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Short communication

The production of the sesquiterpene β-caryophyllene in a transgenic strain of the cyanobacterium *Synechocystis*

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ABSTRACT

The plant secondary metabolite, β-caryophyllene, is a ubiquitous component of many plant resins that has traditionally been used in the cosmetics industry to provide a woody, spicy aroma to cosmetics and perfumes. Clinical studies have shown it to be potentially effective as an antibiotic, anesthetic, and anti-inflammatory agent. Additionally, there is significant interest in engineering phototrophic microorganisms with sesquiterpene synthase genes for the production of biofuels. Currently, the isolation of β-caryophyllene relies on purification methods from oleoresins extracted from large amounts of plant material. An engineered cyanobacterium platform that produces β-caryophyllene may provide a more sustainable and controllable means of production. To this end, the β-caryophyllene synthase gene (*QHS1*) from *Artemisia annua* was stably inserted, via double homologous recombination, into the genome of the cyanobacterium *Synechocystis* sp. strain PCC6803. Gene insertion into *Synechocystis* was confirmed through PCR assays and sequencing reactions. Transcription and expression of *QHS1* were confirmed using RT-PCR, and synthesis of β-caryophyllene was confirmed in the transgenic strain using GC-FID and GC–MS analysis.

Introduction

The sesquiterpene β-caryophyllene is an unsaturated allopathic hydrocarbon, which can be produced in a constitutive or induced manner by some members of the plant kingdom. Research suggests that this terpene is involved in a number of plant defense mechanisms including: deterring herbivory (De Moraes et al., 2001); attracting predators to noxious herbivores (Rasmann et al., 2005); and inhibiting fungal and bacterial infection (Sabulal et al., 2006). The production of the β-caryophyllene precursor, farnesyl diphosphate, is part of larger terpene synthesis pathway that is endogenous to many biological systems, including *Synechocystis* sp. strain PCC6803 (Fig. 1). However, *Synechocystis* has not been found to produce β-caryophyllene or any other sesquiterpenoid, indicating that perhaps the farnesyl diphosphate is used only for further terpenoid elongation reactions (Dewick, 2002). The β-caryophyllene synthase has been isolated and characterized in a number of plant species (Dehal and Croteau, 1988; Cai et al., 2002; Mercke et al., 2004; Cheng et al., 2007; Kollner et al., 2008; Wang et al., 2009).

Traditionally, β-caryophyllene has been used in the fragrance and cosmetic industry. This chemical is also found at high concentrations in a number of natural remedies such as balsam (Cascon and Gilbert, 2000) and ginger oils (Sabulal et al., 2006). Studies have suggested that β-caryophyllene has anti-inflammatory (Baylac and Racine, 2003; Lourens et al., 2004), anti-microbial (Santos et al., 2008), anesthetic (Ghelardini et al., 2001), and anti-carcinogenic activity (Modzelewska et al., 2005). β-Caryophyllene has also been shown to interact with Type-2 cannabinoid receptors at the same hydrophobic pocket in which endogenous and exogenous cannabinoids interact (Gertsch et al., 2008). This may explain the chemical's anti-inflammatory and anesthetic properties (Gertsch et al., 2008). Extraction methods for β-caryophyllene often require large amounts of initial plant biomass in order to yield high enough concentrations for medicinal purposes (Quispe-Condori et al., 2008; Topal et al., 2008).

The cyanobacterium *Synechocysti* ssp. strain PCC6803 has been used as an oxygenic, photosynthetic organism in many transgenic experiments. The numerous advantages of using this popular laboratory strain have been previously highlighted (Vermaas, 2004). An endogenous homologous recombination system makes *Synechocystis* sp. stain PCC6803 an appropriate strain for transgenic

Abbreviations: bp, base pair; GC-FID, Gas Chromatography Flame Ionization Detection; GC–MS, Gas Chromatography–Mass Spectrometry; LB, Lysogeny Broth; PCR, Polymerase Chain Reactions; RPM, Revolutions Per Minute; RT-PCR, Reverse Transcriptase Polymerase Chain Reaction.

