Phytochemistry 72 (2011) 1635–1646

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Review

The biochemistry of homoterpenes – Common constituents of floral and herbivore-induced plant volatile bouquets

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ARTICLE INFO

Article history:
Available online 19 February 2011

Keywords:
Plant volatiles
Indirect defense
Floral scent
Homoterpene
Cytochrome P450
Terpene synthase

ABSTRACT

Volatile organic compounds emitted by plants mediate a variety of interactions between plants and other organisms. The irregular acyclic homoterpenes, 4,8-dimethylnona-1,3,7-triene (DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), are among the most widespread volatiles produced by angiosperms with emissions from flowers and from vegetative tissues upon herbivore feeding. Special attention has been placed on the role of homoterpenes in attracting parasitoids and predators of herbivores and has sparked interest in engineering homoterpene formation to improve biological pest control. The biosynthesis of DMNT and TMTT proceeds in two enzymatic steps: the formation of the tertiary C_{15}-, and C_{20}-alcohols, (*E*)-nerolidol and (*E*,*E*)-geranyl linalool, respectively, catalyzed by terpene synthases, and the subsequent oxidative degradation of both alcohols by a single cytochrome P450 monooxygenase (P450). In *Arabidopsis thaliana*, the herbivore-induced biosynthesis of TMTT is catalyzed by the concerted activities of the (*E*,*E*)-geranyllinalool synthase, AtGES, and CYP82G1, a P450 of the so far uncharacterized plant CYP82 family. TMTT formation is in part controlled at the level of *AtGES* expression. Co-expression of *AtGES* with *CYP82G1* at wound sites allows for an efficient conversion of the alcohol intermediate. The identified homoterpene biosynthesis genes in Arabidopsis and related genes from other plant species provide tools to engineer homoterpene formation and to address questions of the regulation and specific activities of homoterpenes in plant–herbivore interactions.

Contents

1. Introduction

Among the plethora of phytochemicals synthesized by plants, many exhibit high vapor pressures and are released as volatile compounds into the environment. Volatile semiochemicals facilitate short and long distance plant interaction with both beneficial and harmful organisms (Pichersky and Gershenzon, 2002; Raguso, 2004). In addition, plant volatiles such as isoprene have been recognized for their protective role against environmental stresses such as high temperature conditions (Loreto and Schnitzler, 2010; Sharkey and Yeh, 2001).

One of the best studied examples of biotic interactions mediated by plant volatiles is the attraction of pollinating insects. However, floral volatiles are not the only compounds emitted by plants but they may form plumes with volatiles released upon attack by herbivores or other biotic stresses. The emitted volatile blends are received as chemical signals by organisms at different trophic levels,

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doi:10.1016/j.phytochem.2011.01.019

thus generating a complex communication system between plants and guilds of pollinators, herbivores, and their parasites and predators (Dicke and Baldwin, 2010; Unsicker et al., 2009). One of the primary challenges in studying the chemical ecology of plant volatiles has been to elucidate the role of individual volatile compounds as well as that of their varying inter- and intra-specific blends.

Terpenes (or isoprenoids) represent the most abundant group of plant volatiles and are common components of floral scent and herbivore-induced volatile mixtures (Dudareva et al., 2006; Pichersky and Gershenzon, 2002). Recently, special interest has been placed on the class of acyclic homoterpenes, which are constituents of floral odors of night-scented plant species but are also frequently emitted by plants upon feeding damage (Boland et al., 1992; Kaiser, 1991). The common 11-carbon homoterpene 4,8-dimethylnona-1,3,7-triene (DMNT) and the 16-carbon homoterpene 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) are irregular terpenes that are derived by degradation of regular 15-carbon and 20-carbon terpene precursors, respectively (Boland et al., 1998). Besides their possible role as pollinator attractants, DMNT and TMTT have been implicated in attracting natural enemies of arthropod herbivores when released from damaged foliage (Fig. 1). This indirect defense strategy, which uses plant volatiles to "call for help" from parasitoids or predators of herbivores was first discovered by pioneering studies of Turlings et al. (1990) and Dicke et al. (1990a) (see articles by these authors in this special issue) and is thought to reduce feeding damage and enhance plant fitness. Whether homoterpenes are key volatile components in indirect defense or function synergistically with other volatiles is currently not well understood. In contrast to DMNT, TMTT was found not to be attractive to the predatory mite *Phytoselius persimilis* as a single compound (Dicke et al., 1990b; Shimoda et al., 2005). Studies with transgenic tomato also suggested a primary role of the spider mite- (*Tetranychus urticae*) induced benzenoid compound methyl salicylate rather than TMTT in *P. persimilis* attraction (Ament et al., 2010). However, de Boer et al. (2004) demonstrated that TMTT, when emitted in the presence of other induced volatiles from spider mite-infested lima bean leaves, influenced the foraging behavior of *P. persimilis*. Further evidence for the role of homoterpenes in indirect defense was obtained by treatment of lima bean with the terpenoid pathway inhibitor fosmidomycin, which severely reduced the emission of homoterpenes and thereby caused reduced attraction of predatory mites (Mumm et al., 2008). In Arabidopsis, TMTT is synthesized *de novo* in response to feeding damage by the crucifer specialists *Plutella xylostella* and *Pieris rapae* together with the monoterpene β-ocimene, the sesquiterpene (*E,E*)-α-farnesene, and methyl salicylate as the primary volatile components (Herde et al., 2008; Snoeren et al., 2010). The emitted volatile blend attracts the parasitoid *Cotesia rubecula*, which leads to an increase in plant fitness under laboratory conditions by parasitizing *P. rapae* larvae (van Loon et al., 2000). Recent studies with genetically engineered Arabidopsis plants showed that transgenic lines emitting DMNT together with its C_{15}-alcohol precursor (*E*)-nerolidol constitutively or upon treatment with jasmonic acid were more attractive to the predatory mite *P. persimilis* (Kappers et al., 2005). According to these observations, (*E*)-nerolidol by itself can attract predatory mites as has been demonstrated for the analogous C_{10}-alcohol linalool produced in transgenic potato (Aharoni et al., 2006). Thus, the attractive value of volatile mixtures containing homoterpenes appears to be species-specific and may depend on more than a single compound.

Despite the tremendous progress in identifying the enzymes involved in the formation of regular terpenes, the biosynthetic pathway leading to C_{11}- and C_{16}-homoterpenes has long remained elusive. Studies on homoterpene biosynthesis were first conducted ca. 15 years ago by Boland and co-workers. Their experiments using stable-isotope precursors laid the foundation for identifying the genes and enzymes involved in homoterpene formation. In this review, we conduct a survey of constitutive and induced emissions of DMNT and TMTT among land plants. We describe the genes and enzymes responsible for the two-step formation of homoterpenes including a recently discovered cytochrome P450 monooxygenase (P450) with homoterpene synthase activity in Arabidopsis. Furthermore, aspects of the regulation and engineering of the homoterpene biosynthesis pathway will be discussed.

