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BIOENERGY AND BIOFUELS

Genetically engineering cyanobacteria to convert CO_2, water, and light into the long-chain hydrocarbon farnesene

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Abstract Genetically engineered cyanobacteria offer a shortcut to convert CO_2 and H_2O directly into biofuels and high value chemicals for societal benefits. Farnesene, a long-chained hydrocarbon ($C_{15}H_{24}$), has many applications in lubricants, cosmetics, fragrances, and biofuels. However, a method for the sustainable, photosynthetic production of farnesene has been lacking. Here, we report the photosynthetic production of farnesene by the filamentous cyanobacterium *Anabaena* sp. PCC 7120 using only CO_2, mineralized water, and light. A codon-optimized farnesene synthase gene was chemically synthesized and then expressed in the cyanobacterium, enabling it to synthesize farnesene through its endogenous non-mevalonate (MEP) pathway. Farnesene excreted from the engineered cyanobacterium volatilized into the flask head space and was recovered by adsorption in a resin column. The maximum photosynthetic productivity of farnesene was 69.1 ± 1.8 $\mu g \cdot L^{-1} \cdot O.D.^{-1} \cdot d^{-1}$. Compared to the wild type, the farnesene-producing cyanobacterium also exhibited a 60 % higher PSII activity under high light, suggesting increased farnesene productivity in such conditions. We envision genetically engineered cyanobacteria as a bio-solar factory for photosynthetic production of a wide range of biofuels and commodity chemicals.

Keywords Cyanobacteria · Biofuels · Synthetic biology · Hydrocarbon · MEP pathway

Introduction

Fossil fuel depletion and climate change due to elevated global CO_2 emissions are two issues that have far-reaching consequences for the world's future social and economic stability. These problems will be exacerbated by an increasing world population, which reached 7 billion people in 2012 and is projected to increase by another 1 billion by 2025 (Cohen 2003). The concerns regarding fossil fuel consumption and CO_2 emissions have led to biotechnologies that convert renewable biomass feedstocks into biofuels. The production of corn ethanol and soybean biodiesel, known as "first generation" biofuels, has gained considerable interest in the last few decades (Nigam and Singh 2011). Advancements in agricultural engineering and biotechnology have further diversified the plant feedstocks which can be used to produce biofuels, such as cellulose and hemicellulose from switchgrass and corn stover (Gomez et al. 2008). However, there are still concerns over the sustainability of these "second generation" biofuels, since they require clean water, fertilizers, pesticides, arable land, and downstream processing steps that drive up production costs and ultimately the price of the fuel.

Volatile, long-chained alkanes and alkenes are of particular interest as biofuels, considering their high energy density, similarities to existing fuels (gasoline, diesel, jet), and propensity to separate from the processing residues. Alkenes, such as terpenes, have recently gained interest as possible biofuels (Gronenberg et al. 2013; Peralta-Yahya and Keasling 2010; Tippmann et al. 2013). Terpenes represent a large and diverse class of organic compounds found in all living organisms They are essential metabolites in bacteria, plants, and humans, and they play important roles in cell wall and membrane biosynthesis (hopanoids), electron transport (ubiquinones and menaquinones), and the conversion of light into chemical energy (chlorophylls and carotenoids), among other processes (Perez-Gil and Rodriguez-Concepcion 2013).

All terpenes are derived from prenyl diphosphate precursors, synthesized from the mevalonate (MVA) pathway and/or the 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP) pathway (also known as the non-

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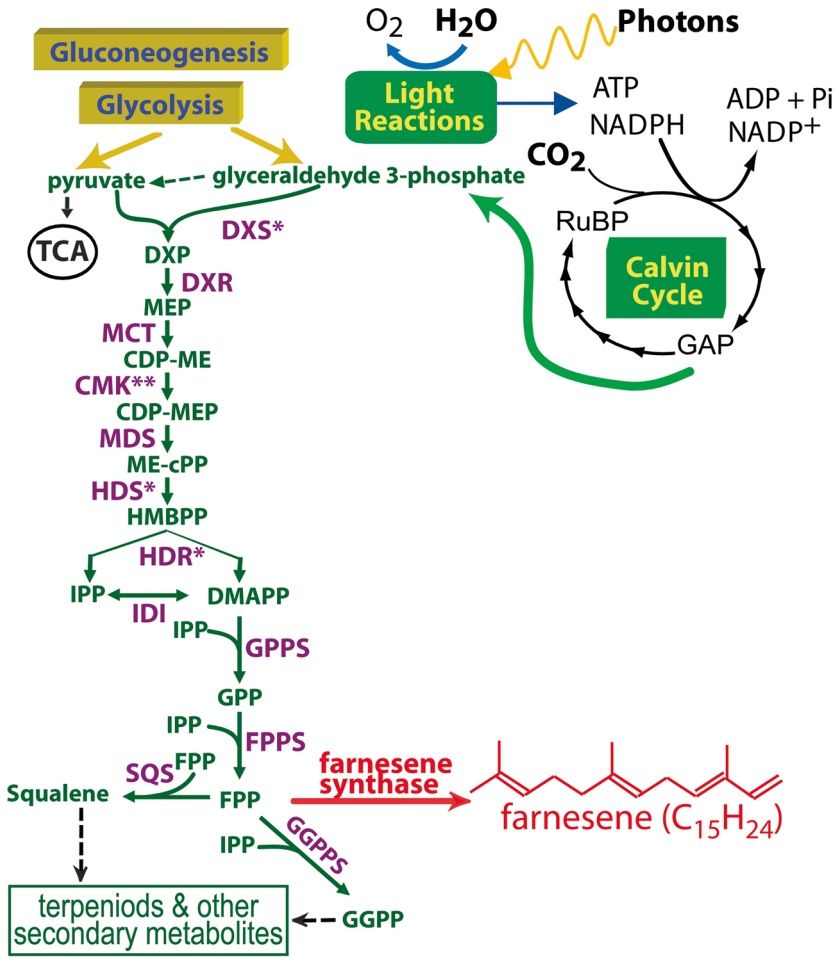
mevalonate pathway). However, with the exception of plants, most organisms have only one of these two pathways. The MVA pathway is mainly present in archaebacteria, some gram-positive bacteria, yeast, and animals (Vranova et al. 2013), while most gram-negative bacteria, cyanobacteria, plant plastids, and green algae have only the MEP pathway (Vranova et al. 2013). In the MEP pathway (Fig. 1), pyruvate and glyceraldehyde-3-phosphate (GAP) are condensed and brought through a series of enzymatic reactions, eventually leading to the basic five-carbon precursors for all terpenes: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP further condense to form geranyl pyrophosphate (GPP), the precursor for all C_{10} monoterpenes and their derivatives. A second condensation step with GPP and IPP forms farnesyl pyrophosphate (FPP), the precursor for all C_{15} sesquiterpenes and their derivatives.