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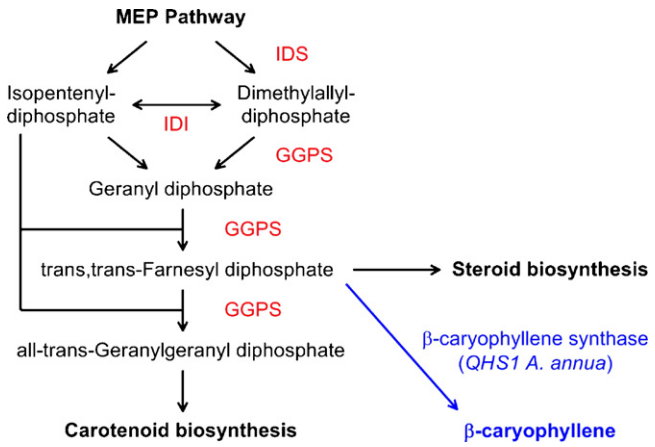

Fig. 1. Overview of isoprenoid biosynthetic pathway in *Synechocystis* sp. strain PCC6803. Native PCC6803 metabolites and pathways shown in black, enzymes shown in red. The engineered sesquiterpene pathway to produce β-caryophyllene shown in blue. Enzyme abbreviations followed by PCC6803 gene names: GGPS, *g*eranylgeranyl *p*yrophosphate *s*ynthase (*crtE*); IDI, *I*sopentenyl diphosphate: *d*imethylallyl diphosphate *i*somerase (*sll1556*); IDS, *I*sopentenyl diphosphate: *d*imethylallyl diphosphate *s*ynthase (*lytB*); MEP pathway, *m*ethyl-erythritol-4-*p*hosphate pathway. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

experiments (Labarre et al., 1989). *Synechocystis* versatility has made it an effective strain for the production of commercially important compounds. For instance, genetic engineering of *Synechocystis* has resulted in strains that produce higher concentrations of commercially important carotenoid anti-oxidants (Lagarde et al., 2000). *Synechocystis* strains have also been designed to produce mammalian proteins, indicating its potential use in the production of protein therapeutics (Chen et al., 1999). Recently, Dexter and Fu (2009) inserted *Zymononas mobluis* fermentative genes into the *Synechocystis* genome, to genetically engineer the production of alcohol. Lindberg et al. (2010) engineered a strain of *Synechocystis* that contained the *Pueraria montana* isoprene synthase, which was able to produce and emit volatile isoprene. In this work, we report the expression of the *Artemisia annua* β-caryophyllene synthase gene and the *in vivo* production of the sesquiterpene β-caryophyllene in *Synechocystis*. This represents the first demonstration of the heterologous production of this class of molecules in a photoautotrophic microorganism.

Materials and methods

Strain and growth conditions

Synechocystis sp. strain PCC803 was obtained from Dr. Sherman at Purdue University. The strain was maintained on BG-11 media with 1.5% agar supplemented with 1 mM of sodium thiosulfate. Subcultures were supplemented with 50 μg mL^{-1} of kanamycin and/or 5% sucrose as needed. Standard liquid cultures were grown in BG-11 liquid media, buffered to pH 8.0, and supplemented with 50 μg mL^{-1} of kanamycin and/or 5% sucrose as needed. Both plates and liquid cultures were grown at 30 °C under constant illumination of 12.5 μE (m^2 s)$^{-1}$. Liquid cultures were aerated using a shaker table set at 130 RPMs. Bioline ElectroShox (Tauton, MA) electrocompetent *E. coli* cells were grown at 37 °C on LB 1.5% agar plates after electroporation and screened with 75 mg L^{-1} of ampicillin. Successful *E. coli* transformants were grown at 37 °C in liquid LB media, buffered to pH 7.0, and supplemented with 75 mg L^{-1} of ampicillin for plasmid extraction, using Qiagen's Miniprep Plasmid Extraction kit (Valencia, CA, USA).

pPSBAIIQHS1 vector construction

Plasmid PSBAII-KS (Lagarde et al., 2000) was obtained from Dr. Vermass at Arizona State University, and the *Artemisia annua* β-caryophyllene synthase gene, housed within plant expression plasmid pET*QHS1* (Cai et al., 2002), was obtained from Dr. Croteau of Washington State University. Cloning primers (5′ and 3′ pET*QHS1* primers) were designed and used to amplify the entire coding region of *QHS1*, along with introducing the *AseI* restriction sites at the 5′ end and a *BamHI* site at the 3′ end (Supplementary data). All PCR assays were run using Dream Taq Master Mix from Fermentas (Glenburie, MD, USA) in a Bio-Rad MJ Mini Personal Thermal Cycler (Hercules, CA, USA). The *QHS1* PCR product was ligated into the digested pPSBAII-KS vector at the *NdeI* and *BamHI* restriction sites using Novagen's T4-Ligation kit (Darmstadt, Germany).

