

Microbial production of plant specialized metabolites

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Received July 22, 2014; accepted October 3, 2014 (Edited by T. Shoji)

Abstract Plant specialized metabolites play important roles in human life. These metabolites, however, are often produced in small amounts in particular plant species. Moreover, some of these species are endangered in their natural habitats, thus further limiting the availability of some plant specialized metabolites. Microbial production of these compounds may circumvent this problem. Considerable progress has been made in the microbial production of various plant specialized metabolites over the past decade. Now, the microbial production of these compounds is becoming robust, fine-tuned, and commercially relevant systems using the methods of synthetic biology. This review describes the progress of microbial production of plant specialized metabolites, including phenylpropanoids, flavonoids, stilbenoids, diarylheptanoids, phenylbutanoids, terpenoids, and alkaloids, and discusses future challenges in this field.

Key words: Phenylpropanoids, flavonoids, terpenoids, alkaloids, synthetic biology.

Introduction

Plants produce various low-molecular-weight organic compounds that can be utilized as medicines, cosmetics, flavors, dyes, seasonings, functional ingredients, and industrial chemicals, all of which play important roles in human life (Facchini et al. 2012). These compounds have long been referred to as plant secondary metabolites. Recently, a new term “plant specialized metabolites” has been proposed to define these compounds, with emphasizing their function in plants’ adaptations for specific ecological situations and their plant lineage-specific distribution (Pichersky and Lewinsohn 2011), although many of these compounds also occur in organisms other than plants. In this review, the term “plant specialized metabolites” will be adopted.

Despite the importance to human life, plant specialized metabolites are often produced in small amounts by particular plant species. Some of these species are endangered in their natural habitats, further limiting the availability of plant specialized metabolites. Although this problem may be overcome by the total organic synthesis of these compounds, organic synthesis often uses heavy metals, toxic organic solvents, and strong acids, many of which have high environmental burdens (Du et al. 2011). Furthermore, it is still difficult to synthesize plant specialized metabolites with

complex structures on a large-scale and at a low cost (Misawa 2011). By contrast, metabolic engineering in microorganisms does not require dangerous chemicals and enables the production of very complex metabolites with the aid of enzymes having high substrate specificities from simple carbon sources such as sugars synthesized during plant photosynthesis (Keasling 2008).

Over the past decade, there has been considerable progress in the metabolic engineering of microorganisms and in identifying genes involved in plant specialized metabolism. This has enabled the construction of sophisticated and fine-tuned metabolic pathways in microorganisms to produce plant specialized metabolites (Chemler and Koffas 2008; Du et al. 2011; Keasling 2008, 2012; Lee et al. 2009; Marienhagen and Bott 2013; Siddiqui et al. 2012). Microbial platforms for the production of plant specialized metabolites can be beneficial to plant scientists because these systems can be used to identify novel enzymes by incorporating the coding genes into microbes (Cyr et al. 2007). Moreover, the reconstitution and investigation of metabolism in a simple system like microbial cells facilitate the quantitation of pathway flux without interference from other related pathways and the characterization of enzyme-enzyme interactions between pathway enzymes (Ralston et al. 2005; Ro and Douglas 2004).

In this context, this review focuses on the progress

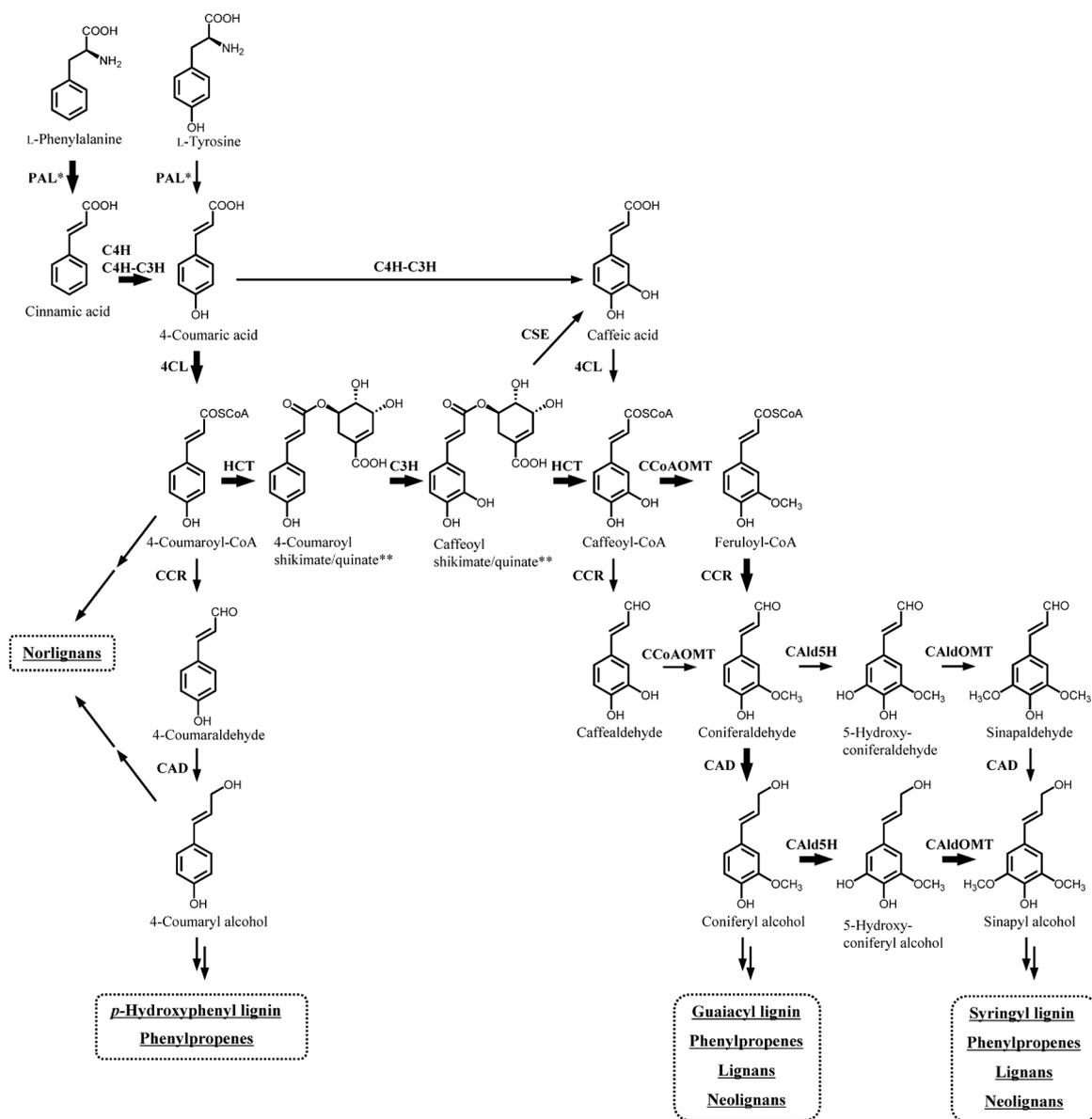


Figure 1. Biosynthetic pathways for phenylpropanoids. The bold arrows represent a major pathway towards coniferyl and sinapyl alcohols formation in *P. trichocarpa* (Wang et al. 2014). 4CL, 4-coumarate:CoA ligase; C3H, coumarate 3-hydroxylase; C4H, cinnamic acid 4-hydroxylase; C4H-C3H, the enzyme complex of C4H and C3H; CAD, cinnamyl alcohol dehydrogenase; CAld5H, coniferaldehyde 5-hydroxylase; CAldOMT, 5-hydroxyconiferaldehyde O-methyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CSE, caffeoyl shikimate esterase; HCT, hydroxycinnamoyl-CoA shikimate/quinic acid hydroxycinnamoyltransferase; PAL, phenylalanine ammonia lyase. *PAL showing strong deaminase activity toward L-tyrosine is often called tyrosine ammonia lyase (TAL). **Note that only chemical structures of the shikimate esters are shown.

of microbial production of major plant specialized metabolites, including phenylpropanoids, flavonoids, stilbenoids, diarylheptanoids, phenylbutanoids, terpenoids, and alkaloids (Limem et al. 2008; Matsumura et al. 2013; Putignani et al. 2013; Sato and Kumagai 2013; Wang et al. 2011). Additionally, future challenges in this field are discussed.