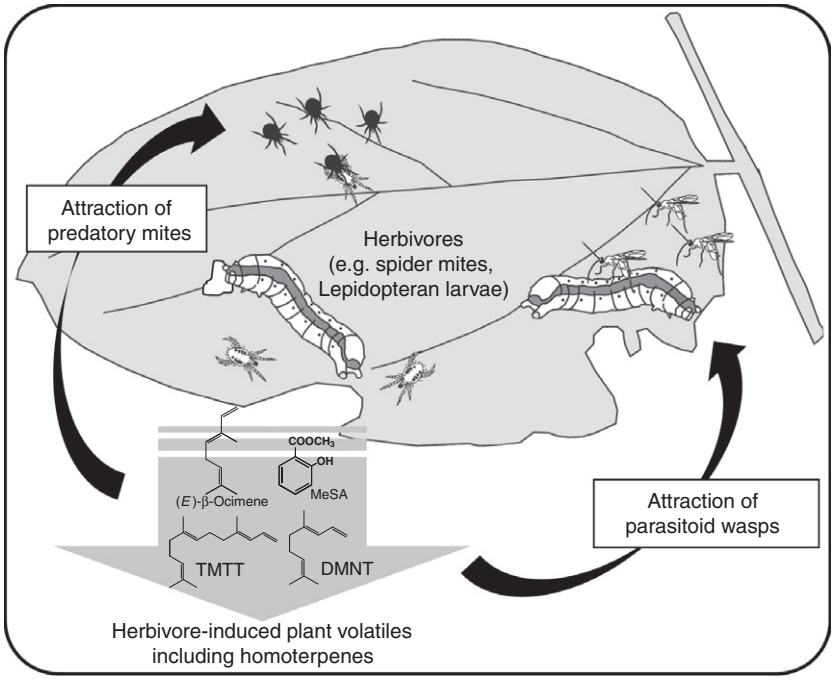

Fig. 1. Simplified scheme of the role of herbivore-induced plant volatiles in indirect defense. DMNT, 4,8-dimethyl-1,3,7-nonatriene; MeSA, methyl salicylate; TMTT, 4,8,12-trimethyltrideca-1,3,7,11-tetraene.

2. Distribution and function of C_{11}- and C_{16}-homoterpenes in land plants: a survey

Growing interest in the biosynthesis and biology of plant volatiles has resulted in a large number of studies reporting the emission of numerous volatile compounds from plant tissues. Terpenes are among the most frequently observed constituents of floral and vegetative odors (Dudareva et al., 2006). In particular, acyclic monoterpenes, sesquiterpenes, and the homoterpenes DMNT and TMTT are common components of volatile blends released in response to herbivore attack. To gain a better understanding of the distribution of homoterpenes and their presumed functions among land plants, we have conducted a survey of the previous and recent literature reporting constitutive and induced emissions of DMNT and TMTT (Fig. 2; Supplementary Table 1).

Although numerous terpenes have been identified from bryophytes including mosses, liverworts and hornworts (e.g. Adio and König, 2007; Saritas et al., 2001), we were unable to find any reports about homoterpenes in these plants. Similarly, no homoterpenes have been detected so far from pteridophytes, which are known for producing a variety of terpenes, especially triterpenes (Shinozaki et al., 2010). Nevertheless, the formation of the DMNT precursor nerolidol has been observed in a few cases in hornworts and ferns (Fons et al., 2010; Gao et al., 2008; Sonwa and König, 2003).

Gymnosperms typically produce resins, which are mixtures of monoterpenes, sesquiterpenes and diterpene acids. However, only little is known to what extent conifers synthesize homoterpene volatiles. Su et al. (2009) described the emission of DMNT amongst monoterpenes and sesquiterpenes from needles of *Pinus massoniana*, which were damaged mechanically or by feeding of larvae of the masson pine moth *Dendrolimus punctatus*. In contrast to the rare detection of homoterpenes in gymnosperms, homoterpene emissions have been reported from numerous angiosperms suggesting that the biosynthesis of these irregular terpenes emerged primarily during the evolution of flowering plants. Indeed, primitive angiosperms such as species in the Magnoliaceae family produce DMNT and TMTT as floral odor compounds (Azuma et al., 2001). Both homoterpenes occur as floral volatiles, often together with their precursors, nerolidol and geranyl linalool, in many other families of monocots and dicots. For a detailed overview of these families, we refer the reader to an excellent review by Knudsen et al. (2006). Most characteristically, homoterpenes are constituents of the so-called "white floral image" of night-scented plants such as members of the Orchidaceae, Cactaceae, Magnoliaceae, and Liliaceae. For example, Kaiser (1991) described TMTT as the main floral volatile of the highly fragrant, moth-pollinated African orchid *Aerangis friesiorum* and of floral scent emitted from *Selenicereus hamatus* (queen of the night). DMNT also represents the primary constituent of floral scent bouquets from yucca species that are associated with moths in an obligate pollination mutualism (Svensson et al., 2005, 2006). Homoterpenes may play an active role in attracting pollinators to the flowers of the described species although their specific activities in these interactions remain to be determined.

Besides their presumed beneficial role in mutualistic relationships, homoterpenes can function as kairomones by attracting herbivorous insects (Supplementary Table 1). DMNT emitted from flowers of the crucifer *Iberis amara* (candytuft) was found amongst other terpene and benzenoid compounds to elicit an olfactory response and the attraction of the pollen beetle *Meligethes aeneus* (Bartlet et al., 2004). Similar attractive effects of homoterpenes have been demonstrated for the strawberry blossom weevil, *Anthonomus rubi* (Bichao et al., 2005), and for fruit pests such as the apple maggot fly, *Rhagoletis pomonella* (Nojima et al., 2003), and the grapevine moth *Lobesia botrana* (Tasin et al., 2006).

Some plants that produce homoterpenes constitutively in flowers also release these compounds as induced odors in response to herbivore damage of vegetative tissue (e.g. magnolia; Azuma et al., 1997). Herbivore-induced emissions of homoterpenes are wide-spread among many monocots and dicots including trees such as poplar and walnut and a variety of crops such as maize, rice, cotton, lima bean, cucumber, tomato, and cabbage (Supplementary Table 1). As described above, it is thought that these induced responses benefit plants as indirect defenses, although studies on the effect of individual compounds or defined odor blends require further attention. Interestingly, homoterpenes may also be involved in the attraction of egg-parasitizing insects. For example, egg deposition by the elm leaf beetle *Xanthogaleruca luteola* on leaves of the European elm *Ulmus minor* induces a volatile blend that contains several terpenes, including DMNT, and leads to attraction of the egg parasitoid *Oomyzus gallerucae* (Wegener and Schulz, 2002).

One of the rather intriguing activities of herbivore-induced plant volatiles is their ability to induce or prime defense responses in unattacked neighboring plants or within the same plant (Frost et al., 2007). The role of volatile terpenes in plant–plant interaction was first described in lima bean, where homoterpenes released upon feeding of *T. urticae* induced the expression of defense genes encoding lipoxygenase (LOX) and the pathogenesis-related protein PR-2 (β-1,3-glucanase) in non-wounded control plants (Arimura et al., 2000). While the fitness advantage gained by plants eavesdropping on volatiles emitted by their neighbors has been discussed controversially, systemic responses mediated by volatiles *in planta* might have physiological benefits by transmitting internal signals independent of vascular transport (Frost et al., 2008). In addition to these air-borne effects, it seems possible that homoterpenes also serve as stress-responsive signals that diffuse into cell layers immediately surrounding a wound site. In this context, it will be important to determine whether the enzymatic degradation of homoterpene precursors leads to the formation of electrophilic α,β-unsaturated C_4-carbonyls such as but-3-ene-2-one (see below), which may function as independent signaling molecules similar to other small reactive lipid-derived electrophiles that induce stress responses (Almeras et al., 2003).

