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Pathway engineering for functional isoprenoids

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Pathway engineering is to engineer biosynthetic pathways for compounds of interests in heterologous organisms such as microbes and higher plants, which has also been one of the most important fields in metabolic engineering and synthetic biology. This review focuses on pathway engineering researches for the production of functional isoprenoids containing monoterpenes, sesquiterpenes, diterpenes, and triterpenes as well as carotenoids and for the elucidation of relevant biosynthesis genes and enzymes, which have been performed in the last two years. As microbial hosts, *Escherichia coli* and *Saccharomyces cerevisiae* have often been employed, since they, specifically the former, are fully amenable to genetic manipulations with extensive molecular resources. Various crops have also been used as the hosts for engineering pathways of functional isoprenoids of the plant origin, particularly carotenoids.

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Introduction

Isoprenoids, also referred to as terpenes or terpenoids, are the most diverse class of natural products consisting of over 40,000 structurally different compounds, which have been isolated from animal and microbial species as well as a wide variety of plant organs [1,2]. Isoprenoids, which contain monoterpenes, sesquiterpenes, diterpenes, and triterpenes as well as carotenoids (tetraterpenes), can exert a wide range of functions in the plant kingdom and in human health and societal issues, and have extensively been applied to pharmaceuticals (e.g., artemisinin, sesquiterpene; paclitaxel (Taxol); diterpene), herbal medicines (e.g., glycyrrhizin and ginsenosides; triterpenes), nutraceuticals (e.g., astaxanthin and lycopene; carotenoids), flavors (e.g., limonene and linalool; monoterpenes), fragrances (e.g., citronellol and geraniol; monoterpenes), cosmetics (e.g., astaxanthin), colorants (e.g., β-carotene; carotenoid), or agrichemicals (e.g., gibberellins; diterpenes). However, many of such functional isoprenoids are present in minute quantities in nature or low yielding from their natural sources, which has hindered their wide industrial use. 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 [3,4]. Past work on their increased production through traditional breeding approaches remained in limited success. Modern biotechnology should offer an alternative and promising approach for the economical production of such functional isoprenoids. Pathway engineering is to engineer biosynthetic pathways for compounds of interests in heterologous organisms such as microbes and higher plants, which has also been one of the most important fields in metabolic engineering and synthetic biology [1,5]. Understanding biosynthetic pathways of the isoprenoids via the incorporation of biosynthesis genes and enzymes that are often uncovered is of great importance for their heterologous production. As microbial hosts, *Escherichia coli* and *Saccharomyces cerevisiae* have often been employed in pathway engineering of functional isoprenoids, since they, specifically the former, are fully amenable to genetic manipulations with extensive molecular resources. *E. coli* is also likely to possess considerable solvent-resistance [6•,7]. Various crops have also been used as the hosts for engineering pathways of functional isoprenoids of the plant origin, particularly carotenoids. This review focuses on pathway engineering researches for the production of functional isoprenoids and for the elucidation of relevant biosynthesis genes and enzymes, which have been performed in the last two years.

Biosynthetic pathways for monoterpenes, sesquiterpene, diterpenes, triterpenes and carotenoids

Biosynthetic routes to isoprenoids are regularly and 'simply' organized, despite their enormous structural diversity. They are all biosynthesized from the same basic isoprene units, isopentenyl diphosphate (pyrophosphate) (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Figure 1a) [1,8]. Most prokaryotes including *E. coli* and plant plastids synthesize IPP and DMAPP through the non-mevalonate [2-*C*-methyl-D-erythritol 4-phosphate (MEP)] pathway starting with the reaction between pyruvate and glyceraldehyde-3-phosphate, while some bacteria and all eukaryotes in the cytoplasm synthesize IPP via the mevalonate pathway [2,9,10]. DMAPP and IPP are condensed to generate geranyl diphosphate (GPP, C_{10}), which is further converted with

