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Terpene synthases and the regulation, diversity and biological roles of terpene metabolism

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Terpene synthases are the primary enzymes in the formation of low-molecular-weight terpene metabolites. Rapid progress in the biochemical and molecular analysis of terpene synthases has allowed significant investigations of their evolution, structural and mechanistic properties, and regulation. The organization of terpene synthases in large gene families, their characteristic ability to form multiple products, and their spatial and temporal regulation during development and in response to biotic and abiotic factors contribute to the time-variable formation of a diverse group of terpene metabolites. The structural diversity and complexity of terpenes generates an enormous potential for mediating plant–environment interactions. Engineering the activities of terpene synthases provides opportunities for detailed functional evaluations of terpene metabolites *in planta*.

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Introduction

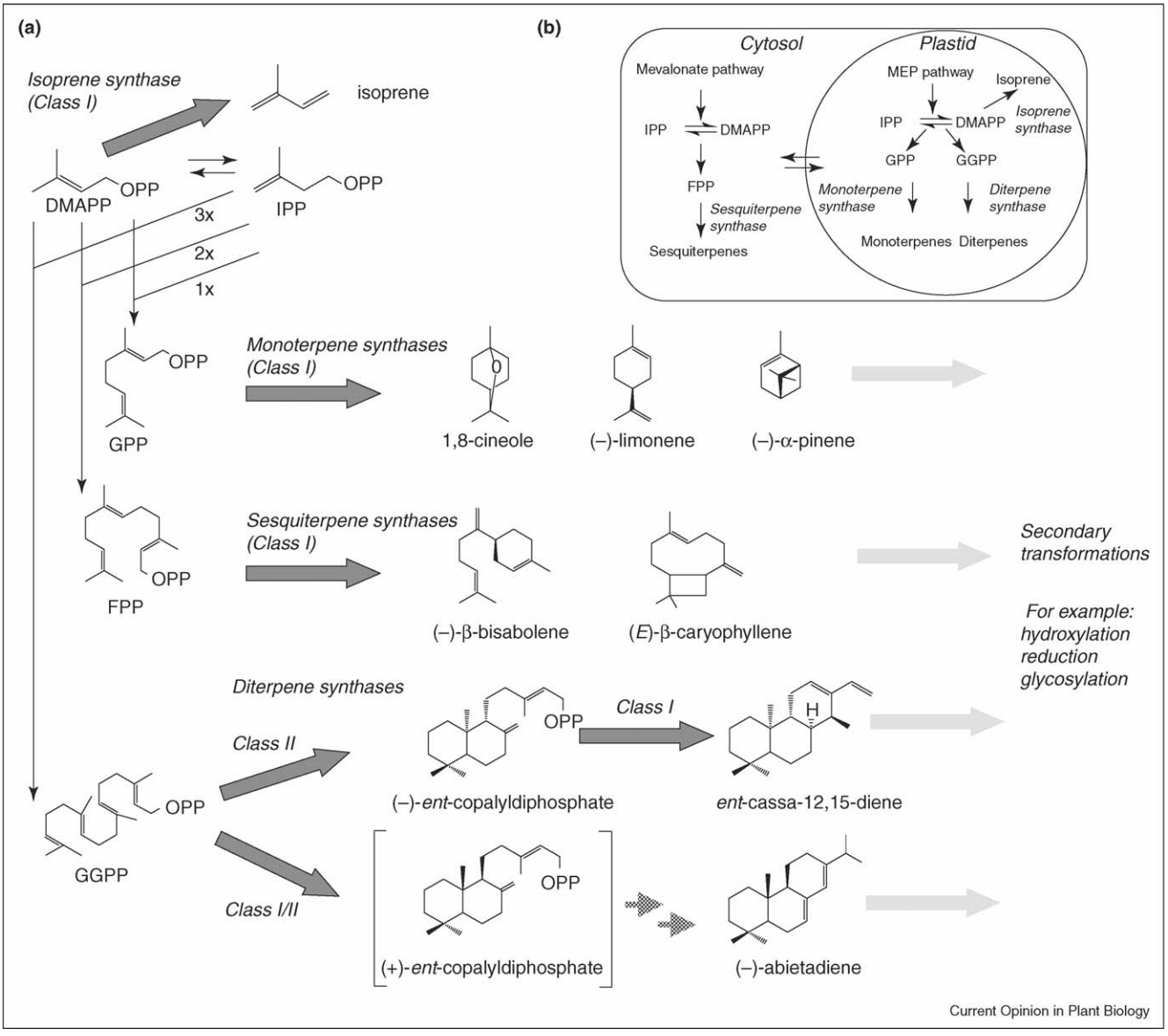
Mankind has used terpenes that are extracted from plants for many different purposes — as fragrances and flavors, as pharmaceutical agents and as insecticides. Aside from their immense commercial value, terpene products have important biological functions in plants. Terpene metabolites not only are essential for plant growth and development (e.g. gibberellin phytohormones) but also represent important tools in the various interactions of plants with the environment. Volatile and non-volatile terpenes are implicated in the attraction of both pollinators and predators of herbivores, in protection against photooxidative stress, in mediating thermotolerance, and in direct defense against microbes and insects [1–3].