The last decade has seen a surge of research exploring the potentials of using engineered microorganisms as "cellular factories" to produce sesquiterpenes and their derivatives as precursors for biofuels and therapeutic drugs. A majority of this effort has focused on enhancing terpenoid pathways in *Escherichia coli* and *Saccharomyces cerevisiae* through genetic engineering (Buijs et al. 2013; Martin et al. 2003;



DXP	1-deoxy-D-xylulose 5-phosphate	DXS	DXP synthase
MEP	methylerythritol-4-phosphate	DXR	DXP reductoisomerase
CDP-ME	diphosphocytidylyl methylerythritol	MCT	CDP-ME synthase
CDP-MEP	CDP-methylerythritol-2-phosphate	CMK	CDP-ME kinase
ME-cPP	methylerythritol-2,4-cyclodiphosphate	MDS	ME-cPP synthase
HMBPP	hydroxymethylbutenyl diphosphate	HDS	HMBPP synthase
IPP	isopentenyl diphosphate	HDR	HMBPP reductase
DMAPP	dimethylallyl-diphosphate	IDI	IPP:DMAPP isomerase
GPP	geranyl-diphosphate	GPPS	GPP synthase
FPP	farnesyl-diphosphate	FPPS	FPP synthase
GGPP	geranylgeranyl-diphosphate	GGPPS	GGPP synthase
		SQS	squalene synthase

Fig. 1 The farnesene production pathway in the engineered cyanobacteria. Known MEP pathway exists in plants and cyanobacteria (*highlighted in green*). The farnesene synthase and farnesene production that are not present in cyanobacteria and targeted for engineering are *highlighted in red*. *NADPH as cofactor **ATP dependent reaction

Peralta-Yahya et al. 2012; Pitera et al. 2007; Wang et al. 2011). Significant advancements in pathway engineering have recently allowed for scale up to industrial-sized facilities. For example, Amyris Biotechnologies, a renewable products company headquartered in Emeryville CA, developed an engineered *S. cerevisiae* to convert sugarcane sucrose to farnesene ($C_{15}H_{24}$), a volatile sesquiterpene with commercial applications in lubricants, cosmetics, fragrances, and biofuels (Buijs et al. 2013). This system was able to produce 1 million liters of farnesene during a 45-day period (Velasco 2013). However, using sugar feedstocks for farnesene production is an added cost factor that puts these facilities at an economic disadvantage compared to the direct photosynthetic production of farnesene.

Photosynthetic microorganisms such as algae and cyanobacteria hold a distinct advantage in biofuel production over plants and yeast. They have faster growth rates and higher photosynthetic efficiencies than plants (>10 % photosynthetic efficiency in cyanobacteria compared to 1–3 % in plants), are able to directly utilize CO_2 as a carbon source, and are genetically amenable (Ducat et al. 2011; Parmar et al. 2011; Ruffing 2011). Cyanobacteria with rewired metabolic pathways have recently been created through genetic engineering, and they possess the ability to convert CO_2 into chemicals and biofuels that can be secreted directly from their cells. Examples include cyanobacteria engineered to produce ethanol (Dexter and Fu 2009), isobutyraldehyde (Atsumi et al. 2009), isoprene (Lindberg et al. 2010), fatty acids (Liu et al. 2011), and hydrogen (Sakurai et al. 2013).

Anabaena sp. PCC 7120 (hereinafter referred to as *Anabaena* 7120) is a filamentous, nitrogen-fixing cyanobacterium. We have previously engineered this strain to produce and secrete limonene, a cyclic C_{10} monoterpene that could potentially be used as a biofuel (Halfmann et al. 2014). Here, we report the viability of using this cyanobacterium to synthesize farnesene, a C_{15} sesquiterpene, from CO_2, mineralized water, and light by introducing a codon-optimized farnesene synthase (*FaS*) gene from Norway spruce into the *Anabaena* 7120 genome. We envision this platform of using CO_2, water, and sunlight as a sustainable feedstock to be applicable for the production of a wide range of industrial chemicals and drop-in fuels.

