Appl Microbiol Biotechnol
DOI 10.1007/s00253-017-8409-z

Pathway engineering for the production of β-amyrin and cycloartenol in *Escherichia coli*—a method to biosynthesize plant-derived triterpene skeletons in *E. coli*

Miho Takemura[1] · **Rie Tanaka**[1] · **Norihiko Misawa**[1]

Received: 18 January 2017 / Revised: 20 June 2017 / Accepted: 27 June 2017

Abstract Cycloartenol is biosynthetically the first sterol skeleton, which is metabolized to phytosterols such as β-sitosterol and stigmasterol. β-Amyrin is the most commonly occurring aglycone skeleton for oleanane-type saponins such as glycyrrhizin and saikosaponins. It has been regarded that these cyclic triterpenes are unable to be produced in *Escherichia coli*, while no reports are available on their production with *E. coli*. Here, we describe a method to synthesize triterpene skeletons from higher plants, including cycloartenol and β-amyrin. We introduced into *E. coli* the biosynthetic pathway genes from farnesyl diphosphate (FPP) to cycloartenol or β-amyrin, which contained Arabidopsis (*Arabidopsis thaliana*)-derived squalene synthase (*AtSQS*) and squalene epoxidase (*AtSQE*) genes in addition to the Arabidopsis cycloartenol synthase (*AtCAS1*) gene, or the β-amyrin synthase (*EtAS*) gene of the petroleum plant *Euphorbia tirucalli*, along with the isopentenyl diphosphate isomerase (*HpIDI*) gene from a green algae *Haematococcus pluvialis*. The order of genes, *HpIDI*, *AtSQS*, *AtSQE*, driven by transcriptional read-through from a *tac* promoter to an *rrnB* terminator, was crucial for their functional expression in *E. coli* to produce cycloartenol or β-amyrin. The co-expression of a bacterial NADPH-regenerating gene (*zwf* or *gdh*) as well as bacterial redox partner protein genes (*camA* and *camB*, or *NsRED* and *NsFER*) was found to increase the amounts of these triterpenes several fold. The present study could open up opportunities not only to carry out functional analysis of a higher-plant-derived oxidosqualene cyclase (*OSC*) gene in *E. coli* but also to produce functional triterpenes that originate from medicinal or herbal plants.

✉ Norihiko Misawa
n-misawa@ishikawa-pu.ac.jp

[1] Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi-shi, Ishikawa 921-8836, Japan

Keywords β-Amyrin · Cycloartenol · Triterpene biosynthesis · *Escherichia coli* · Pathway engineering

Introduction

Terpenes, also referred to as terpenoids or isoprenoids, are a series of compounds composed of C_5 isoprene units, and they constitute the vast group of more than 50,000 structurally different compounds, which have been isolated not only from the plant kingdom but also from animals and microbial species (Roberts 2007; Suzuki et al. 2014). The biosynthetic routes to terpenes are regularly and "simply" organized, despite their enormous structural diversity (Misawa 2011). All terpenes are initially biosynthesized from isopentenyl diphosphate (IPP; C_5) and its isomer dimethylallyl diphosphate (DMAPP; C_5). This isomerization step is catalyzed by IPP isomerase (IDI). IPP and DMAPP are supplied by two independent pathways, the mevalonate (MVA) pathway by way of D-mevalonate, and the methylerythritol 4-phosphate (MEP) pathway by way of 2-C-methyl-D-erythritol 4-phosphate (also referred to as the non-mevalonate pathway) (Ajikumar et al. 2008; Muntendam et al. 2009). The MVA pathway exists in the cytosol of eukaryotes and in some of actinobacteria and archaea, whereas the MEP pathway is present in prokaryotes and in the plastids (chloroplasts and chromoplasts) of plants (Kirby and Keasling 2009; Wu et al. 2006). DMAPP is typically condensed with each molecule of IPP sequentially to yield geranyl diphosphate (GPP; C_{10}), farnesyl diphosphate (FPP; C_{15}), and geranylgeranyl diphosphate (GGPP; C_{20}) by GPP synthase, FPP synthase, and GGPP synthase (generally called prenyl transferases), respectively (Kurokawa and Koyama 2010). In the case of *Escherichia coli*, the MEP pathway is used to synthesize IPP and DMAPP simultaneously, which are synthesized from their common precursor 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-

Published online: 15 July 2017

diphosphate (HMBPP) by HMBPP reductase (Jiang et al. 2012). IDI (Idi) converts excess IPP to DMAPP. The synthesis of FPP in *E. coli* occurs through sequential condensation of two molecules of IPP to DMAPP, which is catalyzed by only one enzyme, FPP synthase, encoded by the *ispA* gene (Fujisaki et al. 1990).

Terpenes typically contain monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), and carotenoids (C_{40}). These biosynthetic pathways are summarized in Fig. 1. Terpene synthases (TPSs; also called terpene cyclases) release diphosphate from GPP, FPP, or GGPP, and the remaining carbonated forms are intra-molecularly cyclized to generate ring-skeletal diversity (Degenhardt et al. 2009). Subsequent terpene-modifying enzymes, particularly cytochrome P450s, and further tailoring enzymes often metabolize the generated cyclic terpene skeletons, yielding a huge variety of monoterpenes, sesquiterpenes, or diterpenes (Ajikumar et al. 2010; Dewick 2009; Ginglinger et al. 2013; Misawa 2011). The biosynthesis of triterpenes starts from the condensation reaction of two molecules of FPP to synthesize squalene by squalene synthase (SQS) (Kushiro and Ebizuka 2010) (Fig. 1). This linear hydrocarbon is the first triterpene, which is predominantly converted to (3*S*)-2,3-oxidosqualene by squalene epoxidase (SQE). Next, the multigene family of oxidosqualene cyclases (OSCs), which are represented with β-amyrin synthase (AS), dammarendiol II synthase (DS), cycloartenol synthase (CAS), and lanosterol synthase (LAS), generate various cyclized forms of triterpene alcohols via cations (Augustin et al. 2011; Husselstein-Muller et al. 2001; Kajikawa et al. 2005; Kushiro et al. 1998; Sawai et al. 2006; Suzuki et al. 2006; Tansakul et al. 2006). Cytochrome P450s and subsequent modifying enzymes such as UDP-glycosyltransferases, are responsible for the production of a large diversity of triterpenes (saponins) (Dewick 2009; Seki et al. 2008; Seki et al. 2011). On the other hand, carotenoid biosynthesis is in contrast to that of the other terpenes: Phytoene is generated from two molecules of GGPP by phytoene synthase, and are in turn dehydrogenated to form conjugated double bonds without cyclization to generate a linear carotene (hydrocarbon carotenoid), usually lycopene (Misawa 2010) (Fig. 1). Lycopene is frequently cyclized and subjected to a series of modifications to form a variety of xanthophylls (oxygen-containing carotenoids), in which non-heme oxygenases are often involved (Fraser and Bramley 2004; Misawa 2010).

