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Metabolic engineering of *Escherichia coli* for α-farnesene production

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ABSTRACT

Sesquiterpenes are important materials in pharmaceuticals and industry. Metabolic engineering has been successfully used to produce these valuable compounds in microbial hosts. However, the microbial potential of sesquiterpene production is limited by the poor heterologous expression of plant sesquiterpene synthases and the deficient FPP precursor supply. In this study, we engineered *E. coli* to produce α-farnesene using a codon-optimized α-farnesene synthase and an exogenous MVA pathway. Codon optimization of α-farnesene synthase improved both the synthase expression and α-farnesene production. Augmentation of the metabolic flux for FPP synthesis conferred a 1.6- to 48.0-fold increase in α-farnesene production. An additional increase in α-farnesene production was achieved by the protein fusion of FPP synthase and α-farnesene synthase. The engineered *E. coli* strain was able to produce 380.0 mg/L of α-farnesene, which is an approximately 317-fold increase over the initial production of 1.2 mg/L.

1. Introduction

Sesquiterpenes belong to a large and diverse isoprenoid family with important medical and industrial properties (Berger, 2009; Dhingra et al., 2000; Muntendam et al., 2009). α-Farnesene (3,7,11-trimethyldodeca-1,3*E*,6*E*,10-tetraene) is one of the simplest acyclic sesquiterpenes (Fig. 1A); it was first discovered in apple peels and was found to play a role in plant defense (Huelin and Murray, 1966; Pare and Tumlinson, 1999; Yang et al., 2011). α-Farnesene has also been developed as a biofuel precursor due to its inherent properties of low hygroscopicity and high energy density. Fanesane, which is reduced from farnesene, is a promising bio-jetfuel candidate (Renninger and Mcphee, 2008). However, the sesquiterpenes, including α-farnesene, are naturally produced in limited quantities (Wallaart et al., 2000). Hence, the metabolic engineering of organisms is an alternative and attractive route for the production of these rare and valuable compounds (Munoz-Bertomeu et al., 2008).

Here, we describe the production of α-farnesene from *E. coli*, a tractable bacterial host. To date, only a few α-farnesene synthases, including apple (*Malus x domestica*) α-farnesene synthase, have been characterized (Pechous and Whitaker, 2004). In most cases, sesquiterpene production was limited by the poor expression of plant synthases (Hohn and Plattner, 1989; Martin et al., 2001). Thus, we designed an artificial apple α-farnesene synthase gene for improved expression of the synthase (Hernandez et al., 2007; Tokuoka et al., 2008). α-Farnesene biosynthesis begins with the formation of precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), generated from the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in *E. coli* and the mevalonate (MVA) pathway in eukaryotes (Bloch et al., 1959; Rohdich et al., 2002). These precursors are condensed into the direct precursor of α-farnesene, farnesyl diphosphate (FPP), by FPP synthase (Fig. 1A). The size of the FPP pool was a determinate factor in sesquiterpene production (Asadollahi et al., 2010). MEP pathway Engineering has been utilized to increase carotenoid production in *E. coli* (Farmer and Liao, 2001; Kajiwara et al., 1997; Kim and Keasling, 2001; Rodriguez-Villalon et al., 2008). For example, the overexpression of 1-deoxy-D-xylulose-5-phosphate synthase (DXS, encoded by *dxs*) and IPP isomerase (IDI, encoded by *idi*) resulted in the improvement of lycopene production (Kajiwara et al., 1997; Matthews and Wurtzel, 2000). Heterologous expression of the MVA pathway in *E. coli* was also an efficient method to increase the precursor supply for high-level production of terpenoids in *E. coli* (Kroll et al., 2009; Martin et al., 2003). We also reported the enhancement of farnesol (FOH) production via the introduction of MVA pathway into *E. coli*, where the additional accumulation of intracellular FPP by the co expression of FPP synthase (IspA, encoded by *ispA*) resulted in a 2-fold increase in FOH production (Wang et al., 2010). The overproduction of α-farnesene in *E. coli* also requires the augmentation of FPP synthesis.

FPP is not only the precursor of α-farnesene but also an essential constituent of some cellular components such as quinones and lipid carriers (Asai et al., 1994; Bouhss et al., 2008; Okada et al., 1997).

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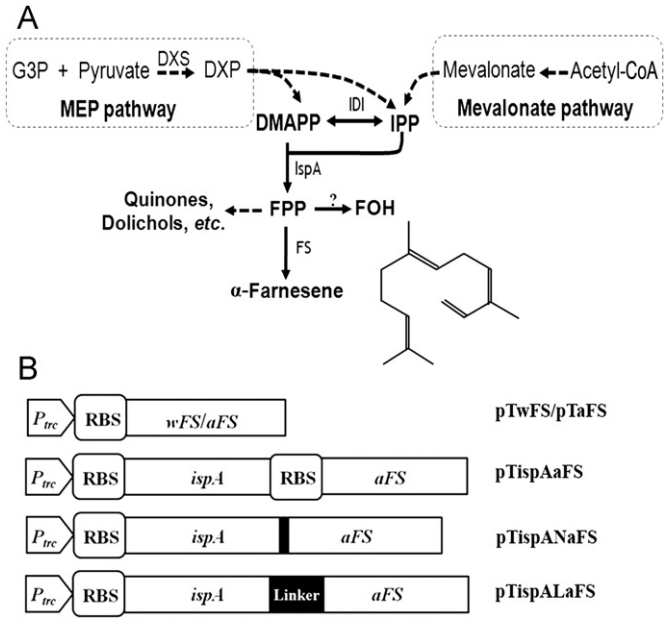