Phenylpropanoids

Phenylpropanoids are composed of C₆-C₃ units. This group includes a variety of low-molecular-weight

metabolites, including phenylpropenes, lignans, neolignans, and norlignans. Polymer lignins are also classified phenylpropanoids (Umezawa 2010). Monolignols (4-hydroxycinnamyl alcohols) are shared as representative monomer precursors by these phenylpropanoids. Monolignols are biosynthesized via the cinnamate/monolignol pathway, and major pathways are proposed in angiosperms, with some variation among plant species (Suzuki and Suzuki 2014; Umezawa 2010; Vanholme et al. 2010; Vanholme et al. 2012) (Figure 1). In a major pathway towards coniferyl alcohol biosynthesis recently proposed in

Populus trichocarpa based on proteomic-based enzyme reaction and inhibition kinetics (Wang et al. 2014), L-phenylalanine is deaminated by phenylalanine ammonia lyase (PAL) to produce cinnamic acid, which is converted to 4-coumaric acid by a cytochrome P450 (CYP) enzyme, cinnamic acid 4-hydroxylase (C4H). 4-Coumaric acid is then activated by 4-coumarate:CoA ligase (4CL) to yield 4-coumaroyl-CoA, which is coupled to shikimic acid or quinic acid to yield 4-coumaroyl shikimate or 4-coumaroyl quinate by a hydroxycinnamoyltransferase (HCT). The resulting 4-coumaroyl shikimate or quinate is converted to caffeoyl shikimate or quinate by coumarate 3-hydroxylase (C3H). These caffeoyl esters are further hydrolyzed by HCT, producing caffeoyl-CoA. The resulting caffeoyl-CoA is sequentially O-methylated and reduced by caffeoyl-CoA O-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD), respectively. On the other hand, it was proposed that another major monolignol, sinapyl alcohol, is mainly synthesized by the hydroxylation of coniferyl alcohol and the O-methylation of 5-hydroxyconiferyl alcohol in *P. trichocarpa* (Wang et al. 2014). However, several by-path pathways were also described regarding coniferyl and sinapyl alcohols biosynthesis in both *P. trichocarpa* and other plant species: 1) the conversion of cinnamic acid to caffeic acid by a C4H–C3H complex and subsequent caffeoyl-CoA formation (Chen et al. 2011), 2) the transformation of caffeoyl shikimate to caffeate by caffeoyl shikimate esterase (CSE) and subsequent caffeoyl-CoA formation (Vanholme et al. 2013), 3) coniferaldehyde formation from caffeoyl-CoA via caffealdehyde (Zhou et al. 2010), and 4) sinapyl alcohol formation from coniferaldehyde via 5-hydroxyconiferaldehyde and sinapaldehyde (Humphreys et al. 1999; Osakabe et al. 1999).

Phenylpropanoids include important phytochemicals with various biological activities, such as antioxidant, antitumor, and antibacterial activities (Marienhagen and Bott 2013). Therefore, the production of these compounds by microbes has attracted much attention. A phenylpropanoid intermediate on the cinnamate/monolignol pathway, *p*-coumaric acid, was first produced in *Saccharomyces cerevisiae* by Ro and Douglas (2004). Later, a PAL showing strong deaminase activity towards L-tyrosine (a tyrosine ammonia lyase, TAL) from the yeast *Rhodotorula glutinis* was expressed in *S. cerevisiae* and *Escherichia coli*, resulting in the production of *p*-coumaric acid. Furthermore, synthetic microbes were constructed to produce 4-coumaric acid and 4-coumaroyl-CoA, which act as intermediates in alkaloid, flavonoid, and stilbenoid production (Nakagawa et al. 2011; Santos et al. 2011; Trantas et al. 2009; Watts et al. 2004). Caffeic and ferulic acids were also reported to be produced by microbes. For example, Choi et al. (2011) reported the conversion

of L-tyrosine to caffeic and ferulic acids in *E. coli* by the coexpression of actinomycete *Saccharothrix espanaensis* TAL (sam8), *S. espanaensis* 4-coumaric acid 3-hydroxylase (sam5), and *Arabidopsis thaliana* 5-hydroxyconiferaldehyde O-methyltransferase (CALdOMT). Subsequently, caffeic and ferulic acids were produced by a tyrosine-overproducing strain of *E. coli* in the absence of L-tyrosine supplementation (Kang et al. 2012). Lin and Yan (2012) reported that a bacterial PAL from *Rhodobacter capsulatus* and an *E. coli* hydroxylase (4-hydroxyphenylacetate 3-hydroxylase) were expressed in *E. coli* JW1316, a strain in which a chromosomal regulatory gene for tyrosine biosynthesis, *tyrR*, was deleted to enhance tyrosine biosynthesis. Additionally, they overexpressed *tyrA* (encoding chorismate mutase and prephenate dehydrogenase), modified *aroG* (encoding 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase), *ppsA* (encoding phosphoenolpyruvate synthase), and *tktA* (encoding transketolase). As a result, caffeic acid was produced, up to 50.2 mg L⁻¹ after fermentation for 48 h. Later, Zhang and Stephanopoulos (2013) optimized the similar system, which resulted in higher titer (108 mg L⁻¹) of caffeic acid production.

Very recently, 4-coumaryl alcohol production was reported in *E. coli* (Jansen et al. 2014). They incorporated *Rhodobacter sphaeroides* TAL, *Pteroselinum crispum* 4CL, *Zea mays* CCR and CAD into *E. coli* cells. The final yields of 4-coumaryl alcohol after 30 h fermentation were 20 mg L⁻¹ without feeding and 105 mg L⁻¹ with 4-coumaric acid supplementation. However, the supplementation of L-tyrosine only slightly increased the yield, suggesting that the deamination by TAL was a rate-limiting step.

Hydroxycinnamoyl anthranilates were produced by feeding 4-coumaric acid to *S. cerevisiae* coexpressing 4CLs and acyltransferases. For example, Moglia et al. (2010) reported that coexpression of 4CL and *Cynara scolymus* HCT resulted in *N*-(4'-coumaroyl)-3-hydroxyanthranilic acid production. Similarly, coexpression of 4CL5 from *A. thaliana* and hydroxycinnamoyl/benzoyltransferase from *Dianthus caryophyllus* led to the production of hydroxycinnamoyl anthranilates including antiallergic tranilast [*N*-(3',4'-dimethoxycinnamoyl)-anthranilic acid] after the addition of hydroxycinnamic acids (Eudes et al. 2011).

Flavonoids, stilbenoids, diarylheptanoids, and phenylbutanoids

Flavonoids have a C₆–C₃–C₆ structural skeleton. To date, more than 7,000 flavonoids have been described (Arita and Suwa 2008). Flavonoids originate from one molecule of 4-coumaroyl-CoA in the cinnamate/monolignol pathway and three molecules of

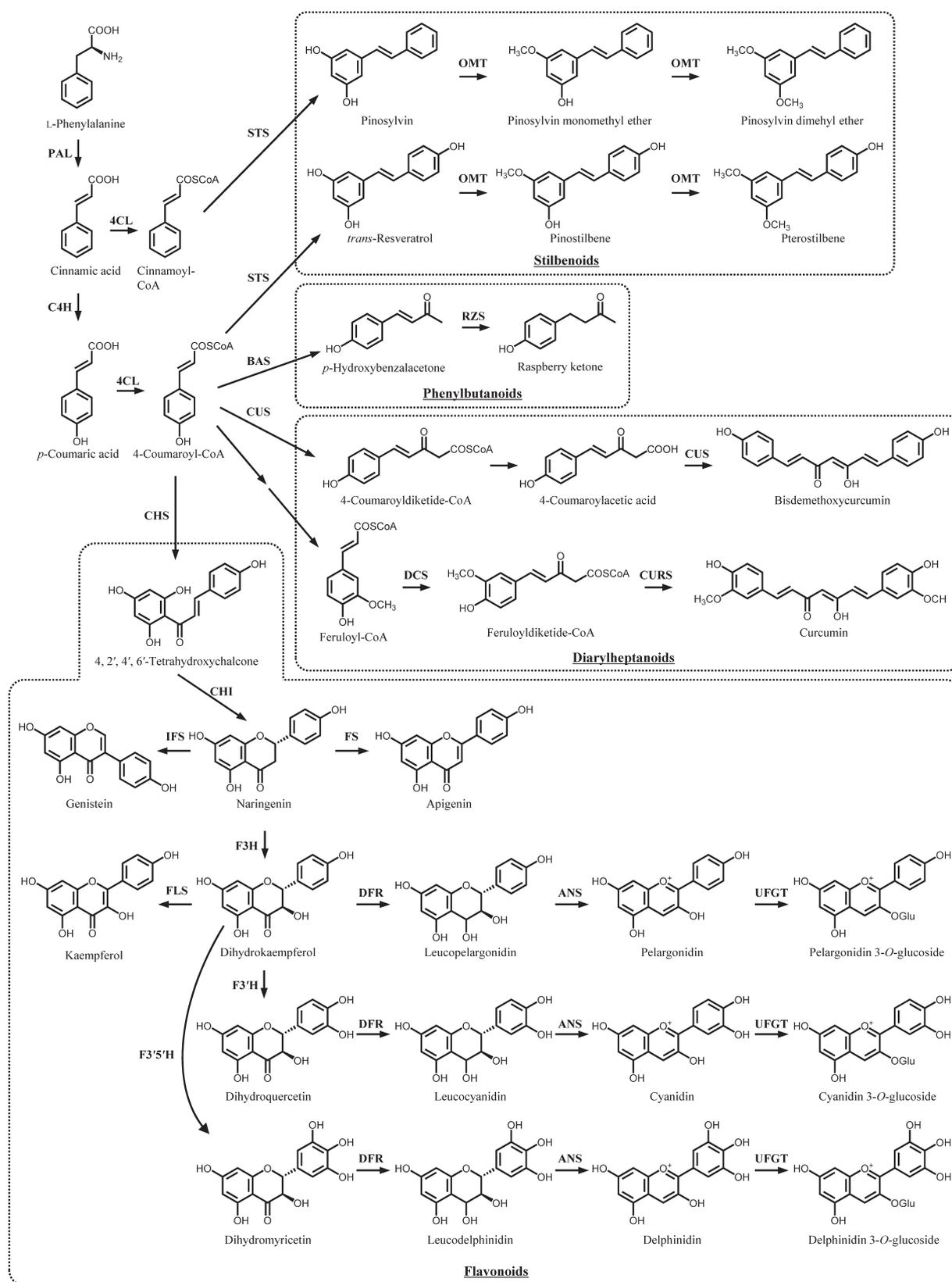


Figure 2. Biosynthetic pathways for flavonoids, stilbenoids, diarylheptanoids, and phenylbutanoids. Only major pathways are shown. 4CL, 4-coumarate:CoA ligase; ANS, anthocyanidin synthase; BAS, benzalacetone synthase; C4H, cinnamic acid 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; CURS, curcumin synthase; CUS, curcuminoid synthase; DCS, diketide-CoA synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; FLS, flavonol synthase; FS, flavone synthase; IFS, isoflavone synthase; OMT, *O*-methyltransferase; PAL, phenylalanine ammonia lyase; RZS, raspberry ketone/zingerone synthase; STS, stilbene synthase; UFGT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase.