Taken together, homoterpenes appear to serve several functions in plants. While, to the best of our knowledge, no specific antimicrobial or insect repellent activities of these compounds have been described to date, many studies suggest that homoterpenes promote defense responses directly or indirectly in concert with other volatile compounds, but they can also be exploited by herbivores as attractive signals. Since homoterpene formation was found to be inducible in primitive flowering plants such as magnolia, it seems plausible that these compounds exhibited defensive activities in early angiosperm evolution most likely in overlap with their function in pollinator attraction. It is intriguing to speculate to what extent homoterpenes produced in flowers prime defense responses in vegetative tissues or interfere with the attraction of pollinators upon their induction in foliage.

3. Biosynthesis of C_{11}- and C_{16}-homoterpene precursors

Initial insight into the formation of homoterpenes came from studies applying stable isotope precursors. Boland and co-workers showed that the deuterium-labeled tertiary C_{15}-sesquiterpene and C_{20}-diterpene alcohols, (*E*)-nerolidol and (*E*,*E*)-geranyl linalool, were converted to DMNT and TMTT, respectively, when administered to floral tissues and to excised lima bean plantlets treated

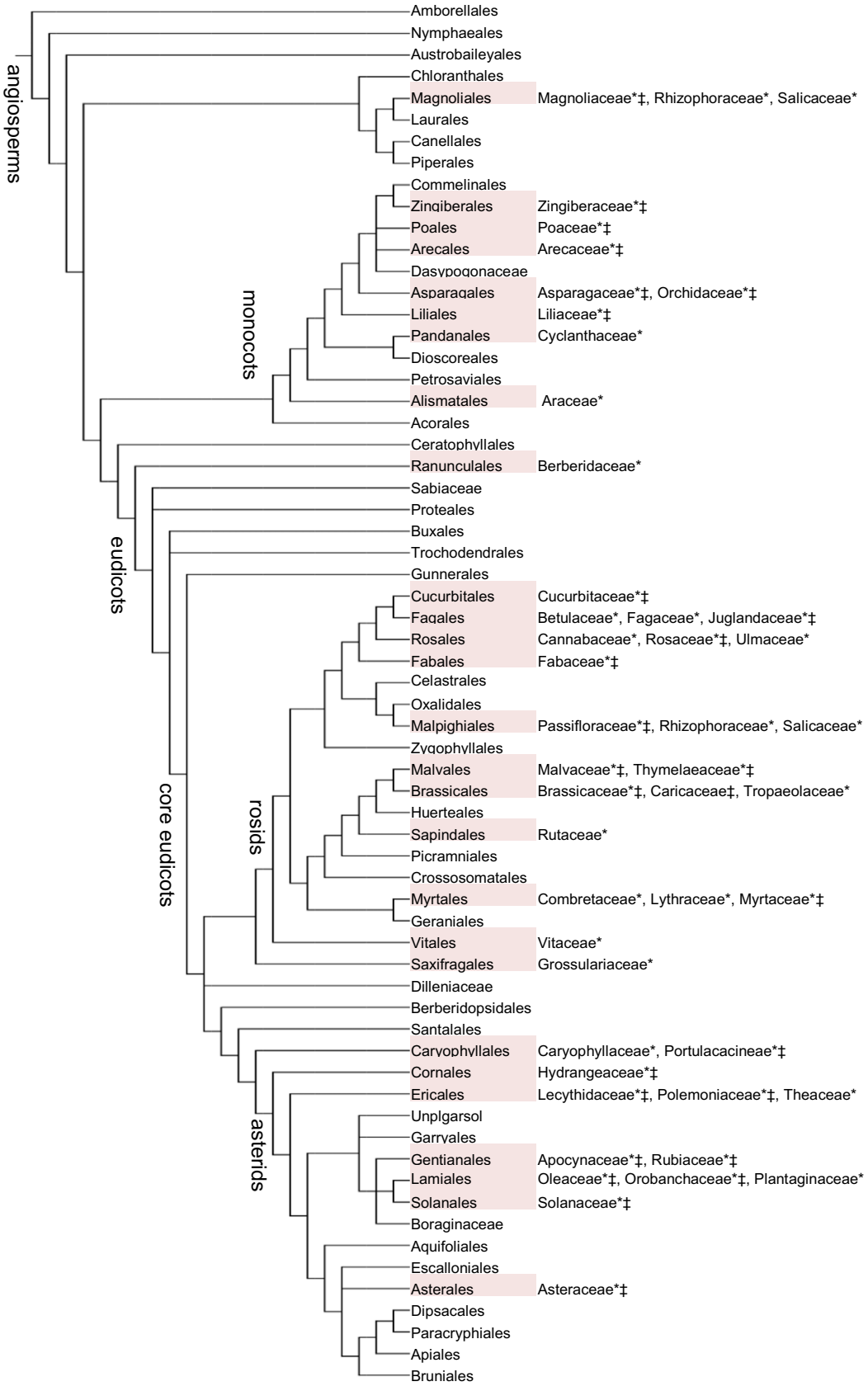

Fig. 2. Angiosperm orders and families with reported emission of DMNT and TMTT. The phylogenetic tree was obtained from the phylodiversity network (http://www.phylodiversity.net) and modified accordingly. For details of the emission of DMNT and TMTT see Supplementary Table 1. *DMNT; ‡TMTT.

with jasmonic acid (Gäbler et al., 1991). It was therefore predicted that the first dedicated step in the biosynthesis of both homoterpenes was the formation of a tertiary alcohol precursor catalyzed by a terpene synthase enzyme. Generally, terpene synthases (TPSs) convert prenyl diphosphate substrates, which are derived from condensation of the universal C_5-isoprenoid building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), into various terpene hydrocarbons or alcohols (Gershenzon and Kreis, 1999). The formation of (*E*)-nerolidol and (*E*,*E*)-geranyllinalool is supposed to proceed via hydrolysis of the diphosphate moiety from C_{15}-(*E*,*E*)-farnesyl diphosphate (FPP) and C_{20}-all-*trans*-geranylgeranyl diphosphate (GGPP), respectively, followed by an allylic rearrangement to yield a tertiary alcohol with a terminal olefin (Fig. 3).

