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Efficient functional analysis system for cyanobacterial or plant cytochromes P450 involved in sesquiterpene biosynthesis

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Abstract Tractable plasmids (pAC-Mv-based plasmids) for *Escherichia coli* were constructed, which carried a mevalonate-utilizing gene cluster, towards an efficient functional analysis of cytochromes P450 involved in sesquiterpene biosynthesis. They included genes coding for a series of redox partners that transfer the electrons from NAD(P)H to a P450 protein. The redox partners used were ferredoxin reductases (CamA and NsRED) and ferredoxins (CamB and NsFER), which are derived from *Pseudomonas putida* and cyanobacterium *Nostoc* sp. strain PCC 7120, respectively, as well as three higher-plant NADPH-P450 reductases, the *Arabidopsis thaliana* ATR2 and two corresponding enzymes derived from ginger (*Zingiber officinale*), named ZoRED1 and ZoRED2. We also constructed plasmids for functional analysis of two P450s, α-humulene-8-hydroxylase (CYP71BA1) from shampoo ginger (*Zingiber zerumbet*) and germacrene A hydroxylase (P450NS; CYP110C1) from *Nostoc* sp. PCC 7120, and co-transformed *E. coli* with each of the pAC-Mv-based plasmids. Production levels of 8-hydroxy-α-humulene with recombinant *E. coli* cells (for CYP71BA1) were 1.5- to 2.3-fold higher than that of a control strain without the mevalonate-pathway genes. Level of the P450NS product with the combination of NsRED and NsFER was 2.9-fold higher than that of the CamA and CamB. The predominant product of P450NS was identified as 1,2,3,5,6,7,8,8a-octahydro-6-isopropenyl-4,8a-dimethylnaphth-1-ol with NMR analyses.

Keywords Cytochrome P450 · NADPH-P450 reductase · Sesquiterpene · Germacrene A · Ginger

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Introduction

Sesquiterpenes that consist of three isoprene (C_5) units are isoprenoids with a huge structural diversity (>7,000 compounds), and have been isolated from a variety of natural sources (Sacchettini and Poulter 1997). They are synthesized from farnesyl diphosphate (farnesyl pyrophosphate;

FPP) with (sesqui)terpene synthases [also referred to as (sesqui)terpene cyclases] and subsequent processing enzymes, typically cytochromes P450 (P450s). Sesquiterpenes can exert important physiological functions in a wide range of organisms. For example, gossypol is produced as a phytoalexin in cotton in response to bacterial or fungal infections (Chen et al. 1995; Townsend et al. 2005). Artemisinin from annual wormwood *Artemisia annua* has been used as the anti-malarial drug (Enserink 2005). Zerumbone, which is abundantly contained in "shampoo ginger" (the tropical ginger; *Zingiber zerumbet* Smith), is a promising chemopreventive agent for suppressing atherosclerosis as well as HIV and tumor (Dai et al. 1997; Eguchi et al. 2007; Murakami et al. 2004). β-Eudesmol included in Chinese herbs was shown to be an antidote for organophosphate intoxication in mouse (Chiou et al. 1995). Understanding biosynthetic pathways of such functional sesquiterpenes via the incorporation of biosynthesis genes and enzymes is important not only for studying their physiological effects but also for their efficient production with heterologous host organisms. Many genes involved in sesquiterpene biosynthesis have been isolated from various organisms, including higher plants and prokaryotes such as actinomycetes and cyanobacteria, and functionally characterized (e.g., Agger et al. 2008; Ajikumar et al. 2008; Cane and Watt 2003; Daum et al. 2009; Dengenhardt et al. 2009; Muntendam et al. 2009; Portnoy et al. 2008; Wang et al. 2008). On the other hand, many of the genes or enzymes involved in sesquiterpene biosynthesis still remain unknown particularly in the plant kingdom generating a large variety of sesquiterpenes. It is typically difficult to examine catalytic functions of such enzymes (especially P450s), since resulting metabolites, which contain volatile compounds, are often not allowed to exist with amounts required for their structural identification through nuclear magnetic resonance (NMR) analyses. Engineering *E. coli* by the introduction of heterologous mevalonate-pathway genes was revealed to be an elegant approach for generating increased amounts of FPP as the substrate of a sesquiterpene synthase (Chang et al. 2007; Harada and Misawa 2009; Tsuruta et al. 2009). This approach enabled us to confirm the functions of novel sesquiterpene synthases, whose genes were isolated from higher plants (Fujisawa et al. 2010; Yu et al. 2008a, b). For example, with this efficient functional expression system, the *ZSS2* gene from shampoo ginger and the *ZoTPS1* gene from ginger (*Zingiber officinale* Roscoe) were identified as those coding for β-eudesmol synthase and (*S*)-β-bisabolene synthase, respectively (Fujisawa et al. 2010; Yu et al. 2008a). In the present study, we expanded such a system for sesquiterpene synthases into an efficient functional analysis system for P450s that may function as sesquiterpene monooxygenases.