malonyl-CoA. The basic pathway that generates core flavonoid skeletons is illustrated in Figure 2. First, 2-phenylchroman skeleton formation is mediated by chalcone synthase (CHS) and chalcone isomerase (CHI). Subsequently, the resulting flavanones are converted to flavones and dihydroflavonols by flavone synthase (FS) and flavanone 3-hydroxylase (F3H), respectively. Dihydroflavonols are then transformed to flavonols and leucoanthocyanidins by flavonol synthase (FLS) and dihydroflavonol 4-reductase (DFR), respectively. Anthocyanins are formed from leucoanthocyanidins by the catalysis of anthocyanidin synthase (ANS) and UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) (Dixon and Pasinetti 2010; Saito et al. 2013). On the other hand, isoflavones, which share a 3-phenylchroman structure, are formed from flavanones by the action of a CYP enzyme, isoflavone synthase (IFS) (Akashi et al. 1999). These flavonoids are further modified by hydroxylation, glycosylation, methylation, and prenylation to yield structurally diverse flavonoids.

The biosynthesis of stilbenoids ($C_6-C_2-C_6$), diarylheptanoids ($C_6-C_7-C_6$), and phenylbutanoids (C_6-C_4) are closely related to flavonoid biosynthesis (Figure 2). These core carbon skeletons are synthesized from cinnamoyl-CoAs and malonyl-CoA by stilbene synthase (STS), a combination of diketide-CoA synthase (DCS) and curcumin synthase (CURS) or curcuminoid synthase (CUS) alone, and benzalacetone synthase (BAS), respectively, all of which are members of the type III polyketide synthase (PKS) superfamily, similar to CHS (Abe and Morita 2010; Yu et al. 2012).

A lot of flavonoids, stilbenoids, and diarylheptanoids function as antioxidants in foods, with some of these compounds showing antibacterial and antitumor activities (Cushnie and Lamb 2011; Kanadaswami et al. 2005; Lin et al. 2014). Some isoflavones are investigated as estrogen receptor agonists and antagonists to modulate estrogen metabolism (Cress et al. 2013). In addition, anthocyanins are potential replacements for artificial dyes that have adverse health effects (Cress et al. 2013). The microbial system enables the readily scalable, cost-effective, and environmental-conscious production of these compounds (Putignani et al. 2013).

Flavonoids

Flavonoid production by bacteria was first described by Hwang et al. (2003). An artificial pathway for chalcone production was constructed in *E. coli* by introducing *PAL* from the yeast *Rhodotorula rubra*, *4CL* from the actinomycete *Streptomyces coelicolor*, and *CHS* from licorice *Glycyrrhiza echinata*. Because *R. rubra* *PAL* can deaminate both L-phenylalanine and L-tyrosine, and because *S. coelicolor* *4CL* shows activity towards both cinnamic and 4-coumaric acids, the coexpression of these enzymes with *CHS* yielded two flavanones,

pinocembrin and naringenin, after non-enzymatic cyclization from the corresponding chalcones. Later, Watts et al. (2004) also reported the production of a flavanone, naringenin, with a yield of 20.8 mg L^{-1} , by the coexpression of *R. sphaeroides* *TAL*, *A. thaliana* *4CL*, and *A. thaliana* *CHS* in *E. coli*. An artificial pathway generating anthocyanins from flavanones was constructed in *E. coli* by the coexpression of *Malus domestica* *F3H*, *Anthurium andraeanum* *DFR*, *M. domestica* *ANS*, and *Petunia hybrida* *UFGT* (Yan et al. 2005a). The *E. coli* cells converted naringenin and eriodictyol to the corresponding anthocyanins, pelargonidin 3-O-glucoside and cyanidin 3-O-glucoside, respectively.

Although several core flavonoids could be synthesized in *E. coli*, the yield was too low for industrial applications. Therefore, the artificial flavonoid pathway and/or the supply of precursor (e.g., L-tyrosine, L-phenylalanine, or malonyl-CoA) were optimized. Miyahisa et al. (2005) showed that the coexpression of five enzymes, *R. rubra* *PAL*, *S. coelicolor* *4CL*, *G. echinata* *CHS*, *Pueraria lobata* *CHI*, and heterodimeric acetyl-CoA carboxylase (*ACC*) from *Corynebacterium glutamicum*, an enzyme involved in malonyl-CoA synthesis, resulted in the efficient generation of naringenin from L-tyrosine and pinocembrin from L-phenylalanine. Further introduction of *P. crispum* *FS* enabled the *E. coli* cells to produce flavones, including apigenin (13 mg L^{-1}) from L-tyrosine and chrysin (9.4 mg L^{-1}) from L-phenylalanine. Introduction into the *E. coli* cells of *F3H* from *Citrus sinensis* and *FLS* from *C. unshiu* led to the production of flavonols, including kaempferol (15.1 mg L^{-1}) from L-tyrosine and galangin (1.1 mg L^{-1}) from L-phenylalanine (Miyahisa et al. 2006).

High-yield production of anthocyanins was optimized by various factors, including coexpressed genes, supplementation of the medium, and the pH of the medium (Yan et al. 2008). This resulted in the production of 78.9 mg L^{-1} pelargonidin 3-O-glucoside and 70.7 mg L^{-1} cyanidin 3-O-glucoside from flavan-3-ols. To efficiently supply malonyl-CoA to the artificial flavonoid pathway, Leonard et al. (2007) overexpressed four *ACC* subunits from *Photobacterium luminescens* under a constitutive promoter. In addition, the levels of expression of *P. lobata* *ACC*-biotin ligase (*BirA*) and the chimeric protein of *P. lobata* *ACC* were increased to enhance acetate assimilation. These modifications resulted in the production of 429 mg L^{-1} pinocembrin, 119 mg L^{-1} naringenin, and 52 mg L^{-1} eriodictyol from the corresponding phenylpropanoic acids. Later, genes (*matB* and *matC*) involved in malonate assimilation in *Rhizobium trifolii* were introduced to utilize exogenous malonate, and a competitive pathway, fatty acid synthesis, was inhibited by the addition of cerulenin. This strategy resulted in the production of 700 mg L^{-1} flavanones and

113 mg L⁻¹ anthocyanins from phenylpropanoic acids and flavan-3-ol precursors (Leonard et al. 2008). Using evolutionary computation coupled with constraint-based modeling to investigate the impact of multiple gene deletions, a process termed the cipher of evolutionary design (CiED), Fowler et al. (2009) modeled the genes that should be deleted to improve the productivity of flavonoids in *E. coli*. Targeted deletion of the genes predicted by CiED and overexpression of the genes involved in flavanone biosynthesis, acetate assimilation, malonyl-CoA biosynthesis, and CoA biosynthesis, yielded 15 to 100 mg L⁻¹ OD⁻¹ naringenin and 13 to 55 mg L⁻¹ OD⁻¹ eriodictyol.

Although microbial flavonoid production systems have been much improved, two limitations were prohibitive during process scale up. First, the fermentation protocols often required two separate cultivation steps to achieve high flavonoid titers. Second, the system heavily relied on precursor feeding to achieve high levels of flavonoid production. To circumvent these problems, Santos et al. (2011) constructed an artificial flavanone synthetic pathway composed of yeast *R. sphaeroides* TAL, *S. coelicolor* 4CL, *A. thaliana* CHS, and *P. lobata* CHI in *E. coli* strains (P2 and rpoA14^R) that produce large amounts of L-tyrosine. The constructed strains were found to be capable of producing 29 mg L⁻¹ naringenin from glucose and up to 84 mg L⁻¹ by the addition of the fatty acid enzyme inhibitor, cerulenin.