With more than 20 000 different terpene metabolites known, the immense structural diversity of these natural products is both fascinating and puzzling. What are the biochemical basis and the biological significance of such product complexity? The initial substrates for the biosynthesis of the 20 000 terpenes are the simple C_5-unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). The activities of three prenyltransferases produces the direct precursors of terpenes, the linear prenyl diphosphates geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}) and geranylgeranyl diphosphate (GGPP, C_{20}). As shown in Figure 1, terpene synthases (TPS) are the primary enzymes responsible for catalyzing the formation of hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}) or diterpenes (C_{20}) from the substrates DMAPP, GPP, FPP or GGPP, respectively.

Plant hemiterpene, monoterpene, sesquiterpene and diterpene synthases are evolutionarily related to each other and are structurally distinct from triterpene or tetraterpene synthases, which are not the focus of this article. Numerous terpene synthases have been characterized and their rapid functional identification is supported by novel experimental improvements, such as surrogate splicing using genomic-sequence information for the recovery of correct full-length clones [4]. There are TPS enzymes that catalyze the formation of just one terpene compound, but there are also many TPS enzymes that have the astonishing capability to synthesize complex product mixtures with high regio- and stereospecificity [5]. Much of the progress achieved in recent years has centered on the structural elucidation of TPS polypeptides and on the identification and biochemical characterization of members of the large *TPS* gene families in a variety of model plants. These discoveries have led to a better understanding of the structural properties of TPS proteins that drive the reactive mechanisms leading to the formation of multiple products and that are the foundation for the molecular evolution of terpene diversity.

In this article, I describe research over the past two to three years that has refined our knowledge of how terpene product formation, terpene variation within single species, and terpene diversity across the plant kingdom have been defined by terpene synthase structure and evolution, and by the developmental and environmental regulation of *TPS* gene expression. I show how these insights, together with the ability to manipulate terpene formation, can be applied to dissect the functions of terpene metabolites *in planta*.

Figure 1



Outline of the formation of plant terpenes. **(a)** All terpenes are derived from the allylic prenyl diphosphates dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) by the action of terpene synthases activities. GPP, FPP, and GGPP are synthesized by prenyltransferases, which fuse DMAPP with varying numbers of isopentenyl diphosphate (IPP) units. Examples of different monoterpene, sesquiterpene, and diterpene synthase products are shown. The enzymatic reactions of all class I terpene synthases involve an initial step in which the prenyl diphosphate substrate is ionized and carbocation intermediates are formed. Class II diterpene synthases, such as *ent*-copalyl diphosphate (CPP) synthases, catalyze a protonation-induced cyclization of the substrate GGPP to CPP. Bifunctional (class I/class II) diterpene synthases, such as abietadiene synthase, catalyze an initial cyclization of GGPP to enzyme-bound (+)-CPP, followed by a typical ionization-initiated cyclization of (+)-CPP and subsequent reaction steps to form abietadiene. Class I diterpene synthases form their products from CPP or GGPP (not shown). All terpene synthase products can be subject to further secondary transformations. **(b)** Compartmentation of terpene biosynthesis in the plant cell. Two independent pathways, the mevalonate and the methylerythritol phosphate (MEP) pathway, form the C5-units IPP and DMAPP in the cytosolic and plastidic compartments, respectively. The biosynthesis of FPP and sesquiterpene metabolites occurs primarily in the cytosol, whereas the enzymes responsible for isoprene, monoterpene and diterpene formation are mostly located in plastids. OPP indicates the diphosphate moiety.