Materials and methods

Plant material, bacterial strains, plasmid, and genetic manipulation

Seed rhizomes of ginger (*Z. officinale* Roscoe, Japanese cultivar "Kintoki") were kindly provided by Sakata Co. Ltd. (Kochi, Japan). *Arabidopsis thaliana* (L.) suspension-cultured cell line T87 was obtained from Riken Bioresource Center (Wako, Japan). *E. coli* DH5α and BL21 (DE3) [*ECOS* Competent *E. coli* DH5α and BL21 (DE3); Nippon Gene, Tokyo, Japan] were used as the hosts for DNA manipulations and for the functional expression of foreign genes, respectively. List of plasmids used or constructed in the present study is exhibited in supplementary information (Table S1). Plasmid pAC-Mev, which carries the 6.5-kb gene cluster for the mevalonate pathway isolated from *Streptomyces* sp. strain CL190 (accession no. AB037666; Harada et al. 2009; Takagi et al. 2000), is available from the corresponding author. Restriction enzymes and DNA-modifying enzymes were purchased from New England BioLabs (Beverly, CA, USA) or Takara Bio (Ohtsu, Japan). PCR amplifications were performed using PrimeSTAR® Max DNA Polymerase (Takara Bio) and a thermal cycler (Takara Bio). In-Fusion™ Advantage PCR Cloning Kit (Clontech, San Jose, CA, USA) was also used for gene cloning. Plasmid DNA was prepared with QIAprep Miniprep Kit (Qiagen, Hilden, Germany). Nucleotide sequences were confirmed by Bigdye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Foster city, CA, USA) and model 3730 DNA analyzer (Applied Biosystems). Other recombinant DNA techniques were conducted as described (Sambrook and Russell 2001).

Construction of plasmids carrying mevalonate-utilizing genes

Two genes encoding 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase and HMG-CoA synthase were removed from plasmid pAC-Mev (Harada et al. 2009) by digesting with *Sac*I and *Eco*RV. A partial DNA fragment of the IPP isomerase gene (*idi*; type 2) from *Streptomyces* sp. strain CL190 (Kaneda et al. 2001) was amplified by PCR using primers CL190idi-F and CL190idi-R described in Table 1, digested with *Sac*I and *Eco*RV, and ligated with the above plasmid fragment to generate plasmid pAC-Mv, in which the *idi* gene is regenerated. Two complementary oligonucleotides, MAPS1 and MAPS2 (Table 1), which contain multiple cloning site (*Eco*RV, *Mfe*I, *Asc*I, *Pac*I, *Spe*I, *Hin*dIII), were annealed and inserted into the *Eco*RV-*Hin*dIII site of pAC-Mv to generate plasmid pAC-Mv (MAPS).

Table 1 List of PCR primers used in this study

Primer name	Sequence (5′→3′)
CL190idi-F	ATCGTCAAGGAGGTCGGCAAC
CL190idi-R	TAGATATCTCATCGTGTGCTTCCCGTCG
MAPS1	ATC CAATTG GGCGCGCC TTAATTAA ACTAGT A
MAPS2	AGCTT ACTA GT TTAATTAA GGCGCGCC CAATTG GAT
camAB-F	AAAGATATC AGGAGG CAGCT **ATG** AACGCAAACGACAACG
camAB-R	AAACAATTGTTACCATTGCCTATCGGGAACAT
NsRED-F	AAAGATATC AGGAGG CAGCT **ATG** TCTAATCAAGGTGCTTTTGATG
NsRED-R	AAACAATTGTTAGTATGTTTCTACGTGCCAGC
NsFER-F	AAACAATTG AGGAGG CAGCT **ATG** GCAACCTTTAAAGTTACATT
NsFER-R	AATCTAGATTAGTAGAGGTCTTCTTCTTTGTGG
ATR2-F	AGTTTAAAGGAGGCAGCT**ATG**CGTCGCTCCGGTTCTGGGAATTCAAAACGTG
ATR2-R	AGCAATTGTTACCATACATCTCTAAGATATCTTCCACTG
RedC199-F	ACCGTGTTGTATTCCTAAATCGTGA
RedC199-R	AAAGAAAACACCAAGAGGAGGTTTG
RedP17-F	AGGTGCATCTACCCCATATACTGCT
RedP17-R	CTCCTTGTCAGCATGGATAGAGAAA
ZoRED1-F	ACACAATTG AGGAGG CAGCT **ATG** CAGCCCGGCGACGTT
ZoRED1-R	GGACTAGTTACCACACATCACGCAGG
ZoRED2-F	AAACAATTG AGGAGG CAGCT **ATG** CAGACGGGTTCCGAG
ZoRED2-R	GGACTAGTCACCATACATCTCTTAGGTATCTCC
ATR2-MfeI-F	GAGCAATTGGAGGAGATCCGGTTCTGG
ATR2-SalI-R	ACGCGTCGACTTACCATACATCTCTAAGATATC
NS1-F	CATGCC **ATG** GAAAAAATTACTTTCCCAA
NS1-R	AACTGCAGTCAAGAAGCCATTAATTCTAGTTT
ZSS1-F	AGGAGATATACC **ATG** GAGAGGCAGTCGATGG
ZSS1-R	ATGCGGCCGCAAGCTTAAATAAGAAAGGATTCAACAAATATG
Alr4686-F	AAGGAGATATACAT **ATG** AAATATCAAATACAGAGACCTAATC
Alr4686-R	GGTGCAGCACGAATTCTGCGTTGAATGTTGTTGAG
CYP71BA1-F	GGAATTCCAT **ATG** GAAGCTATTTCCCTCTTCTC
CYP71BA1-R	GGGGTACCTATGGCAGAGGAATTATTAGCTTG

Underlines and double underlines represent restriction enzyme sites and the artificial SD sequence, respectively. The start codons are shown in bold letters

Construction of plasmids carrying various redox partner genes in addition to the mevalonate-utilizing genes

The *camA* and *camB* (*camAB*) genes were amplified from genome DNA of *Pseudomonas putida* ATCC17453 by PCR using a specific primer set, camAB-F and camAB-R (Table 1). This fragment was digested with *Eco*RV and *Mfe*I, and ligated into the corresponding site of pAC-Mv (MAPS) to generate plasmid pAC-Mv camAB (Fig. 1a). The *NsRED* and *NsFER* genes were isolated from genome DNA of *Nostoc* (also referred to as *Anabaena*) sp. strain PCC 7120 by PCR using specific primer sets, NsRED-F and NsRED-R, and NsFER-F and NsFER-R, respectively (Table 1). These amplified fragments were respectively digested with *Eco*RV and *Mfe*I, and *Mfe*I and *Xba*I, and tandem ligated into the *Eco*RV-*Spe*I site of pAC-Mv (MAPS) to construct plasmid pAC-Mv NsRED NsFER (Fig. 1a). In these plasmids, the artificial Shine-Dalgarno (SD) sequence (AGGAGG) is designed to exist precedent to the start codon of each gene (except for *camB*), as shown in Table 1.