Fig. 1. Schematic diagram of the α-farnesene synthesis pathway and expression constructs containing the α-farnesene synthase gene. (A) IPP and DMAPP are synthesized by the MEP or MVA pathway. The MEP pathway is initiated by the condensation of glyceraldehyde 3-phosphate and pyruvate. MVA pathway utilizes acetyl-CoA as the starting substrate. FPP is synthesized by IspA through the condensation of 1 molecule of DMAPP and 2 molecules of IPP. α-Farnesene synthase catalyzes α-farnesene formation from the FPP precursor. The solid and dashed arrows represent single and multiple enzyme reactions, respectively. (B) The open arrows and rectangles represent promoters and genes, respectively. The closed rectangles indicate fusion junctions between the *ispA* and *aFS* genes. RBSs are shown as rounded rectangles. The abbreviations of the pathway intermediates are as follows: G-3-P, glyceraldehyde 3-phosphate; DXP, 1-dexoy-D-xylulose-5-phosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; FPP, (*E*,*E*)-farnesyl pyrophosphate; and FOH, farnesol. The abbreviations of the enzymes are as follows: DXS, DXP synthase; IDI, IPP isomerase; IspA, FPP synthase; (?), endogenous phosphatases of *E. coli*; and FS, α-farnesene synthase. *aFS* represents the artificial codon-optimized α-farnesene synthase gene.

It has been postulated that the co localization of FPP synthase and α-farnesene synthase, while catalyzing consecutive reactions from IPP and DMAPP to α-farnesene, would minimize FPP intermediate flux away from the desired α-farnesene product (Dueber et al., 2009; Kourtz et al., 2005; Meynial Salles et al., 2007). Multifunctional *Phomopsis amygdale* fusicoccadiene synthase produces fusicoccadiene, a C20 precursor of fusicoccins from IPP and DMAPP (Toyomasu et al., 2007). An FPP synthase and *epi*-aristolochene synthase fusion chimera has exhibited more efficient conversion of IPP and DMAPP to *epi*-aristolochene than the single enzymes (Brodelius et al., 2002). The fusion of FPP synthase and α-farnesene synthase was promoted by these naturally occurring and artificially created fusion enzymes that provide a metabolic pathway with little free diffusion of intermediates and an efficient and exclusive catalytic reaction (Huang et al., 2001; Hyde et al., 1988). The present study thus investigated α-farnesene production using a FPP synthase and α-farnesene synthase fusion with the introduction of the MVA pathway.

2. Materials and methods

2.1. Media and culture conditions

E. coli DH5α cells were grown in 2YT medium (16 g tryptone, 10 g yeast extract, and 5 g sodium chloride per 1 L) at 37 °C for plasmid construction, and at 29 °C for α-farnesene production. The seed culture grown overnight at 37 °C was inoculated to an optical density at 600 nm (OD_{600}) of 0.1 in 2YT medium containing 1% (v/v) glycerol, unless otherwise specified, and was grown at 29 °C for α-farnesene production. *E. coli* strains harboring the pSSN12Didi plasmid were cultured with an additional 5 mM mevalonate unless otherwise noted. All strains are listed in Table 1. Ampicillin (100 μg/mL), chloramphenicol (50 μg/mL), and 0.1 mM IPTG were added as required. To harvest α-farnesene produced during culture, 1 mL of decane was initially overlaid on 5 mL of culture broth (Fig. S1 in Supplementary information). Cell growth was determined by measuring the OD_{600}. All experiments were carried out in triplicate.

2.2. Construction of plasmids

Basic molecular biology procedures, including restriction enzyme digestion and bacterial transformation, were carried out as described in the literature (Sambrook and Russell, 2001). DNA fragments were amplified by PCR using *Pfu* DNA polymerase (SolGent, Daejeon, Korea) according to the manufacturer's instructions. The pET-30a/MdAFS1 plasmid, which was kindly donated by Dr. Green (Horticultural and Food Research Institute, Auckland, New Zealand) (Green et al., 2007), was digested with EcoRI and XhoI to obtain the wild-type apple α-farnesene synthase gene (*wFS*). The resulting fragment was ligated into the EcoRI–SalI sites of pTrc99A (Amann et al., 1988) to produce pTwFS. An artificial α-farnesene synthase gene (*aFS*) was synthesized by GenScript Corp. (NJ, US), according to *E. coli* codon usage, and inserted into pUC57 to produce pUC57-aFS. The *aFS* gene amplified from pUC57-aFS with primers aFS-F2 and aFS-R was digested with BamHI and HindIII and ligated into the corresponding restriction sites of pTrc99A to generate pTaFS. For the construction of the pTispAaFS plasmid, the *ispA* gene was amplified from *E. coli* genomic DNA with primers ispA-F and ispA-R and inserted into the pTaFS plasmid digested using EcoRI and BamHI. To fuse *ispA* and *aFS*, two *ispA* products amplified with primers ispA-F and ispAN-R or ispA-F and ispAL-R were cloned into the EcoRI–BamHI sites of pTrc99A to produce pTispAN (containing the *ispA* gene with the TAA stop codon removed) and pTispAL (containing the *ispA* gene with the TAA stop codon removed but a $(GGGGS)_2$ linker introduced), respectively. The *aFS* gene amplified with primers aFS-F1 and aFS-R was digested with BamHI and HindIII and subsequently inserted into pTispAN and pTispAL to produce pTispANaFS and pTispALaFS, respectively. The schematic diagram of the constructs containing the α-farnesene synthase gene is shown in Fig. 1B. PCR primers and plasmids are also listed in Table 1.