The role of terpene synthases in the evolution and diversity of terpene formation

The tremendous structural variety of terpene metabolites observed in the plant kingdom is mostly due to the evolution of a large terpene synthase superfamily, which includes more than a hundred *TPS* genes known today. A recent phylogenetic analysis compared the deduced amino-acid sequences of terpene synthase genes from

the conifer *Picea abies* with those of other gymnosperms and angiosperms. This work affirmed earlier studies stating that most gymnosperm terpene synthases form a family distinct from those in angiosperms [6]. Nevertheless, all plant TPS appear to have arisen from an ancestral diterpene synthase involved in primary metabolism that existed prior to the division and functional specialization of TPS in angiosperms and gymnosperms [6,7].

The genomic sequence and expressed sequence tag (EST) data sets of model plants such as *Arabidopsis*, maize, rice, tomato, *Medicago*, and *Picea* show large gene families of terpene synthases, resulting from cycles of gene duplication, multiple mutations and presumably functional divergence. The *Arabidopsis TPS* gene family represents a good example of these evolutionary processes [8]. Two root-expressed genes have been isolated that encode 1,8-cineole synthase and (*Z*)-γ-bisabolene synthase [9•,10]. Each gene exists in the form of two copies, which share 100 and 91% amino-acid sequence identity, respectively, indicating evolutionarily recent tandem duplications. In addition, another *Arabidopsis TPS* gene has been identified that has high amino-acid sequence identity (78%) to the two 1,8-cineole synthases, but the encoded enzyme does not form 1,8-cineole as its main product and is specifically expressed in floral tissues [11]. These observations indicate rapid divergence of the tissue-specific expression patterns and catalytic activities of closely related *TPS* genes.

Many monoterpene, sesquiterpene or diterpene synthases of different plant species form TPS subfamilies (TPSa–g) within the angiosperm or gymnosperm clades [6]. However, the rapid evolution of *TPS* genes has also established species-specific paralogous *TPS* gene clusters. In *Arabidopsis*, more than 10 sesquiterpene and putative diterpene synthases are more closely related to each other than to the sesquiterpene and diterpene synthases of other angiosperms [8,12•]. In addition to the divergent evolution of such species-specific genes, convergent evolution of *TPS* gene function can frequently be observed. For example, convergent evolutionary patterns have been suggested for isoprene synthase and other terpene synthases that form non-cyclic products, such as (*E*,*E*)-α-farnesene, by relatively simple reaction mechanisms [13•]. These enzymes apparently evolved independently from monoterpene synthases in both angiosperms and gymnosperms.

Aside from the rapid evolution of terpene synthases, the structural features of these enzyme catalysts are another major cause of terpene diversity. The reaction mechanisms of class I monoterpene, sesquiterpene and diterpene synthases (Figure 1a) involve a divalent-cation-dependent ionization of the prenyl diphosphate substrate and the formation of carbocation intermediates [5]. The intermediates have several different metabolic fates, leading to the synthesis of structurally diverse products. It is not usually possible to predict the product profile of terpene synthases on the basis of their primary structure alone. Therefore, the elucidation of enzyme structure–function relationships depends on relating three-dimensional (3D) structures and the position of amino-acid residues to the catalytic process. Previous structural analyses of the tobacco sesquiterpene synthase *epi*-aristolochene synthase [14] and the monoterpene synthase bornyl diphosphate synthase from *Salvia officinalis* [15] have defined a relationship between the catalytic mechanism and the topology of the active-site pocket located at the carboxy-terminal domain.

