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Review

The phenylpropene synthase pathway and its applications in the engineering of volatile phenylpropanoids in plants

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Abstract Phenylpropenes such as eugenol, isoeugenol, chavicol, and anethole are C_6–C_3 volatile compounds derived from phenylalanine by modification of its benzene ring and reduction of its propyl side chain, with the final reduction step catalyzed by a product-specific phenylpropene synthase. Recent advances in the biochemical and molecular analysis of phenylpropene synthases have improved our understanding of their evolution, structural properties, and reaction mechanism, providing insights into how plants produce and regulate the many types of phenylpropene volatiles. Since phenylpropenes are important in determining the flavor of foods and the quality of essential oils in cosmetics, the identification of the genes and enzymes responsible for the biosynthesis of phenylpropene volatiles has also provided us with tools to meet the challenge of improving plant aromas through genetic engineering.

Key words: Plant volatiles, specialized natural products, genetic engineering, phenylpropenes.

Introduction

Like terpenes, benzenoids, and fatty acid derivatives, phenylpropenes constitute a major group of volatiles emitted from plants. Phenylpropenes play an important role in attracting both pollinators, through floral fragrances, and seed-dispersers, through scents emitted by fruits. For example, methyleugenol was identified as the major volatile in orchid flowers (genus *Bulbophyllum*) that attract male fruit flies as pollinators. These flies ingest the compound and convert it into pheromones that attract female flies. Hence, for the orchid flowers and fruit flies methyleugenol serves as a synomone, a signaling chemical that benefits both the sender and receiver (Tan and Nishida 2000). Moreover, when phenylpropenes are produced and stored in vegetative tissues such as trichomes, they can act as toxicants, deterrents, and antifeedants that discourage herbivores and bacterial pathogens (Pasay et al. 2010; Raguso and Pichersky 1995; Stuurman et al. 2004).

Phenylpropenes consist of a modified benzene ring (C_6) bearing a propenyl side chain (C_3). The structural diversity of many phenylpropenes stems from variation both in the position of the propenyl double bond and in the substituents on the benzene ring (Figure 1A). These structural differences sometimes influence the biological activity of the phenylpropenes. For example, phenylpropenes such as eugenol, isoeugenol, and chavicol, with a *para*-hydroxyl group and sometimes also a *para*-methoxyl group on the benzene ring, exhibit antifungal activity against filamentous pathogens such as *Botrytis cinerea*, while prenylated phenylpropenes, in which the hydroxyl group is masked with a dimethylallyl group, have no antifungal activity (Koeduka et al. 2014).

Phenylpropenes are not only essential for physiological function in plants, but also make important contributions to the fragrances and flavors of many plant species. Humans have historically used herbs and spices containing phenylpropene volatiles, which have a characteristic pungent or herbaceous aroma, for many different purposes—as seasoning when cooking, as pharmaceutical agents, and as pleasant aromatics in cosmetics. Therefore, plant biochemists have been eager to isolate the genes and enzymes responsible for phenylpropene production, and to utilize these for genetic modification of phenylpropene biosynthesis.

In this review, I highlight the latest advances in phenylpropene biosynthetic research and discuss recent challenges in the modification of phenylpropene profiles through genetic engineering of the phenylpropene pathway.

Phenylpropene biosynthesis

Phenylpropenes are normally formed from phenylalanine, which is also a precursor of flavonoids,