Flavanones have also been produced in *S. cerevisiae*. For example, Jiang et al. (2005) expressed *Rhodospiridium toriloides* PAL, *A. thaliana* 4CL, and *Hypericum androsaemum* CHS, while Yan et al. (2005b) expressed *P. crispum* 4CL, *P. hybrida* CHS and CHI, and *A. thaliana* C4H. Later, Trantas et al. (2009) reported that the enzymes involved in the entire flavonoid synthetic pathway (PAL, C4H, 4CL, CHS, CHI, F3H, FLS, and F3'H) were expressed in *S. cerevisiae* to produce flavonols, kaempferol and quercetin, from supplied L-phenylalanine. Koopman et al. (2012) recently achieved naringenin production from glucose by deregulation of aromatic amino acid biosynthesis and reduction of byproduct (phenylethanol) formation in *S. cerevisiae* as well as by introduction of the naringenin biosynthetic pathway from L-phenylalanine.

Various flavonoids, not limited to basic compounds, have also been produced in microbial systems. For example, Yan et al. (2007) showed that a 5-deoxyflavanone liquiritigenin, commonly found in leguminous plants, could be produced from 4-coumaric acid in *E. coli* by coexpression of *P. crispum* 4CL, *P. hybrida* CHS, *Medicago sativa* chalcone reductase and CHI. Construction of the same artificial pathway in *S. cerevisiae* resulted in a lower yield than in *E. coli*. On the other hand, Malla et al. (2012) reported that a methylated dihydroflavonol, 7-*O*-methylaromadendrin, was

produced in *E. coli* by expressing *Streptomyces avermitilis* 7-*O*-methyltransferase and *A. thaliana* F3H, as well as the other enzymes involved in flavanone biosynthesis (4CL, CHS, and CHI) and in malonyl-CoA biosynthesis (ACC and acetyl-CoA synthase). Naringenin feeding of a recombinant yeast expressing a prenyltransferase isolated from *Sophora flavescens* resulted in the synthesis of a prenylated flavonoid 8-dimethylallylnaringenin (Sasaki et al. 2009).

Isoflavones including genistein and daidzein have been produced from L-tyrosine using *E. coli* and *S. cerevisiae* co-culture (Katsuyama et al. 2007c), from flavanones using *E. coli* alone (Leonard and Koffas 2007), or from flavanones, 4-coumaric acid, and L-phenylalanine using *S. cerevisiae* alone (Trantas et al. 2009). Among them, the isoflavone production from flavanones by expressing IFS (Leonard and Koffas 2007) is noticeable. Although functional eukaryotic CYP expression in prokaryotic *E. coli* system is generally unsuitable (Paddon and Keasling 2014), Leonard and Koffas expressed *G. max* IFS having a modified membrane recognition signal and fusing to *Catharanthus roseus* CYP reductase (CPR).

Stilbenoids

Beekwilder et al. (2006) introduced *Nicotiana tabacum* cv. Samsun 4CL2 and *Vitis vinifera* STS into *E. coli* and obtained resveratrol (16 mg L⁻¹) from 4-coumaric acid. Watts et al. (2006) reported that the coexpression of peanut (*Arachis hypogaea*) STS and *A. thaliana* 4CL1 in *E. coli* and the addition of 1 mM 4-coumaric acid to the medium yielded over 100 mg L⁻¹ resveratrol. Feeding of these cells with 1 mM caffeic acid resulted in over 10 mg L⁻¹ piceatannol. However, feeding of ferulic acid did not result in a cyclized stilbenoid. Rather, triketide and tetrakedide lactone intermediates were identified, indicating that substrate utilization by *A. thaliana* 4CL1 was limited. Substitution of *A. thaliana* 4CL1 with *A. thaliana* 4CL4 resulted in a substrate preference for ferulic acid, but no detectable isorhapontigenin. Thus, feruloyl-CoA utilization by STS is the limiting step in the pathway. A study assessing the production of various stilbenoids from their corresponding phenylpropanoic acids in *E. coli* resulted in 155 mg L⁻¹ pinosylvin and 171 mg L⁻¹ resveratrol (Katsuyama et al. 2007b). Incorporating a rice *O*-methyltransferase (OMT) gene (Os08g06100) into stilbenoid-producing *E. coli* cells yielded mono- and di-*O*-methylated resveratrols (pinostilbene and pterostilbene) and pinosylvins (pinosylvin monomethyl ether and pinosylvin dimethyl ether) (Katsuyama et al. 2007a). This OMT gene actually encodes CALDOMT (Figure 1), an OMT involved in syringyl lignin biosynthesis (Koshiba et al. 2013). In *E. coli* cells, however, the OMT methylated phenolic hydroxyl groups in stilbenoids. This activity may be due to its broad substrate specificity, inasmuch as the rice

OMT could *O*-methylate flavonoids (Lin et al. 2006). Instead of expensive 4-hydroxycinnamic acids, Wu et al. (2013) used *L*-tyrosine as a supplemental precursor to produce resveratrol. They incorporated *R. glutinis* *TAL*, *P. crispum* *4CL*, *V. vinifera* *STS*, and *R. trifolii* *matB* and *matC* into *E. coli*. The optimum strain was capable of producing 35 mg L⁻¹ resveratrol from *L*-tyrosine in a single medium.

There are several examples of stilbenoid production in yeasts. However, the level of production of resveratrol is usually lower in yeast than in *E. coli* (Jeandet et al. 2012). For example, Beekwilder et al. (2006) integrated *N. tabacum* *4CL* and *V. vinifera* *STS* into *LEU2* locus of *S. cerevisiae* and obtained resveratrol (6 mg L⁻¹) from 4-coumaric acid. Later, the complete pathway for resveratrol production from *L*-phenylalanine was constructed in *S. cerevisiae* by incorporating *PAL* and *CPR* from *Populus trichocarpa* × *deltoids*, *C4H* and *4CL* from *Glycine max*, and *STS* from *V. vinifera* 'Soultanina' (Trantas et al. 2009). They fed 10 mM *L*-phenylalanine to the engineered yeast and obtained 0.29 mg L⁻¹ resveratrol. Wang et al. (2011b) optimized *TAL* codons for yeast and introduced low-affinity and high-capacity *E. coli* *araE* transporter to enhance resveratrol production up to 2.3 mg L⁻¹. More recently, Wang and Yu (2012) constructed synthetic scaffolds to recruit *4CL* and *STS* and improved resveratrol production in yeast cells.

Diarylheptanoids

Among diarylheptanoids, curcumin and its congeners are called curcuminoids. Because curcumin possesses various pharmacological effects, including anti-inflammatory, antioxidant, anticarcinogenic, and antitumor activities (Esatbeyoglu et al. 2012), and because curcumin has been extracted only from the rhizomes of *Curcuma longa* (turmeric), the biotechnological production of this compound has attracted much attention.

Curcumin is biosynthesized from two molecules of feruloyl-CoA and a molecule of malonyl-CoA in *C. longa*. First, *DCS* catalyzes the formation of feruloyldiketide-CoA from feruloyl-CoA and malonyl-CoA. Next, the resulting feruloyldiketide-CoA reacts with another feruloyl-CoA by the catalysis of *CURS* to produce curcumin. By contrast, *CUS*, a type III PKS identified in *O. sativa*, catalyzes both cinnamoyldiketide-CoA (e.g. 4-coumaroyldiketide-CoA) formation and subsequent curcuminoid formation (Yu et al. 2012) (Figure 2).

An artificial curcuminoid biosynthetic pathway was constructed in *E. coli* by introducing *R. rubra* *PAL*, *Lithospermum erythrorhizon* *4CL*, and rice *CUS* into these cells. Cultivation of these recombinant *E. coli* cells in the presence of *L*-tyrosine and/or *L*-phenylalanine led to the production of bisdemethoxycurcumin,

dicinnamoylmethane, and cinnamoyl 4-coumaroylmethane. Another *E. coli* system carrying *4CL* and *CUS* was also used for high-yield production of curcuminoids from the exogenously supplemented phenylpropanoid acids, 4-coumaric, cinnamic, and ferulic acids, with a yield of curcuminoids as high as 100 mg L⁻¹ (Katsuyama et al. 2008).

Phenylbutanoids

Phenylbutanoids include several important plant flavor compounds such as raspberry ketone for raspberry flavor and zingerone for ginger flavor (Koeduka et al. 2011). Raspberry ketone extracted from natural sources is one of the most expensive flavor components used in the food industry. Raspberry ketone can be chemically synthesized by the condensation of *p*-hydroxybenzaldehyde with acetone; however, this chemically-synthesized compound cannot be regarded as a "natural flavor" according to food laws in the US and Europe. In contrast, raspberry ketone produced by microbes is regarded as a "natural flavor". Because consumers prefer "natural flavors", the microbial production of raspberry ketone has attracted much attention (Vandamme and Soetaert 2002).

Raspberry ketone was produced in *E. coli* and *S. cerevisiae* cells, in the former by introducing tobacco *4CL* and raspberry *CHS* and incubating the cells with 3 mM 4-coumaric acid (Beekwilder et al. 2007). Because of the endogenous reductase activity of *E. coli*, these cells produced naringenin as well as 4-hydroxybenzalacetone and raspberry ketone, suggesting that raspberry *CHS* has both *CHS* and *BAS* activity.