Terpenes can exert a wide range of physiological functions in natural conditions, including communication between different organisms, and their beneficial effects on human health have been applied extensively to pharmaceuticals (e.g., artemisinin and α-santonin, sesquiterpenes; paclitaxel and ginkgolide, diterpenes), herbal medicines (e.g., glycyrrhizin, platycodin D, saikosaponin a, and ginsenosides; triterpenes), nutraceuticals or functional foods (e.g., astaxanthin, lutein, and lycopene; carotenoids), and flavors or fragrances (e.g., L-menthol, D-limonene, L-linalool, D-citronellol, and geraniol, monoterpenes; D-nerolidol, and farnesol, sesquiterpenes) (Augustin et al. 2011; Dewick 2009; Fraser and Bramley 2004). However, many such functional terpenes are present in minute quantities in nature or at low yields from their natural sources, which has hindered their widespread industrial use. The chemical synthesis approach of these metabolites has been hampered because of high cost due to their structural complexity, although it was successful for some monoterpenes and carotenoids (Kusama et al. 2000; Jackson et al. 2008). Previous work on increased production through traditional breeding approaches has met with limited success. Pathway engineering, i.e., to engineer biosynthetic pathways for compounds of interest in heterologous organisms, should offer an alternative and promising approach for the economical production of such terpenes (Kirby and Keasling 2009; Misawa 2011). Two representative microbial hosts for terpene production are *E. coli* (Carter et al. 2003; Harada et al. 2009; Immethun et al. 2013; Jiang et al. 2012; Lemuth et al. 2011; Misawa and Shimada 1998; Morrone et al. 2010; Sandmann 2002) and *Saccharomyces cerevisiae* (Engels et al. 2008; Immethun et al. 2013; Jiang et al. 2012; Kirby et al. 2008; Rico et al. 2010; Sun et al. 2016; Yamano et al. 1994). These microorganisms, specifically the former, are fully amenable to genetic manipulation and with extensive molecular resources. *E. coli* is also likely to possess considerable solvent resistance (Girhard et al. 2009; Schewe et al. 2009). Pathway engineering approaches in *E. coli* have been performed for the efficient production of monoterpenes, sesquiterpenes, and diterpenes, as well as carotenoids (Ajikumar et al. 2010; Harada and Misawa 2009; Immethun et al. 2013; Jiang et al. 2012; Kirby et al. 2015; Lemuth et al. 2011; Misawa 2011). Acyclic triterpene squalene was shown to be efficiently produced in *E. coli* (Ghimire et al. 2009; Katabami et al. 2015). However, for cyclic triterpenes production, *E. coli* has been an inadequate and unsuccessful host, despite the industrial importance of these compounds (Ajikumar et al. 2008; Li et al. 2016; Suzuki et al. 2014). The present study describes a new method to produce cyclic triterpenes in *E. coli*, which are catalyzed by OSCs.

Materials and methods

Bacterial strains and genetic manipulation

Escherichia coli DH5α was utilized as the host for DNA manipulations. *E. coli* BL21 (DE3) were used for the functional expression of plasmids in the present study. PCR amplifications were performed using PrimeSTAR GXL DNA Polymerase (Takara Bio, Ohtsu, Japan). All recombinant DNA experiments were carried out in accordance with the suppliers' instructions or Sambrook and Russell (2001).

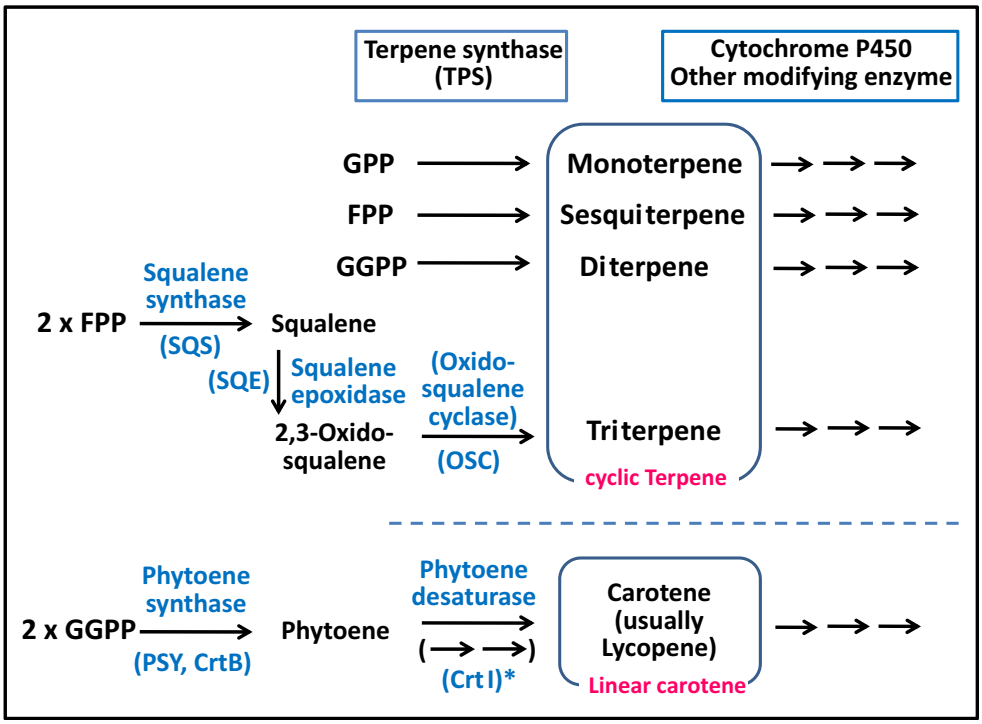


Fig. 1 Biosynthesis system of monoterpenes, sesquiterpenes, diterpenes, triterpenes, and carotenoids (tetraterpene) from prenyl diphosphates. *The route from phytoene to lycopene is mediated by CrtI in bacteria (excluding cyanobacteria) and fungi, or by PDS, CRTISO, ZDS, and Z-ISO in plants

Construction of plasmids for expressing triterpene biosynthesis genes

A base expression vector for *E. coli*, named pAC-PtacTrrnB, was constructed as follows: The *tac* artificial promoter (accession no. E02582) and the *E. coli rrnB* terminator (accession no. V00348) were amplified by polymerase chain reaction (PCR) using plasmid pAC-Mev (Harada et al. 2009) as the template. These fragments were inserted into the chloramphenicol resistance gene of the cloning vector pACYC184 (Rose 1988; accession no. X06403), yielding pAC-PtacTrrnB with the tetracycline resistance gene. The coding region of the *Haematococcus pluvialis IDI* gene (Kajiwara et al. 1997; *HpIDI*, accession no. AB019034) was amplified by PCR with plasmid pACHP-Beta (Takemura et al. 2015) as the template. The Arabidopsis (*Arabidopsis thaliana*) *SQS* (*AtSQS*, accession no. ATU79159) and *SQE* (*AtSQE*, accession no. AY099643) genes were amplified by PCR using Arabidopsis leaf complementary DNA (cDNA) as the template. The amplified *HpIDI*, *AtSQS*, and *AtSQE* genes were inserted into pAC-PtacTrrnB simultaneously in this order. The resultant plasmid was named pAC-HpIDI/AtSQS/SQE (Fig. 2a). Plasmid pAC-HpIDI/AtSQE/SQS (Fig. 2a) was also constructed, which contained *HpIDI*, *AtSQE*, and *AtSQS* in this order. In these plasmids, the artificial Shine-Dalgarno (SD) sequence (AGGAGG) was designed to precede the start codon of each gene. The gene-specific primers used in this study are listed in Table 1.

The coding regions of the *A. thaliana CAS* gene (*AtCAS1*, accession no. U02555) and the AS gene (accession no. AB206469) from the petroleum plant *Euphorbia tirucalli* were amplified by PCR with Arabidopsis stem cDNA and pPICZA-EtAS (Kajikawa et al. 2005) as the templates, respectively. These fragments were individually cloned into the pETD vector (Merck Millipore, Darmstadt), creating plasmids pETD-AtCAS1 and pETD-EtAS (Fig. 2b), respectively.