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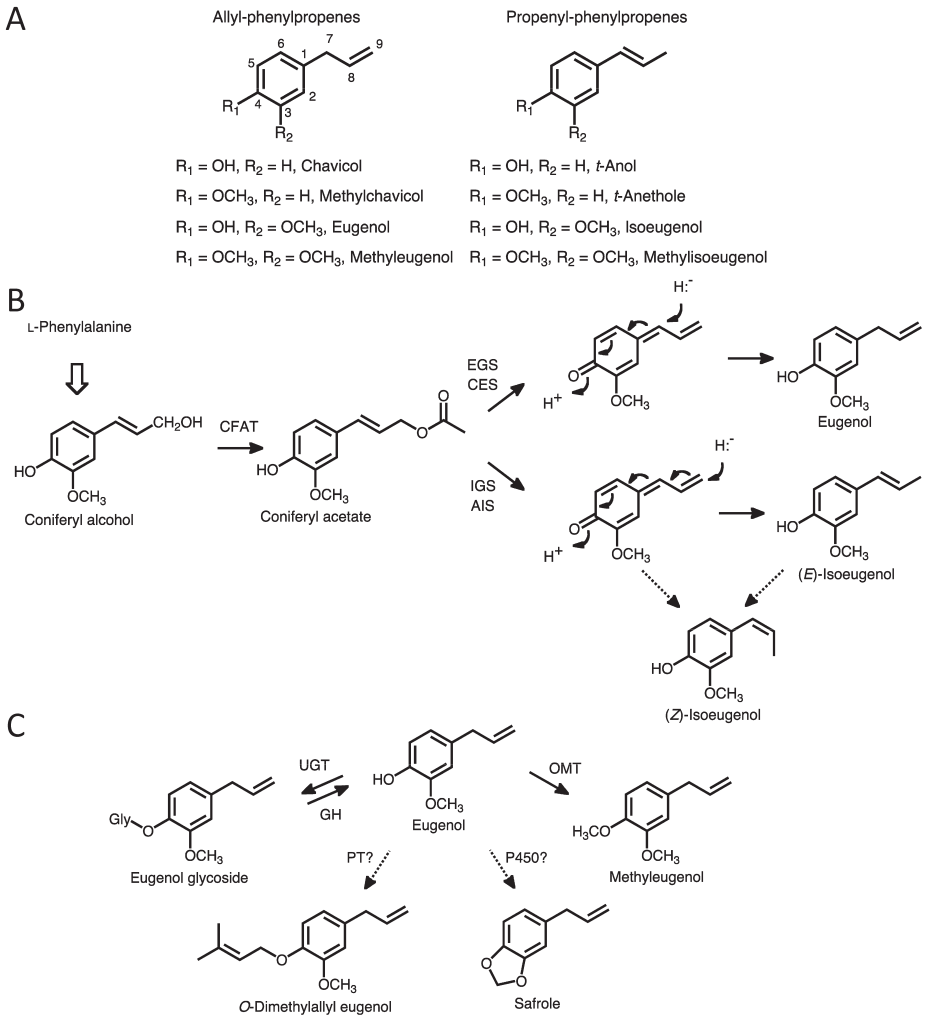

Figure 1. Structures and biosynthetic pathways for phenylpropene volatiles. (A) Structures of chavicol, eugenol, *t*-anol, isoeugenol, and their methylated derivatives. The carbon numbering system used in the text is shown. (B) The biosynthetic pathway leading to eugenol and isoeugenol from phenylalanine. CFAT, coniferyl alcohol acetyltransferase; EGS, eugenol synthase; CES, chavicol/eugenol synthase; IGS, isoeugenol synthase; AIS, *t*-anol/isoeugenol synthase. (C) Phenylpropene derivatives formed from eugenol. UGT, UDP-glycosyltransferase; GH, glycosyl hydrolase; PT, prenyltransferase; OMT, *O*-methyltransferase.

benzenoids, curcuminoids, stilbenoids, and lignin. The phenylpropene biosynthetic pathway diverges from the lignin pathway after the formation of monolignols such as *p*-coumaryl alcohol or coniferyl alcohol. At this point, an acyltransferase acetylates these monolignols to form *p*-coumaryl acetate and coniferyl acetate, respectively. These monolignol acetates are reduced by two distinct NADPH-dependent reductases, eugenol synthase (EGS) or isoeugenol synthase (IGS), to form an allyl-phenylpropene or propenyl-phenylpropene (Figure 1B). Further modifications of the benzene ring might then occur, including additional methylation, prenylation, or the formation of a methylenedioxy bridge (Figure 1C). These steps are described in more detail in the following sections.