Enzymes of the plant TPS superfamily show frequent species-specific radiation, a process thought to occur in adaptation to diverse biotic (and abiotic) selective pressures (Martin et al., 2004). Accordingly, the ability of TPSs to produce (*E*)-nerolidol and (*E*,*E*)-geranyllinalool by a rather simple reaction mechanism has arisen several times. (*E*)-Nerolidol synthases and (*E*,*E*)-geranyllinalool synthases have been identified in two TPS subfamilies, TPS-g and TPS-e/f (Fig. 4). Enzymes of the TPS-g subfamily are class I TPSs, which consist of a catalytically active C-terminal domain or α-fold with a highly conserved DDxxD motif and an inactive N-terminal domain. The DDxxD motif is generally required to position the diphosphate substrate for ionization. TPS-g proteins lack a RRX_8W motif, which is highly conserved near the N-terminus of monoterpene synthases (Dudareva et al., 2003). Many TPS-g type (*E*)-nerolidol synthases also produce (*E*,*E*)-geranyllinalool from GGPP and/or the C_{10}-analog linalool from geranyl diphosphate (GPP). For example, the TPS3 protein from *Medicago truncatula* (MtTPS3) (Arimura et al., 2008) synthesizes all three alcohols *in vitro*, although (*E*)-nerolidol and (*E*,*E*)-geranyllinalool are the primary products. Since expression of the *MtTPS3* gene is induced in *M. truncatula* leaves together with the emission of DMNT and TMTT upon feeding by the beet armyworm *Spodoptera exigua*, MtTPS3 is thought to contribute to the formation of one or both homoterpenes. Other multifunctional or bi-functional TPS-g enzymes producing all three alcohols or (*E*)-nerolidol and linalool have been characterized from rice (Yuan et al., 2008), snapdragon (AmNES/LIS1 and 2) (Nagegowda et al., 2008), Arabidopsis (AtTPS14) (Chen et al., 2003), tomato (LeMTS1) (van Schie et al., 2007), grape (VvPNLNGl1; VvPNLISNES1) (Martin et al., 2010), and strawberry (FaNES1) (Aharoni et al., 2004) (Fig. 4). However, with the exception of the rice TPS, the products of these enzymes do not seem to be further converted to homoterpenes and are directly emitted as components of floral scent (snapdragon, Arabidopsis), trichomes (tomato) or fruit aroma (grape, strawberry).

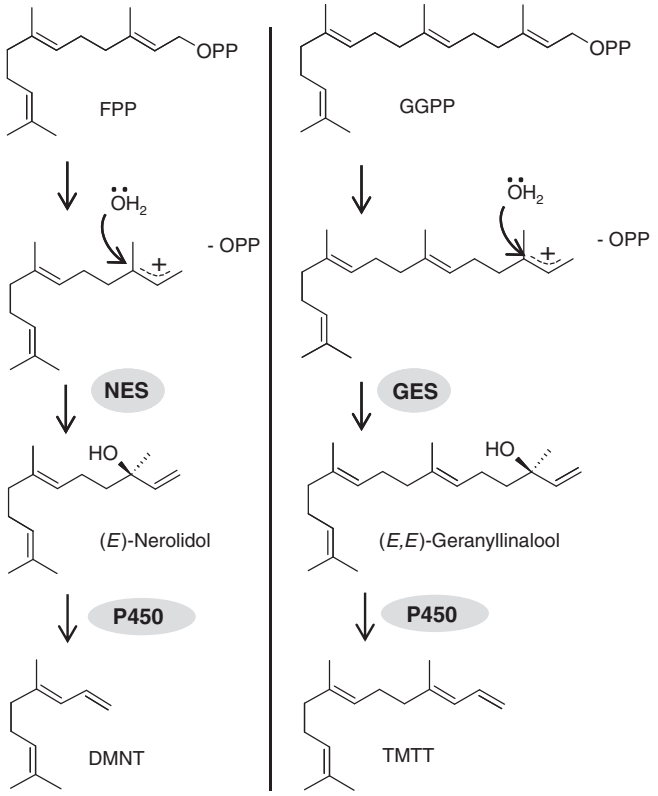

Fig. 3. Enzymatic steps in homoterpene biosynthesis. FPP, (*E*,*E*)-farnesyl diphosphate; GGPP, (*E*,*E*,*E*)-geranylgeranyl diphosphate; DMNT, 4,8-dimethyl-1,3,7-nonatriene; TMTT, 4,8,12-trimethyltrideca-1,3,7,11-tetraene; NES, (*E*)-nerolidol synthase; GES, (*E*,*E*)-geranyllinalool synthase; P450, cytochrome P450 monooxygenase.

Whether a TPS enzyme produces more than one terpene alcohol most likely depends on the size of the prenyl diphosphate substrate that can be accommodated by the protein active site cavity. Moreover, the product-specificity of a multi-functional TPS enzyme *in vivo* is determined by the subcellular localization of the TPS protein (Fig. 5). TPS enzymes targeted to plastids usually synthesize monoterpenes (linalool in this case) from GPP or diterpenes (geranyl linalool) from GGPP, because GPP and GGPP represent the primary prenyl diphosphate substrates in this organelle. Absence or loss of the targeting sequence result in cytosolic localization and the formation of sesquiterpenes (nerolidol) from the predominant pool of FPP in this cellular compartment. A differential subcellular localization was observed for the two nearly identical (*E*)-nerolidol/linalool synthase isoforms (AmNES/LIS1 and 2) (Nagegowda et al., 2008) from snapdragon. Similarly, strawberry FaNES1 produced primarily linalool in transgenic Arabidopsis when the protein was targeted to the plastid (Aharoni et al., 2003). The Medicago TPS3 enzyme described above also carries a plastidial transit peptide (Arimura et al., 2008) and, therefore, is thought to catalyze the formation of (*E*,*E*)-geranyllinalool from GGPP.

In addition to the cytosolic formation of (*E*)-nerolidol, the biosynthesis of (*E*)-nerolidol from FPP in mitochondria has been discussed (Fig. 5). Indications for this assumption came from subcellular localization studies of a FaNES1-related nerolidol synthase protein and from observed DMNT emissions of transgenic Arabidopsis expressing FaNES1targeted to the mitochondria (Aharoni et al., 2003; Kappers et al., 2005). (*E*)-Nerolidol produced in the mitochondria may be converted to DMNT in the same compartment or transported to the cytosol and the endoplasmic reticulum (ER) for degradation into DMNT.

Besides the formation of a single tertiary alcohol, some TPSs make several additional terpene products from the same substrate. For example, the maize terpene synthase, TPS1, synthesizes (*E*)-nerolidol as a major product together with the sesquiterpene olefin (*E*)-β-farnesene and the primary alcohol (*E*,*E*)-farnesol (Schnee et al., 2002). The TPS1 enzyme, which forms the (3*R*)-stereoisomer of (*E*)-nerolidol and a previously identified (3*S*)-(*E*)-nerolidol synthase activity (Degenhardt and Gershenzon, 2000) are presumably involved in the induced formation of DMNT in response to feeding damage by the cotton leafworm, *Spodoptera littoralis*. A similar correlation of induced DMNT emission and (3*S*)-(*E*)-nerolidol synthase activity was observed in lima bean and cucumber in response to feeding damage by the spider mite *T. urticae* (Bouwmeester et al., 1999). The volatile blends induced from the foliage of all these plants contain no (*E*)-nerolidol or only trace amounts, indicating a rapid conversion of this terpene alcohol into DMNT.

