot in (**a**), shown as a control for protein loading

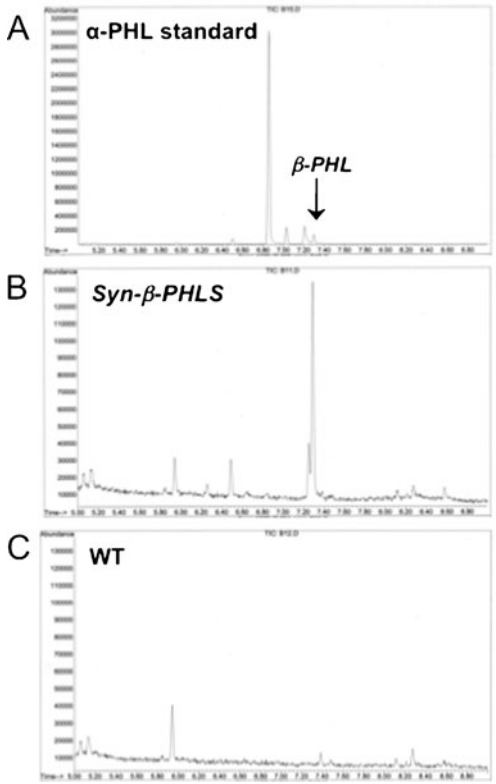

Fig. 7 GC-MS analyses of heptane-extracted samples from WT and Syn-*β*-PHLS transformant cultures. Following 48 h of photoautotrophic growth, a heptane overlay was applied to the liquid cultures to extract *β*-phellandrene and subjected to GC-MS analysis. **a** The GC profiles of the α-phellandrene standard showed a small *β*-phellandrene component with a retention time of 7.3 min. **b** GC profile of heptane extracts from *Syn-β-PHLS* liquid cultures showed a major *β*-phellandrene component with a retention time of 7.3 min. **c** GC profile of heptane extracts from WT cultures

retention time peak in the α-phellandrene standard for our purposes. *β*-phellandrene with a retention time of 7.3 min in the α-phellandrene standard was thus used as a reference for identification of *β*-phellandrene produced by the *Synechocystis* cultures. A prominent peak, with a retention time of 7.3 min, was observed in the heptane extraction from the *Syn-β-PHLS* transformant (Fig. 7b), which is comparable to the retention time of the *β*-phellandrene peak observed in the α-phellandrene standard. This peak was not observed in the heptane extract from the WT culture (Fig. 7c).

Positive identification of the peak with the 7.3-min retention time as *β*-phellandrene was obtained upon examination of the mass spectral lines from the GC-MS measurements. Both the *β*-phellandrene peak in the α-phellandrene standard (Fig. 8a) and the heptane extract from the surface of the *Synechocystis* culture (Fig. 8b) showed distinct mass spectral lines (lines 77, 93, and 136) that signify *β*-phellandrene hydrocarbons. As the bioreactor was sealed during the 48 h period of growth and biomass/*β*-phellandrene accumulation, we further analyzed a sample of the reactor gaseous headspace to test if *β*-phellandrene also accumulated as a volatile product. However, only a low amplitude *β*-phellandrene peak was detected in the headspace gasses of *Syn-β-PHLS* transformants (results not shown), suggesting that the vast majority of the *β*-phellandrene product accumulated in the liquid form on the surface of the culture. Disruption of *Syn-β-PHLS* transformant cells by French Press, and the subsequent heptane extraction of the crude homogenate did not yield any further amounts of *β*-phellandrene (results not shown), indicating that *β*-phellandrene does not accumulate within the cell, nor does it adhere to the cell wall or cell exterior.

Taken together, these results provide evidence that the *Syn-β-PHLS* transgene and its encoded *β*-phellandrene synthase enzyme are responsible for the catalysis of *β*-phellandrene synthesis in the transformant *Synechocystis* strains. Furthermore, the results support the notion of a process whereby photosynthetically generated *β*-phellandrene spontaneously diffuses though the cyanobacterial plasma membrane and cell wall, and thus accumulate in the extracellular media as a non-miscible lighter-than-water liquid product.