Total RNA was extracted from *A. thaliana* T87 cultured cells or young rhizomes of ginger, and subsequent cDNA synthesis was performed as described (Fujisawa et al. 2010). An N-terminally truncated form of the NADPH-P450 reductase 2 gene (*ATR2*; Hull and Celenza 2000; Urban et al. 1997) was isolated from the *A. thaliana* cDNA by PCR using a primer pair, ATR2-F including the SD sequence and ATR2-R (Table 1). This fragment was digested with *Dra*I and *Mfe*I, and ligated into the *Eco*RV-*Mfe*I site of pAC-Mv (MAPS) to generate plasmid pAC-Mv ATR2 (Fig. 1a). In order to isolate two NADPH-P450 reductase genes from ginger, 3′- and 5′-RACE methods were carried out with the cDNA using primer pairs RedC199-F and RedC199-R, and RedP17-F and RedP17-R (Table 1), which were designed based on EST contigs (accession no.

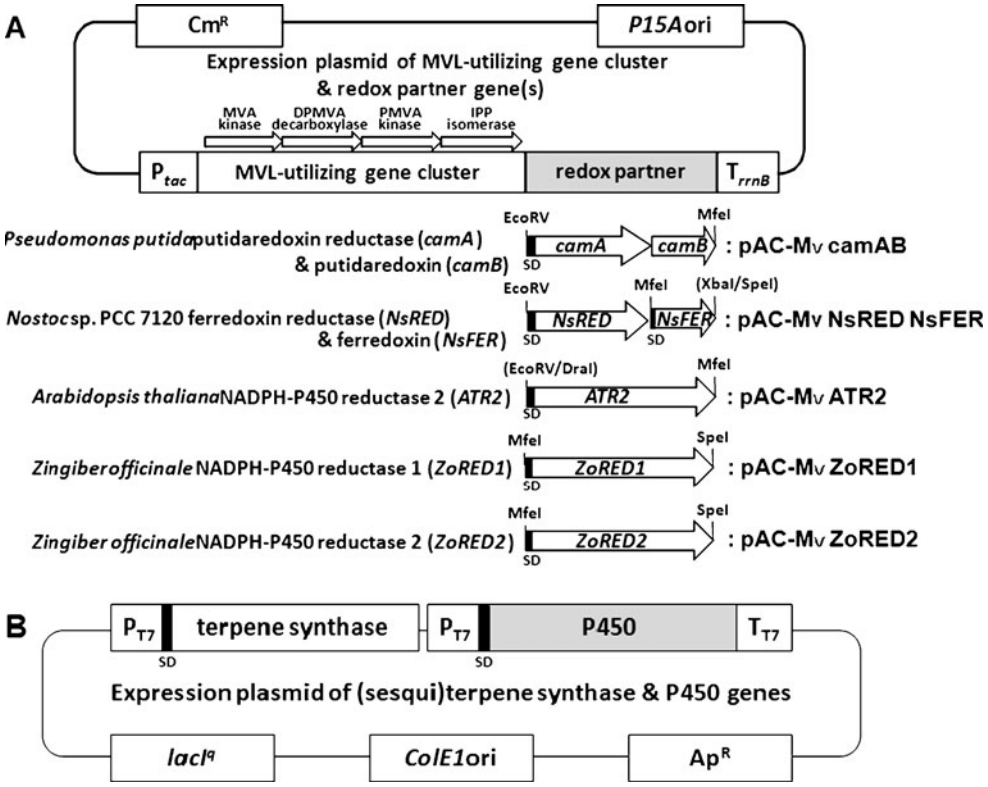

Fig. 1 Structure of plasmids constructed in this study. **a** MVA kinase, D-mevalonate kinase; DPMVA decarboxylase, diphosphomevalonate decarboxylase; PMVA kinase, phosphomevalonate kinase; IPP isomerase, isopentenyl diphosphate isomerase; P_{tac}, the *tac* promoter; T_{rrnB}, the *rrnB* terminator. **b** pET-NS1-P450NS and pET-ZSS1-CYP71BA1, respectively contain *NS1* and *ZSS1* as the sesquiterpene synthase genes, and *P450NS* and *CYP71BA1* as the P450 genes. The functions of the enzymes encoded with these genes are shown in Fig. 3. P_{T7}, the T7 promoter; T_{T7}, the T7 terminator

DY344852 and DY344854, and DY360384 and DY360385, respectively). Two complete genes (designated as *ZoRED1* and *ZoRED2*) were isolated by PCR with specific primer sets, ZoRED1-F and ZoRED1-R, and ZoRED2-F and ZoRED2-R, respectively (Table 1). The artificial SD sequence is also adjacently added to the start codon of each gene. These redox partner genes were digested with *Mfe*I and *Spe*I, and inserted into the corresponding site of pAC-Mv (MAPS) to construct plasmids pAC-Mv ZoRED1 and pAC-Mv ZoRED2, respectively (Fig. 1a).