2.3. Identification and quantification of α-farnesene

The decane phase of the two-phase culture was collected and centrifuged for 10 min at 12,000 rpm to remove cell debris, and subsequently subjected to gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). The production of α-farnesene and FOH was quantified using an Agilent Technologies 7890A Gas Chromatograph equipped with a flame ionization detector (FID). Samples were injected at a split ratio of 1:10. Each analyte from the 1 μl samples was separated on a 19091J HP-5 column (length, 30 m; internal diameter, 0.32 mm; film thickness, 250 μm). The oven temperature was initially held at 80 °C for 1 min and was increased at a rate of 10 °C/min to 250 °C, where it was held for 1 min. Nitrogen was used as the carrier gas with an inlet pressure of 39 psi. The detector temperature was maintained at 260 °C. GC–MS analysis was run on a Double-Focusing Mass Spectrometer GC Mate II (JEOL, Tokyo, Japan). *Trans*-β-farnesene (Sigma–Aldrich, US) was used as the standard compound to construct the standard curve ($R^2 > 0.99$) for the estimation of α-farnesene production (Fig. S2 in Supplementary information).

Table 1
Primers, plasmids and strains used in this study.

Name	Description	Reference or source
Primers		
aFS-F1	GGGATCC**ATG**GAATTTCGTGTCCACCTG	This study
aFS-F2	GGGATCC*AGGAGG*TAATAATA**ATG**GAATTTCGTGTCCACCTG	This study
aFS-R	CCCAAGC**TTA**GTTGACCAGCGGCTGAAACAG	This study
ispA-F	GCGAATTC**ATG**GACTTTCCGCAGCAAC	This study
ispA-R	GGGATCC**TTA**TTTATTACGCTGGATGTAG	This study
ispAN-R	GCGGATCCTTTATTACGCTGGATGTAGTC	This study
ispAL-R	GCGGATCCGCCGCCACCCGAGCCACCGCCACCTTTATTACGCTGGATGTAGTC	This study
Plasmids		
pSTV28	P_{lac} expression vector, pACYC184 origin, *lacZ*, Cm^r	Takara Co., Ltd.
pSdxs	pSTV28 vector containing *dxs* from *E. coli*	This study
pSidi	pSTV28 vector containing *idi* from *E. coli*	This study
pSSN12Didi	pSTV28 vector containing *mvaK1*, *mvaK2* and *mvaD* from *S. pneumoniae*, and *idi* from *E. coli*	Yoon et al. (2006)
pSNA	pSTV28 vector containing *mvaE* and *mvaS* from *E. faecalis*, *mvaK1*, *mvaK2*, and *mvaD* from *S. pneumoniae*, and *idi* from *E. coli*	Yoon et al. (2006)
pET-30a/MdAFS1	pET-30a vector containing *wFS*	Green et al. (2007)
pTrc99A	P_{trc} expression vector, pBR322 origin, $lacI^q$, Amp^r	Amann et al. (1988)
pTwFS	pTrc99A vector containing *wFS*	This study
pUC57aFS	pUC57 vector containing *aFS*	This study
pTaFS	pTrc99A vector containing *aFS*	This study
pTispAaFS	pTrc99A vector containing *aFS* and *ispA* from *E. coli*	This study
pTispANaFS	pTrc99A vector containing a fusion gene of *aFS* and *ispA* without the linker between *aFS* and *ispA*	This study
pTispALaFS	pTrc99A vector containing a fusion gene of *aFS* and *ispA* with a $(GGGGS)_2$ linker between *aFS* and *ispA*	This study
Strains		
E. coli DH5α	F^-, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *recA*1, *endA*1, *hsdR*17(r_K^- m_K^+), *phoA*, *supE*44, λ^-, *thi*-1	ATCC
E. coli wFS	*E. coli* DH5α harboring pTwFS	This study
E. coli aFS	*E. coli* DH5α harboring pTaFS	This study
E. coli aFS-CT	*E. coli* DH5α harboring pTaFS and pSTV28	This study
E. coli aFS-dxs	*E. coli* DH5α harboring pTaFS and pSdxs	This study
E. coli aFS-idi	*E. coli* DH5α harboring pTaFS and pSidi	This study
E. coli aFS-SN	*E. coli* DH5α harboring pTaFS and pSSN12Didi	This study
E. coli ispAaFS-CT	*E. coli* DH5α harboring pTispAaFS and pSTV28	This study
E. coli ispAaFS-SN	*E. coli* DH5α harboring pTispAaFS and pSSN12Didi	This study
E. coli ispANaFS-SN	*E. coli* DH5α harboring pTispANaFS and pSSN12Didi	This study
E. coli ispALaFS-SN	*E. coli* DH5α harboring pTispALaFS and pSSN12Didi	This study
E. coli ispALaFS-NA	*E. coli* DH5α harboring pTispALaFS and pSNA	This study

Note: the start codon and stop codon are in bold, the enzyme sites are underlined, and the ribosome binding site is in italics.

2.4. Quantification of mevalonate and glycerol

Mevalonate concentration was determined by GC analysis. Culture filtrate was adjusted to pH 2 with 3 M HCl, incubated at 45 °C for 1 h, saturated with anhydrous Na_2SO_4, and extracted with ethyl acetate. The resulting samples were analyzed for mevalonate concentration using an Agilent Technologies 7890A Gas Chromatograph. The analytical temperature of the GC was controlled at an initial temperature of 180 °C for 1 min, then ramped to 200 °C gradually at 2.5 °C/min and held for 2 min. The detector temperature was maintained at 260 °C. Glycerol concentration was determined by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) with a refractive index detector and Kromasil KR100-5NH2 column (250 mm × 4.6 mm, Eka Chemicals, Bohus, Sweden). The supernatant obtained after centrifugation of the culture broth was injected for the quantification of residual glycerol. The mobile phase was 75% acetonitrile in water at 35 °C and was run at a flow rate of 1.5 mL/min.