IPP into farnesyl diphosphate (FPP, C_{15}), with prenyl transferase such as FPP synthase (Figure 1a). FPP is further condensed with IPP to form geranylgeranyl diphosphate (GGPP, C_{20}) with GGPP synthase. GPP, FPP, and GGPP are the precursors of monoterpenes, sesquiterpenes, and diterpenes, respectively. Two molecules of FPP and GGPP are typically condensed to squalene and phytoene, which are the first hydrocarbon precursors of triterpenes (and sterols) and carotenoids, respectively (Figure 1b). The chemical diversity of the monoterpenes, sesquiterpenes, diterpenes, and triterpenes is primarily determined with the functional diversity of terpene synthases (terpene cyclases), which has been evolutionally acquired. Subsequent terpene-modifying enzymes, particularly cytochromes P450 (P450s), are secondarily responsible for the structural diversity of these chemicals (Figure 1b). On the other hand, carotenoid biosynthesis is interestingly contrasting, that is, phytoene is dehydrogenated to form conjugated double bonds to generate acyclic carotenes (hydrocarbon carotenoids) such as lycopene, frequently cyclized, and subjected to series of modifications to form a variety of xanthophylls (oxygen-containing carotenoids) (Figure 1b) [5]. Thus, the structural diversity of carotenoids attributes the major part to the functional diversity of carotene-modifying enzymes and subsequent tailoring enzymes, following to the biosynthesis of relatively small numbers of carotenes.

Pathway engineering for functional monoterpenes

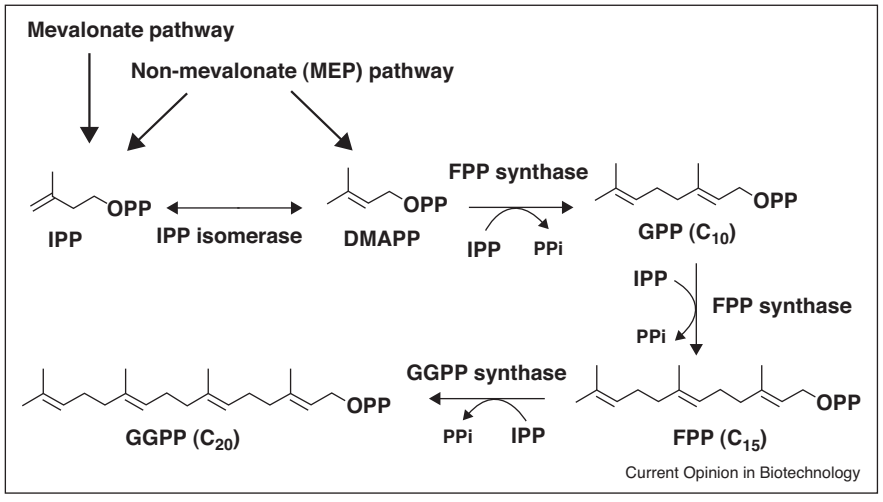
The essential oils of herbal plants contain a variety of monoterpenes, for example, limonene, citronellol, L-menthol, linalool, and geraniol, which have been used as flavors or fragrances with aroma (and pharmaceutical) value. Various monoterpene synthase genes have been identified in plants [11]. Chemical synthesis of functional monoterpenes has often been successful to generate low-priced materials, since they have relatively simple structures among isoprenoids. Thus, little pathway-engineering research has been conducted for their economical production, since the research by Carter *et al.* [12], that is, nearly 5 mg/L of limonene was produced with a pathway-engineered *E. coli*, which expressed the GPP synthase gene from grand fir (*Abies grandis*) and the (−)-limonene (L-limonene) synthase gene from spearmint (*Mentha spicata*). This limonene synthase gene was also constitutively expressed in spike lavender (*Lavandula latifolia*) plants [13]. Their developing leaves were shown to contain increased limonene contents, which corresponded to more than 450% increase compared with non-transformed controls [13]. Herrero *et al.* [14] revealed that a recombinant wine yeast strain of *S. cerevisiae*, which expressed the (*S*)-linalool synthase gene from a higher plant *Clarkia breweri*, efficiently excreted linalool to levels exceeding the threshold of human perception under micro-brewing conditions. A gene encoding the catalytic domain of endogenous 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rare-limiting enzyme in the mevalonate pathway, was overexpressed in the yeast strain expressing the linalool synthase genes, and the resultant yeast was shown to double linalool production [15].