β-phellandrene Quantification and Carbon-Partitioning Analysis in *Syn-β-PHLS* Transformants

β-phellandrene was reported to have a spectrophotometric signature with a specific absorbance maximum at 231 nm [26, 27]. Accordingly, spectrophotometric analyses were conducted as an approach for independent *β*-phellandrene identification and quantification. Figure 9a shows representative

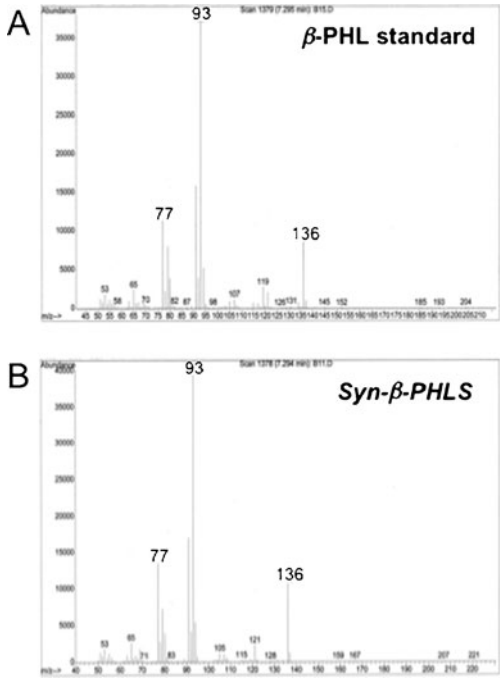

Fig. 8 Mass spectrum analysis of β-phellandrene. MS analysis of the GC peaks with a retention time of 7.3 min attributed to β-phellandrene in Fig. 7a (β–PHL standard, upper panel) and Fig. 7b (Syn–β–PHLS extract, lower panel). Both MS analyses showed the signature MS lines (*77*, *93*, and *136*) of β-phellandrene

absorbance spectra of heptane-extracted samples from WT and *Syn-β-PHLS*-transformant cultures. A well-defined peak at 231 nm was measured in the heptane extracts from the *Syn-β-PHLS* transformants, while this absorbance band was absent from the extracts of the WT samples. Importantly, this method allowed β-phellandrene to be easily distinguished from α-phellandrene, as the latter has a specific absorbance maximum of 260 nm, and lacks an absorbance peak at 231 nm (Fig. 9b). The absence of absorbance peaks at 260 nm in *Syn-β-PHLS* transformants (Fig. 9a) indicates that the recombinant β-phellandrene synthase enzyme specifically and exclusively catalyzed the formation of β-phellandrene, and not the α-phellandrene isomer, in agreement with the results from the GC-MS analysis.

Quantification of the amount of β-phellandrene extracted with heptane from the surface of the liquid cultures of *Syn-β-PHLS* transformants was determined according to the Beer–Lambert Law, using the absorbance values measured at 230 nm and the known molar extinction coefficient $\varepsilon_{231}=9.12\ mM^{-1}cm^{-1}$ of β-phellandrene [27]. During 48 h of active photoautotrophic growth in the presence of CO_2 in a sealed gaseous/aqueous two-phase bioreactor [25], a 700-mL culture of *Syn-β-PHLS* transformant produced, on average, 35 μg of β-phellandrene in the form of a non-miscible product floating on the surface of the culture. This corresponds to a yield of 50 μg β-phellandrene L^{-1} culture over the 48 h incubation period, and a rate of production of 1.0 μg β-phellandrene L^{-1} culture h^{-1} during the actively dividing phase of photoautotrophic growth. Biomass accumulation (as dry cell weight) was also measured over the 48-h period to allow a calculation of assimilated carbon that partitioned toward β-phellandrene production. Assuming that carbon contributes to about half the weight of dry biomass, the average assimilated carbon partitioning as non-miscible β-phellandrene in the *Syn-β-PHLS* transformant was estimated to be 0.03 %.

Continuous β-phellandrene Production Process

A semi-continuous culturing system was employed to maintain high photosynthetic growth rates with the aim of testing continuity of β-phellandrene production over a prolonged period of time. Cultures in gaseous/aqueous two-phase reactors [25] were initially inoculated with cells to establish a density of about 0.05 g dry weight L^{-1}. The headspace of the reactor was filled with CO_2 to support photoautotrophic growth. Under these conditions, biomass accumulation doubled every 24 h, necessitating dilution with fresh growth media every 48 h, down approximately to the original 0.05 g dry weight L^{-1} (Fig. 10a). β-phellandrene was also produced and accumulated in a linear fashion. It was removed and quantified every 48 h of cultivation. Using this approach, a constant rate of β-phellandrene production equal to 1 μg L^{-1} culture h^{-1} was achieved over 192 h of experimentation