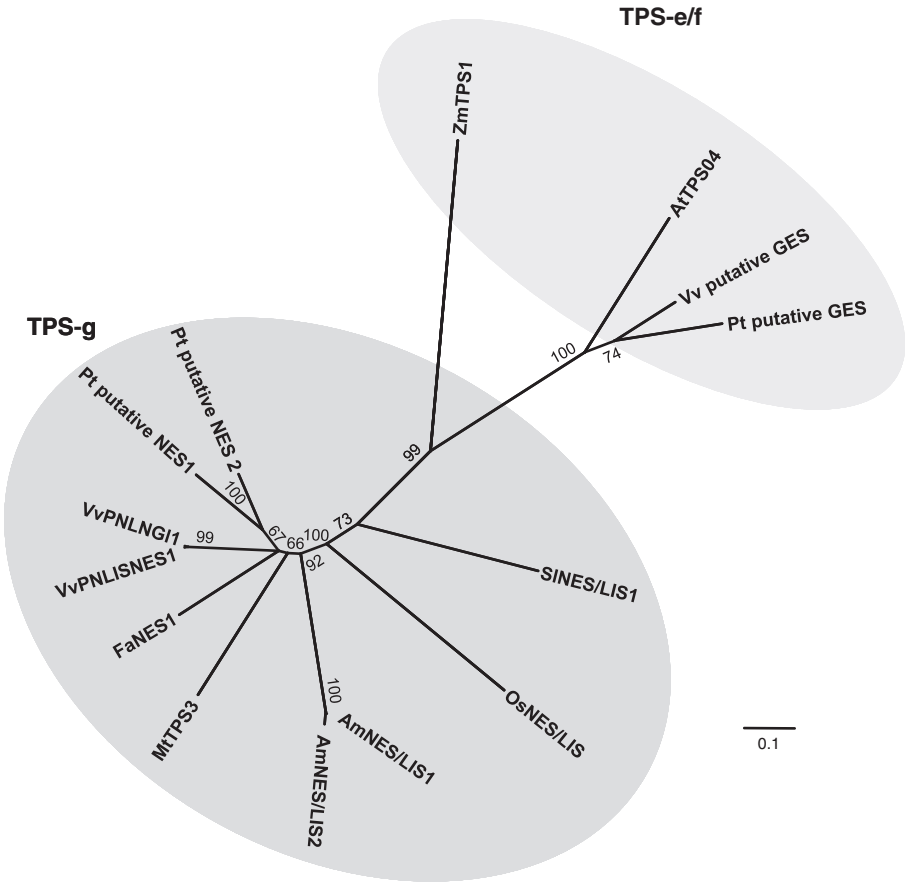

Fig. 4. Characterized and putative TPSs of the TPS-g and TPS-e/f subfamilies that produce two or more of the tertiary alcohols linalool, (*E*)-nerolidol and (*E*,*E*)-geranyllinalool *in vitro*. ZmTPS1 produces (*E*)-nerolidol, (*E*)-β-farnesene, and (*E*,*E*)-farnesol. Protein sequences were aligned using MUSCLE and a neighbor-joining tree was constructed with the GENEIOUS program (Biomatters Ltd.). Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*; Fa, *Fragaria* × *ananassa*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; Pt, *Populus trichocarpa*; Vv, *Vitis vinifera*; Zm, *Zea mays*; Sl, *Solanum lycopersicum*. LIS, linalool synthase; NES, (*E*)-nerolidol synthase; GES, (*E*,*E*)-geranyllinalool synthase. Accession numbers: AmNES/LIS1, EF433761; AmNES/LIS2, F433762; FaNES1, AX529067; MtTPS3, AY766249; OsNES/LIS, OS02G02930; Pt putative NES1, XM_002305597; Pt putative NES2, XM_002305599; Pt putative GES, XM_002305640; SlNES/LIS1, AY840091; VvPNLNGI1, VvTPS57; VvPNLISNES1, VvTPS54; Vv putative GES, XM_002268557; ZmTPS1, NM_001111627.

Among the enzymes of the TPS-e/f subfamily, an (*E*,*E*)-geranyllinalool synthase from Arabidopsis (TPS04, AtGES) has previously been identified (Herde et al., 2008). The single *AtGES* gene is induced locally at the site of feeding damage and is responsible for the *de novo* formation of (*E*,*E*)-geranyllinalool in herbivore-induced TMTT biosynthesis. The response is regulated by jasmonic acid or its derivatives but is independent of salicylic acid and ethylene (Herde et al., 2008). Other TPSs with sequence similarity to the AtGES protein are a (*E*)-nerolidol/(*E*,*E*)-geranyllinalool synthase from grape (Martin et al., 2010) and a related uncharacterized TPS from poplar (Fig. 4). Together, these proteins and the related linalool synthases from *Clarkia breweri* and *Clarkia concinna* share structures with similarity to kaurene synthases in gibberellin biosynthesis consisting of a functionally active class I (C-terminal, α) domain, a functionally inactive class II-type (β) domain, and an insertional (γ) domain (Cao et al., 2010; Dudareva et al., 1996). The protein and gene structures of AtGES (and related enzymes) resemble those of the presumed progenitor of plant TPSs, although TPS e/f type geranyllinalool synthases have so far only been found in angiosperms (Cao et al., 2010; Fig. 4). Another (*E*,*E*)-geranyllinalool synthase activity has been described in tomato, which is induced by jasmonate-treatment in stringent correlation with the formation of TMTT (Ament et al., 2006). Also, hydroxygeranyllinalool glycosides with potent anti-herbivore defense activity were recently detected in the wild tobacco *Nicotiana obtusifolia* (Jassbi et al., 2010). However, genes with similarity to *AtGES* have not been identified in these species and may reside in a different TPS subfamily.

In contrast to the common localization of diterpene synthases in plastids, AtGES is missing a plastidial transit peptide and resides in the cytosol or the ER (Herde et al., 2008). The enzyme either produces geranyllinalool from GGPP that is synthesized by cytosolic GGPP synthase isoforms or from plastidic GGPP transported to the cytosol (Fig. 5). The latter case is supported by studies investigating the prenylation of proteins in tobacco BY-2 cells and by experiments applying fosmidomycin, an inhibitor of the methylerythritol phosphate pathway that provides the C_5-precursors in the plastid-specific biosynthesis of terpenes (Fig. 5) (Lee and Tholl, unpublished results).

4. Degradation of the alcohol precursors: a single enzyme does the trick

The enzymatic step(s) responsible for the fragmentation of nerolidol and geranyllinalool into DMNT and TMTT, respectively, has long remained unknown. Comprehensive studies by Boland and coworkers using a variety of deuterium-labeled metabolic probes shed first light on the mechanism and stereochemistry of this reaction (Boland et al., 1998; Boland and Gäbler, 1989; Gäbler et al., 1991). As a result, it became evident that the transformation of the tertiary alcohols into homoterpenes proceeds via an oxidative C–C bond cleavage