A control plasmid for expressing only the truncated *ATR2* gene without the mevalonate-pathway genes was as follows: The truncated *ATR2* gene was amplified with the *A. thaliana* cDNA by PCR using primers ATR2-MfeI-F and ATR2-SalI-R (Table 1). This fragment was digested with *Mfe*I and *SalI*, and ligated into the *Eco*RI-*Sal*I site of vector pSTV28 (Takara Bio) to generate pSTV28-ATR2. This truncated *ATR2* gene was designed to be fused to the sequence encoding the 7 amino acid terminus from β-galactosidase (LacZ) in pSTV28.

Construction of plasmids carrying sesquiterpene synthase and P450 genes

Plasmids for expressing a sesquiterpene synthase gene and a P450 gene were constructed by inserting these two genes into two multiple cloning sites of vector pETDuet-1 (Novagen; Darmstadt, Germany). This vector contains the multiple cloning sites for the insertion of two genes, which are designed to have the transcriptional read-through from the T7 promoter, and includes artificial SD sequence precedent to the ATG of a foreign gene. Two sesquiterpene synthase genes, the *NS1*gene of *Nostoc* sp. strain PCC 7120 encoding germacrene A synthase (alr4685; Agger et al. 2008), and the *ZSS1* gene of *Z. zerumbet* encoding α-humulene synthase (Yu et al. 2008b), were amplified by PCR using primer pair sets, NS1-F and NS1-R, and ZSS1-F and ZSS1-R, respectively (Table 1). Similarly, two P450 genes, the *Nostoc* sp. strain PCC 7120 *P450NS* gene (*CYP110C1*; alr4686; Agger et al. 2008), and the *Z. zerumbet* α-humulene hydroxylase gene (*CYP71BA1*, accession no. AB331234; Yu et al. 2010), were amplified by PCR using primer pair sets, Alr4686-F and Alr4686-R, and CYP71BA1-F and CYP71BA1-R, respectively (Table 1). Plasmid pET-NS1-P450NS (Fig. 1b) was constructed by inserting the *Nco*I-*Pst*I *NS1* fragment and the *Nde*I-*Eco*RI *P450NS* fragment into the respective corresponding sites of vector pETDuet-1. Plasmid pET-ZSS1-CYP71BA1 (Fig. 1b) was constructed by inserting the *Nco*I-*Hin*dIII *ZSS1* fragment and the *Nde*I-*Kpn*I *CYP71BA1* fragment into the respective corresponding sites of pETDuet-1.

Production of hydroxylated sesquiterpenes in *E. coli*

E. coli BL21 (DE3) was co-transformed with a pETDuet-based plasmid (Fig. 2b) and a pAC-Mv-based plasmid

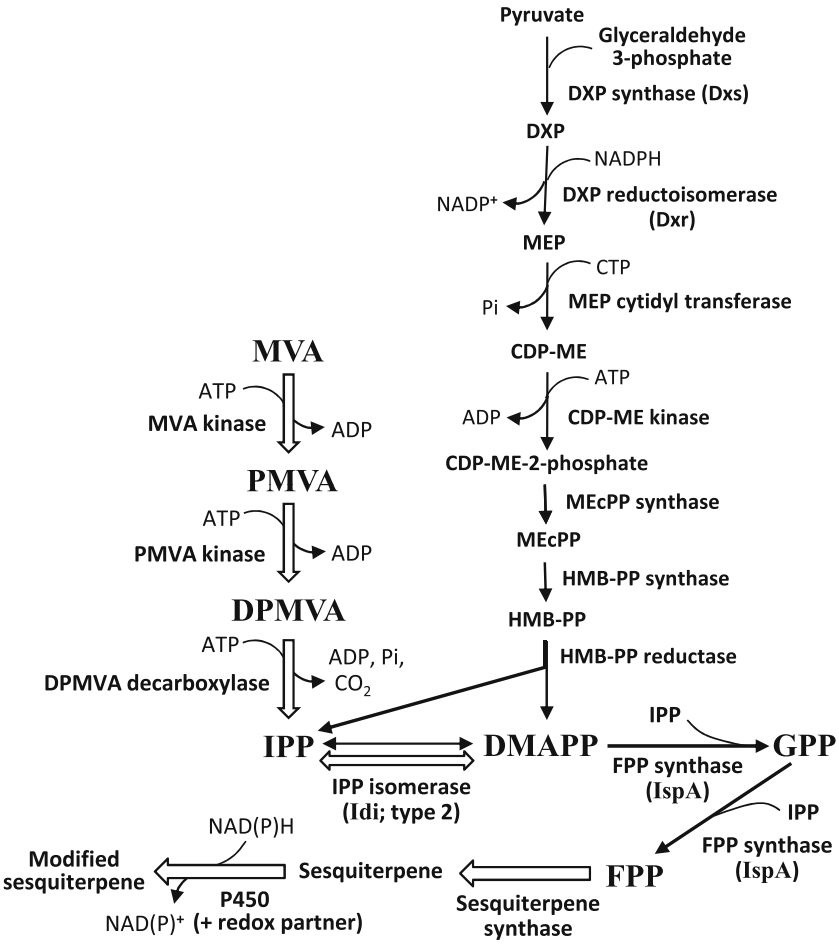

Fig. 2 Isoprenoid biosynthetic pathway in *E. coli* and biosynthetic routes reconstituted in this study. Enzymes encoded with genes introduced into *E. coli* are shown with *box arrows*. IPP and DMAPP are originally synthesized through the MEP pathway. DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; CDP-ME, CDP-2-*C*-methyl-D-erythritol; MEcPP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; HMB-PP, (*E*)-4-hydroxy-3-metyl-but-2-enyl diphosphate