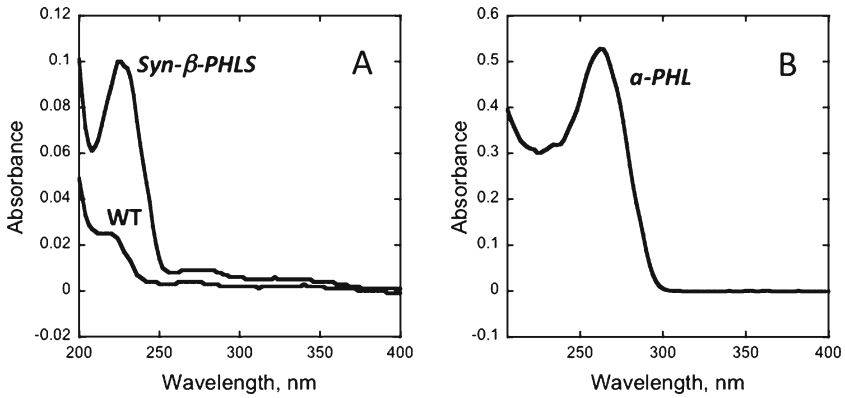

Fig. 9 Absorbance spectra of phellandrene hydrocarbons in heptane. **a** Absorbance spectra of heptane-extracted samples from the surface of wild-type (*WT*) and *Syn-β-PHLS* transformant liquid cultures. The β-phellandrene absorbance peak is observed at 231 nm. **b** Absorbance spectra of the α-phellandrene standard diluted in heptane. The α-phellandrene absorbance peak is observed at 260 nm

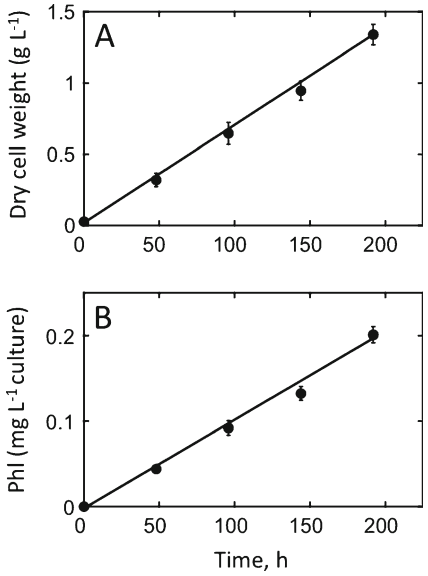

Fig. 10 Continuous growth of *Syn-β-PHLS* transformants for biomass and β-phellandrene accumulation. *Syn-β-PHLS* transformant cultures were administered aliquots of 100 % CO_2 every 48 h to maintain photoautotrophic growth while the bioreactor was sealed for β-phellandrene accumulation. Cultures were diluted with fresh growth media every 48 h to a dry cell weight of approximately 0.05 gL^{-1} to maintain active cellular metabolism. **a** Culture dry cell weight accumulation as a function of time during continuous growth. Dilutions of the culture were implemented every 48 h. Results shown are the average of three independent experiments. **b** Cumulative β-phellandrene production over the time course of this experiment

(Fig. 10b), coupled with the linear rate of biomass accumulation (Fig. 10a). The constant rate of β-phellandrene production in the absence of antibiotic pressure over the course of such long-term experimentation indicated that the β-phellandrene synthase gene is stably incorporated in the cyanobacterial genome, and that it can be stably expressed in a photosynthetic microorganism for continuous product generation

Fitness of the *Syn-β-PHLS* Transformants

The possibility of β-phellandrene-induced adverse effects on cell growth and fitness were addressed by comparative cell growth and light saturation curves of photosynthesis between WT and transformant cultures. Photoautotrophic cell growth kinetics of the *Syn-β-PHLS* transformants were the same as that of the WT, with a doubling time of 16 h in batch culture under a light intensity of 20 μmol photons $m^{-2}s^{-1}$ and continuous bubbling with air (Fig. 11a). The light saturation curves of photosynthesis of WT and the *Syn-β-PHLS* transformants were also similar to one another (Fig. 11b), where oxygen evolution saturated at about 200–300 μmol photons $m^{-2}s^{-1}$. Interestingly, the average P_{max} was slightly higher in the *Syn-β-PHLS* transformants compared with the WT. These results clearly showed that deletion of the endogenous *psbA2* coding region from the *Synechocystis* genome, and subsequent replacement/integration and expression of the *Syn-β-PHLS* transgene in the cell, as well as the generation and accumulation of significant amounts of β-phellandrene, had no adverse effects on the growth and photosynthetic productivity parameters of the transformant cells.