Genes and enzymes of phenylpropene metabolism

Acetyltransferases

The first committed enzyme in phenylpropene biosynthesis is an acyltransferase, belonging to the BAHD family that transfers the acetyl moiety of acetyl-CoA to the hydroxyl group of a monolignol such as *p*-coumaryl alcohol or coniferyl alcohol. The resulting monolignol acetate serves as the substrates for EGS or IGS in a subsequent reductive reaction that produces a phenylpropene volatile. Several acetyl-CoA-dependent transacylation reactions have been documented in various plant species and biosynthetic pathways (D'Auria 2006). For example, benzyl acetate, geranyl acetate, and (*Z*)-3-hexen-1-yl acetate, which are known as fruit flavor and green leaf volatiles, are formed by BAHD acetyltransferases, including benzyl alcohol acetyltransferase (BEAT) in *Clarkia breweri*,

acetyl-CoA geraniol/citronellol acetyltransferase (AAT) in *Rosa hybrida*, and acetyl CoA:(*Z*)-3-hexen-1-ol acetyltransferase (CHAT) in *Arabidopsis thaliana* (D'Auria et al. 2007; Dudareva et al. 1998; Shalit et al. 2003). Recently, it was demonstrated that coniferyl alcohol acetyltransferase (CFAT) shows the substantial specificity for coniferyl alcohol, and that biosynthesis of isoeugenol, a prominent floral scent component in petunia, was inhibited by RNAi suppression of the *CFAT* gene (Dexter et al. 2007). In the isolated glandular trichomes of basil (*Ocimum basilicum*), efficient conversion of labeled coniferyl alcohol into its acylated form was observed when *p*-coumaroyl-CoA, instead of acetyl-CoA, was added into the assay mixtures (Koeduka et al. 2006). Thus, the acyltransfer reactions in basil leaves might be catalyzed by acyltransferases that can also utilize *p*-coumaroyl-CoA as the acyl-donor.

NADPH-dependent reductases

The monolignol acetates produced by the acetyltransfease, as described above, are subsequently reduced to phenylpropenes by one of two distinct NADPH-dependent enzymes, EGS or IGS. EGS converts the monolignol acetate into an allyl-phenylpropene eugenol (with the double bond between C8 and C9), whereas IGS produces a propenyl-phenylpropene isoeugenol (with the double bond between C7 and C8) from the same substrate (Figure 1B). These enzymes have been isolated from several plant species, basil (*Ocimum basilicum*), petunia, and *Clarkia breweri* (Koeduka et al. 2006, 2008). Enzymes other than EGS and IGS can also perform this reduction; for example, it was reported that a *t*-anol/isoeugenol synthase (AIS) from anise (*Pimpinella anisum*) and a chavicol/eugenol synthase (CES) from *Larrea tridentata* can use both *p*-coumaryl acetate and coniferyl acetate to synthesize either allyl- or propenyl-phenylpropenes (Koeduka et al. 2009a; Vassão et al. 2007).

All EGS and IGS enzymes identified to date belong to the PIP reductase family, the name of which derives from the first letters of the names of its first three identified members, pinoresinol-lariciresinol reductase (PLR), isoflavone reductase (IFR), and phenylcoumaran benzylic ether reductase (PCBER). The reaction mechanism of both EGS and IGS involves the reductive cleavage of acetate from the propenyl side chain of the monolignol acetate substrate by the formation of a quinone methide intermediate (Figure 1B). Detailed examination of substrate specificity using coniferyl acetate analogs with different side chains has demonstrated that coniferyl propionate, coniferyl butyrate, and coniferyl benzoate are converted to eugenol, whereas several analogs lacking a free *p*-hydroxyl group or a double bond in the propenyl side chain, including 4-bromo-cinnamyl acetate and dihydroconiferyl acetate, did not undergo any measurable reduction (Koeduka et al. 2006, 2009b). Therefore, EGS and IGS require both the propenyl double bond and the hydroxyl group of benzene ring in their substrate in order to form the quinone methide intermediate that leads to catalysis.

A naturally occurring frameshift mutation in the *IGS* gene of *Petunia axillaris* ssp. *parodii* has recently been discovered. Flowers of plants that carry this mutation do not produce isoeugenol; instead, they accumulate high levels of dihydroconiferyl acetate, a substrate derivative from the reduction of the propenyl double bond of coniferyl acetate (Koeduka et al. 2009b). Therefore, it is most likely that the reductive reaction occurs through a quinone methide intermediate occurred in planta as well as in in vitro assays using recombinant enzymes.