Construction of plasmids carrying redox partner protein genes or a NADPH-regenerating enzyme gene

As redox partner protein genes, we used the putidaredoxin reductase (*camA*) and putidaredoxin (*camB*) genes from *Pseudomonas putida* belonging to the class γ-Proteobacteria (*camAB*, accession no. PSECAMABA), the ferredoxin reductase (*NsRED*) and ferredoxin (*NsFER*) genes from cyanobacterium *Nostoc* sp. strain PCC7120 (*NsRED/NsFER*, accession no. WP_010998287 and NP_488161), and the ferredoxin reductase (*SoFNR*) and ferredoxin I (*SoFER*) genes from spinach (*Spinacia oleracea*) (SoFNR/FER, accession no. X07981 and SPIFERRI). The *A. thaliana* NADPH-P450 reductase 2 gene (*ATR2*, *AtATR2*, accession no. AF325101) was also used as a redox partner protein gene. The *camAB*, *NsRED/NsFER*, and *AtATR2* coding regions were amplified by PCR with pAC-MvcamAB, pAC-MvNsREDNsFER, and pAC-MvATR2 as the respective templates (Harada et al. 2011). The SoFNR/FER fragment was digested from the plasmid pET-SoFNR/FER containing the spinach ferredoxin reductase (*SoFNR*) and ferredoxin I (*SoFER*) genes (a gift from T. Otomatsu) using *Spe*I and *Not*I. The PCR fragments or digested fragment were inserted into the multiple cloning sites

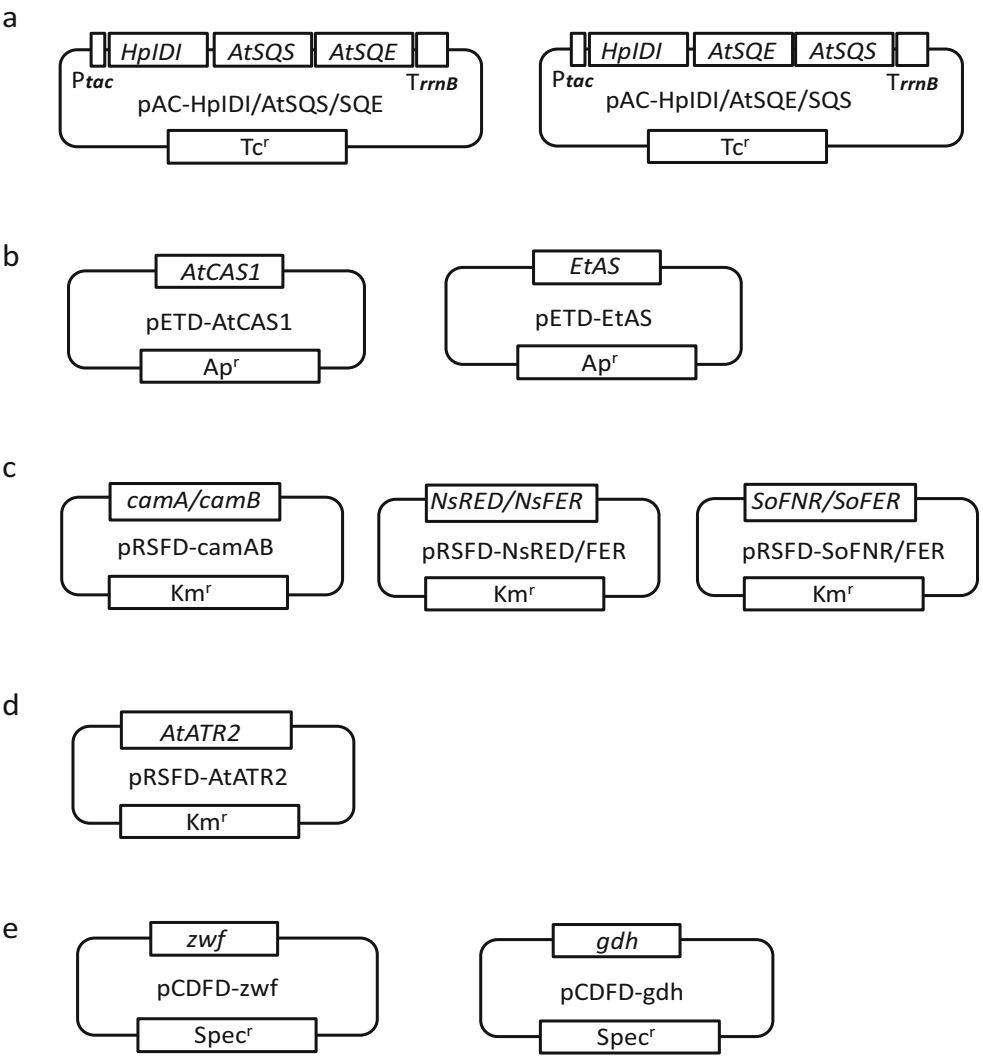


Fig. 2 Plasmids constructed and used in the present study. **a** Base plasmids for 2,3-oxidosqualene synthesis. *Tc*^{r}, tetracycline resistance gene. **b** Plasmids for the expression of each *OSC* gene. *Ap*^{r}, ampicillin resistance (β-lactamase) gene. **c** Plasmids for the supply of ferredoxin reductase and ferredoxin as redox partner proteins. *Km*^{r}, kanamycin resistance gene (*nptII*). **d** Plasmid for the supply of NADPH-P450 reductase as a redox partner protein. **e** Plasmids for the supply of NADPH by NADPH regeneration system. *Spec*^{r}, spectinomycin resistance gene (*aadA*)

of a pRSFDuet vector (Merck Millipore, Darmstadt) to create pRSFD-camAB, pRSFD-NsRED/FER, pRSFD-SoFNR/FER (Fig. 2c), and pRSFD-AtATR2 (Fig. 2d).

In order to introduce the NADPH-regenerating system, we used genes encoding glucose 6-phosphate dehydrogenase from *E. coli* (*zwf*, accession no. ECU13788) and glucose dehydrogenase from *Bacillus subtilis* (*gdh*, accession no. EF626962). These coding regions were amplified from genomic DNA of *E. coli* and *B. subtilis* as the templates, respectively, and cloned into a pCDF vector (Merck Millipore, Darmstadt). The resulting plasmids were named pCDFD-zwf and pCDFD-gdh (Fig. 2e), respectively.

Cultivation of recombinant *E. coli* and extraction of generated triterpenes

E. coli cells transformed with several plasmids were grown in 2× YT medium (Sambrook and Russell 2001) containing 10 mg/L tetracycline, 40 mg/L ampicillin, 40 mg/L kanamycin, and 100 mg/L spectinomycin as needed, at 37 °C with reciprocal shaking for 3–4 h until the absorbance at OD 600 nm had reached approximately 0.8–1.0. Then, 0.05 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added, and culturing at 21 °C was continued for 2 days.

The cultures were centrifuged and bacterial pellets were collected. Chloroform/methanol (2:1) solution was added, and mixed by vortexing for 10 min. After centrifugation, the organic phase was transferred to another tube. This extraction was repeated three times, and all organic phases were collected into one tube. These extracts were evaporated, and dissolved in methanol.

Gas chromatography–mass spectrometry analysis of the *E. coli* extracts

A 3-μL aliquot of the solution was subjected to gas chromatography–mass spectrometry (GC-MS) analysis as described previously (Kajikawa et al. 2005). In brief, we used

Table 1 Primers used in this study

Primer	Sequence
AtSQSF	CGGATCCAGGAGGCAGCTATGGGGAGCTTGGGGACG
AtSQSR	TGGTACCCAGATCTTCAGTTTGCTCTGAGATA
AtSQEF	CGGATCCAGGAGGCAGCTATGGAGTCACAATTATGG
AtSQER	TGGTACCCAGATCTCTATGAACATTTGGTTTC
HpidiF	ACTCGAGGAGGCAGCTATGCTTCGTTCGTTGCTC
HpidiR	AGGTACCCAGATCTCACGCTTCGTTGATGTG
AtCAS1F	CGGATCCATGTGGAAACTGAAGATC
AtCAS1R	CGTCGACTCATTCTCCTTGTTGCAA
EtASF	CCATGGGGAAGCTGAAGATAG
EtASR	AAGCTTTTAAAGAGTAGTGGAAGG
AtATR2F	CCATGGGGAGGAGATCCGGTTCTG
AtATR2R	GGATCCTTACCATACATCTCTAAG
camABF	CCATGGACGCAAACGACAACGTGG
camABR	GGATCCTTACCATTGCCTATCGGG
NsRED/FERF1	CCATGGCTAATCAAGGTGCTTTTG
NsRED/FERR1	GGATCCTTAGTAGAGGTCTTCTTC
zwfF	GCCATGGCGGTAACGCAAACAG
zwfR	TGCGGCCGCTTACTCAAACTCATTCCAG
gdhF	GCCCATGGATCCGGATTTAAAAGG
gdhR	AGTCGACTTAACCGCGGCCTGCCTG

a GCMS-QP2010 (Shimadzu, Kyoto, Japan) equipped with DB-5ms column (φ0.25 mm × 30 m, 0.25 μm film thickness) (JW Scientific, Folsom, CA). The column temperature was maintained at 50 °C for 1 min, elevated to 300 °C at a rate of 20 °C min^{-1}, and held at 300 °C for 15 min. The ionization was operating at 70 eV with a scan range of 45–500 Da. Quantification was performed using standard calibration lines constructed for each analysis.