E. coli transformation

Bioline ElectroShox electrocompetent *E. coli* cells were mixed with the ligated pPSBAII*QHS1* plasmid and electroporated at 1800 V for 5 ms, with a capacitance of 25 μF, a resistance of 200 Ω, using a Bio-Rad GenePulse Xcell Electroporator (Hercules, CA, USA). Colonies that grew on ampicillin plates were screened by colony PCR with the pET*QHS1* primers, at an annealing temperature of 60 °C for 35 cycles. Further screening was done through a sequencing reaction (MCLAB DNA Sequencing Services; San Francisco, CA, USA) to determine *QHS1* retention and orientation. The colony that showed the correct sequence and orientation of the *QHS1* gene inside the pPSBAII*QHS1* vector was used for the subsequent plasmid extractions.

Synechocystis double homologous recombination reaction

Synechocystis sp. strain PCC6803 was grown until mid-log phase, then aliquots were centrifuged, washed, and resuspended in fresh BG-11 medium. The pPSBAII-KS plasmid was added to the resuspension and incubated at 30 °C for 6 h. Aliquots of the cell:plasmid mixture were transferred onto 80 mm filter paper, placed on top of non-selective BG-11 agar plates and allowed to incubate for 24 h at 30 °C. The filter paper was then moved onto kanamycin (50 μg mL^{-1}) BG-11 agar plates and grown for 4 days. Successful transformants were transferred to separate kanamycin selective plates. Isolated colonies were grown in liquid BG-11 cultures and screened for the presence of the *sacB*/*aphX* cassette by testing sucrose sensitivity on 5% sucrose BG-11 plates and full segregation by running a PCR using the 5′^{UP} pPSBAII*QHS1*$_{sq}$ and 3′^{DOWN} pPSBAII*QHS1*$_{sq}$ primers (Supplementary data). After establishing segregation, the *sacB*/*aphX* transformant strain was grown to mid-log phase, at which point the liquid culture was concentrated and the pPSBAII*QHS1* vector was added. The cell:plasmid mixture was incubated for 6 h before being diluted 20:1 with non-selective BG-11 liquid media. This mix was incubated for 48 h at normal growth conditions before a sterile-filtered sucrose solution was added, bringing the final concentration to 5% sucrose. The sucrose selective liquid culture was incubated for 4 days before small aliquots were streaked on selective 5% sucrose, BG-11 agar plates. After 1 week, individual colonies that grew on the sucrose were screened for presence of the *QHS1* using a PCR assay with either the pET*QHS1* primers or the 5′^{UP} pPSBAII*QHS1*$_{sq}$ and 3′^{DOWN} pPSBAII*QHS1*$_{sq}$ primers (Supplementary data). One successful transformant colony was further subcultured, verified for segregation, and used for RNA and terpene extraction. Genomic DNA from this colony, along with the pPSBAII*QHS1*$_{sq}$ sequencing primers were used for sequencing at MCLAB DNA Sequencing Services, to confirm orientation and position of the *QHS1* gene within the *Synechocystis* genome.