Previous studies have shown that a number of varieties of *Acorus* species contain (*Z*)-propenyl phenylpropenes, including β-asarone, but no enzyme responsible for the formation of these (*Z*)-propenyl phenylpropenes has thus far been found (Du et al. 2008). According to the reaction mechanism of EGS and IGS based on the quinone methide intermediate, advance rotation at the single bond between C7 and C8 in the propyl side chain of the quinone methide intermediate before hydride attack at C9, could presumably lead to the formation of (*Z*)-isoeugenol (Figure 1B). Alternatively, it may be possible that isomerization of (*E*)-propenyl phenylpropenes by unknown enzymes leads to the formation of a (*Z*)-propenyl side chain. Further experiments are required to better understand this isomerization step.

In *Clarkia* flowers, eugenol and isoeugenol, along with their methylated derivatives, are produced. Their respective phenylpropene synthases (CbEGS1 and CbIGS1) were found to be very similar to each other; site-directed mutants in which two amino acid residues in CbIGS1 (V84F and Y87I) were converted into those found in CbEGS1, and the corresponding double mutant of CbEGS1 (F84V and I87Y), exhibited product preferences opposite for those of the natural enzymes (Koeduka et al. 2008). These two *Clarkia* EGS and IGS enzymes are members of Class I in the EGS/IGS subfamily of the PIP family, together with basil EGS and petunia IGS. On the other hand, phylogenetic analysis has revealed that CbEGS2 clusters together with members of the Class II subfamily, such as PhEGS1, FaEGS1a, and FaIGS1b, all of which show high similarity to *P. taeda* PCBER and *L. japonicus* PTR (Figure 2). It has been noted that EGS and IGS enzymes belonging to the Class II subfamily in Figure 2 show less substrate specificity than those in Class I. For example, PhEGS1 and CbEGS2 are able to use the same substrate used by PCBER, although their turnover rate on this alternate substrate is very low (Koeduka et al. 2008). While most EGS or IGS enzymes produce only one product, FaEGS2 from garden strawberry (*Fragaria*×*ananassa*) catalyzes