Terpenoids

Terpenoids are a class of isoprenoids composed of five-carbon isoprene units, the largest class of plant specialized metabolites. Although their chemical structures are very diverse, with >40,000 known to date (Bohlmann and Keeling 2008), the early steps of terpenoid biosynthesis are very simple (Chang and Keasling 2006). Regardless of species, terpenoids are biosynthesized from only two common precursors, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP). In plants, IPP and DMAPP (IPP/DMAPP) are biosynthesized through two different pathways, the mevalonate (MVA) and non-mevalonate [2-*C*-methyl-*D*-erithritol 4-phosphate (MEP)] pathways. IPP/DMAPP for the biosynthesis of monoterpenes (C₁₀), diterpenes (C₂₀), and carotenoids (C₄₀) are derived from the MEP pathway in plastids. In contrast, IPP/DMAPP for the biosynthesis of sesquiterpenes (C₁₅) and triterpenes (C₃₀) are produced via the MVA pathway localized to the cytosol. IPP and DMAPP couple to yield geranyl diphosphate

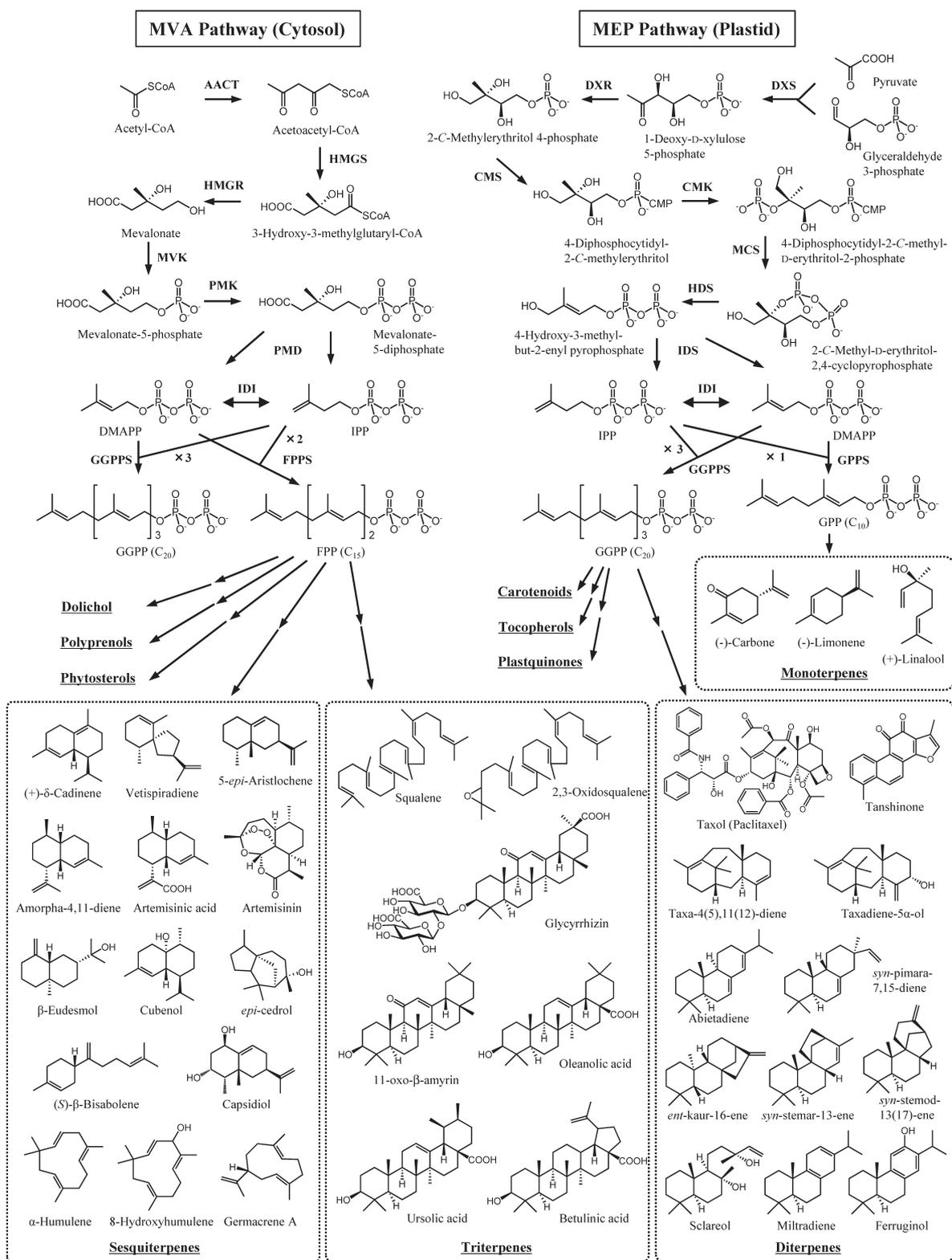


Figure 3. Biosynthetic pathways for terpenoids and other isoprenoids. The chemical structures of terpenoids targeted for microbial production are shown. AACT, acetyl-CoA acetyltransferase; CMK, 4-diphosphocytidyl-methylerythritol kinase; CMS, 4-diphosphocytidyl-methylerythritol synthase; DMAPP, dimethylallyl diphosphate; DXR, 1-deoxyxylulose 5-phosphate reductoisomerase; DXS, 1-deoxyxylulose 5-phosphate synthase; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; GGPP, geranylgeranyl diphosphate; GGPPS, geranylgeranyl diphosphate synthase; GPP, geranyl diphosphate; GPPS, geranyl diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HDS, hydroxymethylbutenyl 4-diphosphate synthase; IDI, isopentenyl diphosphate isomerase; IDS, isopentenyl diphosphate synthase; IPP, isopentenyl diphosphate; MCS, methylerythritol 2,4-cyclodiphosphate synthase; MVK, mevalonate kinase; PMD, mevalonate diphosphate decarboxylase.

(GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), and these precursors are cyclized by various terpene cyclases to yield various types of terpenoid carbon skeletons (Misawa 2011). Many of the resulting terpenoids are further modified by other enzymes such as CYPs and glycosyltransferases (Misawa 2011) (Figure 3).

In *E. coli*, as in other bacteria, IPP and DMAPP are synthesized via the MEP pathway, and are coupled to yield GPP (C_{10}), a reaction catalyzed by GPP synthase (GPPS). Non-engineered *E. coli* cells can synthesize a small amount of FPP (C_{15}) from the coupling of IPP and GPP, or the coupling of two molecules of IPP and DMAPP, but cannot synthesize GGPP (C_{20}) and other higher-carbon isoprenoid precursors. Therefore, it is necessary to introduce a GGPP synthase gene (GGPPS) into *E. coli* to produce diterpenes. In contrast, IPP and DMAPP are synthesized via the MVA pathway in *S. cerevisiae*. Because ergosterol (C_{30}) is an essential sterol component of cell membranes, and because protein prenylation including farnesylation and geranylgeranylation have been reported, *S. cerevisiae* can produce isoprenoid precursors including GPP (C_{10}), FPP (C_{15}), GGPP (C_{20}), and squalene (C_{30}). This characteristic is beneficial for producing various terpenoids in yeasts including *S. cerevisiae*.

Monoterpenes

Because monoterpenes have rather simple structures among the terpenoids, the chemical synthesis of monoterpenes has often been successful. Therefore, few plant monoterpenes have been synthesized by microbes (Misawa 2011). However, microbial production of plant-specific monoterpenes may be important because it could enrich wine aroma in brewery (Herrero et al. 2008), supply abundant “green solvents”—solvents with low environmental burdens (Gu and Jérôme 2013), and contribute to the substitution of high energy liquid fuel such as JP-10 for monoterpene dimers (Meylemans et al. 2012).

In the earlier report on the construction of monoterpene biosynthetic pathway in microorganisms, four genes required for (–)-carvone synthesis were introduced into *E. coli*: a GPP synthase gene (GPPS) from grand fir (*Abies grandis*); and (–)-limonene synthase, (–)-limonene-6-hydroxylase, and (–)-carveol dehydrogenase genes from spearmint (*Mentha spicata*) (Carter et al. 2003). Only (–)-limonene, an intermediate in (–)-carvone biosynthesis, was detected, although (–)-limonene-6-hydroxylase and (–)-carveol dehydrogenase were functional. Using a wine yeast strain of *S. cerevisiae* as a host, Herrero et al. (2008) expressed *Clarkia breweri* (+)-linalool synthase. The production of linalool was increased by overexpressing 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, a rate-

limiting enzyme in the MVA pathway. More recently, Alonso-Gutierrez et al. (2013) reported the production of (+)-limonene and its hydroxylated product, (+)-perillyl alcohol in *E. coli*. A strain containing all the MVA pathway genes (*AACT*, *HMGS*, *HMGR*, *MVK*, *PMK*, *PMD*, and *IDI*; Figure 3), *GPPS*, and limonene synthase gene in a single plasmid was found to produce (+)-limonene (over 400 mg L^{-1}) from glucose. Further introduction of a *CYP* from *Mycobacterium* sp. HXN-1500 and two genes encoding electron transfer proteins (ferredoxin and ferredoxin reductase) into the strain resulted in the production of (+)-perillyl alcohol at the titer of 100 mg L^{-1} . Similarly, Sarria et al. (2014) expressed the entire MVA pathway enzymes and further introduced three *GPPS*s and three pinene synthase genes (*PS*s) from *Abies grandis*, *Picea abies*, or *Pinus taeda* combinatorially. As a result, the pair of *A. grandis* *GPPS* and *PS* was found to be the best one for pinene production (ca. 28 mg L^{-1}). Moreover, to increase the yield, they engineered *GPPS*/*PS* fusion proteins to channel GPP at the *GPPS* active site directly into the *PS* active site. Among the clones they tested, the clone harboring *A. grandis* *GPPS*-*PS* fusion produced pinene at the titer of 32 mg L^{-1} .