3. Results and discussion

3.1. α-Farnesene production in E. coli and synthase gene optimization

To evaluate α-farnesene synthesis, *E. coli* wFS was cultured in 5 mL of 2YT medium with an overlay of 1 mL of decane at 29 °C for 24 h (Table 1). The functional expression of wild-type apple α-farnesene synthase did result in α-farnesene synthesis (Fig. S3 in Supplementary information); however, *E. coli* wFS produced only a low amount of α-farnesene (Fig. 2). This result was likely due to the poor expression of α-farnesene synthase. To overcome the expression problem, a codon-optimized artificial α-farnesene synthase gene (*aFS*) was designed for improved expression in *E. coli* (Table S1 and Fig. S5 in Supplementary information). Indeed, the improvement of synthase expression resulted in a 1.3-fold increase in α-farnesene production. However, a severe reduction in cell growth was induced by the addition of 0.1 mM IPTG. This growth inhibition was not likely due to protein overexpression or rare codon depletion but to a shortage of essential metabolites (IPP, DMAPP, and FPP). The expression level of α-farnesene synthase was not high enough to cause a protein burden (Fig. S5 in Supplementary information), and the rare codons were absent from the codon-optimized *aFS* gene; however, the metabolite consumption of IPP, DMAPP, and FPP would be exacerbated by induction of α-farnesene synthase expression. Therefore, the enhancement of IPP, DMAPP, and FPP synthesis was required for α-farnesene production.

3.2. Enhancement of the IPP and DMAPP supply for α-farnesene production

E. coli aFS-dxs and *E. coli* aFS-idi were created to enhance the expression of DXS and IDI, two MEP pathway rate-limiting enzymes (Table 1 and Fig. 3). The α-farnesene production of *E. coli* aFS-idi was increased 7-fold over *E. coli* aFS-CT, which produced only 0.9 mg/L α-farnesene with 0.1 mM IPTG induction. The synthesis of one molecule of α-farnesene requires two

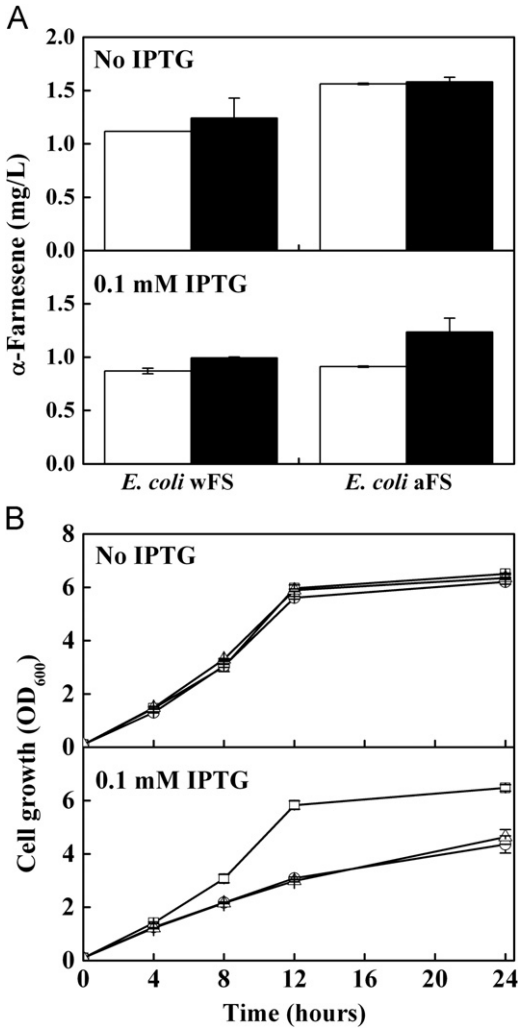

Fig. 2. Effect of the codon optimization of the α-farnesene synthase gene on α-farnesene production and cell growth. (A) α-Farnesene production by *E. coli* wFS and *E. coli* aFS was compared. The open and closed bars indicate the α-farnesene production obtained at 24 and 48 h, respectively. (B) Cell growth of recombinant *E. coli* harboring empty pTrc99A vector, pTwFS and pTaFS is represented by squares (□), circles (○) and triangles (Δ), respectively. Cells were cultured for 24 h in 2YT medium containing 1.0% (v/v) glycerol at 29 °C, with either no IPTG or 0.1 mM IPTG.

molecules of IPP and one molecule of DMAPP; IPP and DMAPP are produced in a 5:1 ratio by the MEP pathway (Adam et al., 2002; Rohdich et al., 2002). Overexpression of IDI would result in a compromise between the consumption and formation of IPP and DMAPP, which contributed to the significant increase in α-farnesene production in *E. coli* aFS-idi. Moreover, co-expression of DXS or IDI alleviated the growth inhibition observed in *E. coli* aFS. This result confirmed that the availability of IPP and DMAPP is of great importance for α-farnesene production.

Introduction of an exogenous MVA pathway is another method to improve the availability of IPP and DMAPP (Martin et al., 2003; Yoon et al., 2006). Here, we transformed plasmid pSSN12Didi, which encodes the proteins from the bottom portion of the MVA pathway that converts mevalonate to IPP and DMAPP, into *E. coli* aFS to produce *E. coli* aFS-SN for α-farnesene production. This recombinant strain was cultivated as previously described, except for supplementation with 5 mM mevalonate. The imported MVA pathway resulted in a dramatic increase in α-farnesene production to 17 mg/L after 48 h whether *E. coli* aFS-SN was induced with IPTG or not (Fig. 3). However, cell growth of *E. coli* aFS-SN was inhibited with IPTG induction. Given the low level of cell growth, the cell specific production (6.5 mg/L/OD_{600}) was increased 3.2-fold by IPTG induction. The growth inhibition of *E. coli* aFS-SN was presumably due to the accumulation of IPP and DMAPP, which are toxic (Martin et al., 2003), rather than the shortage observed in *E. coli* aFS. The next limitation for α-farnesene production would be the step coupling the conversion of IPP and DMAPP to FPP, the direct precursor of α-farnesene, as a single chromosomal copy of the FPP synthase gene (encoded by *ispA*) would not be sufficient to synthesize a considerable amount of FPP for α-farnesene production in *E. coli* aFS-SN.