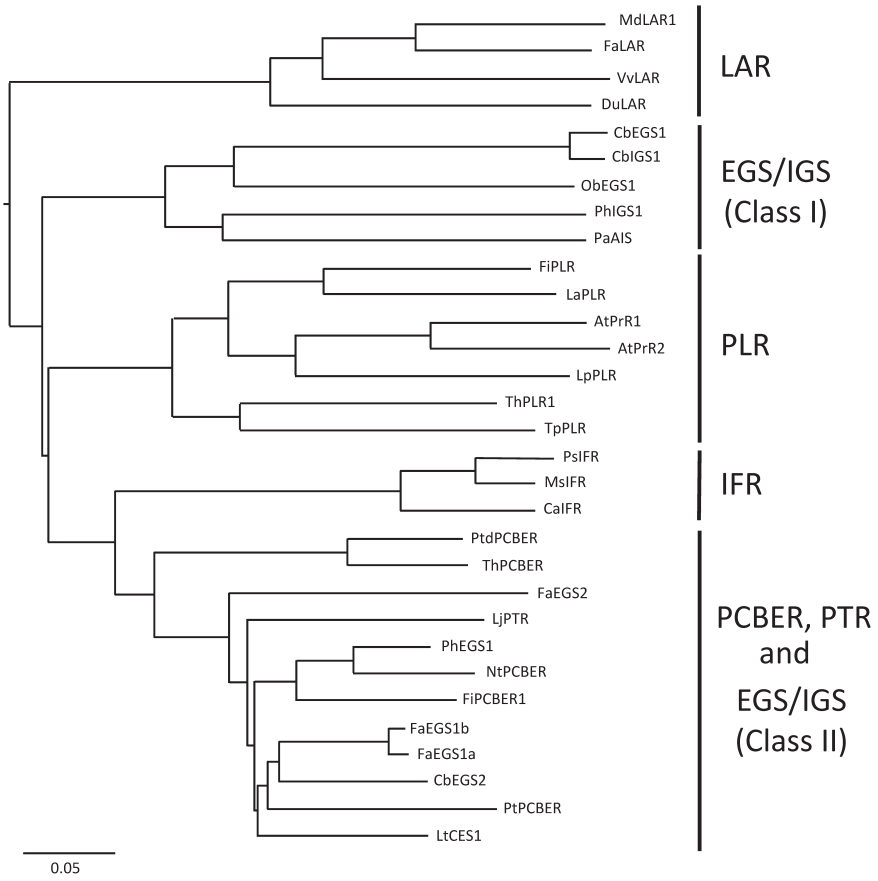

Figure 2. Phylogenetic tree of eugenol- and isoeugenol-forming enzymes together with selected protein sequences from the PIP family of NADPH-dependent reductases. Particular reductase clades are labled: LAR, leucoanthocyanidin reductases; PLR, pinoresinol lariciresinol reductases; IFR, isoflavone reductases; PTR, pterocarpan reductase; PCBER, phenylcoumaran benzylic ether reductases. Accession numbers corresponding to the sequences used in this study are: Md, *Malus domestica* LAR1 (AAZ79364); Fa, *Fragaria×ananassa* LAR (ABH07785), EGS1a (KF56224), EGS1b (KF562265), EGS2 (KF562266); Vv, *Vitis vinifera* LAR1 (NP_001267887); Du, *Desmodium uncinatum* LAR (Q84V83); Cb, *Clarkia breweri* EGS1 (ABR24113), EGS2 (ABR24114), IGS1 (ABR24112); Ob, *Ocimum basilicum* EGS1 (Q15GI4); Ph, *Petunia hybrida* IGS1 (Q15GI3), EGS1 (ABR24115); Pa, *Pimpinella anisum* AIS (ACL13526); Lt, *Larrea tridentata* CES1 (published in Vassao et al. 2007); Fi, *Forsythia×intermedia* PLR (AAC49608), PCBER1 (AAF64174); La, *Linum album* PLR (AAC49608); At, *Arabidopsis thaliana* PrR1 (NP_174490), PrR2 (NP_193102); Lp, *Linum perenne* PLR (ABM68630); Th, *Tsuga heterophylla* PLR1 (AAF64184), PCBER (AAF64179); Tp, *Thuja plicata* PLR (AAF63509); Ps, *Pisum sativum* IFR (P52576); Ms, *Medicago sativa* IFR (CAA41106); Ca, *Cicer arietium* (Q00016); Ptd, *Pinus taeda* PCBER (AAC32591); Nt, *Nicotiana tabacum* PCBER (BAG84267); Lj, *Lotus japonicus* PTR (BAF34844).

the formation of eugenol and also isoeugenol, but the latter with a lower catalytic efficiency (Aragüez et al. 2013). The kinetic parameters for monolignol acetate on CbEGS2 and PhEGS1 indicate that these enzymes, members of the Class II subfamily, are *bona fide* EGS. However, phylogenetic analysis suggests that the enzymes that are ancestral to the Class II EGS subfamily are unlikely to be EGS/IGS. Taken together with the distinct substrate specificities, it thus appears likely that the Class I and II subfamilies have evolved independently. Such convergent evolution has often been observed in other biosynthetic enzymes responsible for the formation of plant volatiles (Pichersky et al. 2006).

Enzymes that modify the phenyl ring of phenylpropenes

O-Methyltransferases

The formation of some phenylpropenes, such as methyleugenol, methylisoeugenol, methylchavicol, and *t*-anethole, requires methylation of the *para*-hydroxyl group on their benzene rings. Several *O*-methyltransferases involved in the biosynthesis of phenylpropenes have been characterized (Table 1). The first such enzyme to be characterized was (iso)eugenol *O*-methyltransferase (IEMT), found in *Clarkia breweri* (Wang et al. 1997). This enzyme can use both eugenol and isoeugenol as substrates, to form methyleugenol and methylisoeugenol respectively, but cannot use chavicol or *t*-anol, which do not have a methoxyl group at the *meta*-position on their benzene rings (unlike eugenol and isoeugenol). Next to be characterized were a chavicol *O*-methyltransferase (CVOMT) and a eugenol *O*-methyltransferase (EOMT) from *Ocimum basilicum*, which were shown to be highly specific for chavicol and eugenol, respectively (Gang et al. 2002). Site-directed mutagenesis to convert a specific amino acid residue

Table 1. Biochemically characterized Phenylpropene *O*-methyltransferases (OMT).