Sesquiterpenes

Sesquiterpenes also show various biological activities, with one, the sesquiterpene lactone artemisinin from *Artemisia annua* being of great importance because artemisinin is used as an anti-malarial medicine effective in patients with multidrug-resistant malaria (Paddon and Keasling 2014; Ro et al. 2008).

Using an *E. coli* system, plant sesquiterpenes were synthesized in vivo by expressing (+)-cadinene cyclase from *Gossypium arboreum*, 5-*epi*-aristolochene cyclase from *N. tabacum*, and vetispiradiene cyclase from *Hyoscyamus muticus* (Martin et al. 2001). However, the yields were very low: $10.3 \mu\text{g}$ for (+)-cadinene, $0.24 \mu\text{g}$ for 5-*epi*-aristolochene, and $6.4 \mu\text{g}$ for vetispiradiene per liter of culture. These sesquiterpene production levels were much lower than carotenoid production, suggesting that the limiting factor for sesquiterpene synthesis in *E. coli* was not the supply of the FPP precursor but the poor expression of the cyclase enzymes. Therefore, a codon-optimized amorphadiene (amorphadiene) synthase gene, which encodes a sesquiterpene cyclase involved in artemisinin biosynthesis, was introduced into *E. coli* to enhance protein expression. Additionally the MVA pathway genes from *S. cerevisiae* were introduced to bypass the endogenous *E. coli* MEP pathway (Martin et al. 2003). This resulted in concentrations of amorphadiene, the precursor of artemisinin, as high as $24 \text{ mg caryophyllene equivalent L}^{-1}$ culture medium (Martin et al. 2003). The yield of amorphadiene was subsequently increased by using a two-phase partitioning bioreactor (Newman et al. 2006).

The availability of MVA was regarded as a limiting step in amorphadiene production through the artificial MVA pathway in *E. coli*. However, enhancing the conversion of acetyl-CoA to MVA resulted in the accumulation of HMG-CoA, which is toxic to *E. coli* cells (Pitera et al. 2007). This toxicity was mitigated by introducing an additional copy of the HMG-CoA reductase (HMGR) gene (Pitera et al. 2007), step by step optimization of the expression of each gene involved in MVA production (Pfleger et al. 2006), and high-level chromosomal expression of the artificial MVA pathway (Yuan et al. 2006). Amorphadiene production in *E. coli* was further modified by strain engineering and optimizing fermentation, resulting in commercially relevant titers ($>25 \text{ g L}^{-1}$) of amorphadiene (Tsuruta et al. 2009).

In addition to amorphadiene, several hydroxylated sesquiterpenes were produced in *E. coli*. These included artemisinic acid, an immediate downstream metabolite of amorphadiene towards artemisinin, and 8-hydroxycadinene. The production of these metabolites was achieved by coexpression of engineered CYPs, CPR, and terpene synthases as well as exogenous MVA pathway genes from yeast (Chang et al. 2007). β -Eudesmol and α -humulene were produced by expressing the β -eudesmol synthase and α -humulene synthase genes respectively from shampoo ginger (*Zingiber zerumbet*), and an MVA pathway gene cluster from *Streptomyces* sp. (Yu et al. 2008a; 2008b). Use of this *E. coli* system resulted in the identification of a new terpene cyclase (S)- β -bisabolene synthase from ginger (*Z. officinale*) (Fujisawa et al. 2010) and the identification of a CYP, α -humulene-8-hydroxylase (CYP71BA1) from shampoo zinger (Yu et al. 2011).

In yeast *S. cerevisiae*, Jackson et al. (2003) showed slight ($370 \mu\text{g L}^{-1}$) production of *epi*-cedrol by expressing *epi*-cedrol synthase from *A. annua*, and Asadollahi et al. (2009) showed cubenol production by expressing cubenol synthase from *Citrus paradisi*. *S. cerevisiae* has been often used for the coexpression of terpene synthases and CYPs, resulting in the construction of terpene carbon skeletons and subsequent modification. For example, Ro et al. (2006) engineered the MVA pathway of *S. cerevisiae* and heterologously expressed amorphadiene synthase, amorphadiene oxidase (CYP71AV1), and CPR from *A. annua*, resulting in artemisinic acid production (ca. 100 mg L^{-1}). On the other hand, Takahashi et al. (2007) individually expressed three terpene cyclases (*epi*-aristolochene synthase from tobacco, premnaspirodiene synthase from *H. muticus*, and valencene synthase from *Citrus*) in three yeast strains engineered for sterol biosynthesis. Further introduction of a CYP, 5-*epi*-aristolochene dihydroxylase gene and CPR into the *epi*-aristolochene synthase-expressing yeast led to the production of a dihydroxylated 5-*epi*-aristolochene, capsidiol.

Although the artemisinic acid production from glucose was achieved in *S. cerevisiae* (Ro et al. 2006), the titers (ca. 100 mg L^{-1}) were much lower than those of amorphadiene produced in *E. coli* ($>25 \text{ g L}^{-1}$) (Tsuruta et al. 2009). However, *E. coli* system is typically unsuitable for the functional expression of eukaryotic CYPs. Therefore, they compared the *E. coli* and *S. cerevisiae* systems and found that *S. cerevisiae* was the superior (Paddon and Keasling 2014). By optimizing the production pathway in yeast (Lenihan et al. 2008), they produced 2.5 g L^{-1} artemisinic acid. In addition, they fermented glucose instead of expensive galactose into artemisinic acid (1.6 g L^{-1}) and amorphadiene ($>40 \text{ g L}^{-1}$) using a yeast strain, CEN.PK2 (Westfall et al. 2012). To produce artemisinic acid at the commercially relevant titers, Paddon et al. (2013) engineered the complete biosynthetic pathway for artemisinic acid from acetyl-CoA in the yeast strain. The most noticeable modification is that they newly introduced following genes: 1) *ADH1*, the gene encoding an NAD-dependent alcohol dehydrogenase responsible for the conversion of artemisinic alcohol to artemisinic aldehyde, 2) *ALDH1*, the gene encoding an NAD-dependent aldehyde dehydrogenase that catalyzes the formation of artemisinic acid from artemisinic aldehyde, and 3) *CYB5*, the gene encoding a cytochrome *b*₅ that enhances the reaction rate of some CYPs. Eventually, they demonstrated artemisinic acid production (25 g L^{-1}) in glucose and ethanol-containing medium. The resulting artemisinic acid was further chemically converted to artemisinin by the method modified for scalable and practical production.

The *S. cerevisiae* system was also useful for the functional identification of new sesquiterpene synthases and CYPs involved in sesquiterpene biosynthesis. Göpfert et al. (2009) identified sesquiterpene synthases, including germacrene A synthases and a multiproduct synthase generating δ -cadinene as a major product in sunflower (*Helianthus annuus*). Use of the yeast germacrene A production system also identified a new CYP, germacrene A oxidase from lettuce (*Lactuca sativa*) (Nguyen et al. 2010).

Diterpenes

Diterpenes also include various clinically and commercially important metabolites, such as Taxol[®] (paclitaxel), tanshinones, and diterpene resin acids. Paclitaxel and its structural analogs are among the most potent and commercially successful anticancer drugs (Ajikumar et al. 2010). Tanshinones have a variety of pharmaceutical activities, including antibacterial, anti-inflammatory, and antitumor properties. They are found in the Chinese medicinal plant *Salvia miltiorrhiza* (Gao et al. 2009). Diterpene resin acids are major components of the oleoresins produced by conifers (Hamberger et al.

2011).

The first example of diterpene production in *E. coli* was reported by Huang et al. (2001). Because *E. coli* does not typically produce GGPP, GGPPS from a bacterium, *Erwinia herbicola*, was introduced into *E. coli* cells after removal of the N-terminal plastid targeting peptide sequence to generate a pseudomature form. Overexpression of 1-deoxyxylulose 5-phosphate synthase (DXS) in the MEP pathway, isopentenylidiphosphate isomerase (IDI), and taxadiene synthase, yielded 1.3 mg L^{-1} of the paclitaxel intermediate taxa-4(5),11(12)-diene (taxadiene). Efficient production of taxadiene (1 g L^{-1}) was also achieved by engineering the upstream MEP pathway native to *E. coli* and the downstream diterpene-forming pathway (Ajikumar et al. 2010). In addition, a CYP, taxadiene 5 α -hydroxylase gene, was introduced into the system to produce taxadiene 5 α -ol, a precursor of paclitaxel immediately following taxadiene.