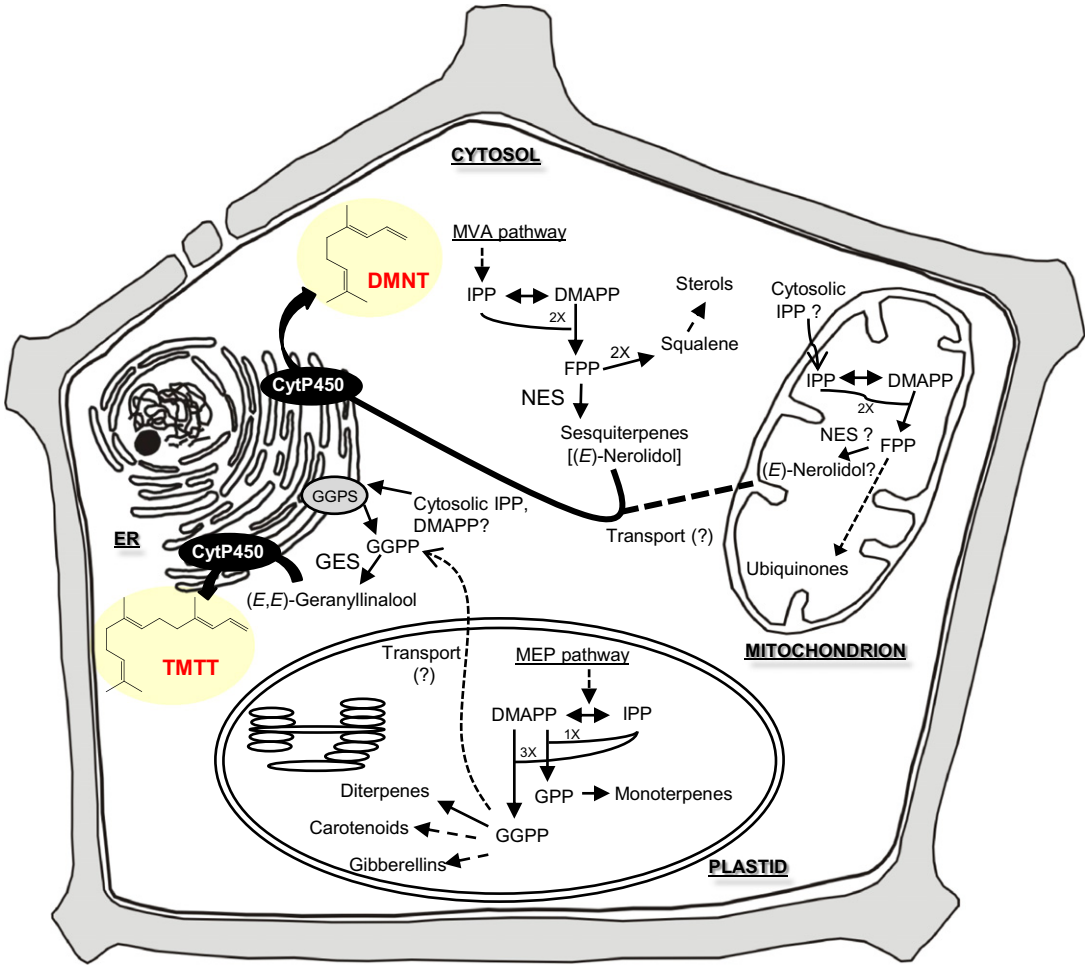

Fig. 5. Schematic of the subcellular compartmentation of DMNT and TMTT biosynthesis. MVA, mevalonate; MEP, methylerythritol phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; NES, nerolidol synthase; GES, geranyllinalool synthase; ER, endoplasmic reticulum; P450, cytochrome P450 monooxygenase.

reaction analogous to the dealkylation reaction of 22-hydroxycholesterol into androstenolone (Donath and Boland, 1994) and the formation of the furanocoumarin psoralen by oxidative degradation of its precursor (+)-marmesin in Apiaceae (*Ammi majus*) (Larbat et al., 2007). These reactions are catalyzed by one or two enzymes of the cytochrome P450 monooxygenase (P450) family and it was, therefore, assumed that P450-type enzymes are responsible for the final degradation step(s) in homoterpene biosynthesis. Examples from triterpenoid and other specialized metabolic pathways have shown that P450s can be tightly co-expressed with other biosynthetic enzymes of the same pathway (Field and Osbourn, 2008). In addition, studies by Bruce et al. (2008) suggested a role of CytP450s in the formation of TMTT in Arabidopsis. Exploiting the CYPedia expression database (http://www-ibmp.u-strasbg.fr/~CYPedia/) and the Arabidopsis co-expression databases from BAR (http://bar.utoronto.ca/) and ATTED (http://atted.jp/), we searched for P450 genes that are co-induced with the geranyllinalool synthase gene *AtGES* under various stress conditions. This approach, together with refined RT-PCR analysis experiments using elicitor-treated leaf tissue, led to the identification of a single gene candidate. Further volatile analysis of gene knockout and complementation lines confirmed that CYP82G1 (At3g25180), a member of the so far uncharacterized plant CYP82 family, is responsible for the herbivore-induced formation of TMTT in Arabidopsis (Lee et al., 2010). Similar to *AtGES*, expression of *CYP82G1* is induced by treatment with the peptaibol fungal elicitor alamethicin, the jasmonate mimic coronalon, and at sites of feeding damage by larvae of *P. xylostella*, all of which cause emission of TMTT (Fig. 6).

Further characterization of CYP82G1 revealed that the enzyme converts (*E*)-nerolidol and (*E*,*E*)-geranyllinalool to DMNT and TMTT, respectively, (Fig. 6) but otherwise has a narrow substrate specificity (Lee et al., 2010). DMNT is not emitted from Arabidopsis leaves (or only in negligible amounts according to our analysis) because of the absence of a prominent (*E*)-nerolidol synthase activity in this tissue. CYP82G1 accepts the 3*S*-enantiomer of (*E*)-nerolidol as a substrate in agreement with a predicted enzymatic preference for this enantiomer (Donath and Boland, 1995). Neither linalool, nor (*Z*)-nerolidol, the primary terpene alcohols (*E*,*E*,*E*)-geranylgeraniol, (*E*,*E*)-farnesol, and (*E*)-geraniol, or fully saturated analogs of (*E*)-nerolidol and (*E*,*E*)-geranyllinalool (3,7,11-trimethyl-3-dodecanol, isophytol) were found to be functional substrates of the CYP82G1 enzyme (Lee et al., 2010). These observations indicated a critical role for the position of the hydroxyl group and the configuration and degree of saturation of the aliphatic chain and were largely consistent with the feeding experiments conducted by Boland and co-workers (Gäbler et al., 1991). Since (*Z*)-, (*Z*,*Z*)-, and (*Z*,*E*)-isomers of DMNT and TMTT have been found as constituents of floral scent (Kaiser, 1991), related P450s from other species may also accept the respective *cis*-alcohol isomers as substrates. While a precise analysis of the reaction mechanism catalyzed by CYP82G1 still requires further investigation, previous studies by Boland's group have provided significant insight into several mech-

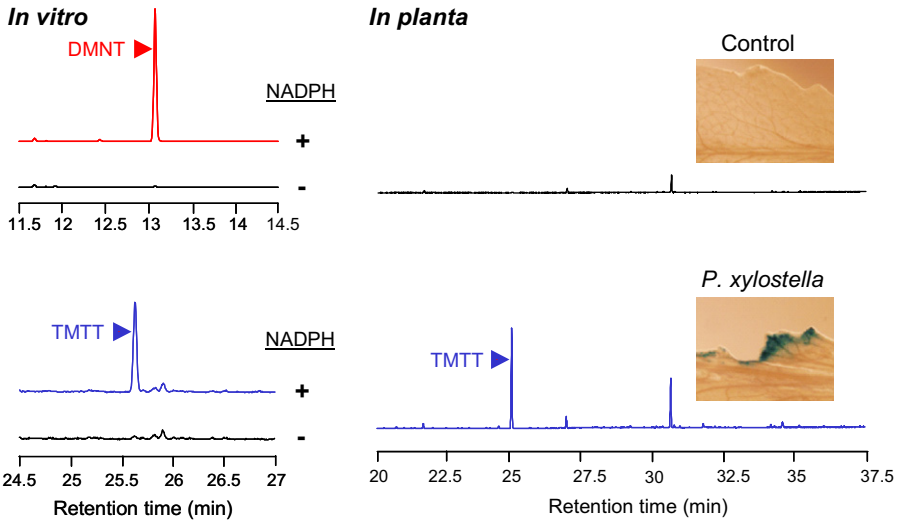

Fig. 6. *In vitro* and *in vivo* activity of Arabidopsis CYP82G1. Left panel: GC–MS chromatograms of DMNT and TMTT produced by yeast microsomal preparations of recombinant CYP82G1 from (*E*)-nerolidol and (*E*,*E*)-geranyllinalool, respectively. Right panel: emission of TMTT and CYP82G1 promoter-GUS expression in Arabidopsis leaves in response to feeding damage by *P. xylostella*.