Pathway engineering for functional sesquiterpenes

Identification and utilization of sesquiterpene synthase genes

Sesquiterpenes perhaps comprise the largest group (>7000 compounds) in isoprenoids, and can exert important

Figure 1



(a) Biosynthesis of basic prenyl diphosphates (prenyl pyrophosphates). *Abbreviations*: MEP, 2-*C*-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate. **(b)** Biosynthesis of monoterpenes, sesquiterpenes, diterpenes and triterpenes, and caroteniods from prenyl diphosphates.

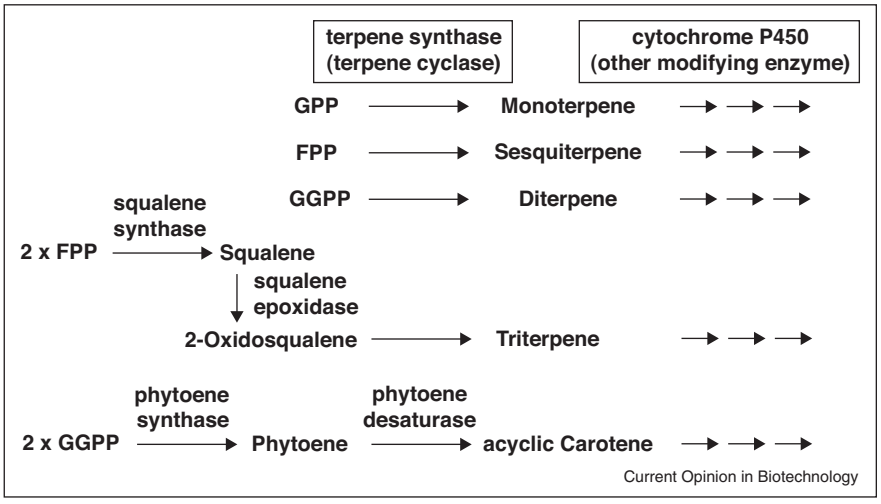


Figure 1. (*Continued*).

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 [16]. Artemisinin, the sesquiterpene endoperoxide lactone, extracted from sweet wormwood *Artemisia annua* is a greatly important medicinal agent for the anti-malarial treatment [17]. Zerumbone, contained in shampoo ginger (*Zingiber zerumbet*), is a promising chemopreventive agent for suppressing atherosclerosis [18]. Sesquiterpene synthases catalyze the first committed step in sesquiterpene biosynthesis, that is, the conversion from FPP to a large variety of sesquiterpene skeletons that are usually volatile cyclic olefins included in the essential oils (Figure 1b). For the last three years, novel sesquiterpene synthase genes were isolated from higher plants, snapdragon (*Antirrhinum majus*), Southern Magnolia (*Magnolia grandiflora*), maize (*Zea mays*), melon (*Cucumis melo*), shampoo ginger, hop (*Humulus lupulus*), and oregano (*Origanum vulgare*) plants, and were functionally identified by GC–MS analysis of the *in vitro* assay products with the encoded enzymes that were prepared from *E. coli* cells expressing the respective genes [11,19–24,25•,26•]. However, such *in vitro* enzyme assays often result in low product yields, which make their structural identification difficult. Göpfert *et al.* [27] identified a sesquiterpene synthase gene isolated from sunflower (*Helianthus annuus*) glandular trichomes as the gene (designated *HaCS*) encoding an enzyme that synthesizes mainly δ-cadinene, using a *S. cerevisiae* strain (EPY300) that was engineered to synthesize copious amount of FPP from simple carbon source. Harada *et al.* [28] reported an efficient production system for a sesquiterpene, using recombinant *E. coli* cells that express the corresponding sesquiterpene synthase gene along with heterologous D-mevalonate-utilizing or acetoacetate-utilizing genes. This system was adopted to the functional identification of a novel gene isolated from ginger (*Zingiber officinale*) young rhizomes as the (*S*)-β-bisabolene synthase gene [29], as well as the functional confirmation of the β-eudesmol synthase and α-humulene synthase (*ZSS1*) genes form shampoo ginger [23,24]. Several sesquiterpene synthase genes derived from a mushroom-forming fungus (*Coprinus cinereus*) were revealed by functionally expressing the respective genes in *E. coli* and/or *S. cerevisiae* [30•]. The biosynthetic route from FPP to the sesquiterpene antibiotic albaflavenone was examined in detail in *Streptomyces coelicolo*r A3(2), which is committed by (+)-epi-isozizaene synthase and CYP170A1 (encoded with *sco5222* and *sco5223*, respectively) [31].