Materials and methods

Strains and growth conditions

Anabaena sp. PCC 7120 was grown in BG11 medium supplemented with 20-mM HEPES buffer under constant white light (50 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the culture surface) while bubbling with a 0.22-μm filtered CO_2 and air (1:100 ratio) at a rate of 100 $mL \cdot min^{-1}$. Cultures were grown at 30 °C and shaken at 120 rpm in a temperature-controlled Innova 44R lighted incubator (New Brunswick Scientific). Light intensity of growing cultures was measured using a Li-Cor 250A light meter. *E. coli* strain NEB 10β (New England Biolabs) was grown in Luria-Bertani (LB) medium at 37 °C and 250 rpm. Kanamycin (Km; 50 $\mu g \cdot mL^{-1}$) was used for antibiotic selection for *E. coli*, and Neomycin (Nm; 100 $\mu g \cdot mL^{-1}$) was used for transgenic *Anabaena* 7120 harboring plasmid bearing farnesene synthase gene (pFaS).

Plasmid construction

A codon-optimized version of a farnesene synthase gene (GenBank access #: KJ847279) from Norway spruce (Martin et al. 2004) with a deleted stop codon and an N-terminal His_6 tag was synthesized by DNA 2.0 (Menlo Park, CA) and cloned into the *Bgl*II-*Sal*I sites of the shuttle vector pZR1188 (Halfmann et al. 2014). The final construct, pFaS (Fig. 2a), was transformed into *E. coli* strain NEB-10β, which was used for conjugal transfer of the plasmid into *Anabaena* 7120.

Conjugal transfer of pFaS into *Anabaena* 7120

The transfer of pFaS into *Anabaena* 7120 was performed via bacterial conjugation using a biparental *E. coli* mating system (Elhai et al. 1997) with the following modifications. *E. coli* HB101 bearing helper (pRL623) and conjugal (pRL443) plasmids were mated with *E. coli* NEB 10β bearing pFaS and selected on LB plates containing triple antibiotic selection for the three plasmids. Selected colonies were grown overnight in 2 mL of LB containing appropriate antibiotics, subcultured by adding 200 μL of overnight culture to 2 mL of fresh LB containing appropriate antibiotics, and grown for additional 3 h. Cells were harvested by centrifugation at 3000×g, washed three times with 1 mL of LB to completely remove antibiotics, and resuspended with 200-μL LB and subjected for mating with *Anabaena* 7120.

A 10-mL culture of wild-type (WT) *Anabaena* 7120 was grown to early exponential stage ($O.D._{700\ nm}$ of 0.3) and then sonicated (Branson 1510 water bath sonicator) for 60–120 s to break filaments into 2–4 cell lengths, which were confirmed under light microscopy. Cells were harvested by centrifugation at 5000×g for 10 min, resuspended in 200 μL of fresh BG11, and then mixed with the above *E. coli* harboring three plasmids for conjugal transfer. This mixture was placed under lighted conditions at 25 °C for 30 min, micropipetted on to an autoclaved Millipore Immobilon nitrocellulose filter (HATF08550) placed on a BG11 with 5 % LB agar plate and grown under white light for 3 days at 30 °C. The filter was then transferred to a BG11 plate containing Nm (100 $\mu g \cdot mL^{-1}$) to select for positive exconjugants. Individual exconjugant colonies were further purified by restreaking onto fresh BG11 plates containing 100 $\mu g \cdot mL^{-1}$ Nm, and routine

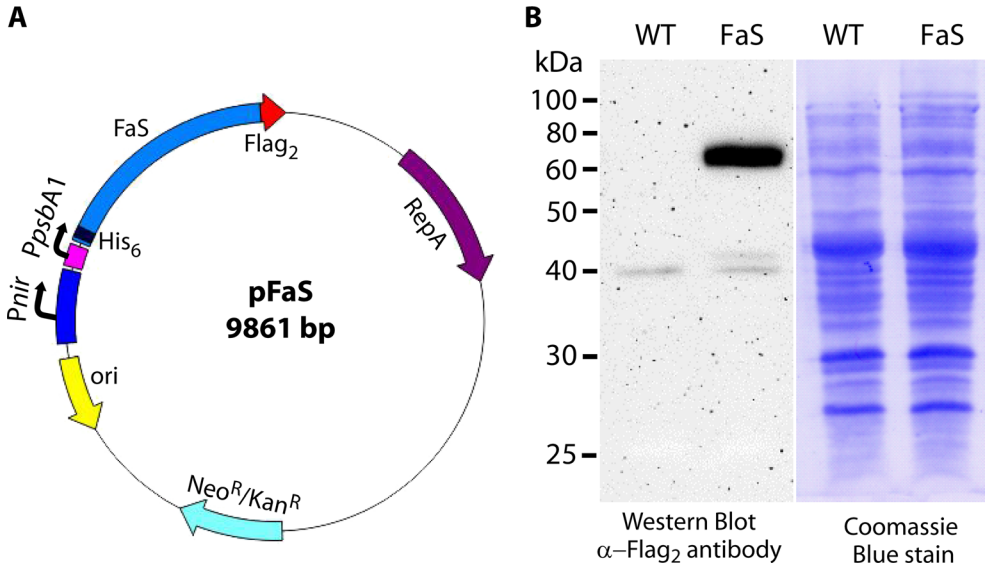


Fig. 2 Expression of FaS in *Anabaena* 7120. **a** Diagram of pFaS plasmid. *Pnir*, *nir* promoter of *Anabaena* 7120; *PpsbA1*, *psbA1* promoter of *Anabaena* 7120; *His_6*, 6X histidine tag; *$Flag_2$*, 2XFLAG tag; *FaS*, farnesene synthase ORF; *RepA*, replication protein A; *Neo^R/Kan^R*, neomycin/kanamycin antibiotic cassette; *ori*, origin of replication. **b** Western blot analysis revealed a 70-kDa FaS-$FLAG_2$ protein in FaS *Anabaena* 7120 bearing pFaS plasmid (*lane 2*) but absent in the WT sample (*lane 1*). The same PVDF membrane was stained with Coomassie blue R250 (*lanes 3* and *4*) to show equal protein loading

colony PCR was used for verification of positive exconjugants.