anistic aspects: first, the reaction proceeds with an exclusive *syn*-elimination of the oxygen-carrying group together with an allylic hydrogen atom at the β-carbon atom (Donath and Boland, 1994). Second, the double bond positioned closest to the β-carbon atom is essential for the reaction, while the vinyl group is not. Third, the reaction may proceed in two consecutive steps via the removal of a C_2-moiety (possibly by an initial epoxidation of the vinyl group) to form a C_{13}-geranylacetone or a C_{18}-farnesylacetone intermediate, which is then converted via deacetylation into the homoterpene end product (Fig. 7). This mechanism was suggested based on the conversion of the ketones into homoterpenes in various feeding experiments, as well as by the detection of small amounts of geranylacetone in floral and leaf volatile blends of DMNT-emitting plants, and by the absence of the C_4-product but-3-ene-2-one resulting from a one-step cleavage reaction. While but-3-ene-2-one is a highly reactive compound and can easily escape detection, Gäbler et al. (1991) could not detect the more stable butan-2-one cleavage product by conversion of a saturated nerolidol analog to DMNT in magnolia flowers or lima bean leaves.

To gain further insight into substrate binding and conversion by the CYP82G1 enzyme, a protein model was constructed based on multiple mammalian P450 templates with closest sequence similarity (Lee et al., 2010). Subsequent molecular docking confirmed a position of (*E*)-nerolidol and (*E*,*E*)-geranyllinalool in the active site cavity that allows the suggested oxidative bond cleavage reaction to proceed by a *syn*-elimination (β-elimination) mechanism. In particular, the carbonyl oxygen of Thr313 in one of the substrate recognition sites appears to be essential for anchoring the substrates by forming a strong hydrogen bond with the hydroxyl group at C-3. The model also supported the observed differences in substrate specificities. For example, a docked position for linalool made the hydrogen atoms at C-5 inaccessible to molecular oxygen. In contrast to the conversion of the ketone intermediates found in experiments by Gäbler et al. (1991), docking of these compounds suggested an *anti*-orientation of the carbonyl-group relative to the C-4 hydrogen, which does not allow *syn*-elimination. Moreover, both compounds were poor substrates in assays with recombinant CYP82G1 enzyme. It cannot be excluded though that

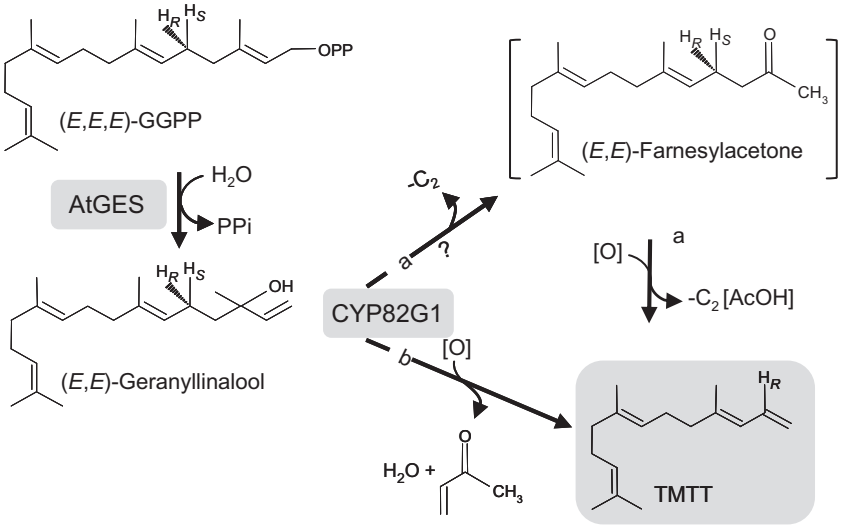

Fig. 7. Scheme of putative reaction mechanisms for the conversion of (*E*,*E*)-geranyllinalool to TMTT catalyzed by Arabidopsis CYP82G1. Path a is a two-step fragmentation with a consecutive loss of two C_2-moieties and formation of a C_{18}-ketone intermediate. Path b is a single-step fragmentation starting with the abstraction of the C-5 H_S atom followed by an immediate cleavage of the allylic radical intermediate to TMTT and but-1-en-3-one. GGPP, geranylgeranyl diphosphate.

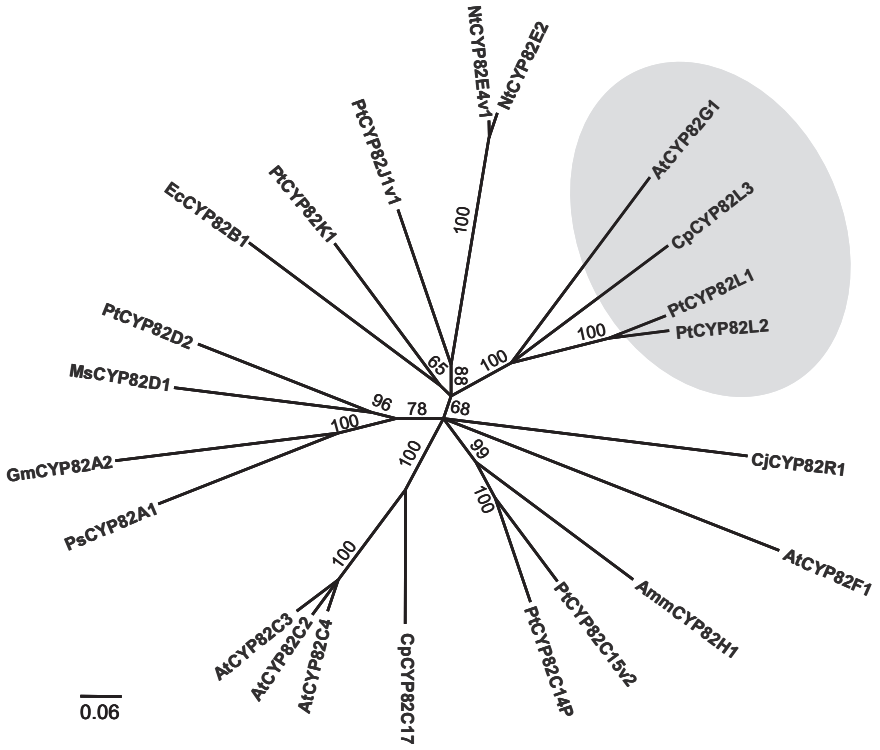

Fig. 8. P450s of the plant CYP82 family. Arabidopsis CYP82G1 and closely related P450s from papaya and poplar are marked in gray. Sequence alignment and construction of the tree were conducted as described for Fig. 4. Amm, *Ammi majus*; Cp, *Carica papaya*; Cj, *Coptis japonica* var. *dissecta*; Ec, *Eschscholzia californica*; Gm, *Glycine max*; Ms, *Medicago sativa*; Nt, *Nicotiana tabacum*; Ps, *Pisum sativum*; Pt, *Populus trichocarpa*.

the active site of the enzyme might undergo conformational changes after initial binding of the alcohol substrate, which may allow subsequent cleavage of a ketone intermediate. More detailed studies testing wild type and mutant forms of the Arabidopsis enzyme and related enzymes from other plants together with the previously applied deuterated substrates are necessary to further elucidate the mechanism of DMNT and TMTT formation.