Introduction of a GGPPS from fir (*A. grandis*) and various diterpene cyclase genes into *E. coli* produced a variety of diterpenes with abietane, cassane, kaurane, pimarane, stemarane, and stemodane-type skeletons (Cyr et al. 2007). Initially, abieta-7,13-diene (abietadiene) was synthesized by coexpressing GGPPS and a diterpene cyclase, abietadiene synthase, from fir (Peters et al. 2000), as pseudomature forms lacking N-terminal transit peptides. Combinations of a class II diterpene cyclase [*ent*-copalyl diphosphate synthase (*ent*-CPS)] from *A. thaliana*, which is responsible for the cyclization of GGPP to *ent*-copalyl diphosphate, and each class I diterpene cyclase [*A. thaliana* kaurene synthase (AtKS), *Oryza sativa* kaurene synthase like-6 (OsKSL6), OsKSL7, OsKSL5j, or OsKSL10], which catalyzes dephosphorylation and further cyclization, were tested. Under optimized conditions, *ent*-kaur-16-ene was produced, at a yield of ca. $100 \mu\text{g L}^{-1}$, by expression of three enzymes, GGPPS, *ent*-CPS, and AtKS. A similar strategy was used to produce *syn*-type diterpenes. A rice *syn*-copalylidiphosphate synthase (OsCPS4) was expressed instead of *ent*-CPS, and each class I diterpene cyclase (OsKSL4, OsKSL8, or OsKSL11) was expressed, along with GGPPS, to generate *syn*-pimara-7,15-diene, *syn*-stema-13-ene, or *syn*-stemod-13(19)-ene, respectively (Cyr et al. 2007).

This modular metabolic engineering system in *E. coli* was later used to identify new diterpene cyclases and a CYP. For example, two diterpene cyclases, SmCPS and SmKSL, were identified in a Chinese medicinal plant *S. miltiorrhiza* (Gao et al. 2009). SmCPS, a class II diterpene cyclase, was found to catalyze the specific formation of normal copalyl diphosphate (neither *ent*- nor *syn*-copalyl diphosphate) from GGPP, whereas SmKSL, a class I diterpene cyclase, mediated the formation of miltiradiene from normal copalyl diphosphate. Miltiradiene was later shown to be an intermediate en route to tanshinones

(Guo et al. 2013). On the other hand, a rice CYP, CYP701A8 was found to convert *ent*-kaurene to *ent*-kauren-19-oic acid using the *ent*-kaurene production system in *E. coli* (Wang et al. 2012).

Using the *S. cerevisiae* system, GGPPS and five sequential enzymes in the paclitaxel biosynthetic pathway between taxadiene and taxadien-5 α -acetoxy-10 β -ol were installed in a single yeast host. Although its yield was low (ca. 1 mg L^{-1}), taxadiene was detected in the yeast; however, the downstream metabolites were hardly detected, probably because of limited expression of taxadiene 5 α -hydroxylase (DeJong et al. 2006). Coexpression of codon-optimized taxadiene synthase and *Sulfolobus acidocaldarius* GGPPS, the latter of which does not show feedback inhibition, as well as a modified HMGR and a mutant of a regulatory protein involved in steroid uptake, UPC2-1, yielded about 8.7 mg L^{-1} taxadiene (Engels et al. 2008).

In vivo formation of diterpene resin acids was observed following the introduction of CYP720B from Sitka spruce (*Picea sitchensis*) into engineered yeast expressing GGPPS, CPR, and diterpene cyclases (Hamberger et al. 2011). The coexpression in yeast of GGPPS and novel class I and II diterpene cyclases from *Salvia sclarea* produced sclareol, a diterpene alcohol valuable for the fragrance industry (Caniard et al. 2012). Regarding tanshinone production, the production of miltiradiene was achieved in the engineered yeast harboring the fusion of SmCPS and SmKSL as well as the fusion of BTS1 (GGPPS) and ERG20 (FPPS) at the titer of 365 mg L^{-1} (Zhou et al. 2012). Similar engineering but additional introduction of *S. acidocaldarius* GGPPS into the miltiradiene production system were performed (Dai et al. 2012). Guo et al. (2013) transformed a new CYP76AH1 from the *S. miltiorrhiza* into the miltiradiene-producing yeast and detected the formation of ferruginol, indicating that miltiradiene is a precursor of ferruginol. They also showed the intermediacy of miltiradiene in tanshinone biosynthesis by tracer experiment and suggested that ferruginol is also an intermediate of tanshinones.

Triterpenes

Triterpenes are aglycons of triterpenoid saponins with a wide range of pharmacological activities. Glycyrrhizin from licorice (*Glycyrrhiza* spp.) is industrially important, because it shows anti-inflammatory, antiulcer, and antiallergy activities. In addition, glycyrrhizin is 150 times sweeter than sucrose. Many forms of licorice are commercially available worldwide as medicinal materials and sweetening agents (Seki et al. 2011). Triterpene biosynthesis is initiated by the formation of squalene from two FPP molecules by squalene synthase. Squalene is subsequently oxidized to 2,3-oxidosqualene by squalene epoxidase, and 2,3-oxidosqualene is cyclized

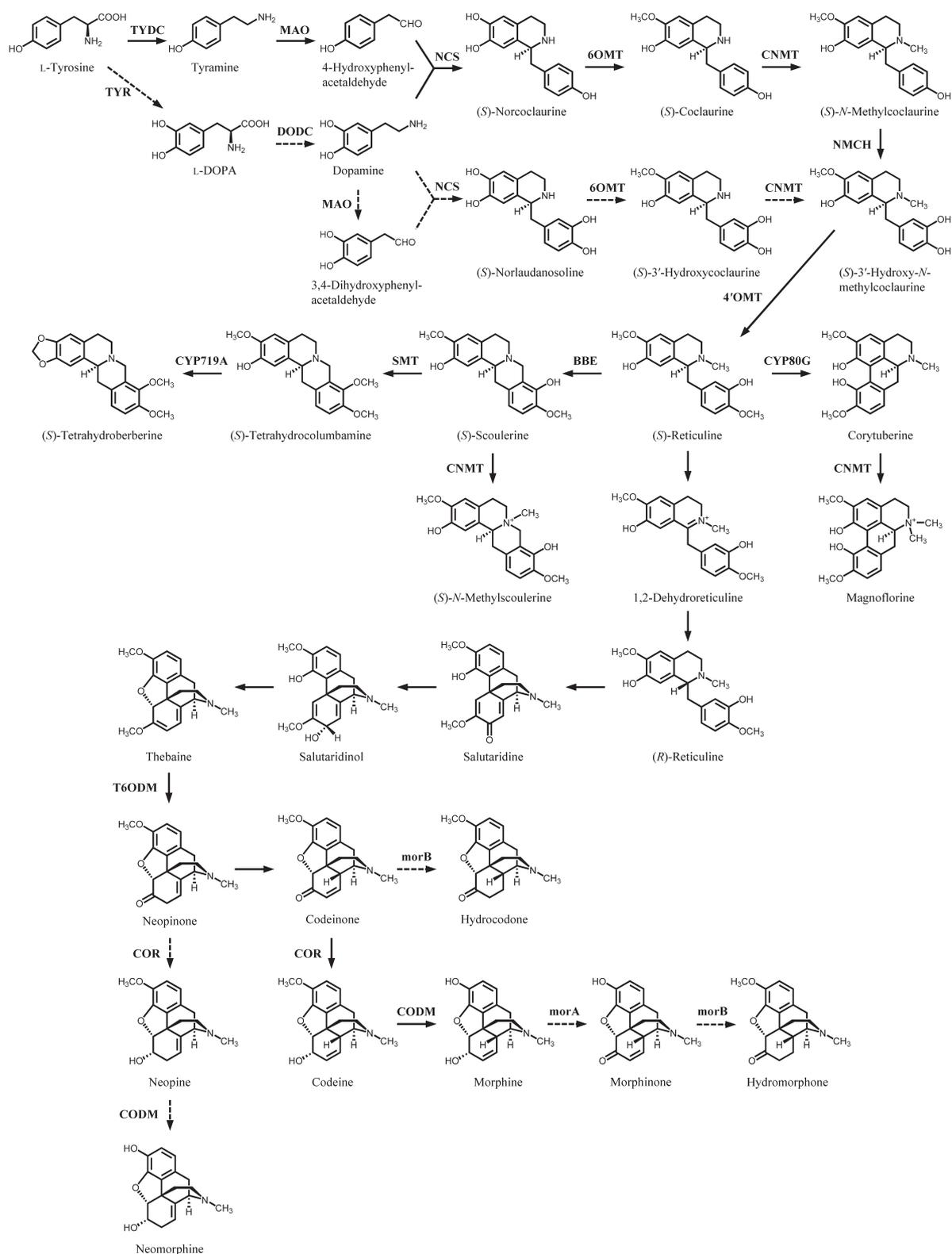


Figure 4. Biosynthetic pathways for benzyloisoquinoline alkaloids. Solid lines, pathways in plants; dotted lines, artificial pathways in microorganisms. 4'OMT, 3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase; 6OMT, norcoclaurine 6-O-methyltransferase; BBE, berberine bridge-forming enzyme; CNMT, coclaurine N-methyltransferase; CODM, codeine O-demethylase; COR, codeinone reductase; DODC, DOPA decarboxylase; MAO, monoamine oxidase; morA, morphine dehydrogenase; morB, morphinone reductase; NCS, norcoclaurine synthase; NMCH, (S)-N-methylcoclaurine 3-hydroxylase; T6ODM, thebaine 6-O-demethylase; TYDC, tyrosine decarboxylase; TYR, tyrosinase; SMT, scoulerine O-methyltransferase.