No 3D structure is yet available for diterpene synthases, although this group of enzymes is particularly known to catalyze both class II and bifunctional types of reaction mechanisms. Class II diterpene synthases, such as *syn*- and *ent*-copalyl diphosphate (CPP) synthases, which are involved in gibberellin and phytoalexin biosynthesis, catalyze a protonation-induced cyclization of the substrate GGPP to CPP (Figure 1a; [16]). Bifunctional (class I/class II) diterpene synthases, such as abietadiene synthase from *Abies grandis*, form enzyme-bound (+)-CPP from GGPP in an initial cyclization step. This step is followed by a typical ionization-initiated cyclization of (+)-CPP and subsequent reactions that form abietadiene isomers (Figure 1a). The two cyclization reactions occur at separate active sites in a central region (containing a DxxD motif for the protonation reaction) and a carboxy-terminal region; however, enzyme activities in a series of truncated polypeptides showed that both regions are structurally interdependent and cannot be dissected into catalytically distinct domains [17]. Both, bifunctional and class II-type enzymes possess an additional, approximately 250 amino acids long, amino-terminal 'insertional' element that is necessary for correct folding [16]. Knowledge of the 3D structure of this region will clarify its specific function.

Models for related TPS polypeptides, that are based on the solved structures of terpene synthases, have been tested using domain swapping and site-directed mutagenesis to identify regions and amino-acid residues in the active side and surrounding layers that control product specificity. Despite the overall high structural similarity of TPS enzymes, the identification of individual amino acids that are correlated with specific mechanistic steps has been achieved to date in only a few cases, and might remain a difficult task given the diversity of TPS proteins. In terpene synthases, which produce multiple products, it often appears that several amino acids act collectively in controlling the reaction cascades. This phenomenon was observed in domain-swapping and site-directed mutagenesis studies of multi-product monoterpene synthases from *A. grandis*; for example, in the attempt to convert

a (−)-pinene synthase into the highly homologous (−)-camphene synthase [18] and by altering the product outcome of a (−)-limonene synthase and a 91% identical (−)-limonene/(−)-α-pinene synthase [19]. Recent studies of two closely related maize terpene synthases, TPS4 and TPS5, have demonstrated the role of four amino acids that are located in the catalytic center in the stereoselectivity of these enzymes. The mixture of sesquiterpene products formed by each of the enzymes corresponds to the sesquiterpene volatile blends of two different maize cultivars whose genomes contain both *TPS* genes but only one functional allele [20••]. This work clearly revealed how terpene diversity can evolve in the process of breeding cultivars, thanks to allelic variation and subtle differences in the primary structures of closely related *TPS* genes. Further mechanistic analyses of TPS4 by modeling and site-directed mutagenesis suggested that the sequential parts of the reaction that leads to sesquiterpene formation occur in two different pockets of the catalytic center. One pocket appears to carry out the binding of the substrate (*E,E*)-FPP, the isomerization to the cisoid nerolidyl diphosphate intermediate, and the first cyclization step in the formation of (*S*)- and (*R*)-bisabolyl cations. These cations then seem to undergo a conformational change, causing a shift from one pocket to the other, where they are subsequently converted into the different sesquiterpene products [21].

Studies of the maize TPS4 and TPS5 enzymes also demonstrated that alleles encoding non-functional terpene synthases are apparently transcribed. A similar situation was found by analyzing the natural variation of floral terpene volatile profiles in different *Arabidopsis* ecotypes. Here, the loss of sesquiterpene volatile emissions from flowers of particular ecotypes was attributed to mutations or to posttranscriptional or translational regulation of two florally expressed *TPS* alleles rather than to differences in their transcription [12•]. On the other hand, in basil, quantitative variation of monoterpene and sesquiterpene mixtures in the peltate glands of different cultivars is primarily controlled by intraspecific variation of the expression of several functional *TPS* genes, which are responsible for the formation of the detected terpene compounds [22•]. Aharoni *et al.* [23•] described the differential expression of a linalool/nerolidol synthase (FaNES1) and a pinene/myrcene synthase gene, which determines the abundance of their terpene products in the fruits of cultivated and wildtype strawberry, respectively. FaNES1 is apparently targeted to the cytosol because of the absence of a plastidic transit peptide that is encoded by the corresponding allele of wild strawberry. The enzyme is responsible for the formation of nerolidol and linalool *in vivo*, which suggests the presence of GPP and FPP substrate pools in the cytosolic compartment. This is an unusual finding as, in most plants, GPP and GGPP are synthesized in plastids whereas FPP is formed in the cytosol with limited crosstalk occurring between both compartments (Figure 1b). Because many terpene synthases, such as FaNES1, accept more than one substrate, an intriguing aspect of the regulation of terpene diversity and variation is that the subcellular sites of terpene synthase activities and substrate pools determine product specificity *in vivo*.