Comparative sequence analyses identified P450s from poplar (CYP82L1 and 2) and papaya (CYP82L3) that share approximately 50% sequence identity with CYP82G1 in the CYP82 family (Fig. 8). It can be assumed that the related proteins from poplar are involved in homoterpene formation since herbivory by forest tent caterpillars on *Populus trichocarpa* × *deltoides* induces a volatile blend with DMNT as a predominant constituent (Arimura et al., 2004). The other Arabidopsis P450s in the CYP82 family seem to have hydroxylase rather than homoterpene synthase activities (Gavilano and Siminszky, 2007). Given the widespread occurrence of homoterpenes among angiosperms, it is possible that homoterpene synthases have emerged independently in other P450 families within the stress-responsive CYP71 clan (which includes the CYP82 family) or in other P450 clans. For example, homoterpene formation in monocots such as maize does not depend on CYP82-type P450s since this family is absent in monocots. Alternatively, Mercke et al. (2004) suggested previously, based on gene expression datasets from cucumber, that peroxidases could be involved in homoterpene biosynthesis. While this assumption cannot be entirely ruled out it is not supported by our current findings.

5. Regulation of homoterpene formation and prospects for engineering homoterpene biosynthesis

The role of plant volatiles in attracting natural enemies of herbivores has spurred growing interest in improving natural plant defenses via the genetic engineering of volatile compounds. First promising steps introducing or increasing the emissions of sesquiterpenes in tissues above- and below-ground have been made in Arabidopsis, rice and maize (Cheng et al., 2007; Degenhardt et al., 2009; Kappers et al., 2005). Such manipulations of volatile blends may also be applicable in integrated pest management strategies that employ volatiles attracting herbivore enemies in so-called push–pull systems (Khan et al., 2008). To optimize the attraction of specific parasitoids or predators under field conditions, an adjustment or fine-tuning of volatile blends to the preferences of these biocontrol agents will be required. Transgenic approaches using plants with defined volatile blends will therefore be essential to corroborate the function of individual volatiles such as homoterpenes and improve their efficacy together with other volatile compounds.

Engineering or enhancing the formation of homoterpene volatiles depends on a better understanding of the regulation of the homoterpene biosynthetic pathway. TMTT formation in Arabidopsis is in part controlled by transcription of the *AtGES* gene (Herde et al., 2008; Lee et al., 2010) similar to the regulation of herbivore-induced DMNT biosynthesis by the formation of (*E*)-nerolidol (Bouwmeester et al., 1999; Degenhardt and Gershenzon, 2000). Constitutive expression of *AtGES* in transgenic Arabidopsis can lead to TMTT emissions at rates as high as those observed from elicitor-induced wild-type plants because of a basal expression level of the geranyllinalool-converting enzyme CYP82G1 (Herde et al., 2008). On the other hand, constitutive expression of CYP82G1increases TMTT emission above wild type levels only when the activity of AtGES is induced upon elicitor-treatment (Lee et al., 2010).

The tight co-expression of *AtGES* and *CYP82G1*, which is observed under wild type conditions, allows an efficient conversion of geranyllinalool to TMTT. Transgenic plants that overexpress only the *AtGES* gene, accumulate geranyllinalool, which leads to reduced growth and the temporary formation of lesions at the cotyledon stage because of possible toxic or stress effects (Herde et al., 2008). Therefore, the expression of both enzymatic steps, ideally

under the control of a herbivore-inducible promoter, is desired to successfully engineer homoterpene biosynthesis in plants. Interestingly, none of the transgenic Arabidopsis plants expressing either *AtGES* or *CYP82G1* produced TMTT at levels that are significantly above those of wild type plants, even when challenged by elicitor treatment (Herde et al., 2008; Lee et al., 2010), which indicates additional regulatory mechanisms such as feedback inhibition and/or possible substrate limitations. Whether geranyllinalool synthesized by AtGES is in part glycosylated is not known. Glycosylation can critically reduce the formation of free terpene alcohols as was shown in experiments engineering the formation of linalool in petunia, Arabidopsis, and potato (Aharoni et al., 2003; Aharoni et al., 2006; Lucker et al., 2001). Moreover, subcellular segregation of the biosynthesis of the GGPP precursor in plastids and the formation of TMTT by CYP82G1 at the endoplasmic reticulum may contribute to a more tightly regulated substrate pool. Increasing the metabolite flow through the central plastidial and/or cytosolic terpenoid pathways or redirecting precursor formation (e.g. by enhancing the formation of GGPP in the cytosol) as demonstrated recently by Wu et al. (2006) may aid in elucidating and circumventing regulatory constraints in homoterpene biosynthesis. Since CYP82G1 accepts both, nerolidol and geranyllinalool, as substrates, these approaches can be tested for the DMNT and TMTT biosynthetic routes.

6. Conclusions and outlook

The homoterpenes, DMNT and TMTT, have been recognized for their widespread occurrence in angiosperms. Emission from flowers and herbivore-damaged tissues has suggested multiple functions of these irregular terpenes in the attraction of pollinators and natural enemies of herbivores. A two-step enzymatic pathway for the biosynthesis of C_{11}- and C_{13}-homoterpenes consisting of a tertiary terpene alcohol synthase and a terpene-degrading P450 enzyme has been elucidated in Arabidopsis allowing the first biochemical characterization of an enzyme of the plant CYP82 family. Enzymes of both biosynthetic steps appear to have evolved independently in different angiosperm lineages. Why homoterpene biosynthesis occurs primarily in flowering plants is not well understood. Homoterpene formation might have evolved with P450 enzymes that efficiently degrade terpene alcohols into more volatile olefinic products. Such enzymatic activities might prevent possible autotoxic effects in plant tissues that cannot store terpenes in specialized cell structures. Attempts to genetically modify homoterpene biosynthesis will provide further insight in the specific role of these compounds at different trophic levels and their possible application in agricultural pest control. It will also be important to gain a better understanding of how homoterpenes, together with other volatile compounds, affect and prime defense responses within and between plants and how these volatile signals are perceived at cellular and molecular levels.

Acknowledgement

We thank Janet Webster for helpful comments on the manuscript. Our work was supported by the National Research Initiative competitive grant no. 2007-35318-18384 from the USDA National Institute of Food and Agriculture, a National Science Foundation AdvanceVT research seed grant, and by the Thomas and Kate Jeffress Memorial Trust (J-850).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.01.019.

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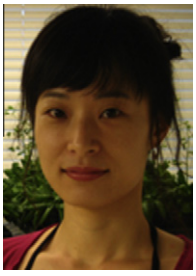

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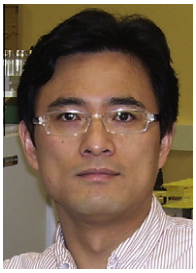

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