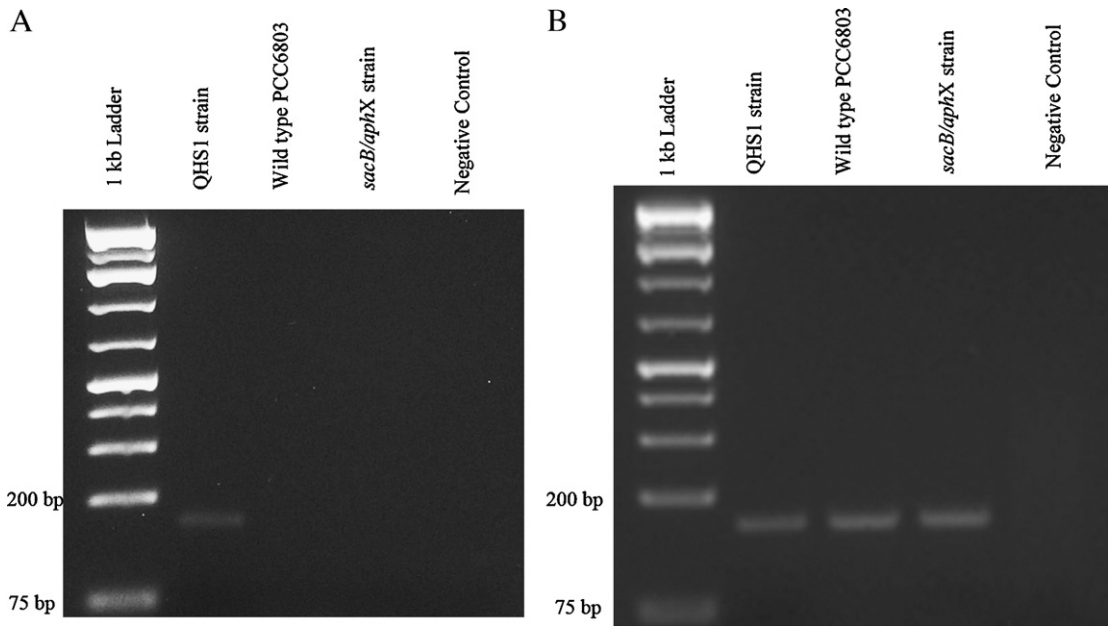

Fig. 2. Reverse transcriptase PCR gel images. (A) RT-PCR results using $QHS1_{RT}$ primers as a probe for *QHS1* transcripts. The pPSBAII*QHS1* lane shows only the appropriately sized band, indicating that *QHS1* is transcribed exclusively in the double recombinant Synechocystis*QHS1*strain. (B) RT-PCR results using $rnPB_{RT}$ primers as a probe for the constitutively present rnBP transcripts. In both images, the negative control lane was loaded with a RT-PCR reaction prepared with water, instead of cDNA. 'Images' contrast adjusted for printing purposes'.

Reverse transcriptase PCR

DNA and RNA were extracted from mid-log phase liquid cultures of wild type *Synechocystis* sp. strain PCC6803, the *Synechocystis sacB/aphX* strain, and the *Synechocystis QHS1* strain, using a modified Chang et al. (1993)'s extraction protocol. An aliquot of the final extraction was retained and used as a DNA template for sequencing and PCR confirmation. The remainder of the extracted solution was treated with Fermentas' DNaseI (Glenburie, MD, USA). The RNA was reverse transcribed using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA, USA) to produce the template cDNA. The primers $QHS1_{RT}$ were used to detect the transgenic *QHS1* RNA (Supplementary data). The primers $rnPB_{RT}$ were used to detect RNA from the constitutively expressed housekeeping gene (*rnPB*), that codes for subunit B of RNase P (Supplementary data) (Vioque, 1992). The resulting cDNA bands were visualized on ethidium bromide stained, 2% agarose gels.

Extraction and quantification of β-caryophyllene

Wild type *Synechocystis* and the *QHS1* transgenic *Synechocystis* liquid cultures were grown to mid-log phase under standard growing condition. Lipids and terpenes were extracted using a chloroform:methanol:water biphasic separation protocol as described by Bligh and Dyer (1959). Chloroform extracts were washed with deionized water and analyzed directly by GC–MS and GC-FID. β-caryophyllene was identified by comparison of GC retention times to standards (Sigma, MO, USA) and by comparison of mass spectra to the NIST Mass Spectral Library (V 2.0d). Mass spectral analysis was conducted on a Varian 3800 GC and Varian 1200 quadrupole MS/MS operated with the following conditions: Rxi-5 ms column (30 m × 0.25 mm; 0.25 μm film thickness); temperature program of 60–240 °C (at 3 °C min^{-1}); He carrier gas: 1.5 mL min^{-1}; mass spectra, 70 eV, in EI mode; ion source temperature 250 °C; scan mass range, 25–600 (Palmeira et al., 2004). β-Caryophyllene levels were quantified via a GC-FID (Agilent 7890A), equipped with a DB–5-ms column (Agilent Technologies, Santa Clara, CA; 30 m × 0.25 mm; 0.25 μm film thickness), and operated under the following conditions: temperature program: 60–240 °C (at 3 °C min^{-1}); He carrier gas: 1.5 mL min^{-1} (Maia et al., 2005).