Regulation of terpene synthase activities in different plant organs and in response to the environment

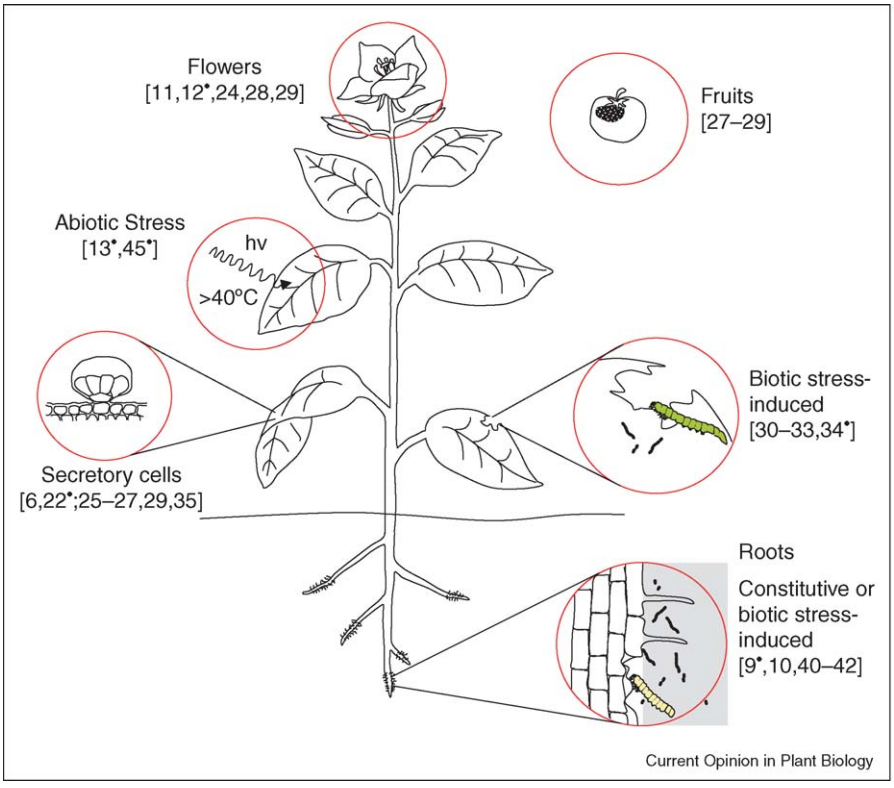
Terpene metabolites are implicated in several ecological and physiological functions on the basis of the differential expression profiles of terpene synthase genes observed throughout plant development and in response to biotic and abiotic environmental factors (Figure 2).

Volatile terpenes are often emitted from specific floral tissues at particular times to attract pollinators. Dudareva *et al.* [24] demonstrated that the biosynthesis and emission of the monoterpenes (*E*)-β-ocimene and myrcene in snapdragon flowers correlate with specific expression patterns of the corresponding monoterpene synthase genes in the upper and lower lobes of flower petals during floral development, with the highest transcript levels detected at day four post-anthesis. The expression of these genes also follows a weak diurnal oscillation that is under the control of a circadian clock. In *Arabidopsis* flowers, monoterpene and sesquiterpene synthases are not expressed in flower petals; instead, their expression is limited to the stigma, anthers, nectaries and sepals [12•]. These expression profiles suggest that the volatile terpenes that are synthesized in the *Arabidopsis* flower might not only function as short range attractants of pollinating insects but might be of equal importance for the defense of floral tissues by warding off microbial pathogens or herbivores from particularly vulnerable sites of the flower.

Many monoterpene and sesquiterpene synthase genes have been isolated and characterized from terpene-accumulating cells and tissues, such as leaf glandular trichomes and from fruits of agriculturally important plants including *Citrus* and grape [22•,25–29]. Some of these *TPS* genes exhibit broader expression in fruits and flowers, whereas others are specifically expressed at particular stages of fruit development or ripening. Such investigations are primarily supported by an interest in the metabolic and genetic regulation of the biosynthesis of flavors and aromas.

