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Terpene Specialized Metabolism in *Arabidopsis thaliana*

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Terpenes constitute the largest class of plant secondary (or specialized) metabolites, which are compounds of ecological function in plant defense or the attraction of beneficial organisms. Using biochemical and genetic approaches, nearly all *Arabidopsis thaliana* (Arabidopsis) enzymes of the core biosynthetic pathways producing the 5-carbon building blocks of terpenes have been characterized and closer insight has been gained into the transcriptional and posttranscriptional/translational mechanisms regulating these pathways. The biochemical function of most prenyltransferases, the downstream enzymes that condense the C_5-precursors into central 10-, 15-, and 20-carbon prenyldiphosphate intermediates, has been described, although the function of several isoforms of C_{20}-prenyltranferases is not well understood. Prenyl diphosphates are converted to a variety of C_{10}-, C_{15}-, and C_{20}-terpene products by enzymes of the terpene synthase (TPS) family. Genomic organization of the 32 Arabidopsis *TPS* genes indicates a species-specific divergence of terpene synthases with tissue- and cell-type specific expression profiles that may have emerged under selection pressures by different organisms. Pseudogenization, differential expression, and subcellular segregation of *TPS* genes and enzymes contribute to the natural variation of terpene biosynthesis among Arabidopsis accessions (ecotypes) and species. Arabidopsis will remain an important model to investigate the metabolic organization and molecular regulatory networks of terpene specialized metabolism in relation to the biological activities of terpenes.

INTRODUCTION

Plants produce a plethora of phytochemicals that have specialized roles in ecological interactions. Terpenes (terpenoids or isoprenoids) represent, by far, the largest and most diverse class of specialized (previously named secondary) metabolites. In comparison to terpene compounds with primary functions in photosynthesis (carotenoids, chlorophylls, plastoquinone), respiration (ubiquinone), and growth and development (cytokinins, sterols, brassinosteroids, gibberellins, abscisic acid), many specialized terpenes have harmful or beneficial effects on other organisms (Dudareva et al., 2006). For example, terpenes can serve as phytoalexins in defense against plant pathogens (Hasegawa et al., 2010) or as direct defenses against herbivores (Keeling and Bohlmann, 2006a; Heiling et al., 2010). Many low-molecular-weight terpene compounds such as 10-carbon monoterpenes and 15-carbon sesquiterpenes are volatile constituents of floral odors that attract pollinators (Pichersky and Gershenzon, 2002). The same compounds are often emitted from vegetative tissues in response to herbivore feeding and function as an indirect defense by attracting natural enemies of herbivores (Dicke et al., 2003; Turlings and Ton, 2006; Unsicker et al., 2009). Furthermore, volatile terpenes released from damaged plants can induce defense responses in neighboring plants (Arimura et al., 2000). Besides their ecological benefits to plants, terpene specialized compounds are widely used by humans as flavors, fragrances, pharmaceuticals, or as potential precursors in the production of biofuels (Bohlmann and Keeling, 2008).

The discovery of specialized terpene metabolites in *Arabidopsis thaliana* (Arabidopsis) was somewhat surprising given the general lack of scent detected from Arabidopsis tissues by the human nose and the absence of specific secretory cell structures frequently occurring in plants that accumulate terpenes at high concentrations. Characterizing the metabolism of specialized terpenes in the Arabidopsis model, therefore, has provided insight into the biochemistry, molecular regulation, and biology of terpene formation in plant lineages that produce and release terpenes without significant storage under tissue-specific and stress-induced regimes. Moreover, elucidation of the Arabidopsis genome has led to the characterization of the core biosynthetic steps producing the precursors for both primary and specialized terpene metabolites. In this book chapter, we describe the biosynthesis of specialized terpene compounds in Arabidopsis. We include the early enzymatic pathways, which have important regulatory function in the biosynthesis of the general C_5-isoprenoid precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), and describe downstream steps in the condensation of these C_5-units to the immediate precursors of terpenes. Further emphasis will be placed on the biochemical function and genomic organization of Arabidopsis terpene synthases that produce C_{10}-, C_{15}-, and C_{20}-terpene compounds. We will address questions concerning the regulation of terpene synthases at different spatial and temporal scales and how these differences relate to the biological functions of terpenes produced in different Arabidopsis tissues. Furthermore, molecular mechanisms controlling the natural variation of terpene metabolism in the genus Arabidopsis will be discussed.