Results and discussion

Construction of pPSBAIIQHS1 vector

The *A. annua QHS1* gene, a cDNA clone that codes for a β-caryophyllene synthase, has been shown to produce a functional a β-caryophyllene synthase when expressed in an *E. coli* (Cai et al., 2002). The ability of this cDNA to produce a functional protein in a prokaryotic system made it an appropriate candidate for expression in *Synechocystis* sp. strain PCC6803, because of the apparent absence of any requisite post-translational modification. Therefore, cloning primers were designed (Supplementary data) to amplify the *QHS1* 1644 bp open reading frame and insert it into the pPSABII *Synechocystis* homologous recombination vector. The *QHS1* sequence was inserted into the multiple cloning site of pPSBAII, placing the gene in front of the strong endogenous promoter for *psbAII* (Lagarde et al., 2000). Sequencing analysis of the plasmid confirmed insertion and correct orientation based on NCBI BLAST analysis (www.ncbi.nlm.nih.gov/blast/) (Supplementary data).

Transformation and segregation of transgenic Synechocystis strain

The double homologous recombination strategy for transgene expression in *Synechocystis* sp. strain PCC6803 allows for targeted insertion of an exogenous gene at a specific site without retaining a selectable marker for continued transgene expression (Lagarde et al., 2000; Dexter and Fu, 2009; Lindberg et al., 2010). However, retention of the transgene is contingent on full segregation after each transformation reaction, in order to prevent reversion back to the wild type genotype. After the first round of homologous recombination, the *Synechocystis* colonies that grew on kanamycin plates were screened using a PCR assay with primers (Supplementary data) that flanked the *psbAII* insertion site. The *Synechocystis* strain transformed with the pPSABII-KS plasmid showed only one band representing the *sacB/aphX* cassette (3729 bp) after full segrega-

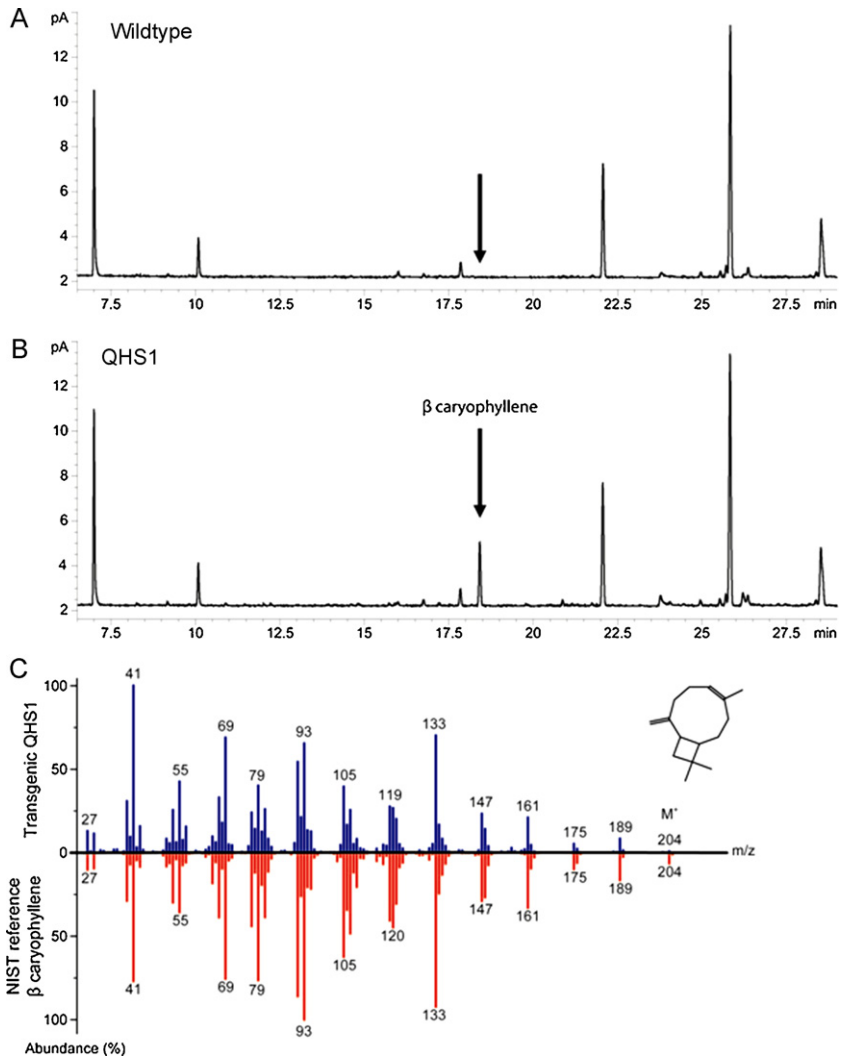

Fig. 3. GC-FID chromatogram of wildtype (A) and transgenic (B) lipid/terpene extracts. Arrow indicates retention time of β-caryophyllene standard (standard GC run not shown). β-Caryophyllene peak is absent in the wildtype (A) but present in the *QHS1* transgenic chromatogram (B). (C) Mass spectrum of the putative β-caryophyllene peak identified in the transgenic *QHS1* (blue) compared to the reference mass spectrum of β-caryophyllene found in the NIST Mass Spectral Library (red). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