(Fig. 2a). Transformed *E. coli* cells were inoculated into 3 mL of LB medium (Sambrook and Russell 2001) supplemented with ampicillin (Ap; 100 μg/mL) and chloramphenicol (Cm; 30 μg/mL), and precultured with agitation at 28 °C for 16 h. One percent (*v*/*v*) of preculture was then added into 10 mL of terrific broth (TB) medium (Sambrook and Russell 2001) containing the reagents of Overnight Express™ autoinduction system (Novagen), Ap (100 μg/

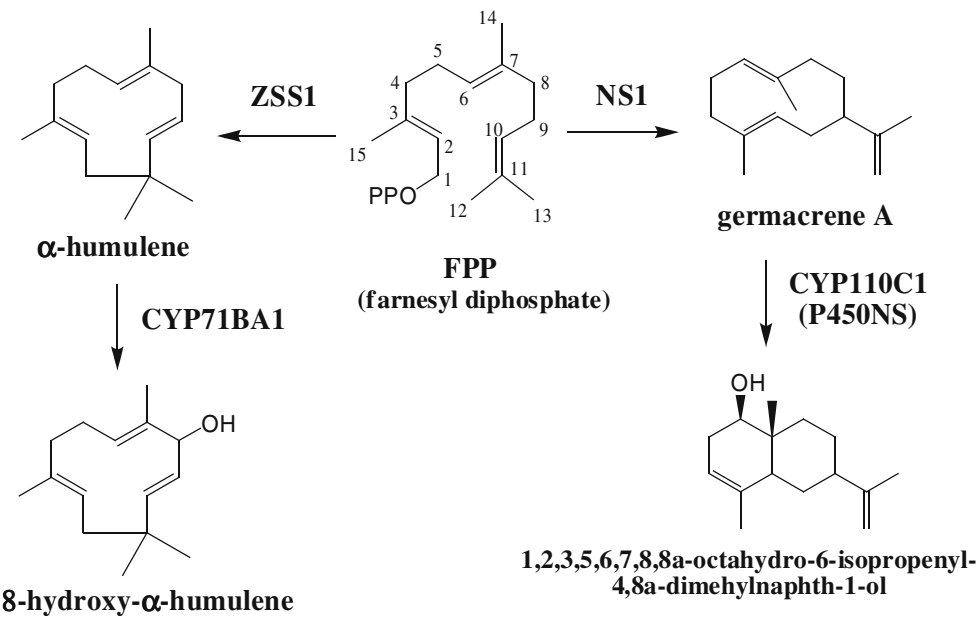

Fig. 3 Names and functions of gene products used in this study. The function of P450NS (CYP110C1) was identified in the present study

mL), Cm (30 μg/mL), D-mevalonolactone (D-mevalonic acid lactone; MVL; 0.5 mg/mL), 5-aminolevulinic acid (80 μg/mL) and $Fe(NH_4)_2(SO_4)_2$ $6H_2O$ (0.1 mM), and incubated at 37 °C until OD_{600} reached 0.8–1.0 (3–4 h). The culture was then incubated at 18 °C with agitation for 72 h. Alternatively, an IPTG induction method was carried out as follows: 1% (*v*/*v*) of preculture was inoculated into 10 mL of TB medium containing 0.025 mM of isopropyl-β-D-thiogalactopyranoside (IPTG), Ap (100 μg/mL), Cm (30 μg/mL), MVL (0.5 mg/mL), 5-aminolevulinic acid (80 μg/mL), and Fe $(NH_4)_2(SO_4)_2$ $6H_2O$ (0.1 mM), and incubated at 18 °C with agitation for 72 h.

After incubation, 2 mL of saturated NaCl and 4 mL of ethyl acetate were added to the culture, vortexed, and centrifuged to separate ethyl acetate and culture medium phases. The ethyl acetate phase was then concentrated and subjected to GC-MS analyses as described (Harada et al. 2009). For identification of a product by NMR analyses, large scale (1 L) culture was carried out at 18 °C with agitation for 72 h, by using the IPTG induction method.

Identification of the product (1)

High-resolution mass spectral data [HREI-MS (Jeol DX505W; Jeol, Tokyo, Japan) or HRAPCI-MS (Jeol JMS-T100LP)], and NMR spectral data (400 MHz, Bruker AMX400) were acquired to determine the structure of the product (1).

Physicochemical properties of the product (1)

The following are the physicochemical properties of the product (1): MS (EI) 220 (M^+). ^1H NMR ($CDCl_3$) δ: 1.03 (s, 3H, H-13), 1.20 (m, 1H, H-8b), 1.50–1.70 (2H, H-7), 1.60 (s, 3H, H-12), 1.67–1.70 (2H, H-2), 1.76 (s, 3H, H-11), 1.83 (m, 1H, H-5b), 2.00 (m, 1H, H-3), 2.05 (m, 1H, H-8a), 2.15 (m, 1H, H-3), 2.53 (dd, 1H, *J*=1.5, 12.0 Hz, H-5a), 3.42 (dd, 1H, *J*=6.2, 9.7 Hz, H-1), 4.72 (brs, 1H, H-10b), 4.73 (brs, 1H, H-10a). ^{13}C NMR ($CDCl_3$) δ: 17.3 (C-13), 19.1 (C-12), 20.8 (C-11), 27.1 (C-7), 27.3 (C-2), 30.9 (C-5), 31.9 (C-3), 38.9 (C-8), 39.7 (C-8a), 46.1 (C-6), 78.4 (C-1), 108.3 (C-10), 124.0 (C-4), 133.7 (C-4a), 150.5 (C-9).