Authentic samples of cycloartenol and β-amyrin were purchased from Extrasynthese (Lyon, France) and Sigma-Aldrich (MO, USA), respectively.

Results

Construction of plasmids for triterpene production in *E. coli*

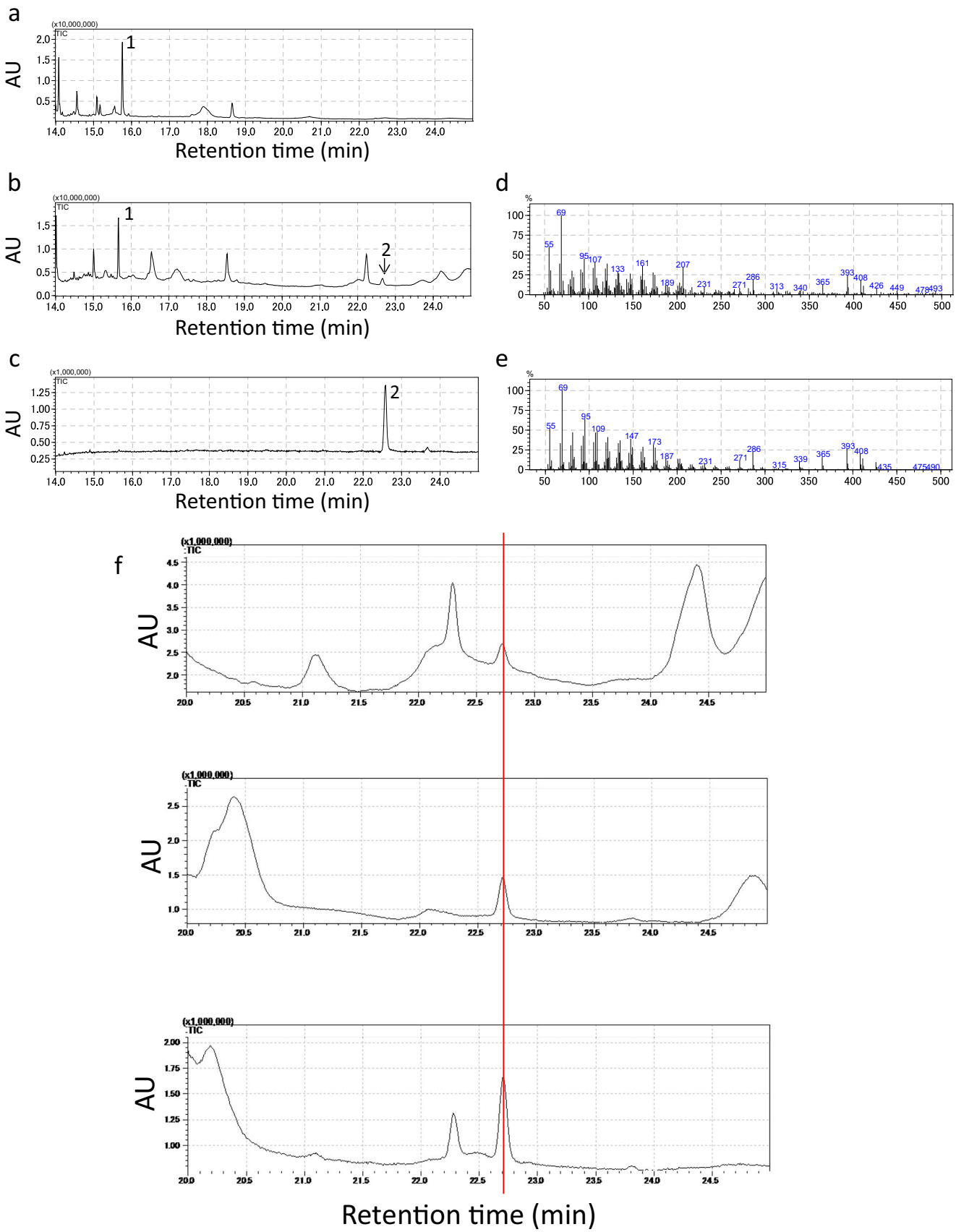
In order to produce (3*S*)-2,3-oxidosqualene, which is the most commonly used substrate for cyclic triterpene skeletons, we selected the *Arabidopsis*-derived *SQS* (*AtSQS*) and *SQE* (*AtSQE*) genes. The *IDI* gene (*HpIDI*) encoding IPP isomerase from the green algae *H. pluvialis* was further added to efficiently increase FPP levels as the substrate of SQS (Kajiwara et al. 1997). Consequently, we obtained plasmids pAC-HpIDI/AtSQS/SQE and pAC-HpIDI/AtSQE/SQS (Fig. 2a).

Previous studies suggested that SQE needs ferredoxin reductase and ferredoxin as redox partner proteins for its activity (Sakakibara et al. 1995; Nagumo et al. 1995). Therefore, we constructed plasmids to produce such electron transport proteins. We selected three distinct sets of ferredoxin reductase and ferredoxin genes, i.e., *camA/camB* (*camAB*) from *P. putida* (Agematsu et al. 2006; Fujita et al. 2009), NsRED/NsFER from *Nostoc* sp. strain PCC7120 (Agger et al. 2008), and SoFNR/SoFER from spinach *S. oleracea*, resulting in pRSFD-camAB, pRSFD-NsRED/FER, and pRSFD-SoFNR/FER (Fig. 2c). A plasmid, pRSFD-AtATR2, containing the NADPH-P450 reductase 2 (*ATR2*, *AtATR2*) gene from *A. thaliana*, was also created for comparison (Fig. 2d).

Furthermore, we selected two NADPH-regenerating enzyme genes, i.e., the *E. coli* glucose 6-phosphate dehydrogenase (*zwf*) gene and the *B. subtilis* glucose dehydrogenase (*gdh*) gene to supply sufficient levels of NADPH, yielding plasmids pCDFD-zwf and pCDFD-gdh, respectively (Fig. 2e).

Cycloartenol biosynthesis in *E. coli*

GC-MS analysis of the *E. coli* cells carrying plasmid pAC-HpIDI/AtSQS/SQE (or pAC-HpIDI/AtSQE/SQS) and pETD vector showed only the peak of squalene at 15.7 min (peak 1; Fig. 3a). A peak for 2,3-oxidosqualene was not detected. We considered the possibility that terpenes, which include the epoxy group in the molecules, are often unstable, and they may be easily degraded or converted to other compounds


a
(x10,000,000)
TIC
AU
1
Retention time (min)
b
(x10,000,000)
TIC
AU
1
2
Retention time (min)
c
(x1,000,000)
TIC
AU
2
Retention time (min)
d
%
69
55
95
107
133
161
189
207
231
271
286
313
340
365
393
408
426
449
478
493
e
%
69
55
95
109
147
173
187
231
271
286
315
339
365
393
408
435
475
490
f
(x1,000,000)
TIC
AU
(x1,000,000)
TIC
AU
(x1,000,000)
TIC
AU
Retention time (min)

◀ **Fig. 3** Cycloartenol production in recombinant *E. coli*. **a** Chromatogram of an extract from *E. coli* (pAC-HpIDI/AtSQS/SQE and pETD); **b** chromatogram of an extract from *E. coli* (pAC-HpIDI/AtSQS/SQE and pETD-AtCAS1); **c** chromatogram of authentic cycloartenol. **d** MS spectrum of peak 2 in **b**; **e** MS spectrum of peak 2 in **c**. Peak 1, squalene; peak 2, cycloartenol. **f** Identification of peak 2. *Upper*, chromatogram of an extract from *E. coli* (pAC-HpIDI/AtSQS/SQE and pETD-AtCAS1) (corresponding to **b**); *middle*, chromatogram of authentic cycloartenol (corresponding to **c**); *lower*, chromatogram of a mixture of the extract (**b**) and authentic standard (**c**). *Red line* represents the position of peak 2 (color figure online)