3.3. Overexpression of IspA to increase α-farnesene production

The overexpression of FPP synthase has been reported to increase the intracellular FPP pool in *S. cerevisiae* (Asadollahi et al., 2010). To overcome the toxicity of IPP and DMAPP accumulation and to increase the intracellular concentration of the FPP substrate supplied to α-farnesene synthase, the FPP synthase gene *ispA* was co-expressed in *E. coli* ispAaFS-SN (Table 1 and Fig. 4). As expected, overexpression of IspA restored cell growth and resulted in the production of 57.6 mg/L of α-farnesene, which is 3.2-fold higher than that of *E. coli* aFS-SN. However, *E. coli* ispAaFS-CT expressing FPP synthase and α-farnesene synthase only produced an amount of α-farnesene comparable to *E. coli* aFS-CT, which further confirmed the importance of IPP and DMAPP availability for α-farnesene production. Predictably, *E. coli* ispAaFS-SN also produced FOH as a by-product. The highest FOH production of 17.8 mg/L was obtained after 48 h in the 0.1 mM IPTG-induced culture. As reported previously, *E. coli* has been engineered to produce FOH through the enhancement of FPP synthesis, in which endogenous phosphatases were thought to catalyze FOH formation (Wang et al., 2010). The endogenous phosphatases seem to compete with α-farnesene synthase for the common FPP substrate in *E. coli* ispAaFS-SN.

3.4. Redirection of FPP flux to α-farnesene

To increase FPP concentration in the proximity of α-farnesene synthase, FPP synthase and α-farnesene synthase were co-localized by in-frame protein fusion. The protein chimera of FPP synthase and α-farnesene synthase was expected to exhibit a superior kinetic property in delivery of FPP to α-farnesene synthase by preventing free diffusion of the intermediate into bulk fluid of cytoplasm. Moreover, substrate channeling has been observed in natural enzyme complexes, such as pyruvate decarboxylase (Huang et al., 2001; James and Viola, 2002). The substrate channeling effect through protein fusion was also anticipated to reduce FPP flux to competing metabolic pathways. Here, *E. coli* ispANaFS-SN and *E. coli* ispALaFS-SN were constructed to determine their abilities to redirect FPP flux to α-farnesene (Table 1 and Fig. 5). As a result of protein fusion, *E. coli* ispALaFS-SN produced 86.8 mg/L of α-farnesene in 48 h. Importantly, FOH formation was not observed in this strain. This result suggests that expression of the FPP synthase–farnesene synthase fusion (IspALaFS) with a $(GGGGS)_2$ linker enables *E. coli* ispALaFS-SN to synthesize α-farnesene from IPP and DMAPP with little loss of the FPP intermediate. However, the fusion of FPP synthase and α-farnesene synthase (IspANaFS) without a linker peptide in *E. coli* ispANaFS-SN did not confer this exclusive increase in α-farnesene production. The failure to improve α-farnesene production was hypothesized to be due to the fact that the IspANaFS fusion likely caused steric hindrance, which impacted both structural configurations and enzyme activities. Additionally, a more than 2-fold increase in α-farnesene production resulted from IPTG induction in *E. coli* ispALaFS-SN.

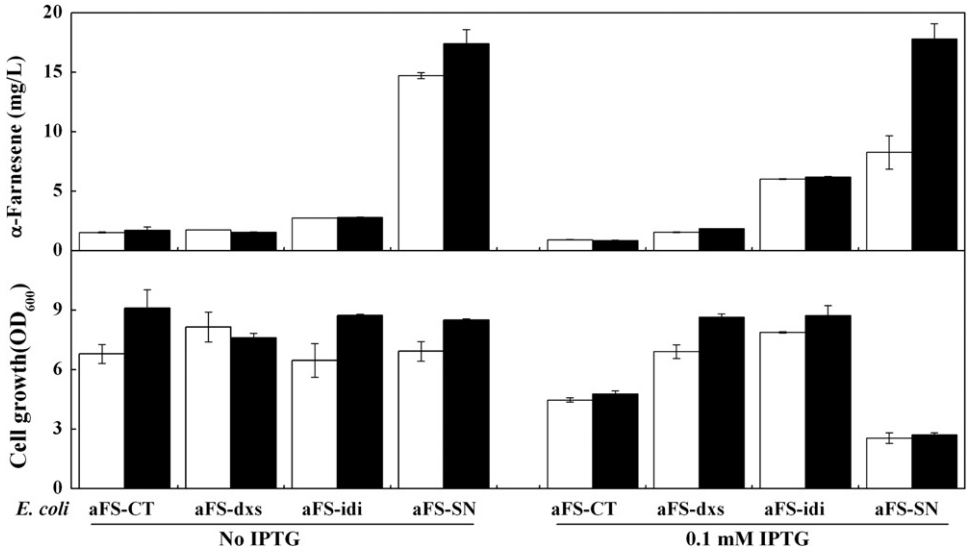

Fig. 3. Effect of the enhancement of IPP and DMAPP synthesis on α-farnesene production and cell growth. *E. coli* strains aFS-CT, aFS-dxs, aFS-idi and aFS-SN were cultivated at 29 °C in 2YT medium containing 1% (v/v) glycerol with either no IPTG or 0.1 mM IPTG. The culture of *E.* coli aFS-SN was supplemented with 5 mM mevalonate. The open and closed bars indicate the results obtained at 24 and 48 h, respectively.