Results

Construction of a tractable plasmid for utilization of D-mevalonate

Figure 2 shows biosynthetic pathway for basic structures of isoprenoids in *E. coli* and biosynthetic routes reconstituted in the present study. In order to confer the ability to utilize D-mevalonate (MVA) on *E. coli* and construct a tractable plasmid, two unnecessary genes encoding HMG-CoA reductase and HMG-CoA synthase were removed from the previously reported plasmid pAC-Mev (Harada et al. 2009), and the multiple cloning sites for *Mfe*I, *Asc*I, *Pac*I, and *Spe*I were inserted between the *Eco*RV and *Hin*dIII sites to generate plasmid pAC-Mv (MAPS). *E. coli* cells harboring this plasmid can supply enough amounts of FPP since high FPP synthase activity is constitutively observed in *E. coli* (data not shown; Fujisaki et al. 1990; Harada et al. 2009). Plasmid pAC-Mv (MAPS) is compatible with most of other *E. coli* vectors such as pET, pUC, and pBluescript due to the presence of the replication origin of vector pACYC184 (accession no. X06403).

Construction of plasmids for functional identification of P450s

In order to function as monooxygenase, usually a P450 must be associated with one or two additional protein(s) to transfer the electrons from NAD(P)H to the heme domain of a P450 protein. The vast majority of P450s that exist in prokaryotes, including cyanobacteria and actinomycetes require a FAD-containing ferredoxin reductase to receive the electrons from NAD(P)H and a ferredoxin, a small iron–sulfur protein, which in turn reduces P450 itself (called the bacterial class I system) (Hannemann et al. 2007). For the functional expression of such P450 genes, plasmids, designated pAC-Mv camAB and pAC-Mv NsRED NsFER (Fig. 1a), were constructed by inserting the putidaredoxin reductase (*camA*) and putidaredoxin (*camB*) genes from *P. putida* (Arisawa and Agematu 2007) and the ferredoxin reductase (*NsRED*) and ferredoxin (*NsFER*) genes from *Nostoc* sp. strain PCC 7120 (Agger et al. 2008) into the respective multipe cloning sites of the plasmid pAC-Mv (MAPS). Plant P450s invloved in the secondary metabolism requires a FAD and FMN-containing NADPH-P450 reductase to receive the electrons from NADPH and reduces P450 itself (called the plant class II system; Hannemann et al. 2007). For the functional expression of such plant P450 genes, three plasmids, designated pAC-Mv ATR2, pAC-Mv ZoRED1, and pAC-Mv ZoRED2 (Fig. 1a), were similarly constructed using the genes encoding the *A. thaliana* NADPH-P450 reductase 2 (*ATR2*) (Urban et al. 1997) and two *Z. officinale* NADPH-P450 reductases (designated *ZoRED1* and *ZoRED2*), respectively. The *ZoRED1* and *ZoRED2* genes were isolated from young rhizomes of ginger, Japanese cultivar "Kintoki," and their nucleotide sequences were deposited in DDBJ/GenBank/EMBL under accession nos. AB566408 and AB566409, respectively. Plasmids pAC-Mv ZoRED1 and pAC-Mv ZoRED2 contain full length of the *ZoRED1* and *ZoRED2* genes. On the other hand, in plasmid pAC-Mv ATR2, we adopted the truncated *ATR2* gene, in which the

predicted chloroplast transit peptide sequence and the proposed motif for anchoring to the endoplasmic reticulum membrane (72 amino acid terminus; Hull and Celenza 2000; Urban et al. 1997) were completely removed.

Functional expression of the *Z. zerumbet CYP71BA1* gene

Names and functions of sesquiterpene synthases and subsequent processing enzymes (P450s), which were used in the present study, are shown in Fig. 3 (Agger et al. 2008; Yu et al. 2008b, 2010). The P450 gene, which was isolated from *Z. zerumbet*, was reported to encode α-humulene hydroxylase (designated *CYP71BA1*; Yu et al. 2010). *E. coli* (DE3) carrying two plasmids pAC-Mv ATR2 and pET-ZSS1-CYP71BA1 synthesized 8-hydroxy-α-humulene (Fig. 3), which was identified by GC-MS analysis in comparison with the authentic sample (obtained from NARD Institute; Yu et al. 2010). In the present study, we compared the production efficiency in the cases that utilize three plasmids—pAC-Mv ATR2, pAC-Mv ZoRED1, and pAC-Mv ZoRED2—when each was co-expressed with plasmid pET-ZSS1-CYP71BA1 in *E. coli* (DE3). The autoinduction method and the IPTG induction method were also compared, as shown in Table 2. As a result, both the methods produced elevated amounts of α-humulene with similar efficiencies of more than 30 times that of a control without the mevalonate-pathway genes. However, the IPTG induction method was superior to the autoinduction method in the conversion of α-humulene to 8-hydroxy-α-humulene. In the IPTG induction method, production levels of *E. coli* carrying the pAC-Mv-based plasmids were 1.5- to 2.3-fold higher than that of the control (Table 2). Their GC-MS chromatographic and spectral data were shown in supplementary information (Fig. S1).

Functional expression of the *Nostoc* sp. strain PCC 7120 *P450NS* gene

Agger et al. (2008) characterized the catalytic functions of three cyanobacterial sesquiterpene synthases (NS1, NP1, NP2), which are derived from *Nostoc* sp. strain PCC 7120 or *Nostoc punctiforme* PCC 73102, using recombinat *E. coli* cells. Further, when the *Nostoc* sp. PCC 7120 *P450NS* gene (*CYP110C1*) was co-expressed with the *NS1* gene (identified as germacrene A synthase gene; Agger et al. 2008), which is present in the same cluster to *P450NS*, along with *NsRED* and *NsFER* in *E. coli*, the generated product was shown to possess the molecular weight (220) of +16 by GC-MS analysis while its chemical structure was not elucidated (Agger et al. 2008).