OMT name	Species	Relative activity (%) for phenylpropenes				Reference
		t-anol	chavicol	isoeugenol	eugenol	
EOMT	*Ocimum basilicum*	0.0	24.0	26.0	100.0	Gang et al., *Plant Cell* (2002)
CVOMT	*Ocimum basilicum*	5.7	100.0	3.4	4.0	Gang et al., *Plant Cell* (2002)
IEMT	*Clarkia breweri*	0.2	1.9	92.3	100.0	Wang et al., *Plant physiology* (1997)
AIMT	*Pimpinella anisum*	45.3	5.9	100.0	9.1	Koeduka et al., *Plant Physiology* (2009)

in one of these enzymes to the amino acid found in the other, and vice versa, caused a corresponding shift in substrate preference, indicating that substrate preference was controlled by a single amino acid residue. Most recently, a *t*-anol/isoeugenol *O*-methyltransferase (AIMT) from *Pimpinella anisum* was reported, with 10-fold higher activity for propenyl phenylpropenes such as *t*-anol and isoeugenol over allyl phenylpropenes such as chavicol and eugenol (Koeduka et al. 2009a). It is likely that these phenylpropene OMTs can discriminate based upon the double-bond position in the propenyl side chain and/or the functional groups on the benzene rings of various potential substrates (Table 1).

Prenyltransferases

O-Prenylated phenylpropenes, such as *O*-dimethylallyleugenol and *O*-dimethylallyl-5-methoxyeugenol, in which the hydroxyl group on the benzene ring is substituted with a dimethylallyl group, are found as major constituents in a few plant species, including *Illicium anisatum* and *Illicium verum* (Howes et al. 2009; Koeduka et al. 2014), with the apparent function of strongly deterring oviposition by mites. The biosynthesis of these *O*-prenylated phenylpropenes is not yet well understood, since no phenylpropene-specific prenyltransferases have yet been characterized. However, recent research has investigated membrane-bound prenyltransferases capable of catalyzing the prenylation of aromatic compounds such as flavonoids and coumarins (Karamat et al. 2014; Yazaki et al. 2009). It is possible that homologous genes might be responsible for the formation of prenylated phenylpropenes in *Illicium* species.

Methylenedioxy bridge-forming enzymes

Some phenylpropenes, including safrole, myristicin, dillapiole, and apiole, contain a methylenedioxy-bridge. In addition to volatile phenylpropenes, methylenedioxy-bridges occur in many other natural products, such as plant isoquinoline alkaloids and lignans. Previous work has found two methylenedioxy bridge-forming cytochrome P450 enzymes, CYP719A and CYP81Q, which catalyze the formation of stylopine and sesamin respectively (Ikezawa et al. 2003, Marques et al. 2013; Ono et al. 2006). It is likely that similar cytochrome P450 enzymes are able to form methylenedioxy-bridges on phenylpropene.

Glycosyltransferases and glycosidases

Phenylpropenes such as eugenol are often sequestered as glycosylated derivatives, such as in tomato fruits, rose flowers, and the leaves of *Camellia sasanqua* (Hammami et al. 2006; Straubinger et al. 1999; Tikunov et al. 2010; Yamada et al. 1967). Recently, it was reported that the UDP-glycosyltransferase gene *SlUGT5* was highly expressed in ripening tomatoes, increasing the eugenol content of the fruit (Louveau et al. 2011). The SlUGT5 protein showed glycosylation activity toward eugenol as well as guaiacol and methyl salicylate in an in vitro assay, although there was no direct evidence that SlUGT5 contributed to accumulation of eugenol glycosides in the fruits. In the leaves of *Viburnum furcatum*, a large amount of chavicol disaccharide was found (Ahn et al. 2004), apparently another instance of the glycosylation and sequestration of a phenylpropene although the enzyme responsible has not yet been found. Ahn et al. have reported the isolation of furcatin hydrolase, which specifically hydrolyzes the β-glycoside bond between chavicol and the disaccharide.