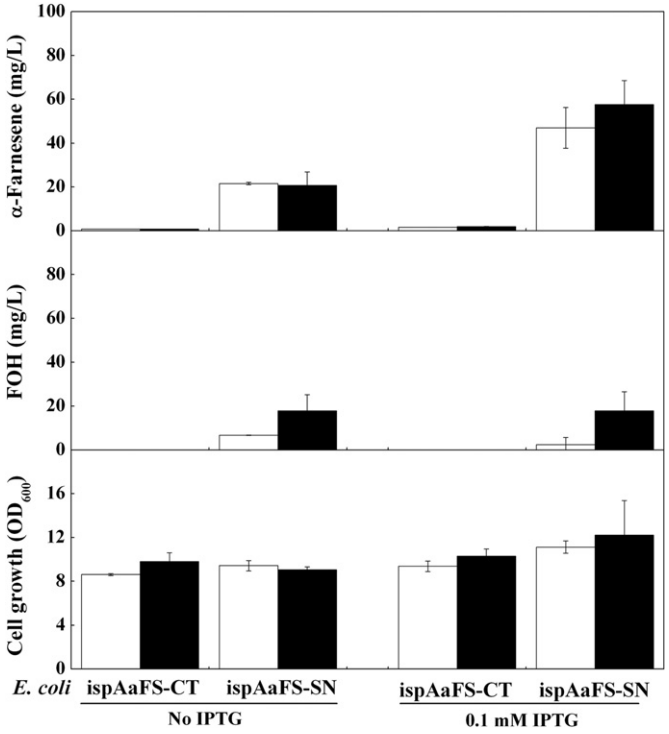

Fig. 4. Effect of the IspA augmentation on α-farnesene production and cell growth. *E. coli* strains ispAaFS-CT and ispAaFS-SN were cultivated at 29 °C in 2YT medium containing 1% (v/v) glycerol with either no IPTG or 0.1 mM IPTG induction. The culture of *E. coli* ispAaFS-SN was supplemented with 5 mM mevalonate. The open and closed bars indicate the results obtained at 24 and 48 h, respectively.

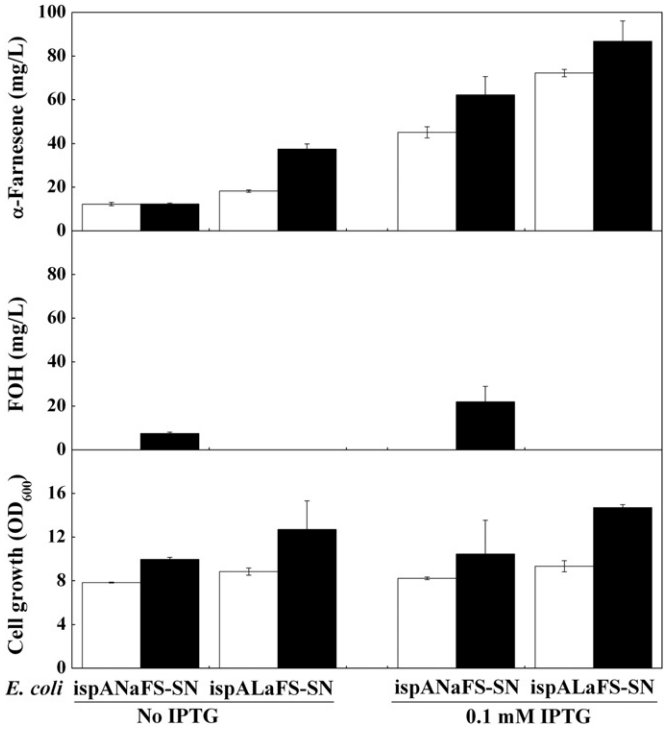

Fig. 5. Effect of the IspA and α-farnesene synthase fusion on α-farnesene production and cell growth. *E. coli* strains ispANaFS-SN and ispALaFS-SN were cultivated at 29 °C in 2YT medium containing 1.0% (v/v) glycerol and 5 mM mevalonate with either no IPTG or 0.1 mM IPTG. The open and closed bars indicate the results obtained at 24 and 48 h, respectively.

Therefore, we wanted to determine whether the mevalonate concentration restricts the ability of *E. coli* ispALaFS-SN to produce α-farnesene when induced with 0.1 mM IPTG.

3.5. Effect of mevalonate concentration on α-farnesene production

The ability of *E. coli* ispALaFS-SN to produce α-farnesene was assessed with various concentrations (0–15 mM) of mevalonate substrate (Fig. 6). Cell growth and α-farnesene production were increased almost proportionally to mevalonate concentration. The culture supplemented with 15 mM mevalonate yielded the highest α-farnesene production of 153.6 mg/L, which was 1.8-fold greater than the production observed with 5 mM mevalonate. However, the withdrawal of mevalonate resulted in a sharp drop in α-farnesene production to 9.8 mg/L and the smallest amount of cell growth. It seemed that the low amount of supplied mevalonate limited the ability of the bottom portion of the MVA pathway to provide ample prenyl pyrophosphate precursors. However, a significant amount of residual mevalonate remained in all

