Pathway engineering is to engineer biosynthetic pathways for compounds of interests in heterologous organisms such as microbes and higher plants, which has 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.

### 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., 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, sesquiterpenes, 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 diphasphate (pyrophosphate) (IPP) and its isomer dimethyallyl diphasphate (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 diphasphate (GPP, C₁₀), which is further converted with
IPP into farnesyl diphosphate (FPP, C15), with prenyl transferase such as FPP synthase (Figure 1a). FPP is further condensed with IPP to form geranylgeranyl diphosphate (GGPP, C20) 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 carotenoids.

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 monoterpenes 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 GGPP synthase gene from grand fir (Abies grandis) and the (−)-limonene (1-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
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 δ-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 coelicolor A3(2), which is committed by (+)-epi-isozizae synthese 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 to 1.0 μg/g fresh weight) [36*].

**Utilization of sesquiterpene-modifying enzyme genes**

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

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**Figure 1.** (Continued)
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 archaean (_Sulfolobus acidocaldarius_) GGPP synthase gene in _S. cerevisiae_, 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 taxadiene 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 CYP450_ gene whose transmembrane region was removed, and produced taxadien-5α-ol (a Taxol precursor) at titer of up to 58 mg/L [49**].

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 (T'696I) 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. cerevisiae_ that overexpressed the HMG-CoA reductase (_HMG1_) gene and a fusion gene for two prenyl diphosphate synthases (ERG20-BTS1) [53].

Pathway engineering for 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 nutraceutical agents, for example, lycopene, a red carotenoid pigment...
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 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′-ketalox (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 monoterpens, 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.

**Acknowledgements**

This work was supported in part by the Research and Development Program for New Bio-industry Initiatives (2006–2010) of the Bio-oriented Technology Research Advancement Institution (BRAIN) of Japan.

**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

15. Rico J, Pardo E, Orejas M: Enhanced production of a plant monoterpene by overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase catalytic domain in
6 Tissue, cell and pathway engineering


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 OstPS3, OstPS4, and OstPS6, respectively, synthesized (–)-germacrene D, bicycle-germacrene, and (E)-β-caryophyllene, predominantly.


Six sesquiterpene synthase genes (cop1 to cop6) and two subsequent P450 genes (cox1 and cox2) were isolated from the mycelium of a basidiomycete Coprinus cinereus, and, except for cop5, functionally expressed in E. coli and/or S. cerevisiae. Consequently, the functions of the encoded enzymes were determined by GC-MS analysis, for example, Cop3 was first identified as α-muurolene synthase.


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 amorphaadiene 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.


A P450 gene homologous to CYP71AV1 (designated GAP4) and germacrene A synthase gene were isolated from lettuce plants, and expressed with the A. annua CPR gene in the S. cerevisiae EBY300 strain. This recombinant yeast was shown to synthesize germacrene A in buffered neutral culture.


A novel P450 (CYP71BA1) from shampoo ginger was shown to convert α-humulene to 8-hydroxy-α-humulene. This compound was demonstrated to accumulate in recombinant E. coli cells that express the Streptomyces sp. CL190 genes for the biosynthesis of IPP and DMAPP from D-mevalonate, when the R. mutans CPR gene was coexpressed with the CYP71BA1 gene.

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A P450 gene (named CYP88D6), isolated from the stolons of licorice plants, was identified as the β-amyrin 11-oxidase gene, and shown to produce 11-oxo-β-amyrin in recombinant S. cerevisiae cells expressing the Lotus japonicus β-amyrin synthase and CPR genes as well as CYP88D6.