by oxidosqualene cyclases to build the basic carbon skeletons of triterpenes and sterols (Abe et al. 1993; Sawai and Saito 2011). Coexpression of genes encoding β -amyrin synthase (bAS) and CPR from *Lotus japonicus* and CYP88D6 from *Glycyrrhiza uralensis* in *S. cerevisiae* resulted in the formation of 11-oxo- β -amyrin as the major product and 11 α -hydroxy- β -amyrin as the minor product (Seki et al. 2008). Introduction of an additional CYP from *G. uralensis*, CYP72A154, into the triple transformant harboring bAS, CPR, and CYP88D6 produced glycyrrhetic acid, an aglycon of glycyrrhizin (Seki et al. 2011). Construction of an expression system of CPR, using each of three different oxidosqualene cyclases (bAS, α -amyrin synthase, and lupeol synthase), and a new CYP from *Medicago truncatula*, CYP716A12, enabled the production of oleanolic acid, ursolic acid, and betulinic acid, respectively (Fukushima et al. 2011).

Alkaloids

Alkaloids are a group of more than 12,000 nitrogen-containing specialized metabolites found in 20% of plant species (Liscombe and Facchini 2008; Minami et al. 2008). Many medicinal plants containing alkaloids were traditionally used as folk medicines, and some alkaloids are clinically used today. The benzyloquinoline alkaloids are one of the largest groups, with 2,500 different structures known to date (Sato and Kumagai 2013). They include clinically important compounds, such as morphine, codeine, berberine, papaverine, and tubocurarine (Liscombe and Facchini 2008).

Benzyloquinoline alkaloids are biosynthesized from L-tyrosine. The biosynthesis of all benzyloquinoline alkaloids is initiated by the norcoclaurine synthase (NCS)-mediated condensation of dopamine and 4-hydroxyphenylacetaldehyde, both of which are derived from L-tyrosine. The resulting (S)-norcoclaurine is then successively modified by norcoclaurine 6-O-methyltransferase (6OMT), coclaurine N-methyltransferase (CNMT), (S)-N-methylcoclaurine 3-hydroxylase (NMCH; CYP80B1), and 3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4'OMT) to yield the central intermediate (S)-reticuline. (S)-Reticuline undergoes diverse intramolecular coupling reactions resulting in the formation of various backbone structures such as protoberberine, benzophenanthridine, morphinan, and aporphine alkaloids (Liscombe and Facchini 2008; Sato and Kumagai 2013) (Figure 4).

To date, various benzyloquinoline alkaloids have been produced in *E. coli* and *S. cerevisiae* systems (Matsumura et al. 2013; Sato and Kumagai 2013). For example, five enzymes required for the conversion of dopamine to (S)-reticuline [i.e., monoamine oxidase (MAO), NCS, 6OMT, CNMT, and 4'OMT] were expressed in *E. coli* cells (Minami et al. 2008).

These engineered *E. coli* cells produced racemic reticuline from supplemented dopamine, although the condensation enzyme NCS, which stereoselectively forms (S)-norlaudanosoline, was expressed, and crude enzymes from the *E. coli* cells catalyzed the specific formation of (S)-reticuline. Co-culture of *S. cerevisiae* expressing CYP80G2 and CNMT with the engineered *E. coli* resulted in the production of magnoflorine and corytuberine. In contrast, co-culture of *S. cerevisiae* expressing berberine bridge enzyme (BBE) and *E. coli* yielded scoulerine and N-methylscoulerine. The reticuline-producing system was further modified to utilize a simple carbon source without precursor supplementation using L-tyrosine-overproducing *E. coli* (Nakagawa et al. 2011). When glycerol was used as a carbon source, this *E. coli* produced ca. 4.37 gL⁻¹ L-tyrosine. Introduction into the L-tyrosine-overproducing *E. coli* of a tyrosinase gene (TYR) from *Streptomyces castaneoglobisporus* to convert L-tyrosine to L-DOPA, a DOPA decarboxylase gene (DODC) from *Pseudomonas putida* to convert L-DOPA to dopamine, and a series of genes responsible for the formation of reticuline from dopamine yielded 2.26 and 6.24 mgL⁻¹ (S)-reticuline using glucose and glycerol, respectively, as carbon sources. Using *Ralstonia solanacearum* TYR instead of ScTYR increased the yield of (S)-reticuline to 46 mg L⁻¹.

Using *S. cerevisiae* as a host, a pathway from norlaudanosoline to reticuline was constructed by introducing 6OMT, CNMT, and 4'OMT, followed by the introduction of four genes, BBE, a scoulerine methyltransferase gene (SMT), CYP719A, and CPR, resulting in the production of (S)-tetrahydroberberine (Hawkins and Smolke 2008). Expression of promiscuous human CYP2D6 by the reticuline-producing yeast resulted in the formation of salutaridine from (R)-reticuline. More recently, Thodey et al. (2014) constructed artificial pathways to produce morphine and neomorphine from thebaine in *S. cerevisiae* by incorporating an episomal vector harboring thebaine 6-O-demethylase (T6ODM), codeinone reductase (COR), and codeine O-demethylase (CODM) genes. Optimizing the gene copy numbers of T6ODM, COR, and CODM and expressing COR fused with an endoplasmic reticulum localization tag at C-terminus resulted in the production of 86% morphine and 14% neomorphine, the latter of which is an unwanted by-product. Moreover, they additionally introduced the genes (*morA* and *morB*) involved in transformation of opiates from *P. putida* M10, a bacterium strain identified in waste from an opium poppy processing factory. When they expressed T6ODM, COR, *morA*, and *morB*, the engineered yeast produced hydrocodone and hydromorphone in addition to morphine and neomorphine.

Summary and future prospects

Plant specialized metabolites have proven beneficial for human life, and microbial production of these metabolites have been pursued over the decade. Among phenylpropanoids, 4-hydroxycinnamic acids such as 4-coumaric, caffeic, and ferulic acids can be produced in microorganisms. However, few reports on the construction of the metabolic pathways towards monolignols in microorganisms have been published. The biosynthetic pathways of flavonoids from L-tyrosine to final metabolites such as anthocyanins and flavones have been well established in microbes. Flavonoid-modifying enzymes such as glycosyltransferases and methyltransferases have also been coexpressed with core flavonoid biosynthetic pathways. Regarding isoprenoids, the first committed step for monoterpene, sesquiterpene, diterpene, and triterpene biosynthesis and subsequent steps catalyzed by CYPs have been reconstituted in microorganisms. Future challenges would be the functional and multiple introduction of CYPs as modifying enzymes towards the production of final complex metabolites. Of the alkaloids, only benzyloisoquinoline alkaloids can be produced using microbial platforms. Because several other classes of alkaloids are as important as benzyloisoquinoline alkaloids, such as indole and tropane alkaloids with high medicinal values, these alkaloids may be the next targets for microbial production.

Considering the high compatibility of eukaryotic membrane-bound CYP and glycosyltransferase expression with eukaryotic system and genetic amenability, *S. cerevisiae* is promising as a chassis organism for microbial production of plant specialized metabolites. In addition, various tools for synthetic biology in *S. cerevisiae* have recently been developed—synthetic promoters (Curran et al. 2014), a terminator library (Yamanishi et al. 2013), strong terminators (Ito et al. 2013), multiple expression systems (Du et al. 2012; Ishii et al. 2014), synthetic metabolons (Chun and Zhang 2013), an RNAi system (Crook et al. 2014), and a genome editing (DiCarlo et al. 2013). These new tools will also strongly assist the development in yeast. The structurally complex plant metabolites with high commercial values (e.g. camptothecin, glycyrrhizin, paclitaxel, and podophylotoxin) will be challenging targets for the production in *S. cerevisiae*.

It should be noted here that, in plants, specialized metabolites often accumulate at high concentrations in particular tissues or specific cell layers, due to the involvement of specific mechanisms for the accumulation of those metabolites. These mechanisms include membrane transport, vesicle transport, and the development of specialized tissues like glandular trichomes (Shitan and Yazaki 2013; Shitan et al. 2013;

Yazaki 2006). Basic understanding of these mechanisms can enable the industrial production of these metabolites; however, little is known about improving the yield of plant specialized metabolites by installing molecular apparatus responsible for these functions in engineered microorganisms. Construction of sophisticated systems consisting of genes involved in both the biosynthesis and accumulation of specialized metabolites remains challenges for future research.

Acknowledgements

This work was partly supported by a grant for explanatory research from the Institute for Sustainability Science, Kyoto University, and a grant for Grant-in-Aid for Scientific Research (C) (no. 25450241) from Japan Society for the Promotion of Science to S. S.

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