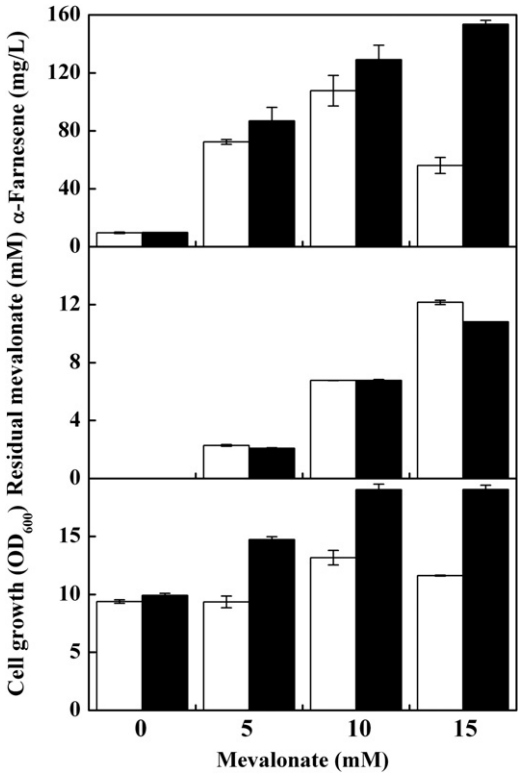

Fig. 6. Effect of the amount of the supplemented mevalonate on α-farnesene production and cell growth of *E. coli* ispALaFS-SN. Cells were cultured at 29 °C in 2YT medium containing 1.0% (v/v) glycerol and 0–15 mM mevalonate with 0.1 mM IPTG. The open and closed bars indicate the results obtained at 24 and 48 h, respectively.

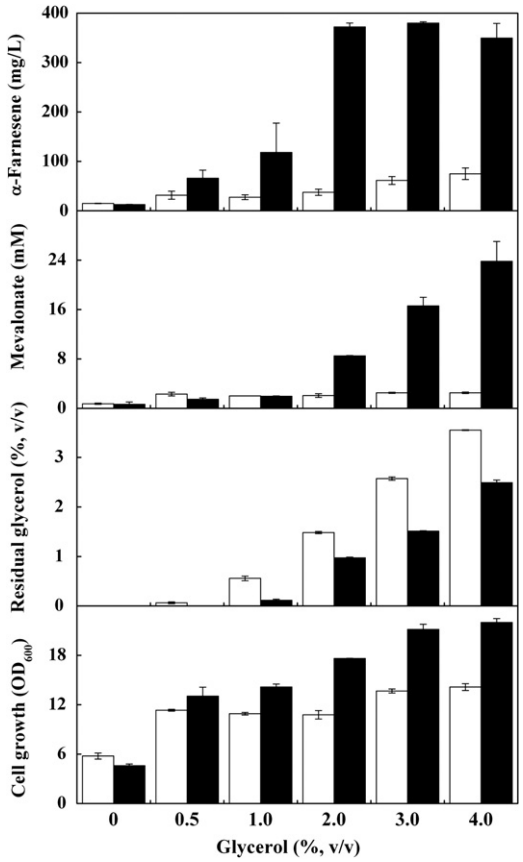

Fig. 7. Effect of glycerol concentration on α-farnesene production and cell growth of *E. coli* ispALaFS-NA. Cells were cultured at 29 °C in 2YT medium containing 0–4.0% (v/v) glycerol with 0.1 mM IPTG. The open and closed bars indicate the results obtained at 24 and 48 h, respectively.

mevalonate supplemented cultures. For example, only 28% of the initially added mevalonate was consumed in 48 h in the 15 mM mevalonate supplemented culture. The increased production was likely due to an enhancement in mevalonate uptake by the cells due to the increasing mevalonate concentration.

Notably, mevalonate consumption and α-farnesene buildup mainly occurred during the initial 24 h. We postulated that a problem with either the bottom portion of the MVA pathway or the IspALaFS fusion in the second 24 h period prevented further increase in α-farnesene production, although the exception of the 15 mM mevalonate supplemented culture was unexplainable. Despite the production improvement from *E. coli* ispALaFS-SN by the addition of mevalonate, the entire MVA pathway was still required to supply IPP and DMAPP due to the high cost of mevalonate substrate.

3.6. α-Farnesene production using the entire MVA pathway

Plasmid pSNA, which encoded the entire MVA pathway, was used to supply prenyl pyrophosphate precursors without the addition of mevalonate (Yoon et al., 2009). *E. coli* ispALaFS-NA was cultivated in a 2YT medium containing 0.1 mM IPTG and various concentrations (0–4% (v/v)) of glycerol (Table 1). Glycerol was chosen as the carbon and energy source due to its abundance, low price, and high degree of reduction (Murarka et al., 2008; Yazdani and Gonzalez, 2007). Glycerol is also a byproduct in the biodiesel production process for which new uses are being sought (da Silva et al., 2009; Fukuda et al., 2001). Indeed, the resulting α-farnesene production was specifically associated with the glycerol concentration up to 3.0% (v/v); but the addition of more than 3.0% (v/v) glycerol was not accompanied by an increase in α-farnesene production (Fig. 7). The addition of 3.0% (v/v) glycerol resulted in the highest production of 380.0 mg/L of α-farnesene, which was 2.5-fold increase over *E. coli* ispALaFS-SN and equivalent to an approximately 317-fold increase over *E. coli* wFS. These results demonstrated that expression of the entire MVA pathway could much more efficiently produce α-farnesene from a cheap carbon source than the expression of the bottom portion of the MVA pathway with addition of mevalonate.

In contrast to the production of α-farnesene during the initial 24 h with *E. coli* ispALaFS-SN, the majority of α-farnesene production occurred during the second 24 h with *E. coli* ispALaFS-NA. For example, approximately 90% of the α-farnesene was produced during the second 24 h with the addition of 2.0% (v/v) glycerol. This result therefore indicated that the IspALaFS fusion was functional and capable of producing α-farnesene during the entire 48 h of culture. We next turned to quantification of the mevalonate intermediate. The use of more than 2% (v/v) glycerol led to a large amount of mevalonate. As much as 23.8 mM mevalonate was accumulated in the presence of 4.0% (v/v) glycerol; moreover, this accumulation was more severe in the second 24 h. These results likely indicate that the activity of the bottom portion of the MVA pathway was decoupled with culture time. Mevalonate kinase, the first enzyme in the bottom portion of the MVA pathway, is subject to feedback regulation by various downstream intermediates, such as diphosphomevalonate, IPP, DMAPP, and FPP (Fu et al., 2008). Diphosphomevalonate has been known to allosterically inhibit mevalonate kinase in *Streptococcus pneumoniae* (Andreassi et al., 2004). We hypothesized that the inefficiency of the bottom portion of the MVA pathway in this study was probably due to such feedback regulation.