(Araki et al. 2016). Thus, we decided to express *OSC* genes simultaneously, which utilize 2,3-oxidosqualene as a substrate, in the above recombinant *E. coli*, and first selected the *AtCAS1* gene among other *OSCs*. The extract from *E. coli* cells that harbored pAC-HpIDI/AtSQS/SQE and pETD-AtCAS1 (Fig. 2b) was analyzed by GC-MS. The result indicated the occurrence of a new small peak at 22.7 min (peak 2 in Fig. 3b), which showed the same retention time as that of the authentic cycloartenol (peak 2 in Fig. 3c). The MS spectrum of this new peak 2 (Fig. 3d) closely resembled that of the authentic standard (Fig. 3e). Moreover, the two peaks (peak 2 in Fig. 3b, c) were observed to overlap completely by GC (Fig. 3f). These results indicated that cycloartenol is produced in the recombinant *E. coli*. This was an unexpected result, because SQE was thought not to work in *E. coli* without the electron transport proteins. On the other hand, the *E. coli* cells, which harbored the other plasmid pAC-HpIDI/AtSQE/SQS along with pETD-AtCAS1, were not found to generate any peak of cycloartenol, indicating that the order of the *HpIDI*, *AtSQS*, and *AtSQE* genes, driven by transcriptional read-through from the *tac* promoter to the *rrnB* terminator, is crucial for their functional expression in *E. coli* to produce cycloartenol. Figure 4 shows routes from FPP to cycloartenol as well as functions of the biosynthesis genes used in the present study.

β-Amyrin biosynthesis in *E. coli*

We next tried to produce the most important and commonly occurring cyclic triterpene, β-amyrin, by introducing pETD-EtAS (Fig. 2b) instead of pETD-AtCAS1 into *E. coli* cells that contained pAC-HpIDI/AtSQS/SQE. GC-MS analysis of the cells of *E. coli* (pAC-HpIDI/AtSQS/SQE and pETD-EtAS) showed a new peak at 22.0 min, which had the same retention time as that of β-amyrin (peak 2; Fig. 5b, c). The MS spectrum of this new peak 2 (Fig. 5d) coincided to that of the authentic β-amyrin (Fig. 5e). The two peaks (peak 2 in Fig. 5b, c) were also found to overlap completely by GC (data not shown). These results indicated that β-amyrin is produced in the recombinant *E. coli* without the introduction of any co-factors. Figure 4 shows routes from FPP to β-amyrin.

Both in Fig. 3 (cycloartenol production) and Fig. 5 (β-amyrin production), several extra peaks appeared in the GC chromatograms, e.g., peaks at 22.3 min in Fig. 3b and 22.2 min in Fig. 5b, which are not present in the control. The MS spectra of these peaks did not correspond to those of any known triterpenes in the database, which included 2,3-oxidosqualene and other cyclic triterpenes such as lanosterol and dammarenediol II (data not shown).

Effects of electron transport proteins and NADPH-regenerating enzymes

We examined the effect of the co-expression of each set of electron transport (redox partner) protein genes, *camAB*, *NsRED/NsFER*, *SoFNR/SoFER*, or *AtATR2*, for comparison, and each NADPH-regenerating enzyme gene (*gdh* or *zwf*) in the cycloartenol- or β-amyrin-synthesizing recombinant *E. coli* cells. The results are shown in Fig. 6. When only the NADPH-regenerating enzyme genes were introduced into the cycloartenol-producing *E. coli* cells, their productivities were not affected (g, z in Fig. 6a). The introduction of the *SoFNR/FER* or *AtATR2* gene into the triterpene-producing cells did not show any effects on productivity (S, A in Fig. 6a, b), whereas that of *camA/camB* (*camAB*) or *NsRED/NsFER* tended to slightly increase production levels (c, N in Fig. 6a, b). On the other hand, the co-expression of the NADPH-regenerating enzyme gene, *gdh* or *zwf*, and two sets of the ferredoxin reductase and ferredoxin genes, *camAB* or *NsRED/NsFER*, improved the productivity of the triterpene

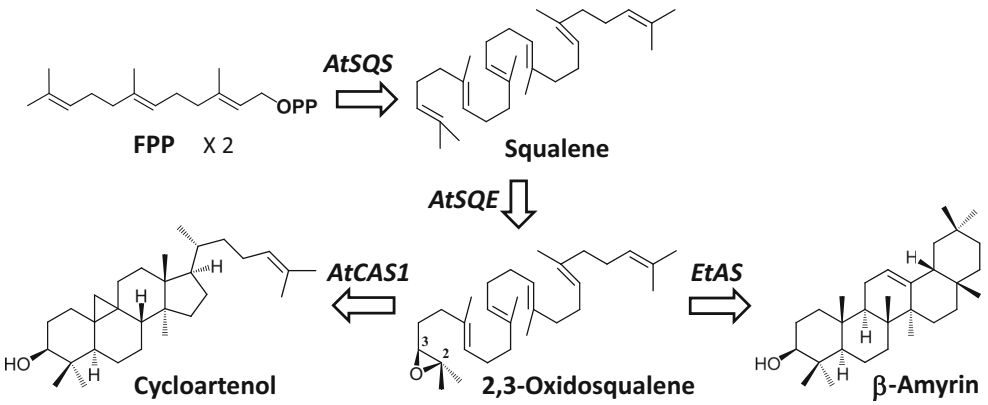


Fig. 4 Pathway from FPP to cycloartenol or β-amyrin and functions of the triterpene biosynthesis genes used in the present study