Protein extraction and Western blot analysis

Total proteins were extracted from both WT and FaS *Anabaena* 7120 cells to determine expression of FaS in the host strains, as described previously (Halfmann et al. 2014). Five-microliter protein supernatants were loaded and separated by 12 % SDS-PAGE at 200 V for 30 min, transferred onto a polyvinylidene fluoride (PVDF) membrane, and FaS-$FLAG_2$ was detected by Western blotting using anti-FLAG (Sigma Aldrich) antibodies at a dilution of 1:5000. The membrane was then stained with Coomassie Blue R250 to visualize total protein content.

Farnesene collection from culture headspace and GC-MS analysis

Starter cultures were set at an $O.D._{700\ nm}$ of 0.02 using a UV–vis spectrophotometer (Genesys 10S, Thermo Scientific). WT and FaS *Anabaena* 7120 strains were grown in 250-mL stoppered Erlenmeyer flasks containing 100-mL BG11 supplemented with 20-mM HEPES buffer and 100 $\mu g \cdot mL^{-1}$ Nm and bubbled with 0.22-μm filtered CO_2 and air (1:100 ratio) at a rate of 100 $mL \cdot min^{-1}$ in the conditions described in Section "Strains and growth conditions" for 15 days, while replacing the culture media every 5 days. A small glass column filled with 100 mg of Supelpak 2SV resin (Sigma Aldrich) was attached to the exhaust port of each flask to capture farnesene and other volatile metabolites from the culture headspace (Fig. 3). Fresh 2SV resin columns were replaced on the culture flasks every 3 days. To analyze volatiles from the resin, samples were eluted twice with 2.5 mL of pentane containing 1 $\mu g \cdot mL^{-1}$ tetracosane as an internal standard (IS), pooled in 5-mL total volumes, evaporated to 1 mL using a gentle stream of N_2 gas (thus concentrating the IS to 5 $\mu g \cdot mL^{-1}$), and subjected to gas chromatography–mass spectrometry (GC-MS) analysis (Agilent 7890A/5975C) at the Functional Genomic Core Facility of South Dakota State University. One-microliter injected samples were separated using a HP-5MS column (30 m×250 μm×0.25 μm), with H_2 as the carrier gas. The oven temperature was initially held at 40 °C for 1 min, increased 5 $°C \cdot min^{-1}$ until 180 °C was reached, and then further increased by 20 $°C \cdot min^{-1}$ to 320 °C. Compounds were identified using the NIST MS library v2.0 and authentic standards.

Quantification of farnesene and absorption test of 2SV resin

A set of trans-β-farnesene standards (Sigma Aldrich no. 73942) at 1, 5, 10, 25, 50, 100, and 200 $\mu g \cdot mL^{-1}$ and 5 $\mu g \cdot mL^{-1}$ tetracosane in pentane was used to create a standard curve to quantify farnesene captured by 2SV columns. To test absorption capacity of the 2SV resin, 1, 2, 3, 4, and 5-μL aliquots of trans-β-farnesene standard were pipetted into separate 10-mL sealed glass vials, with 100 mg of 2SV resin inside a glass column attached the exhaust port on each vial. A gentle stream of air was injected into the glass vial for 3–5 min, allowing the farnesene to evaporate and pass into the resin. The resin was then washed with pentane and mixed with the tetracosane IS as described above. These farnesene/tetracosane peak ratios were compared to the standard curve

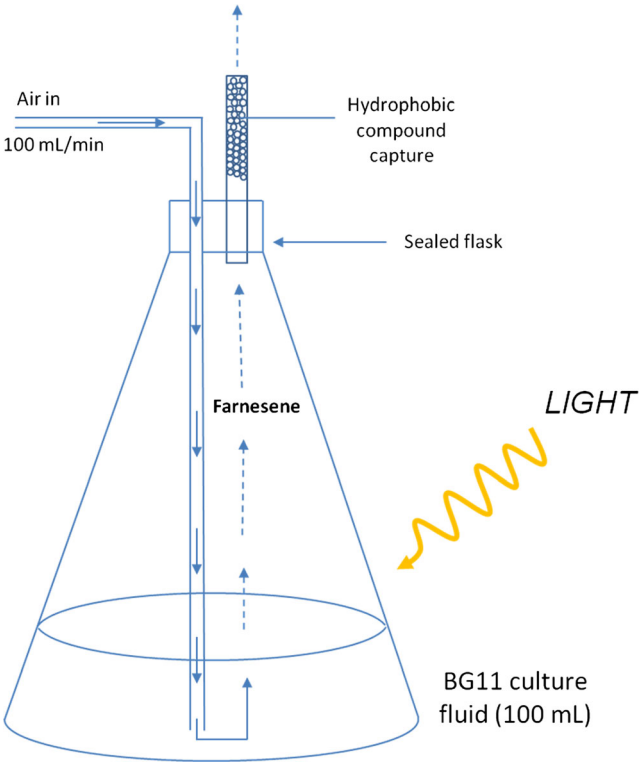


Fig. 3 Diagram of the apparatus for capturing volatile farnesene emitted from cyanobacterial cultures. A small glass column filled with 100 mg of Supelpak 2SV resin (Sigma Aldrich) was attached to the exhaust port of the sealed flask to capture farnesene and other volatile hydrocarbons from the culture headspace

to calculate the recovered amounts of farnesene. An α-farnesene standard peak was generated by grinding approximately 100 mg of Granny Smith apple (*Malus domestica*× *M. sylvestris*) peel with a mortar and pestle and sonicating the grounds in 2 mL of room-temperature pentane with a Branson Digital Sonifier at 70 % amplitude for 30 s. The extracts in organic phase were then concentrated by evaporating pentane into 0.5 mL using a gentle stream of N_2 and analyzed using GC-MS.