We found that α-farnesene production and cell growth were significantly dependent on the addition of glycerol. Residual

Table 2
Stepwise increases in α-farnesene production from the engineered *E. coli* strains.

Strains	Culture conditions	α-Farnesene production (mg/L)	Fold increases
E. coli wFS	2YT medium, 29 °C, 1.0% (v/v) glycerol	1.2	1
E. coli aFS	2YT medium, 29 °C, 1.0% (v/v) glycerol	1.6	1.3
E. coli aFS-dxs	2YT medium, 29 °C, 1.0% (v/v) glycerol, 0.1 mM IPTG	1.9	1.6
E. coli aFS-idi	2YT medium, 29 °C, 1.0% (v/v) glycerol, 0.1 mM IPTG	6.2	5.2
E. coli aFS-SN	2YT medium, 29 °C, 1.0% (v/v) glycerol, 0.1 mM IPTG, 5 mM mevalonate	17.8	14.8
E. coli ispAaFS-SN	2YT medium, 29 °C, 1.0% (v/v) glycerol, 0.1 mM IPTG, 5 mM mevalonate	57.6	48.0
E. coli ispANaFS-SN	2YT medium, 29 °C, 1.0% (v/v) glycerol, 0.1 mM IPTG, 5 mM mevalonate	62.2	51.8
E. coli ispALaFS-SN	2YT medium, 29 °C, 1.0% (v/v) glycerol, 0.1 mM IPTG, 5 mM mevalonate	86.8	72.3
	2YT medium, 29 °C, 1.0% (v/v) glycerol, 0.1 mM IPTG, 15 mM mevalonate	153.6	128.0
E. coli ispALaFS-NA	2YT medium, 29 °C, 3.0% (v/v) glycerol, 0.1 mM IPTG	380.0	316.7

Note: the item "Fold increase" represents the relative production increase normalized by the α-farnesene production of *E. coli* wFS.

glycerol was thus analyzed to investigate carbon source availability during culture. Approximately 1.0% (v/v) glycerol was consumed during the initial 24 h in glycerol supplemented cultures. Given the dramatic increase in α-farnesene production during the second 24 h, the glycerol consumed during initial 24 h was mainly contributed to cell buildup. Therefore, the addition of less than 1.0% (v/v) glycerol would limit α-farnesene production and cell growth. In the 2.0% (v/v) glycerol supplemented culture, α-farnesene yield (α-farnesene production/glycerol consumption) was 28.7 mg/g, which represented 11.6% of the theoretical yield (246.6 mg/g). The yield decreased in the culture supplemented with a larger concentration of glycerol added culture (18.4 mg/g with the addition of 4.0% (v/v) glycerol, 7.5% of the theoretical yield). However, if the accumulated 23.8 mM mevalonate in the 4.0% (v/v) glycerol supplemented culture could be converted to α-farnesene, the yield would be 103.6 mg/g or 42.0% of the theoretical yield. Therefore, this low yield could be attributed to inefficiency in the bottom portion of MVA pathway. An additional study should focus on constructing an efficient bottom portion of the MVA pathway, either by replacement of the rate-limiting enzymes or rational protein engineering. Recent advances in genome scale model are available to predict the cellular behavior and metabolic network in order to rewire regulatory system to enhance sesquiterpene production (Asadollahi et al., 2009). It is also necessary to manipulate the overall cellular metabolism and phenotypes like as optimization of carbon flux toward acetyl-CoA, which is the starting substrate of MVA pathway, to increase the α-farnesene yield.

4. Conclusions

In summary, we have demonstrated the feasibility of producing α-farnesene in metabolically engineered *E. coli*. Stepwise increases in α-farnesene production are summarized in Table 2. Poor expression of plant sesquiterpene synthase genes limited the overproduction of sesquiterpenes from the bacterial hosts (Hohn and Plattner, 1989; Martin et al., 2001). Codon optimization of α-farnesene synthase resulted in a moderate increase (1.3-fold) in α-farnesene production within the background of the native MEP pathway. Overexpression of the MEP pathway rate-limiting enzymes (DXS or IDI) and introduction of the exogenous MVA pathway further increased α-farnesene production from 1.6- to 48.0-fold. The dramatic increase achieved by using the MVA pathway confirmed its great potential to supply IPP and DMAPP (Martin et al., 2003). More recent studies have claimed that the MEP pathway was also an efficient route to supply IPP and DMAPP for terpenoid production; therefore, a high level α-farnesene production could be expected by careful manipulation of the entire MEP pathway rather than overexpression of only the rate-limiting enzymes (Ajikumar et al., 2010; Leonard et al., 2010). The highest α-farnesene production of 380.0 mg/L was achieved with a fusion of FPP synthase and α-farnesene synthase and harnessing of the MVA pathway. This production represented a 316.7-fold increase over the initial level of α-farnesene production (1.2 mg/L). Although a marked increase in α-farnesene production was attained in this study, mevalonate accumulation suggested inefficiency in the bottom portion of the MVA pathway that needs to be reprogrammed. Therefore, future goals include identifying the bottleneck in this pathway and maximizing the supply of IPP and DMAPP. This study can serve as the basis for the construction of much more robust strains for sesquiterpene production in the future.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2011.08.001.

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