Discussion

The expression of recombinant terpene synthases has been described in host organisms such as bacteria and yeast [28–32]. However, only two examples of heterologous expression of a terpene synthase have been documented in a photosynthetic microorganism, comprising an isoprene synthase [22, 25] and a β-caryophyllene synthase [33]. These have been introduced into the cyanobacterium *Synechocystis*, resulting in the photosynthetic generation of isoprene and the sesquiterpene β-caryophyllene, respectively.

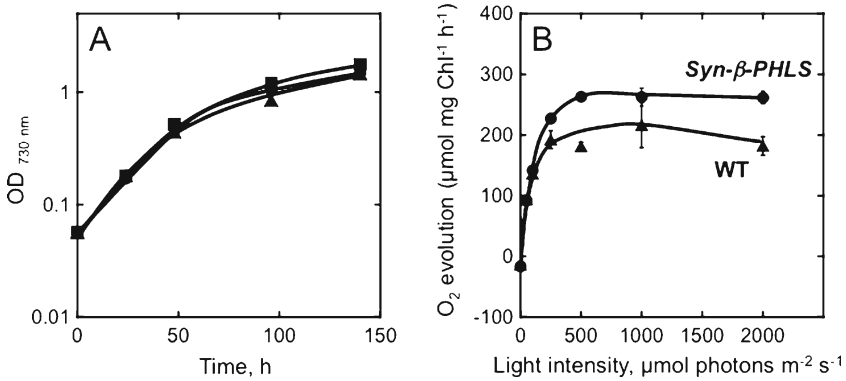

Fig. 11 Growth and photosynthesis characteristics of WT and *Syn-β-PHLS* transformants in liquid culture. **a** Photoautotrophic growth kinetics of WT (*triangles*) and two different *Syn-β-PHLS* transformant lines (*circles* and *squares*), as measured by the optical density of the culture at 730 nm, when grown with continuous aeration and continuous illumination at 20 μmol photons $m^{-2}s^{-1}$. **b**. Light saturation curves of photosynthesis for WT and *Syn-β-PHLS* transformant cells, as measured by the oxygen-evolution activity of an aliquot of the cultures in the presence of 15 mM $NaHCO_3$, pH 7.4 under a range of actinic light intensities

The monoterpene β-phellandrene ($C_{10}H_{16}$) has commercial value as a key ingredient in perfumes, as well as in medicinal, cosmetic and cleaning products. There is potential for β-phellandrene to be developed as a renewable fuel, where the monoterpene itself serves as a supplement to gasoline, whereas dimerization of the ten-carbon units may generate second order fuel molecules that are suitable for supplementing jet fuel and diesel type molecules. Hydrocarbons, such as β-phellandrene, also offer the advantage of being able to store greater relative energy than alcohols [34, 35].

Microbial systems for the generation of terpenoids offer the promise of a more efficient and less costly method of product generation than traditional methods that include chemical synthesis or harvesting the natural product from plants. The generation of high-value plant bio-products upon genetic and metabolic engineering of a photosynthetic microorganism offer the advantage of a system whereby the same organism serves both as photo-catalyst and producer of a ready-made fuel or chemical [5, 22]. Here, we demonstrated the expression of the *L. angustifolia* β-phellandrene synthase in *Synechocystis* and, further, synthesis and accumulation of the monoterpene β-phellandrene, as a non-miscible product floating on the surface of the cyanobacterial liquid culture. This defines the first known example of successful expression of a recombinant monoterpene synthase in a photosynthetic microorganism, free diffusion from the intracellular to the extracellular space, and subsequent accumulation of a C10-class of terpenoid on the surface of the liquid culture.