Chlorophyll extraction and analysis of photosynthetic activity

Chlorophyll was extracted using a 90 % methanol extraction procedure described previously (Meeks and Castenholz 1971). Oxygen evolution was quantified in 1-mL culture samples (adjusted to a cell density that has 10-μg chlorophyll·mL^{-1}) in BG11 with 10-mM $NaHCO_3$ using a Clark-type electrode and DW2 Oxygen Electrode Chamber with O_2 View Oxygen Monitoring software (Hansatech). Light was introduced to samples using an LS2/H tungsten-halogen 100 W light source and adjusted with neutral density filters. Light intensity and sample temperature was monitored using a Quantitherm light-temperature meter during experimentation. All measurements were performed in triplicate.

Results

Expression of a farnesene synthase (FaS) in *Anabaena* 7120

We first sought to utilize the native MEP pathway in *Anabaena* 7120 to produce farnesene via photosynthesis. Although *Anabaena* 7120 possesses an endogenous MEP pathway, a BLAST search of the genome using a characterized FaS protein sequence (Martin et al. 2004) resulted in no similarities (0.1 E value as cutoff). We then acquired a farnesene synthase gene (PaTPS-Far; Genbank accession no. AY473627.1) from Norway spruce (*Picea abies*), which was previously characterized as an E, E-α-farnesene synthase through an in vitro enzymatic activity assay (Martin et al. 2004). PaTPS-Far was then codon-optimized (DNA 2.0, Menlo Park, CA) for optimal expression in the cyanobacterium. We included an N-terminal His_6 tag and a C-terminal $FLAG_2$ tag to verify protein expression and cloned the gene into the replicating shuttle vector pZR1188, a high-copy plasmid with the ability to replicate independently from the chromosome and maintains stability in the cell without chromosomal integration. The resulting construct was named pFaS (Fig. 2a). This construct was then transferred into WT *Anabaena* 7120 using conjugation with an *E.coli* donor. *FaS* transcription was driven by a dual *Anabaena* 7120 *nir-psbA1* promoter to maintain maximal protein expression constitutively in the cells. As seen in Fig. 2b, an expected 70-kDa protein was detected from immunoblotting with anti-FLAG antibodies in FaS (lane 2), but not in WT *Anabaena* 7120 (lane 1), suggesting that FaS was expressed in the transgenic *Anabaena* 7120.

Identification of farnesene emitted from FaS Anabaena 7120 using GC-MS

Our previous studies with engineered *Anabaena* 7120 strains capable of producing limonene have shown that volatile compounds are emitted from the cells and volatilized into the flask headspace (Halfmann et al. 2014). This volatilization is especially rapid when the culture is bubbled with air or CO_2-enriched air to provide carbon. To collect these chemicals, we passed the exhaust gas from the headspace of a culture flask through a resin column filled with Supelpak 2SV resin (Sigma Aldrich) as shown in Fig. 3. Our absorption tests with a trans-β-farnesene standard as described in Section "Quantification of farnesene and absorption test of 2SV resin" showed that 100 mg of 2SV resin could absorb at least 1 mg of farnesene. To determine whether the expressed FaS was functionally active and produce farnesene in *Anabaena* 7120, we collected the volatile compounds emitted from the culture fluid through a glass column filled with 2SV resin and then analyzed the resin pentane extract using GC-MS. Figure 4 shows GC-MS chromatographs of WT *Anabaena* 7120 and FaS *Anabaena* 7120. A