Given the increasing interest of researchers in the biological functions of plant volatiles, many studies are concerned with the role of terpene volatiles in the indirect defense of plants by attracting natural enemies of herbivores and plant parasites. Consequently, several *TPS* genes that are induced in leaves by herbivore attack or spider mite infestation, and which are responsible for the *de novo* biosynthesis of volatile monoterpenes

Figure 2



Plant organs and tissues in which terpene synthases are expressed under constitutive and stress conditions. The numbers in brackets correspond to the references describing terpene synthase gene expression or activities in the particular tissue.

and sesquiterpenes, have been identified in microarray, EST-data and cDNA analyses of maize, *Medicago*, *Lotus* and cucumber [30–33]. Owing to the devastating effects of different insect pests and associated fungi on forest trees, the function of volatile and non-volatile terpenes in the defense of trees has become a growing focus area. In poplar, for example, *TPS* gene expression and terpene volatile emissions are induced locally and systemically in leaves by feeding of forest tent caterpillars [34•]. Induced expression of *TPS* genes was also observed in stems of Sitka spruce following attack by white pine weevils [35]. The increased levels of *TPS* transcripts were accompanied by major changes in terpene accumulation, a response known as traumatic resinosis in conifer defense.

Thanks to the availability of the complete genomic sequence of rice, a comprehensive investigation of the biosynthesis and function of terpene metabolites in this monocot has become possible. In particular, the formation of gibberellins and diterpene defense metabolites (i.e. phytoalexins and allelopathic compounds) has been studied in detail, and several copalyl diphosphate synthase- and kaurene synthase-like genes have been identified [36]. While single *ent*-copalyl diphosphate (*OsCPS1*) and kaurene synthase (*OsKS1*) genes are most probably responsible for gibberellin formation [36,37•,38•], the remaining genes seem to be involved in diterpene phytoalexin synthesis as they are induced in rice leaves by elicitor and UV treatment. For example, a *syn*-CPP synthase gene (known as *OsCyc1* or *OsCPSsyn*) and a second *ent*-CPP synthase isoform gene (*OsCyc2*) have been characterized. These genes produce the CPP precursors from which the diterpene carbon skeletons of momilactones, oryzalexines and phytocassanes are derived [16,37•,38•]. In addition, two class I-type diterpene synthases (*ent*-cassa-12,15-diene synthase [*OsDTCS1*] and *syn*-pimara-7,15-diene [*OsDTS2*]) have been identified that convert *ent*-CPP or *syn*-CPP, respectively, into phytoalexin diterpene precursors [39,40].

In contrast to the above-ground organs of plants, roots represent a rather unexplored area of terpene biosynthesis and function. A few terpene synthases have been identified in plants roots, including the diterpene synthase OsDTS2 from rice [40]. In *Arabidopsis*, *TPS* genes encoding 1,8-cineole synthase and (*Z*)-γ-bisabolene synthase are differentially expressed in the stele of younger root growth zones and in the cortex and epidermis of older roots [9•,10]. These findings raise intriguing questions about the biological functions of volatile

terpenes in roots at different developmental stages and in interactions with root herbivores, parasites and microorganisms. As in aerial plant organs, terpene biosynthesis can be induced in roots under stress conditions, as shown after Douglas fir roots were treated with methyl jasmonate [41] or in the response of maize roots to attack by herbivores [42].

Several studies, including those described above, have confirmed that terpene synthase activities are often controlled at the level of *TPS* gene transcription. However, only one transcription factor, GaWRKY1, has been characterized to date, and it regulates the expression of δ-cadinene synthase (*CAD1-A*) in cotton [43•]. CAD1-A catalyzes the first committed step in the formation of the phytoalexin gossypol. Coordinated expressions of *GaWRKY1* and *CAD1-A* were observed in floral organs and in response to fungal elicitor treatment. W-box elements, the proposed binding sites for WRKY transcription factors, have been identified not only in the *CAD1-A* promoter but also in the promoter regions of other plant *TPS* genes, including tobacco 5-*epi*-aristolochene synthase [44] and putative *TPS* genes from *Arabidopsis* and rice [43•].