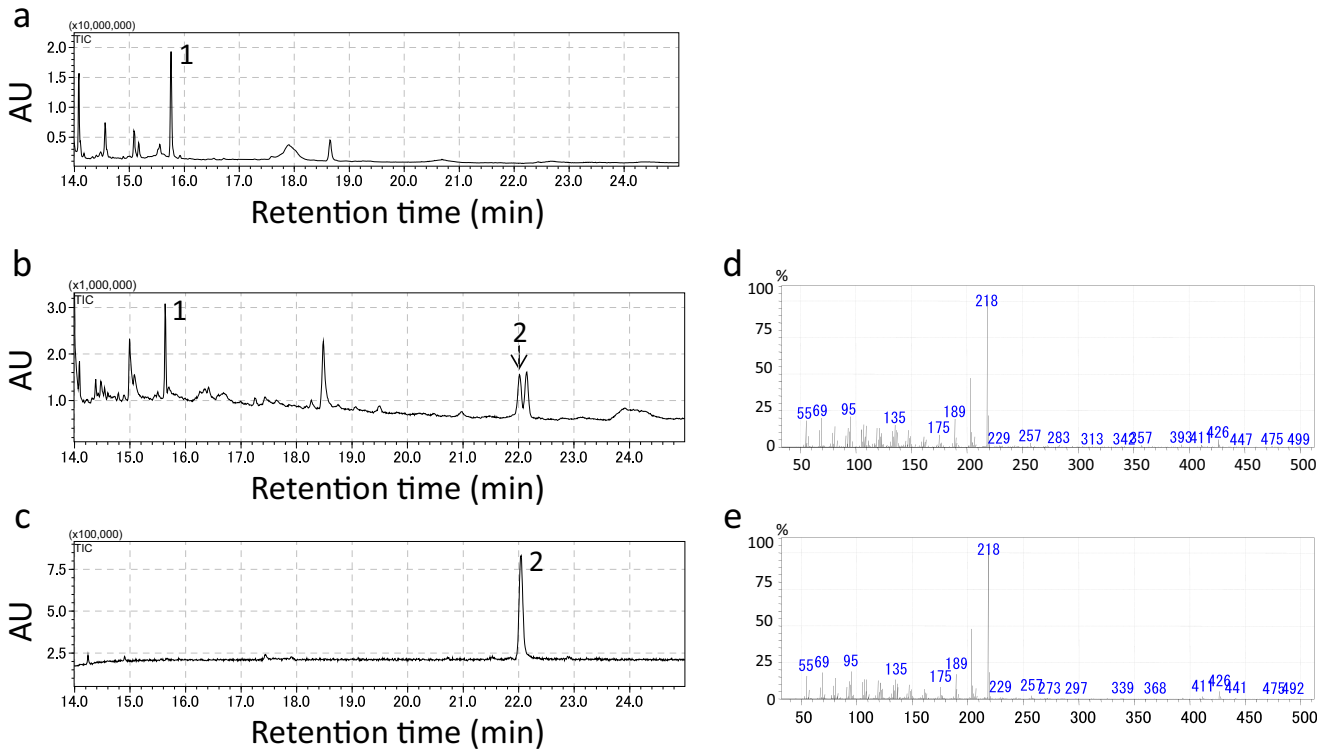


Fig. 5 β-Amyrin production in recombinant *E. coli*. **a** Chromatogram of an extract from *E. coli* (pAC-HpIDI/AtSQS/SQE and pETD). **b** Chromatogram of an extract from *E. coli* (pAC-HpIDI/AtSQS/SQE and pETD-EtAS). **c** Chromatogram of authentic β-amyrin. **d** MS spectrum of peak 2 in **b**. **e** MS spectrum of peak 2 in **c**. Peak 1, squalene; peak 2, β-amyrin

up to several times (g/c, g/N, z/c, z/N in Fig. 6a, b). Meanwhile, neither SoFNR/FER nor AtATR2 had any effects, even with the NAPDH-regenerating enzymes (g/S, g/A, z/S, z/A in Fig. 6a, b). Figure 7 shows a feasible scheme of electron transport from NADPH to the substrate squalene via SQE enzyme.

Discussion

All eukaryotes can produce sterols: in animals and plants, and yeasts and fungi, sterols such as cholesterol and ergosterol are biosynthesized by way of lanosterol, which is cyclized from 2,3-oxidosqualene by lanosterol synthase (LAS) (Dewick 2009). In plants, phytosterols, such as β-sitosterol and stigmasterol, are predominantly biosynthesized via cycloartenol, which is converted from 2,3-oxidosqualene by cycloartenol synthase (CAS). On the other hand, bacteria cannot synthesize sterols, although a methanotroph *Methylococcus capsulatus*, which belongs to the γ-Proteobacteria in the same class as *E. coli*, can produce lanosterol efficiently (Nakano et al. 2007). It is thought that the corresponding fungal genes were transferred into this bacterium by lateral gene transfer. Meanwhile, bacteria including *Zymomonas mobilis*, *Alicyclobacillus acidocaldarius*, and cyanobacteria produce triterpenes via hopene, which is converted directly from squalene not via 2,3-oxidosqualene by squalene-hopene cyclase (Kannenberg and Poralla 1999). Recently, Li et al. (2016) reported the biosynthesis of a triterpene skeleton dammarenediol II in engineered *E. coli*. They introduced the *M. capsulatus SQE* gene into *E. coli* that synthesizes squalene, along with the dammarenediol II synthase (*DS*) gene and the *S. cerevisiae*-derived NADPH-P450 reductase (*CPR*) gene or *Arabidopsis*-derived NADPH-P450 reductase 1 (*ATR1*, *AtATR1*; *CPR*) gene. They also reported that without each *CPR* gene, 2,3-oxidosqualene was not biosynthesized. However, our results clarified that at least *Arabidopsis* SQE (AtSQE) works without the introduction of any other cofactors in *E. coli*. It is likely that further co-expression of the *HpIDI* gene in addition to *AtSQE* increased FPP levels and enabled visible triterpene production.

The effects of several electron transport (redox partner) proteins and NADPH-regenerating enzymes were investigated in the present study. All of them were individually not able to show significant effects on triterpene productivity. Meanwhile, simultaneous introduction of the electron transfer protein and NADPH-regenerating enzyme genes increased the productivity of the triterpenes. As NADPH-regenerating enzymes, Zwf and Gdh showed similar effects on enhancing the levels of the triterpenes. With regard to the redox partner proteins, CamA/CamB (CamAB) and NsRED/NsFER were found to cause similar enhancements of the levels of the

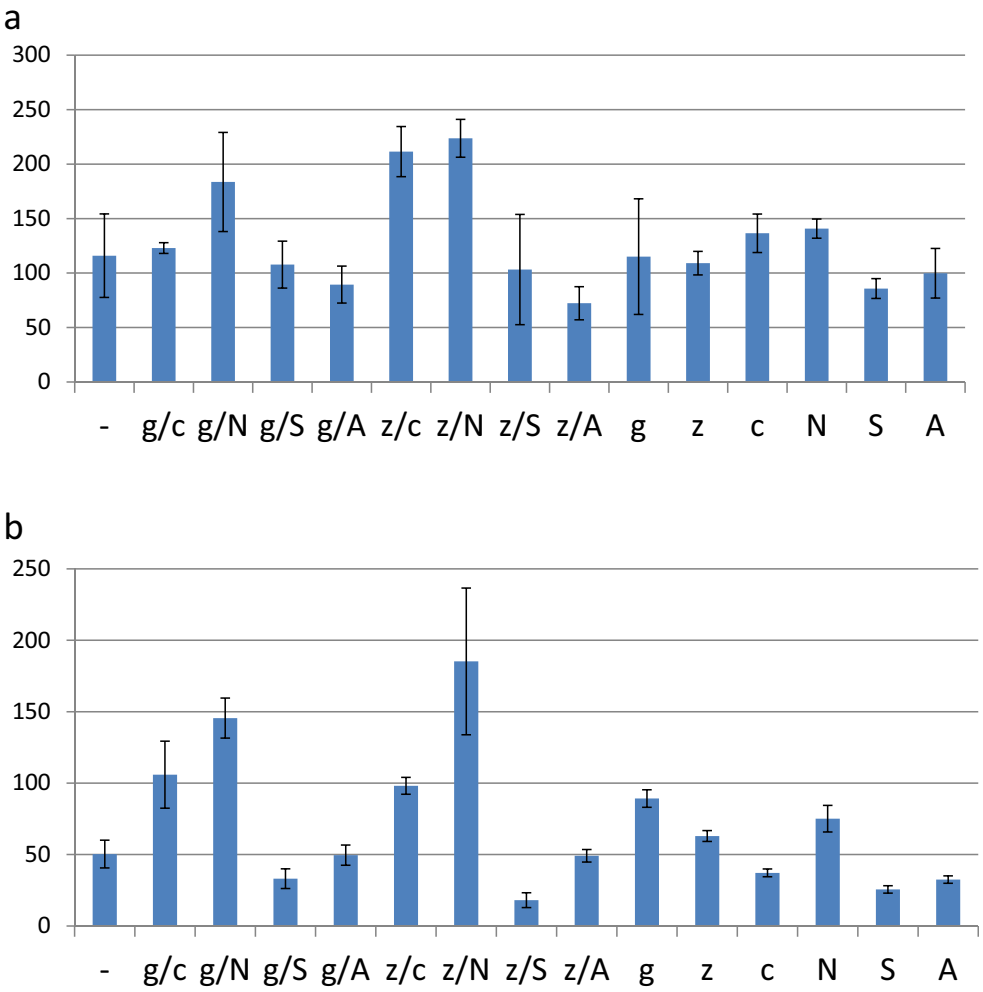


Fig. 6 Effect of further introduction of each set of redox partner protein genes (*camAB*, *NsRED/FER*, or *SoFNR/FER*, or *AtATR2*) and each NADPH-regenerating enzyme gene (*gdh* or *zwf*). **a** Productivity of cycloartenol [μg/g FCW (fresh cell weight)]. **b** Productivity of β-amyrin (μg/g FCW). *g*, *gdh*; *z*, *zwf*; *c*, *camAB*; *N*, *NsRED/FER*; *S*, *SoFNR/FER*; *A*, *AtATR2*. Results are the mean ± SD of the three experiments

triterpenes, whereas the *S. oleracea* FNR/FER system and *Arabidopsis* NADPH-P450 reductase 2 (AtATR2) were unsuitable as the electron-transferring partner protein(s) to AtSQE (Fig. 7). On the other hand, Li et al. (2016) reported that the functional expression of the *AtATR1* gene was effective to the productivity of the *M. capsulatus* SQE, which seems contradictory to our results. Anyway, these results suggest that compatibility between SQE and electron transport proteins is important, and that the addition of a NADPH-regenerating enzyme is effective for increasing triterpene productivity in *E. coli*. Figure 7 shows a feasible scheme of electron transport from NADPH to the substrate squalene via squalene epoxidase (SQE) as a monooxygenation enzyme, which was proposed based on the present results and previous reports (Sakakibara et al. 1995; Nagumo et al. 1995).