Metabolic engineering of phenylpropenes

Phenylpropenes such as eugenol and isoeugenol contribute substantially to the flavors of fruits and the scents of flowers, and higher levels of them are sometimes desired by plant breeders and consumers. Although many genes responsible for phenylpropene biosynthesis have been characterized, progress on the metabolic engineering of phenylpropene production has been limited. To date, there has been only one report of the engineering of phenylpropene production in a microorganism: in *E. coli*, the co-expression of a monolignol acetyltransferase and a chavicol/eugenol synthase (CES) from creosote bush (*Larrea tridentata*) was achieved and the direct conversion of coniferyl alcohol to eugenol was detected (Kim et al. 2014). In strawberries, on the other hand, heterologous overexpression of the basil *EGS* gene or the petunia *IGS* gene increased the formation of eugenol, isoeugenol, and related phenylpropenes (Hoffmann et al. 2011). Increased phenylpropenes production was also obtained by downregulation of the strawberry *chalcone synthase*

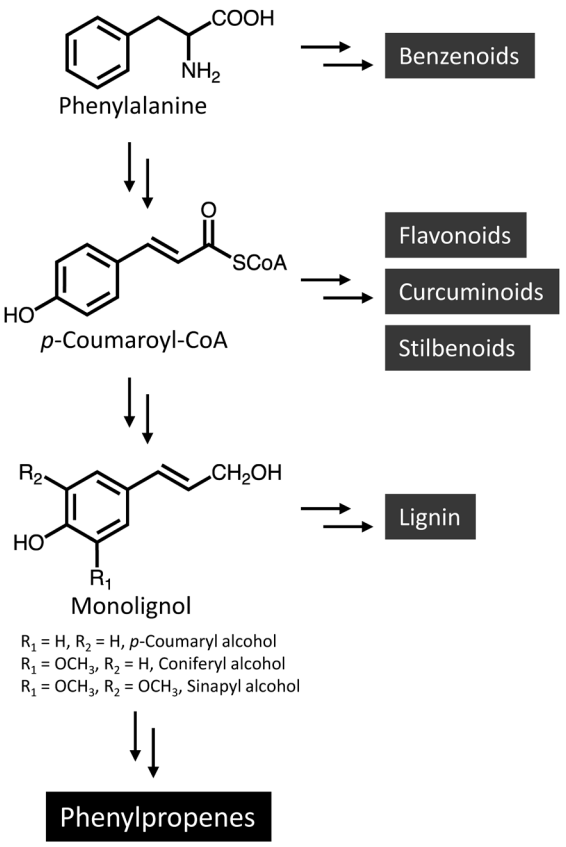

Figure 3. A flowchart showing possibilities for the enhancement of phenylpropene production in transgenic plants through modification of related biosynthetic pathways.

(*CHS*) gene with the simultaneous overexpression of basil *EGS* or petunia *IGS*. Recently, it was reported that overexpression in aspen leaves of petunia *coniferyl alcohol acetyltransferase* (*CFAT*), which catalyzes the first step in phenylpropene biosynthesis as discussed above, greatly increased levels of both eugenol and eugenol glycosides, while the overexpression of petunia *EGS* resulted in only a minor increase in eugenol (Koeduka et al. 2013). In contrast, the overexpression of petunia *CFAT* in transgenic tobacco and *Arabidopsis*, which unlike strawberry and aspen do not naturally produce phenylpropenes, gave no significant formation of eugenol. Thus, in order to increase the production of phenylpropenes, two key steps must be followed: (i) a host plant must be chosen that produces at least small amounts of phenylpropenes naturally, and (ii) the carbon flux of the biosynthetic pathway must be redirected away from some other final product, such as flavonoids, stilbenoids, or lignins, toward phenylpropene synthesis (Figure 3). Alternatively, the metabolic flow from phenylalanine into C_6–C_2 benzenoids, including 2-phenylacetaldehyde and 2-phenylethanol, could be suppressed, resulting in an increase in phenylpropene precursors. Transformation using *Agrobacterium* infection is limited to model plants; the development of a more generally applicable transformation method for phenylpropene-producing plants would allow the genetic engineering of the phenylpropene biosynthetic pathway to progress in a wide range of commercially important plant species.

Conclusions

Many phenylpropene volatiles have been identified in the essential oils and floral scents of a wide variety of plant species and play an important role in the ecological interactions between plants and other organisms such as pollinators, seed dispersers, and herbivores. Recent advances in our understanding of the phenylpropene biosynthetic pathway have revealed many key enzymes, but some of the enzymes responsible for functional modifications on the benzene ring remain to be discovered. Furthermore, our ability to genetically engineer the phenylpropene biosynthetic pathway is still limited, and there is much work yet to come in identifying additional phenylpropene synthases and developing more broadly applicable transformation methods.

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