We compared production levels of two recombinant *E. coli* strains carrying plasmids pAC-Mv camAB and pAC-Mv NsRED NsFER in addition to pET-NS1-P450NS with the IPTG induction method. As a result, content of germacrene A was similar in both the strains (data not shown). On the other hand, the conversion ratio of this sesquiterpene substrate to the P450NS product, which was observed as a single peak at 41.72 min by GC-MS analyses, was 1.8% (for pAC-Mv camAB) and 5.2% (for pAC-Mv NsRED NsFER). Level of the P450NS product with the combination of NsRED and NsFER was 2.9-fold higher than that of the CamA and CamB.

Functional identification of the *Nostoc* sp. strain PCC 7120 P450NS product

An identification experiment of the P450NS product was performed using NsRED and NsFER to demonstrate efficiency in our functional analysis system. Purification of the product was performed from 1 L culture of *E. coli* (DE3) cells carrying plasmids pAC-Mv NsRED NsFER and pET-NS1-P450NS. The fermented broth was extracted with ethyl acetate (500 mL×2) without adjusting pH. The organic layer was concentrated to dryness to give colorless oil (352.8 mg). The oil was subjected to silica gel column chromatography (1×25 cm, Silica Gel 60, Kanto Chemical) using hexane-acetone (10:1). In this chromatography, the product (1) was purified as colorless oil (1.1 mg).

The molecular weight of 1 was proven to be 220 by EI-MS. The ^{1}H NMR of 1 in $CDCl_3$ showed 23 H signals,

Table 2 α-Humulene and 8-hydroxy α-humulene concentration using two different induction systems

Plasmid	Overnight Express™ autoinduction		IPTG induction	
	α-Humulene (mg- L^{-1})	8-Hydroxy-α-humulene (mg L^{-1})	α-Humulene (mg L^{-1})	8-Hydroxy-α-humulene (mg L^{-1})
pSTV28-ATR2	1.014±0.250 (1.0)	0.081±0.019 (1.0)	0.925±0.225 (1.0)	0.193±0.048 (1.0)
pAC-Mv ATR2	39.506±7.025 (39.0)	0.059±0.007 (0.7)	29.065±6.572 (31.4)	0.291±0.074 (1.5)
pAC-Mv ZoRED1	47.972±4.380 (47.3)	0.112±0.019 (1.4)	37.756±3.439 (40.8)	0.443±0.046 (2.3)
pAC-Mv ZoRED2	33.764±5.578 (33.3)	0.042±0.024 (0.5)	28.620±8.228 (30.9)	0.405±0.120 (2.1)

Sesquiterpenes were extracted from recombinant *E. coli* cells carrying respective plasmids in addition to plasmid pET-ZSS1-CYP71BA1 and analyzed with GC-MS. Values in parentheses represent the ratio to sesquiterpene contents extracted from a control strain without the mevalonate-pathway genes (pSTV28-ATR2 and pET-ZSS1-CYP71BA1). Each value is shown as mean ± SE (n=3)

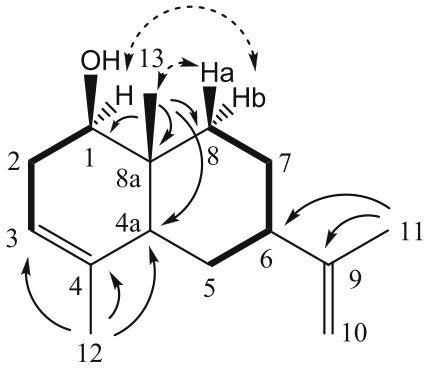

Fig. 4 Structure of 1,2,3,5,6,7,8,8a-octahydro-6-isopropenyl-4,8a-dimethylnaphth-1-ol (1). Key correlations in ^{1}H-^{1}H COSY (*bold lines*), HMBC (*arrows*), and NOESY (*dashed arrows*) spectra were shown

including δ 3.42 (OCH, H-1), and ^{13}C NMR of 1 showed 15 signals including δ 78.4 (OCH, C-1). From these results, the molecular formula of 1 was determined to be $C_{15}H_{24}O$.

Analyses of the HMQC and ^{1}H-^{1}H COSY spectra of 1 proved two ^{1}H-^{1}H vicinal spin net works of OCH (δ 3.42, C-1)–CH_2 (δ 1.67–1.70, C-2)–CH_2 (δ 2.00 and δ 2.15, C-3) and CH_2 (δ 1.83 and δ 2.53, C-5)–CH (δ 1.83, C-6)–CH_2 (δ 1.50–1.70, C-7)–CH_2 (δ 1.20 and δ 2.05, C-8) (Fig. 4).

HMBC experiment on 1 proved the ^{1}H-^{13}C long range coupling from H-9 (δ 1.60) to C-3 (δ 31.9), C-4 (δ 124.0), and C-4a (δ 133.7), and from H-13 (δ 1.03, CH_3) to C-1 (δ 78.4), C-4a, C-8 (δ 38.9), and C-8a (δ 39.7) (Fig. 4). From these results, the presence of 1-hydroxy-4, 8a-dimethyl-4,4a-dehydro-decalin structure in 1 was established. The HMBC experiment also showed the ^{1}H-^{13}C long range coupling from H-11 (δ 1.76) to C-6 (δ 46.1), C-9 (δ 150.5), and C-10 (δ 108.3) (Fig. 4). Thus, an attachment of isopropenyl side chain at C-6 was proven, and the total structure of 1 was determined as shown in Fig. 4. The IUPAC name of 1 is 1,2,3,5,6,7,8,8a-octahydro-6-isopropenyl-4,8a-dimethyl-naphth-1-ol. The OH function at C-1 was clarified to be *equatorially* orientation by the large coupling constant of $J_{1,2}$ (9.7 Hz). The NOEs observed between H-1 and H-8b (δ 1.20), and between H-13 and H-8a (δ 2.05) proved that 1-OH and C-13 (CH_3) were in the same location (Fig. 4). The presence of 1 in cascarilla oil was reported previously, and the reported ^{1}H spectral data were identical to those of 1 (Hagedorn and Brown 1991). Thus, P450NS was identified to synthesize 1 as the main product from germacrene A.