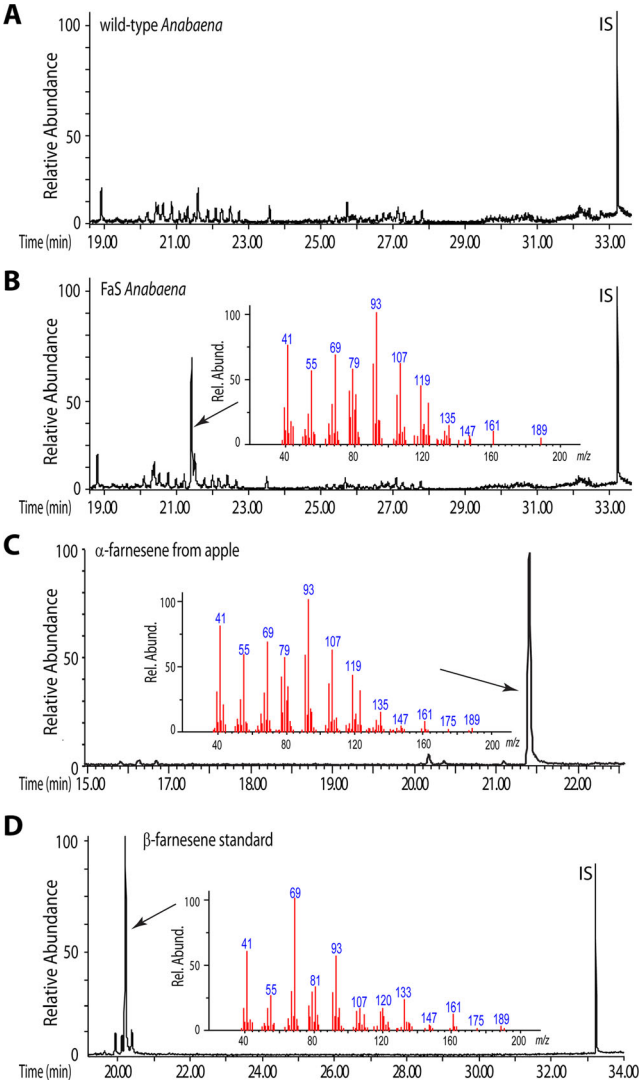


Fig. 4 GC-MS chromatographs of flask headspace samples from WT (**a**) and FaS (**b**) *Anabaena* 7120 cultures. A peak at the retention time 21.4 min (*arrowed*) found in FaS *Anabaena* 7120 matches the α-farnesene peak found in rind of green apple (**c**) but is absent in the WT (**a**). Mass spectra (*inserted red*) of these peaks also display the expected pattern for α-farnesene. Trans-β-farnesene (**d**) was used to create a standard curve for farnesene quantification. Five $\mu g \cdot mL^{-1}$ tetracosane serves as an internal standard (IS)

prominent peak appears at 21.4 min in the FaS *Anabaena* 7120 chromatograph (Fig. 4b), but not in WT *Anabaena* 7120 (Fig. 4a). This new peak in FaS *Anabaena* 7120 was identified as α-farnesene by the MS library (NIST MS Library v2.0).

Since α-farnesene is not commercially available, we compared the probable farnesene peak from the transgenic *Anabaena* 7120 to authentic farnesene from the rind of green apple, which is known to be abundant with E,E-α-farnesene (Pechous and Whitaker 2004). GC-MS revealed a major peak in the rind extract (Fig. 4c), having the same retention time (21.4 min) and ion fragmentation pattern as the probable farnesene peak from FaS *Anabaena* 7120 (Fig. 4b), and identified as α-farnesene by the MS library. This is consistent with the reported enzymatic product being E,E-α-farnesene (Martin et al. 2004). No farnesene was detected in the culture medium extracts or homogenized cell extracts (data not shown), suggesting an efficient transfer of farnesene from the growing culture to the headspace. Taken together, the genetically engineered cyanobacterium bearing the codon-optimized farnesene synthase gene from Norway spruce is capable of producing and emitting α-farnesene.

To quantify farnesene production from Fas *Anabaena* 7120, we used a commercially available trans-β-farnesene as a standard. Although trans-β-farnesene peak gave a shorter retention time (20.2 min, Fig. 4d) than the peak observed in the FaS *Anabaena* 7120, as well as α-farnesene peak from apple extract (21.4 min, Fig. 4b and c, respectively), the fragmentation pattern was similar to α-farnesene (comparing the inserted figure in Fig. 4d to that in 4c). Therefore, trans-β-farnesene standard was used to create a calibration curve with tetracosane serving as an IS.

Farnesene production in FaS Anabaena 7120 using CO_2, light, and mineralized water

We employed a continuous growth and farnesene recovery system to analyze the amount of farnesene produced from FaS *Anabaena* 7120 over a 15-day growth period. To measure farnesene production, the 2SV columns fitted to each culture flask were replaced every three days, and volatiles from the resin samples were eluted with pentane and quantified by GC-MS using a standard curve created by trans-β-farnesene. Our results showed that FaS *Anabaena* 7120 produced a total of 45.7±2.5 $\mu g \cdot L^{-1}$ farnesene from days 0–3, and these amounts continued to increase over the remainder of the growth period to approximately 93.3±9.6 $\mu g \cdot L^{-1}$ from days 13–15 (Fig. 5a). Total farnesene accumulation was measured at 305.4± 17.7 $\mu g \cdot L^{-1}$ during 15 days of growth. We further assessed the specific farnesene production rate by monitoring culture $O.D._{700\ nm}$ every 3 days (Fig. 5b) and comparing it to the amount of farnesene produced during each three-day collection period (Fig. 5c). Water loss due to evaporation was accounted for by refilling culture flasks with sterilized ddH_2O to proper culture volume before culture density analysis. The highest farnesene production rate was observed during the first 3 days, with a rate of 69.1±1.8 $\mu g \cdot L^{-1} \cdot O.D.^{-1} \cdot day^{-1}$. The productivity decreased during the remainder of the growth trial, leveling off at 15–20 $\mu g \cdot O.D^{-1} \cdot L^{-1} \cdot day^{-1}$.