Pathway engineering research on artemisinin precursors has focused on their efficient production with *E. coli* and *S. cerevisiae* [8,32]. Tsuruta *et al.* [33•] expressed the *A. annua* amorphadiene synthase (*ADS*) gene and mevalonate pathway genes in *E. coli*, and achieved the production of 27.4 g/L of amorpha-4,11-diene (amorphadiene), the first committed precursor of artemisinin, with the recombinant *E. coli* cells by optimizing nitrogen delivery in the fed-batch fermentation process. It was also shown that the accumulation of HMG-CoA is cytotoxic to *E. coli* and its deleterious effect can be counteracted by addition of fatty acid such as palmitic acid in the growth medium [34]. A precise and sensitive nonradioactive method was developed for the simultaneous quantification of FPP and GGPP in *E. coli* cells [35]. Concerning a plant host, the α-zingiberene synthase gene from lemon basil (*Ocimum basilicum*) was fruit-specifically overexpressed in tomato (*Solanum lycopersicum*) fruits, and the resultant tomato fruits were shown to accumulate high levels of α-zingiberene (up to1.0 μg/g fresh weight) [36•].

Utilization of sesquiterpene-modifying enzyme genes

Dihydroartemisinic acid, which is an immediate precursor for chemical synthesis of artemisinin, was elucidated to generate from amorphadiene with the *A. annua* P450

(CYP71AV1) and artemisinic aldehyde Δ11(13) reductase (Dbr2) [37]. When the *ADS* and *CYP71AV1* genes were expressed in the *S. cerevisiae* EPY300 strain along with the *A. annua* NADPH-cytochrome P450 reductase (*CPR*) gene, the resultant yeast produced 250 mg/L of artemisinic acid in shake-flask culture, while it was likely to suffer oxidative and drug-associated stresses [38]. A P450 gene homologous to *CYP71AV1*was isolated from lettuce (*Lactuca sativa*) leaves, and the encoded enzyme was confirmed to oxidize germacrene A to form germacrene A acid using the yeast (EPY300 strain) expression system [39•]. A P450 (designated CYP71BA1) from shampoo ginger was revealed to convert α-humulene to 8-hydroxy-α-humulene, the immediate precursor to zerumbone [40•]. When the *ZSS1* and *CYP71BA1* genes were expressed in *E. coli* along with the *Arabidopsis thaliana* NADPH-P450 reductase 2 (*ATR2*) gene and heterologous D-mevalonate-utilizing genes, the resultant *E. coli* produced 3.0 mg/L of 8-hydroxy-α-humulene with D-mevalonolactone supplement [40•].