Indications of the posttranscriptional or posttranslational regulation of terpene synthases were obtained in studies of sesquiterpene volatile biosynthesis in flowers of *Arabidopsis* ecotypes [12•]. In addition, analyses of the seasonal variation of isoprene formation in poplar leaves suggested posttranslational modifications of isoprene synthase activity [45•]. Furthermore, isoprene formation in poplar appears to be regulated at metabolic levels because concentrations of the substrate DMAPP and emissions of isoprene are subject to coordinated diurnal changes without alterations in isoprene synthase activity. In snapdragon flowers, monoterpene biosynthesis is closely correlated with the expression levels of the small subunit of GPP synthase, indicating a tight control of the GPP substrate pool for monoterpene formation during flower development [46].

Manipulating terpene synthase expression for assessing functions of terpenes *in planta*

The biological activities of plant terpene metabolites, such as their toxic, repellent or antimicrobial properties, have been evaluated mostly by *in vitro* assays. However, these studies do not necessarily reflect the effect of terpene products at the cellular and tissue level *in vivo*. Furthermore, many plants form mixtures of terpenes, which are believed to be ecologically more effective than single compounds, for example, in the attraction of pollinators or parasites of insects, or by slowing down the breakthrough of a direct plant defense. One of the most valuable approaches to address these issues is the manipulation of terpene formation in model plants by the expression of *TPS* genes. Bouwmeester and coworkers [47] investigated the effect of ectopic expression of the strawberry nerolidol/linalool synthase gene *FaNES1* in *Arabidopsis* leaves that do not emit terpene volatiles under normal conditions. The formation of linalool from chloroplast-targeted FaNES1 had a repellent effect on the aphid *Myzus persicae*. Targeting of FaNES1 to the mitochondrial cell compartment caused the conversion of the enzyme product nerolidol into the homoterpene 4,8-dimethyl-1,3-(*E*)-7-nonatriene ([*E*]-DMNT) by an uncharacterized mitochondrial enzyme. Emissions of DMNT had an attractive effect on carnivorous predatory mites, supporting the important role of this terpene volatile in indirect plant defense [48••]. A similar approach was taken by Schnee *et al.* [49••] who expressed the maize gene *tps10* in *Arabidopsis*. Transgenic lines that emitted tps10 volatile sesquiterpene products could attract females of the parasitoid *Cotesia marginiventris* that had learned to locate their lepidopteran hosts after prior exposure to these volatiles in association with the hosts.

Another example of the manipulation of terpene volatile emission comes from the ectopic expression of *Kudzu* isoprene synthase in *Arabidopsis*, which is a non-isoprene producing species [13•]. Although these isoprene emitting transgenic lines have not yet been studied in great detail, they represent important tools for future analyses to understand the role of isoprene in mediating thermotolerance and oxidative stress protection. Successful manipulations of terpene formation have also been achieved in other model plants, such as tobacco [50,51]. In concert with *TPS* gene-expression lines, *TPS* gene knock-out lines will make it possible to dissect the biological functions of single endogenous terpene compounds or of defined terpene blends *in planta*. For example, the sesquiterpene volatile mixtures emitted from *Arabidopsis* flowers are dramatically altered in two *Arabidopsis* lines that carry T-DNA insertions in each of the florally expressed sesquiterpene synthase genes [12•]. These lines will help to clarify the physiological, defense and/or attractive roles of these compounds in the *Arabidopsis* flower.

Conclusions

Terpene synthases catalyze the formation of the most abundant and structurally diverse group of natural metabolites in plants. The evolution of terpene synthases in multi-gene families, their ability to form multiple products, and their differential expression that is mediated by developmental and stress-related programs, together drive the complexity and plasticity in terpene production. Although hundreds of terpene synthases have been characterized to date and our knowledge of the structural and mechanistic properties of these enzymes has increased tremendously, elucidating the multiple physiological and/or ecological roles of terpene synthase products remains a challenging task. Further investigations of the organization and regulatory machinery of terpene synthases at

cellular and subcellular levels should be combined with monitoring the ecological or physiological consequences of engineered terpene product profiles in model plants. These approaches will improve our understanding of the role of natural product complexity and diversity in plant–environment interactions.

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