E. coli and *S. cerevisiae* have been utilized as two representative microbial hosts for the efficient production of terpenes, as described in the "Introduction" section. It has been regarded that *S. cerevisiae* is a better host than *E. coli* for the functional expression of cytochrome P450 genes involved in terpene biosynthesis, since *E. coli* possesses no P450 genes. However, recent advance on this field enabled functional

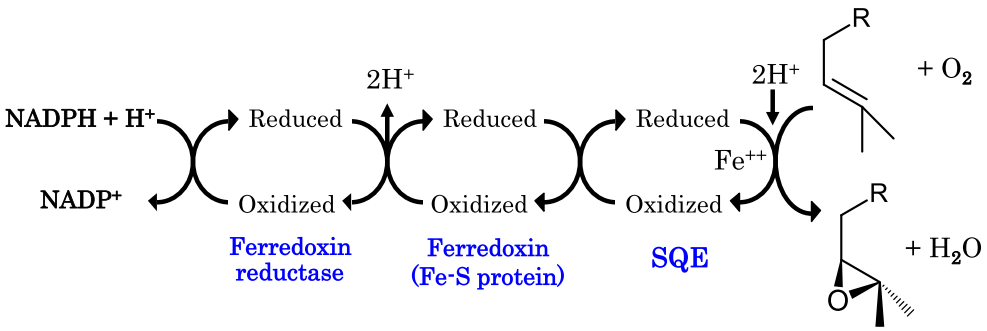


Fig. 7 Feasible scheme of electron transport from NADPH to the substrate squalene via squalene epoxidase (SQE; monooxygenase)

expression of many P450 genes in *E. coli*, e.g., it was found that co-expressing a truncated *AtATR2* gene is often effective to the activation of a P450 of interest (Harada et al. 2011; Jiang et al. 2012). Thus, it would be realized in *E. coli* in the near future to produce P450 products from cyclic triterpenes that were biosynthesized by OSCs.

In the present study, for the first time, we showed that a plant-original *SQE* is functional in *E. coli*, and that two types of important cyclic triterpenes, cycloartenol and β-amyrin, could be produced in *E. coli* cells. This could open up opportunities not only to carry out functional analysis of a higher plant-derived oxidosqualene cyclase (*OSC*) genes in *E. coli* but also to produce functional triterpenes that originate from medicinal or herbal plants.

Acknowledgements The authors thank Mr. Toshihiko Otomatsu, KNC Laboratories Co., Ltd., for the gift of plasmid pET-SoFNR/FER.

Compliance with ethical standards

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

References

Agematu H, Matsumoto N, Fujii Y, Kabumoto H, Doi S, Machida K, Ishikawa J, Arisawa A (2006) Hydroxylation of testosterone by bacterial cytochromes P450 using the *Escherichia coli* expression system. Biosci Biotechnol Biochem 70:307–311

Agger SA, Lopez-Gallego F, Hoye TR, Schmidt-Dannert C (2008) Identification of sesquiterpene synthases from *Nostoc punctiforme* PCC 73102 and *Nostoc* sp. strain PCC7120. J Bacteriol 190:6084–6096

Ajikumar PK, Tyo K, Carlsen S, Mucha O, Phon TH, Stephanopoulos G (2008) Terpenoids: opportunities for biosynthesis of natural product drugs using engineered microorganisms. Mol Pharm 5:167–190

Ajikumar PK, Xiao WH, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. Science 330:70–74

Araki M, Kaku N, Harada M, Ando Y, Yamaguchi R, Shindo K (2016) Production of auroxanthins from violaxanthin and 9-*cis*-violaxanthin by acidic treatment and the antioxidant activities of violaxanthin, 9-*cis*-violaxanthin, and auroxanthins. J Agric Food Chem 64:9352–9355

Augustin JM, Kuzina V, Andersen SB, Bak S (2011) Molecular activities, biosynthesis and evolution of triterpenoid saponins. Phytochemistry 72:435–457

Carter OA, Peters RJ, Croteau R (2003) Monoterpene biosynthesis pathway construction in *Escherichia coli*. Phytochemistry 64:425–433

Degenhardt J, Köllner TG, Gershenzon J (2009) Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochemistry 70:1621–1637

Dewick PM (2009) Medicinal natural products a biosynthetic approach Third Edition. A Wiley

Engels B, Dahm P, Jennewein S (2008) Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (paclitaxel) production. Met Eng 10:201–206

Fraser PD, Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res 43:228–265

Fujisaki S, Hara H, Nishizima Y, Horiuchi K, Nishino T (1990) Cloning and nucleotide sequence of the *ispA* gene responsible for farnesyl diphosphate synthase activity in *Escherichia coli*. J Biochem 108: 995–1000

Fujita N, Sumisa F, Shindo K, Kabumoto H, Arisawa A, Ikenaga H, Misawa N (2009) Comparison of two vectors for functional expression of a bacterial cytochrome P450 gene in *Escherichia coli* using *CYP153* genes. Biosci Biotechnol Biochem 73:1825–1830

Ghimire GP, Lee HC, Sohng JK (2009) Improved squalene production via modulation of the methylerythritol 4-phophate pathway and heterologous expression of genes from *Streptomyces peucetius* ATCC 27952 in *Escherichia coli*. Appl Environ Microbiol 75:7291–7293

Girhard M, Machida K, Itoh M, Schmid RD, Arisawa A, Urlacher VB (2009) Regioselective biooxidation of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. Microb Cell Factories 8:36

Ginglinger JF, Boachon B, Hofer R, Paetz C, Kollner TG, Miesch L, Lugan R, Baltenweck R, Mutterer J, Ullmann P, Beran F, Claudel P, Verstappen F, Fischer MJ, Karst F, Bouwmeester H, Miesch M, Schneider B, Gershenzon J, Ehlting J, Werck-Reichhart D (2013) Gene coexpression analysis reveals complex metabolism of the monoterpene alcohol linalool in Arabidopsis flowers. Plant Cell 25:4640–4657

Harada H, Misawa N (2009) Novel approaches and achievements in biosynthesis of functional isoprenoids in *Escherichia coli*. Appl Microbiol Biotechnol 84:1021–1031

Harada H, Shindo K, Iki K, Teraoka A, Okamoto S, Yu F, Hattan J, Utsumi R, Misawa N (2011) Efficient functional analysis system for cyanobacterial or plant cytochromes P450 involved in sesquiterpene biosynthesis. Appl Microbiol Biotechnol 90:467–476

Harada H, Yu F, Okamoto S, Kuzuyama T, Utsumi R, Misawa N (2009) Efficient synthesis of functional isoprenoids from acetoacetate through metabolic pathway-engineered *Escherichia coli*. Appl Microbiol Biotechnol 81:915–925

Husselstein-Muller T, Schaller H, Benveniste P (2001) Molecular cloning and expression in yeast of 2,3-oxidosqualene-triterpenoid cyclases from *Arabidopsis thaliana*. Plant Mol Biol 45:75–92