Instead of using plant P450 genes that are often problematic to be functionally expressed in heterologous microbes such as *E. coli*, substrate-promiscuous P450s that originate from bacteria can be utilized. P450 BM3 (P450BM-3) from *Bacillus megaterium* is a natural fusion enzyme composed of a P450 part and a eukaryote-type NADPH-P450 reductase domain. Specifically, P450 BM3variants incorporating active site mutations that include a F87V or F87A substitution attract much ongoing attention [7,41•,42]. One of such P450 BM3 variants enabled the *regio*-specific and *stereo*-specific epoxidation of amorphadiene, at titers of 250 mg/L in *E. coli* [41•]. P450 BM3 variant F87V, which was N-terminally fused to an archaeal peptidyl-prolyl *cis-trans* isomerase (PPIase), was shown to elevate the stability of the P450 protein in *E. coli* cells, and biotransform β-eudesomol to 5-hydroxy-β-eudesomol [42]. CYP109B1 from *Bacillus subtilis* was also found to be a substrate-promiscuous P450, and to regioselectively convert (+)-valencene, α-ionone, and β-ionone to (+)-nootkatone, 3-hydroxy-α-ionone, and 4-hydroxy-β-ionone, respectively [6•,43]. CYP109D1 from *Sorangium cellulosum* was reported to perform the same reactions for α-ionone and β-ionone [44].

Pathway engineering for functional diterpenes and triterpenes

Diterpenes and triterpenes comprise a large variety of important chemicals as pharmaceuticals, herbal medicines or agrichemicals, for example, Taxol (paclitaxel), a diterpene including three benzene rings in the molecule, which was first isolated from the Pacific yew (*Taxus brevifolia*), is a successful anti-cancer drug, and glycyrrhizin is a triterpene glycoside (triterpene saponin), extracted from the roots and stolons of *Glycyrrhiza* (licorice) plants, is widely used as a natural sweetener as well as a pharmacological agent.

Pathway engineering for functional diterpenes has focused on their efficient production with *E. coli* and *S. cerevisiae*. Several enzymes involved in Taxol biosynthesis were functionally elucidated even for the last three years, since its biosynthetic pathway is not fully understood [45–47]. Engels *et al.* [48] expressed not only the *Taxus chinensis* taxadiene synthase and truncated HMG-CoA reductase genes but also an archaeal (*Sulfolobus acidocaldarius*) GGPP synthase gene in *S. serevisiae*, and produced 8.7 mg/L of taxa-4(5),11(12)-diene (taxadiene), the first committed precursor of Taxol, with the recombinant yeast cells. Ajikumar *et al.* [49••] succeeded in greatly increasing titers of taxadine in *E. coli*, that is, they achieved the production of 1 g/L of taxadiene by overexpressing genes for the enzymatic bottlenecks in the MEP pathway as well as the taxadiene synthase and GGPP synthase genes. They further introduced there a plasmid for expressing the taxadiene 5α-hydroxylase (P450) gene, which was C-terminally fused to the *Taxus CPR gene* whose transmembrane region was removed, and produced taxadien-5α-ol (a Taxol precursor) at titer of up to 58 mg/L [49••]. Other diterpenes, abietadiene and levopimaradiene, were efficiently produced with recombinant *E. coli* expressing the corresponding diterpene synthase genes, whose early isoprenoid pathways to GGPP were properly engineered, at maximum titers of 100 mg/L and 700 mg/L, respectively [50,51]. Morrone *et al.* [52] reported that one amino acid substitution (T696I) in the *syn*-primara-7,15-diene synthase from rice (*Oryza sativa*; OsKSL4) changed its cyclization-reaction specificity to form aphidicol-15-ene predominantly. (*E*,*E*,*E*)-Geranylgeraniol, a perfume agent, was produced at titers of 134 mg/L in optimized jar fermentations with *S. cereviase* that overexpressed the HMG-CoA reductase (*HMG1*) gene and a fusion gene for two prenyl diphosphate synthases (*ERG20-BTS1*) [53].

Pathway engineering for the efficient production of functional triterpenes has not been active, since many of their relevant biosynthesis genes and enzymes remains unknown. Hopanoid genes encoding squalene/phytoene synthases and FPP synthase from *Streptomyces peucetius* were overexpressed in *E. coli* along with the native *E. coli* 1-deoxy-D-xylulose 5-phosphate synthase (*dxs*) and IPP isomerase (*idi*) genes, and resultant bacteria were shown to produce 11.8 mg/L of squalene [54]. β-Amyrin 11-oxidase gene that encodes the first modification enzyme in the glycyrrhizin pathway was first identified in licorice (*Glycyrrhiza uralensis*) plants [55•].