tion (Supplementary data). This segregated strain was used in the second homologous recombination reaction with the pPSBAIIQ*HS1* vector, in which the *sacB*/*aphx* cassette was replaced with *A. annua's QHS1* gene. This transformation is driven by the selective pressure caused by the addition of 5% sucrose to the growth media, which, in the presence of the *sacB* protein, levansucrase, induces a lethal phenotype (Cai and Wolk, 1990). Colonies that grew on the sucrose media were screened using a PCR assay with the original cloning primers, pETQ*HS1*, to detect *QHS1* insertion (Supplementary data). The colony that showed consistent *QHS1* banding was further screened to determine complete genomic segregation, using a similar PCR assay as described above. The double transformant showed only one band (2091 bp) corresponding to the *QHS1* gene inserted into *psbAII* site (Supplementary data). Genomic DNA of the segregated double transformant was sequenced and the resulting NCBI BLAST results analysis confirmed the correct sequence and orientation (Supplementary data).

Confirmation of QHS1 transcription

To determine if the *A. annuaQHS1* sequence was being transcribed, RNA was extracted from the various strains and cDNA was generated for RT-PCR. As shown in Fig. 2, only the *QHS1* transformant strain showed the corresponding transcript when the $QHS1_{RT}$ primers were used and all strains showed the housekeeping transcript, rnPB. The retention and constitutive expression of the exogenous gene appears to be sustained, as RT-PCR and chemical analysis were conducted on transformant strains that were sub-cultured numerous times over a 6-month period.

Production of β-caryophyllene by the transformant strain

As discussed earlier, Cai et al. (2002) established that the *A. annua* β-caryophyllene synthase could be produced in a prokaryotic system and that this enzyme was active *in vitro*. To determine if this enzyme was active *in vivo*, terpenes were extracted from both the *QHS1* transformant and wild type strains and the resulting extracts were analyzed using GC-FID and GC–MS. Comparison of wildtype and transgenic GC-FID chromatograms revealed a peak present in the transgenic strain at the same retention time as a β-caryophyllene standard (data not shown) but was absent in the wild type strain (Fig. 3A and B). This peak was confirmed as β-caryophyllene by comparison of its mass spectrum to the β-caryophyllene spectrum found in the NIST Mass Spectral Library, which had high similarity (Fig. 3C). Cai et al. (2002) originally reported that the β-caryophyllene synthase, as

expressed in *E. coli*, produced minor amounts of α-humlene, another common sesquiterpene, but we were unable to detect this constituent. Under normal growth conditions we were able to extract 46.4 ± 2.9 ng of β-caryophyllene (mL of culture)$^{-1}$ week^{-1}, which corresponded to approximately 3.7 μg of β-caryophyllene (g of dry cell weight)$^{-1}$ week^{-1}. To our knowledge, this the first time *Synechocystis* has been shown to produce sesquiterpenes, either endogenously or through transgene gene expression.

Conclusion

The ability to transform *Synechocystis* to produce β-caryophyllene, as detailed in this paper, is a promising step towards developing strains of autotrophic microorganisms that can efficiently synthesize important terpenoids, which is of relevance to both the biofuel and pharmaceutical research community. Chang and Keasling (2006) highlighted the potential of *E. coli* and *Saccharomyces cerevisiae* as platforms for pharmaceutical isoprenoid production that may be able to avoid the shortfalls of synthetic chemistry or natural extraction. More recently, Radakovits et al. (2010) have highlighted the potential of engineering phototrophic microalgae for the accumulation of isoprenoids, particularly C10 and C15 products, as these hydrocarbons are excellent fuel surrogates. Our work is a logical descendant of preceding synthetic biological approaches, as it incorporates the production of important terpenoids into an autotrophic microorganism. Increasing our understanding of the interconnected pathways of terpene metabolism and carbohydrate accumulation, as well as continuing research into optimizing transgene expression may lead to more efficient biological systems for the production of important terpenoids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2010.11.006.

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