Farnesene production enhances photosynthetic activity

In order to test the effects of farnesene synthesis on photosynthetic activity and biomass yield in the engineered cyanobacterium, we measured biomass growth and oxygen evolution rates (Fig. 6) of the water-splitting reaction of photosystem II

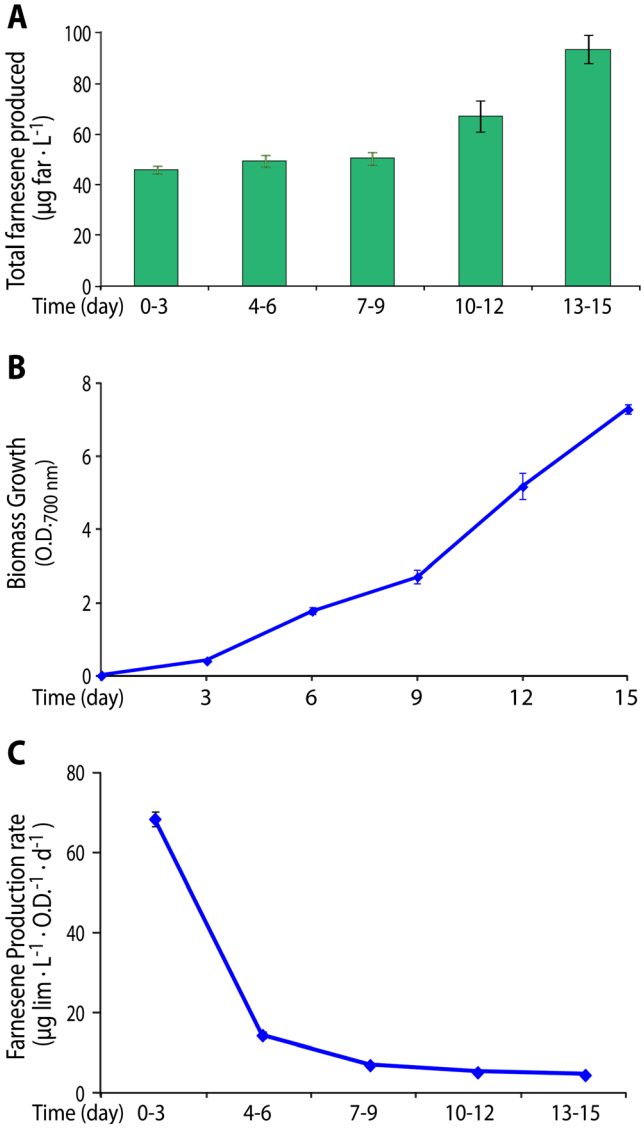


Fig. 5 Growth and farnesene production characteristics of genetically engineered FaS *Anabaena* 7120. **a** Total farnesene captured in 2SV column every 72 h from FaS *Anabaena* 7120. **b** Biomass growth of FaS *Anabaena* 7120. **c** Rate of farnesene produced from FaS *Anabaena* 7120 per optical density. The *horizontal axis* represents time (day). All experiments performed under 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ light conditions. Results are the average of three biological replicates, with *error bars* representing the standard deviation of the mean

(PSII) from WT and FaS *Anabaena* 7120. We found no significant difference in biomass accumulation between WT and FaS *Anabaena* 7120 (data not shown). The rate of oxygen release from the cells of FaS *Anabaena* 7120 was similar to the WT in the light ranges of 50–500 $\mu E \cdot m^{-2} \cdot s^{-1}$. However, at high light intensities (≥1000 $\mu E \cdot m^{-2} \cdot s^{-1}$), oxygen evolution from FaS *Anabaena* 7120 was approximately 60 % higher compared to the WT. Interestingly, FaS *Anabaena* 7120 maintained similar oxygen evolution rates (~3 μmol $O_2 \cdot min^{-1} \cdot mg\ chl^{-1}$) at 1000 and 1800 $\mu E \cdot m^{-2} \cdot s^{-1}$, while the WT exhibited a slight decrease in this light range (Fig. 6).

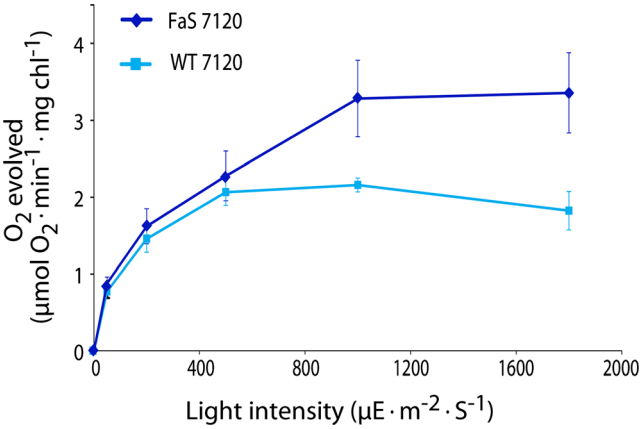


Fig. 6 Photosynthetic oxygen evolution of wild-type *Anabaena* and FaS *Anabaena* 7120 under indicated light intensities. The culture samples adjusted to a cell density containing 10-μg chlorophyll·mL^{-1} were tested in the presence of 10 mM $NaHCO_3$ in BG11 media

Discussion

The conversion of CO_2 to terpenes using a cyanobacterium as a bio-catalyst has been widely researched in recent years. It was first demonstrated in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, with the photosynthetic production of isoprene (C_5H_8) by heterologous expression of a codon-optimized isoprene synthase (IspS) from *Pueraria montana* (Lindberg et al. 2010). Initial production rates using a gaseous/aqueous two-phase bioreactor were 2-μg isoprene·L^{-1}·hr^{-1} during the first 48 h of growth (Bentley and Melis 2012). Our results show slightly lower production rates of farnesene (approximately 0.625 μg·L^{-1}·hr^{-1} during the first 72 h; 1.29 μg·L^{-1}·hr^{-1} during the last 72 h of growth). This is likely due from differences in bioreactor design and isoprenoid extraction methods, catalytic efficiency of the expressed enzyme, or differences in precursor metabolite pools of DMAPP and FPP for isoprene and farnesene, respectively. In addition, the expression of FaS on a replication vector can result in multiple copies of *fas* per chromosome, in contrast to chromosomal integration methods which theoretically produce one gene copy per chromosome in purified cultures. Recent studies have found that pDU1-based plasmids can have widely variable copy numbers in *Anabaena* 7120, ranging from 0.53–1812 per chromosome (Yang et al. 2013). These variables together likely underlie the differences in terpene production rates found between research groups.