Pathway engineering for functional carotenoids

Carotenoids comprise a large group (>750 compounds) in isoprenoids, and can exert important physiological functions in a wide range of organisms. Some of the carotenoids attract on-going attention as neutraceutical agents, for example, lycopene, a red carotenoid pigment

contained in tomato and watermelon, is considered to prevent prostate cancer [56], and another red carotenoid astaxanthin, which is found in red sea animals such as crab, shrimp and red fish (red sea bream and salmon), is likely to prevent cardiovascular disease and (UV-)light aging in human body [57]. Pathway engineering research for the efficient production of such functional carotenoids has widely been performed up to the present, since the biosynthetic pathway via the incorporation of biosynthesis genes and enzymes is well elucidated [5,58].

Analysis on catalytic functions of carotenoid biosynthesis enzymes is facile, since immediate carotenoid precursors of the enzyme products can accumulate in recombinant *E. coli* cells that harbor the biosynthesis gene clusters starting with the GGPP synthase gene. Using this system, some new carotenoid biosynthesis enzymes (genes) that originated from higher plants, cyanobacteria and actinomycetes were functionally identified for the last three years [59–62]. Production of lycopene reached high levels (near to 20 mg/g cell) in 24-h batch flask culture in pathway-engineered *E. coli*, which reflected results of multi-dimensional gene target search or gene-knockout analysis [63]. As for astaxanthin production, although relevant studies are done in heterologous microbes, *E. coli* [28] and *S. cerevisiae* [64], the main research field is likely to have move to higher plants as heterologous hosts [57,65,66•,67,68]. Astaxanthin and other ketocarotenoid intermediates were shown to accumulate in carrot (*Daucus carota*) roots, maize endosperms, and canola (*Brassica napus*) seeds by introducing and expressing there carotenoid 4,4′-ketolase (oxygenase; *bkt1* or *crtW*) gene [65,67,68]. The biosynthesis genes of astaxanthin from β-carotene (*crtZ* and *crtW*) were directly transformed to the chloroplast of tobacco (*Nicotiana tabacum*) leaves, and the resultant tobacco leaves were shown to accumulate the maximum level of astaxanthin (more than 5 mg/g dry weight) [66•]. Such a chloroplast transformation strategy is promising towards efficient astaxanthin production in edible crops, although it is adaptable to limited numbers of crops such as lettuce and tomato plants.

Conclusions

Pathway engineering for the efficient production of functional isoprenoids containing monoterpenes, sesquiterpenes, diterpenes, triterpenes, and carotenoids (tetraterpenes) has been performed with microbial hosts such as *E. coli* and *S. cerevisiae*, or with higher plants as the hosts. Understanding biosynthetic pathways of the isoprenoids via the incorporation of biosynthesis genes and enzymes that are often uncovered is also greatly important for their heterologous production. This review has focused on advances achieved in such pathway engineering fields for the last two years. Semi-synthesis precursors of the crucial terpene drugs, artemisinin and Taxol, were successfully produced in microbial hosts, specifically *E. coli*. On the other hand, carotenoid pigments, lycopene and astaxanthin, were successfully produced in *E. coli* and tobacco leaves, respectively.

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- •• of outstanding interest

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Three sesquiterpene synthase and three monoterpene synthase genes were isolated from glandular trichomes of oregano young leaves, and functionally identified. The three sesquiterpene synthases, designated *Ov*TPS3, *Ov*TPS4, and *Ov*TPS6, respectively, synthesized (−)-germacrene D, bicycle-germacrene, and (*E*)-β-caryophyllene, predominantly.

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The authors constructed *E. coli* expressing the *S. cerevisiae* mevalonate pathway genes, in which the HMG-CoA synthase and reductase genes were replaced with equivalent genes from *Staphylococcus aureus*, in addition to the *A annua* amorphadiene synthase (*ADS*) gene, and achieved the production of 27.4 g/L (more than doubling production) of amorpha-4,11-diene with the recombinant *E. coli* cells.

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