Discussion

CamA and CamB from *P. putida* have been shown to exert broad adaptability to the bacterial class I P450s as their redox partners in *E. coli* cells (Agematu et al. 2006; Arisawa and Agematu 2007; Fujita et al. 2009; Girhard et al. 2009). In the present study, this redox partner pair was also shown to couple with the cyanobacterial P450NS, although its efficiency was inferior to that of the NsRED and NsFER pair from cyanobacterium *Nostoc* sp. strain PCC 7120. P450 genes belonging to this class have been functionally expressed in *E. coli* not only using such plasmids carrying the *camAB* genes, but also using pRED vector (Fujita et al. 2009; Nodate et al. 2006; Otomatsu et al. 2010; Li et al. 2007). The latter vector utilized the redox region derived from the *Rhodococcus* sp. NCIMB 9784 self-sufficient P450, P450RhF (CYP116B2), which contained ferredoxin reductase and ferredoxin domains (Nodate et al. 2006; Roberts et al. 2002). In the present study, plasmid pAC-Mv NsRED NsFER, which contains the cyanobacterial *NsRED* and *NsFER* genes, was added to the above functional expression plasmids for the class I P450 genes in *E. coli*. Using this plasmid system, cyanobacterial P450NS (CYP110C1) was identified as germacrene A hydroxylase, which synthesizes 1 as the

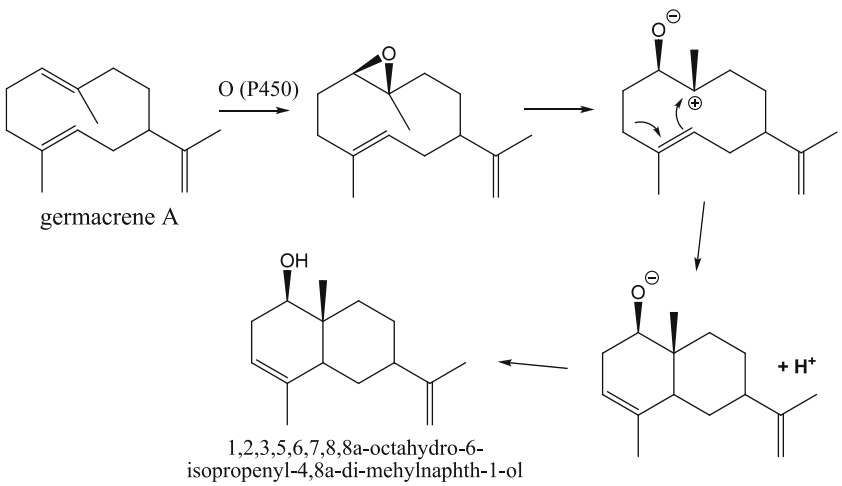

Fig. 5 Putative biosynthetic pathway from germacrene A to 1,2,3,5,6,7,8,8a-octahydro-6-isopropenyl-4,8a-dimethylnaphth-1-ol (1)

predominant product. Putative biosynthetic pathway from germacrene A to this product is shown in Fig. 5.

In the present study, ATR2, ZoRED1, and ZoRED2 were shown to function similarly as NADPH-P450 reductases towards CYP71BA1 in *E. coli* cells, i.e., the relative amounts of the CYP71BA1 product were 1 (for ATR2), 1.52 (for ZoRED1), and 1.39 (for RED2) while ZoRED1 is likely to be the best redox partner for this P450. ZoRED1 and ZoRED2 originated from the same genus (*Zingiber*) to the origin of CYP71BA1 while the genus *Arabidopsis* (the origin of ATR2) is distant from *Zingiber*. Further experiments should be necessary to demonstrate the adaptability of the *ATR2* genes as well as *ZoRED1* and *ZoRED2* towards functional identification of various P450 genes belonging to the plant class II system.

Functional identifications of many P450s belonging to this class II have been performed using yeast *Saccharomyces cerevisiae*, since it possesses the endogenous NADPH-P450 reductases (e.g., Seki et al. 2008). On the other hand, extensive molecular genetic resources are present for *E. coli* and it is amenable to genetic manipulation. Moreover, this bacterium itself does not generate sesquiterpenes and other typical terpenes, and never possesses an endogenous P450. Whereas, the endogenous flavodoxin reductase and flavodoxin in *E. coli* were shown to work well with PtlI (CYP183A1) involved in pentalenolactone (sesquiterpene) biosynthesis from *Streptomyces avermitilis* (Quaderer et al. 2006). Such characteristics of *E. coli* should simplify functional analysis of a foreign P450 involved in (sesqui)terpene biosynthesis. Chang et al. (2007) demonstrated that *E. coli* can be genetically engineered to produce high levels of 8-hydroxycadinene and artemisinic acid (>100 mg L^{-1}) using a series of heterologous genes that include plant P450 genes and a redox partner gene from *Candida tropicalis*. The *E. coli* system, which includes our five pAC-Mv-based plasmids as the parts (Fig. 1a), is likely to work as an efficient functional analysis system for a wide range of P450s that may function as sesquiterpene monooxygenases, while further improvements should be needed in culture conditions and plasmid design.

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