Compared to the other biosynthetic pathways, allocation of fixed carbon into the MEP pathway is low, constituting only 3–5 % of the total biomass in cyanobacteria (Lindberg et al. 2010). In order to increase metabolite flux for enhanced isoprenoid production, overexpression of key rate-limiting MEP pathway enzymes was previously shown to improve the production of limonene in *Anabaena* 7120 (Halfmann et al. 2014). Heterologous expression of the whole MVA

pathway in *Synechocystis* 6803 also increased isoprene production 2.5-fold, as it endowed the cyanobacterium to draw more photosynthetic metabolites into isoprene production alongside its native MEP pathway (Bentley et al. 2014). In order to exploit the full potential of designed pathways in cyanobacteria for farnesene production, researchers will need to modulate transcriptional and translational elements of enzyme networks, in order to relieve bottlenecks, suppress feedback inhibition, and ultimately maximize carbon flow through the MEP pathway. These strategies have been researched in cyanobacteria through modulation of ribosomal binding sites (Oliver et al. 2014) and promoters (Huang et al. 2010; Huang and Lindblad 2013; Qi et al. 2013). The suppression of alternate pathways downstream of FPP synthesis (e.g., squalene sythesis) can also contribute to an increase in carbon flux into sesquiterpenes, previously demonstrated by replacing the native squalene synthesis promoter in engineered *Saccharomyces cerevisiae* with a methionine-repressible promoter, which resulted in a five-time increase in amorphadiene production (Paradise et al. 2008).

Although the total amount of farnesene produced by culture flasks increased every 3 days, we noticed that the specific rate of farnesene production dropped almost 78 % from day 3 to 6, until it leveled off during the remainder of the growth trial. We attribute this decline in farnesene productivity to an elevated cell density (O.D.) in culture flasks towards the end of the growth trial, which shaded the cells and inhibited the amount of photons collected. This could decrease photosynthetic activity and the reducing power needed for carbon flux through the MEP pathway. In cyanobacteria, enzymes in the MEP pathway has been found to interact closely with redox partners involved in light-independent reactions, making the MEP pathway tightly regulated by photosynthesis (Okada and Hase 2005). Low light quality could also affect the transcriptional activity of the engineered *psbA1* promoter, which known to be induced by light in cyanobacteria (Golden 1995). Indeed, increasing the light intensity during growth conditions has shown to significantly improve productivity and yield in biofuel-producing cyanobacteria (Halfmann et al. 2014; Ungerer et al. 2012).

The observed increase in O_2 production from the *Anabaena* 7120 strain endowed with the ability to synthesize farnesene is a phenomenon that has been observed in previous studies on cyanobacteria engineered to produce hydrocarbons (Bentley et al. 2013; Oliver et al. 2013). The observed enhancement in photosynthetic activity of FaS *Anabaena* 7120 under increasing light intensities could be explained by farnesene's role as an added carbon sink in the cyanobacterium. The continuous synthesis of farnesene could help maintain a relatively oxidized electron transport chain and help suppress photoinhibition, caused by imbalances between light absorption and carbon fixation. This opening of a new carbon sink would therefore create a positive feedback in the light reactions for photochemical energy production, resulting in more O_2 production compared to the WT. Similar traits such as increased PSII activity, chlorophyll content, and carbon fixation were also found in a strain of *Synechococcus elongatus* sp. PCC 7942 engineered to accumulate and excrete sucrose (Ducat et al. 2012).

The fine tuning of the modular units in biofuel pathways will be a crucial next step in engineering fourth-generation biofuel systems, as the current rates of fuel precursors produced from engineered cyanobacteria will need to be increased to make large-scale systems economically realistic. Previously, we used parameters from algae biomass production models and a hypothetical "greenhouse-ethanol plant" relay system to calculate an ideal limonene productivity using cyanobacteria (Halfmann et al. 2014). Using the same approach, we calculated that a 6.4-million L PBR capable of converting 1 % of the annual CO_2 emissions from a single ethanol plant (275,000 metric tons) into farnesene could produce roughly 851.3 metric tons (1,047,117 L) of farnesene annually, about 30 % of the annual farnesene yields reported by Amyris in 2013 (Voegele 2014). Assuming rough estimates for capital, labor, and operation, a total production costs of $\$1.30 \cdot L^{-1}$ farnesene is possible, compared to the current $\$4.00 \cdot L^{-1}$ production cost reported by Amyris. This considerable cost difference emphasizes the advantages of fourth-generation biofuels, compared to biofuel production systems that utilize land plants as a feedstock.

Cyanobacteria are ideal organisms for biofuel production, due to their fast growth rates, penchant for converting carbon into energy-rich molecules, and ability to secrete these molecules out of the cell. Their genetic amenability also provides for a way to open new metabolic channels, allowing for the creation of a myriad of chemical products. As a proof of concept, we show that expressing a chemically synthesized farnesene synthase (*faS*) gene in *Anabaena* 7120 led to photosynthetic production of farnesene directly from CO_2 and H_2O. This direct "CO_2 to fuel approach" completely bypasses the expensive, multistep processes currently used to produce biofuels from plant biomass. We envision CO_2 conversion systems using engineered cyanobacteria to pave the way for a greener, post-petroleum era.

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