Immethun CM, Hoynes-O'Connor AG, Balassy A, Moon TS (2013) Microbial production of isoprenoids enabled by synthetic biology. Front Microbiol 4:75 (1-8)

Jackson H, Braun CL, Ernst H (2008) The chemistry of novel xanthophyll carotenoids. Am J Cardiol 101:50D–57D

Jiang M, Stephanopoulos G, Pfeifer BA (2012) Toward biosynthetic design and implementation of *Escherichia coli*-derived paclitaxel and other heterologous polyisoprene compounds. Appl Environ Microbiol 78:2497–2504

Kajikawa M, Yamato KT, Fukuzawa H, Sakai Y, Uchida H, Ohyama K (2005) Cloning and characterization of cDNA encoding β-amyrin synthase from petroleum plant *Euphorbia tirucalli* L. Phytochemistry 66:1759–1766

Kajiwara S, Fraser PD, Kondo K, Misawa N (1997) Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. Biochem J 324:421–426

Kannenberg EL, Poralla K (1999) Hopanoid biosynthesis and function in bacteria. Naturwissenschaften 86:168–176

Katabami A, Li L, Iwasaki M, Furubayashi M, Saito K. Umeno D (2015) Production of squalene by squalene synthases and their truncated mutants in *Escherichia coli*. J Biosci Bioeng 119:165–171

Kirby J, Keasling JD (2009) Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annu Rev Plant Biol 60:335–355

Kirby J, Nishimoto M, Chow RWN, Baidoo EEK, Wang G, Martin J, Schackwitz W, Chan R, Fortman JL, Keasling JD (2015) Enhancing terpene yield from sugars via novel routes to 1-deoxy-D-xylulose 5-phosphate. Appl Environ Microbiol 81:130–138

Kirby J, Romanini DW, Paradise EM, Keasling JD (2008) Engineering triterpene production in *Saccharomyces cerevsiae* -β-amyrin synthase from *Artemisia annua*. FEBS J 275:1852–1859

Kurokawa H, Koyama T (2010) Prenyltransferase. In: Mander L, Lui H-W (eds) Comprehensive natural products II Chemistry and biology, vol volume 1. Elsevier, Oxford, pp 557–584

Kusama H, Hara R, Kawahara S, Nishimori T, Kashima H, Nakamura N, Morihira K, Kuwajima I (2000) Enantioselective total synthesis of (−)-taxol. J Am Chem Soc 122:3811–3820

Kushiro T, Ebizuka Y (2010) Triterpenes. In: Mander L, Lui H-W (eds) Comprehensive natural products II Chemistry and biology, vol volume 1. Elsevier, Oxford, pp 673–708

Kushiro T, Shibuya M, Ebizuka Y (1998) β-Amyrin synthase cloning of oxidosqualene cyclase that catalyzes the formation of the most popular triterpene among higher plants. Eur J Biochem 256:238–244

Lemuth K, Steuer K, Albermann C (2011) Engineering of a plasmid-free *Escherichia coli* strain for improved *in vivo* biosynthesis of astaxanthin. Microb Cell Factories 10:29

Li D, Zhang Q, Zhou Z, Zhao F, Lu W (2016) Heterologous biosynthesis of triterpenoid dammarenediol-II in engineered *Escherichia coli*. Biotechnol Lett 38:603–609

Misawa N (2010) Carotenoids. In: Mander L, Lui H-W (eds) Comprehensive natural products II Chemistry and biology, vol volume 1. Elsevier, Oxford, pp 733–753

Misawa N (2011) Pathway engineering for functional isoprenoids. Curr Opin Biotechnol 22:627–633

Misawa N, Shimada S (1998) Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. J Biotechnol 59:169–181

Morrone D, Lowry L, Determan MK, Hershey DM, Xu M, Peters RJ (2010) Increasing diterpene yield with a modular metabolic engineering system in *E. coli*: comparison of MEV and MEP isoprenoid precursor pathway engineering. Appl Microbiol Biotechnol 85: 1893–1906

Muntendam R, Melillo E, Ryden A, Kayser O (2009) Perspectives and limits of engineering the isoprenoid metabolism in heterologous host. Appl Microbiol Biotechnol 84:1003–1019

Nagumo A, Kamei T, Sakakibara J, Ono T (1995) Purification and characterization of recombinant squalene epoxidase. J Lipid Res 36: 1489–1497

Nakano C, Motegi A, Sato T, Onodera M, Hoshino T (2007) Sterol biosynthesis by a prokaryote: first *in vitro* identification of the genes encoding squalene epoxidase and lanosterol synthase from *Methylococcus capsulatus*. Bisci Biotechnol Biochem 71:2543–2550

Rico J, Pardo E, Orejas M (2010) Enhanced production of a plant monoterpene by overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase catalytic domain in *Saccharomyces cerevisiae*. Appl Environ Microbiol 76:6449–6454

Roberts SC (2007) Production and engineering of terpenoids in plant cell culture. Nat Chem Biol 3:387–395

Rose RE (1988) The nucleotide sequence of pACYC184. Nucleic Acids Res 16:355

Sakakibara J, Watanabe R, Kanai Y, Ono T (1995) Molecular cloning and expression of rat squalene epoxidase. J Biol Chem 270:17–20

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual

Sandmann G (2002) Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. Chem Bio Chem 3:629–635

Sawai S, Shindo T, Sato S, Kaneko T, Tabata S, Ayabe S, Aoki T (2006) Functional and structural analysis of genes encoding oxidosqualene cyclases of *Lotus japonicus*. Plant Sci 170:247–257

Schewe H, Holtmann D, Schrader J (2009) $P450_{BM-3}$-catalyzed whole-cell biotransformation of α-pinene with recombinant *Escherichia coli* in an aqueous-organic two-phase system. Appl Microbiol Biotechnol 83:849–857

Seki H, Ohyama K, Sawai S, Mizutani M, Ohnishi T, Sudo H, Akashi T, Aoki T, Saito K, Muranaka T (2008) Licorice β-amyrin 11-oxidase, a cytochrome P450 with a key role in the biosynthesis of the triterpene sweetener glycyrrhizin. Proc Natl Acad Sci U S A 105: 14204–14209

Seki H, Sawai S, Ohyama K, Mizutani M, Ohnishi T, Sudo H, Fukushima EO, Akashi T, Aoki T, Saito K, Muranaka T (2011) Triterpene functional genomics in licorice for identification of CYP72A154 involved in the biosynthesis of glycyrrhizin. Plant Cell 23:4112–4123

Sun Y, Sun L, Shang F, Yan G (2016) Enhanced production of β-carotene in recombinant *Saccharomyces cerevisiae* by inverse metabolic engineering with supplementation of unsaturated fatty acids. Process Biochem 51:568–577

Suzuki S, Koeduka T, Sugiyama A, Yazaki K, Umezawa T (2014) Microbial production of plant specialized metabolites. Plant Biotechnol 31:465–482

Suzuki M, Xiang T, Ohyama K, Seki H, Saito K, Muranaka T, Hayashi H, Katsube Y, Kushiro T, Shibuya M, Ebizuka Y (2006) Lanosterol synthase in dicotyledonous plants. Plant Cell Physiol 47:565–571

Takemura M, Maoka M, Misawa N (2015) Biosynthetic routes of hydroxylated carotenoids (xanthophylls) in *Marchantia polymorpha*, and production of novel and rare xanthophylls through pathway engineering in *Escherichia coli*. Planta 241:699–710

Tansakul P, Shibuya M, Kushiro T, Ebizuka Y (2006) Dammarenediol II synthase, the first dedicated enzyme for ginsenoside biosynthesis, in *Panax ginseng*. FEBS Lett 580:5143–5149

Wu S, Schalk M, Clark A, Miles RB, Coates R, Chappell J (2006) Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nat Biotechnol 24:1441–1447

Yamano S, Ishii T, Nakagawa M, Ikenaga H, Misawa N (1994) Metabolic engineering for production of β-carotene and lycopene in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 58:1112–1114