Mechanistically, terpene synthases catalyze the formation of highly reactive carbocation intermediates from a prenyl diphosphate substrate [36]. The subsequent rearrangements of the carbon backbone and final stabilization of the molecule by de-protonation constitute the first step in a process that may result in a large array of cyclic and acyclic products from the terpene synthases [36]. Terpene synthases, however, are often promiscuous enzymes, yielding multiple products from a single substrate and enzyme catalytic site. For example, the expression of *L. angustifolia* β-phellandrene synthase in *E. coli* also yielded small quantities of limonene in addition to β-phellandrene as the major product [18]. Similarly, the β-phellandrene synthase from grand fir produced β-pinene, α-pinene, and 4S-limonene as minor products [16], and the tomato β-phellandrene synthase produced small amounts of carene, α-phellandrene, γ-terpinene, and limonene with neryl diphosphate as the substrate in addition to the major β-phellandrene product [17]. Here, upon expression of the *L. angustifolia* β-phellandrene synthase in *Synechocystis* we measured β-phellandrene as the major product upon heptane extraction of hydrocarbons from the surface of the liquid cultures, however much smaller amounts of other products, including β-myrcene and limonene, were also detected that were not naturally present in the WT. The carbocation rearrangement reactions catalyzed by terpene synthases of the same class are often only subtly different, therefore it seems inevitable that small amounts of minor products, with similar chemical structures, will form in any microbial system designed to produce a specific terpenoid product. The purity of the targeted terpenoid, however, is far superior to the essential oils harvested from natural plant sources and also to those products of petrochemical origin.

The question of product separation from the biomass weighs heavily on the economics of a microbial production system, when commercial application is assessed. In our approach, the majority of the photosynthetically generated β-phellandrene accumulated as non-miscible product, floating on the surface of the liquid cyanobacterial culture, suggesting that β-phellandrene efficiently diffuses from the intracellular space through the plasma membrane and cell wall and accumulates in the extracellular environment. Disruption of *Syn-β-PHLS* transformant cells by French Press, and the subsequent heptane extraction of hydrocarbons did not yield any additional amounts of β-phellandrene (results not shown), indicating that β-phellandrene does not accumulate within the cell, nor does it adhere to the cell wall or cell exterior. This is an important consideration for a microbial production system, as spontaneous product separation from the biomass would alleviate issues of product toxicity, which is important for maintaining optimal growth rates for maximum yields. In this instance, and under the conditions of our production system, the boiling point of β-phellandrene (171–172 °C) enabled product accumulation in the liquid form, alleviating the need to apply condensation and trapping of volatile products [5]. Furthermore, the hydrophobic nature of β-phellandrene ensures that it would separate from the liquid medium alleviating the requirement of time-consuming, expensive, and technologically complex biomass dewatering [37, 38] that otherwise would be needed for the separation of larger size fuel molecules. The extraction of β-phellandrene from the surface of the liquid culture with an organic solvent, such as heptane, resulted in a blend that is immediately suitable for fuel supplementation.

This work also described a method suitable for the semi-continuous culturing of photosynthetic microorganisms for the production of β-phellandrene, employing periodic extraction using an organic solvent. We measured sustained rates of 1.0 μg β-phellandrene L^{-1} culture h^{-1} over an 8-day period using this method, which equates to 0.03 % of assimilated carbon partitioning as β-phellandrene (assuming that carbon contributes to approximately half of the weight of the biomass). The titer of engineered photosynthetic products, such as β-phellandrene, in cyanobacterial and microalgal hosts remains a challenge that requires novel strategies for improvement [39].

In summary, monoterpenes, such as β-phellandrene, are a class of ten-carbon hydrocarbon molecules with industrial

applications that range from personal care, to cleaning products to medicinal use. There is also potential for these energy-rich hydrocarbons to be developed as renewable fuel supplements. Harvesting of monoterpenes from natural sources is uneconomical, and synthetic derivation from petroleum resources is unsustainable. In this work, we showed proof of concept for monoterpene production in an engineered photosynthetic microorganism via heterologous expression of a gene encoding a monoterpene synthase. We used β-phellandrene synthase as an example, and showed that the recombinant expression of *L. angustifolia* β-phellandrene synthase in a cyanobacterium leads to β-phellandrene accumulation as a product of photosynthesis through the MEP pathway. Importantly, we also showed that this ten-carbon hydrocarbon is hydrophobic and small enough to move though the cyanobacterial plasma membrane and cell wall, and to accumulate in the extracellular space as a non-miscible product. This "photosynthesis-to-fuels" system, therefore, meets two economically important criteria for the generation of bio-based products: (1) it is a renewable and carbon-neutral, and (2) yields an energy-rich product that spontaneously separates both from the biomass and the culturing medium.

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