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BIOSYNTHESIS OF THE C_5-TERPENOID PRECURSORS IPP AND DMAPP

The Mevalonic Acid Pathway

In plants, the universal C_5-building blocks of all terpenes, IPP and DMAPP, are synthesized by two independent pathways, the mevalonic acid (MVA) pathway and the methylerythritol phosphate (MEP) pathway. The MVA pathway occurs in prokaryotes and in almost all eukaryotes including higher plants. In plants, all enzymatic steps of the MVA pathway are thought to be mainly located in the endoplasmic reticulum (ER)/cytosol compartment of the cell, where they provide the precursors for the biosynthesis of sesquiterpenes, phytosterols, brassinosteroids, and triterpenes (Newman and Chappell, 1999). The cytosolic localization of the MVA pathway was recently challenged by Sapir-Mir et al. (2008) based on the bioinformatic prediction of peroxisomal targeting sites for several enzymes of the MVA pathway in Arabidopsis and evidence for the peroxisomal localization of MVA pathway enzymes in mammals (Kovacs et al., 2007). Furthermore, Leivar et al. (2005) demonstrated the localization of Arabidopsis MVA pathway enzymes in small vesicles.

The pathway begins with two consecutive condensation reactions of three molecules of acetyl-CoA to form the C_6-compound 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (Fig. 1). In animals and plants, both steps are catalyzed by two separate enzymes, acetoacetyl-CoA thiolase (AACT, EC 2.3.1.9), which is responsible for the condensation of two acetyl-CoA molecules to form the intermediate acetoacetyl-CoA (AcAc-CoA), and HMG-CoA synthase (HMGS, EC 2.3.3.10) forming *S*-HMG-CoA by condensation of one molecule of AcAc-CoA with a third molecule of acetyl-CoA. The Arabidopsis genome contains two *AACT* genes (*ACT1* and *ACT2*), which encode the closely related functional AACT isoforms AACT1 and AACT2 (Ahumada et al., 2008) (Table 1). While *ACT2* is highly expressed throughout the plant, low levels of *ACT1* transcript occur in roots and inflorescences. Interestingly, in contrast to a cytosolic localization of the AACT2 enzyme, AACT1 appears to be targeted to peroxisomes based on two alternative peroxisomal targeting sequences (the N-terminal PTS1 or the C-terminal PTS2) resulting from different splicing events (Carrie et al., 2007; Ahumada et al., 2008). *Act2* T-DNA insertion mutants are embryo lethal, which supports an essential role of AACT2 in isoprenoid biosynthesis, while *act1* null mutants are viable with no growth defects, indicating a possibly different metabolic role of AACT1 in peroxisomes (Jin and Nikolau, 2007).

A cDNA encoding HMGS from Arabidopsis was described to functionally complement *erg11* and *erg13* yeast mutants defective for HMGS (Montamat et al., 1995), and an HMGS from *Brassica juncea* was enzymatically characterized (Nagegowda et al., 2004). In contrast to the single *HMGS* gene copy in Arabidopsis (Table 1), HMGS in *Brassica* and other plants is encoded by small gene families with differential tissue-specific expression and wound- or stress hormone-induced responses (Wegener et al., 1997; Alex et al., 2000; Nagegowda et al., 2005).

In the following irreversible step of the MVA pathway, *S*-HMG-CoA is converted into *R*-mevalonate by two reduction steps, each requiring NADPH as the reducing equivalent (Fig. 1). The reaction is catalyzed by the enzyme HMG-CoA reductase (HMGR, EC 1.1.1.34), which has been under intensive investigation due to its key regulatory role in sterol biosynthesis (Goldstein and Brown, 1990). Several genes encoding HMGR isoforms have been cloned from different plant species, providing more detailed information about the structure, location, and regulation of this enzyme (Nelson et al., 1994; Weissenborn et al., 1995; Ha et al., 2001; Ha et al., 2003). The Arabidopsis genome contains two *HMGR* genes (*HMG1*, *HMG2*) encoding two functionally active HMGR isoforms (Caelles et al., 1989; Learned and Fink, 1989; Enjuto et al., 1994; Suzuki et al., 2009) (Table 1).

All plant *HMGR* genes known to date encode membrane-bound class I HMGR proteins (in contrast to class II HMGR occurring in some eubacteria), which share a similar domain structure: a highly variable N-terminal region, a more conserved membrane domain with two membrane-spanning sequences, and a highly conserved catalytic C-terminal region (Enjuto et al., 1994; Campos and Boronat, 1995; Denbow et al., 1995; Re et al., 1997). A refined *in vivo* analysis of HMG1 indicated a localization of this enzyme in vesicle-like structures, which may be derived from segments of the ER (Leivar et al., 2005).

Members of plant *HMGR* gene families exhibit distinct patterns of expression in different plant organs, during development, and in response to phytohormones (Chye et al., 1992) and external stimuli such as light, wounding, elicitor treatment, and pathogen attack (Choi et al., 1992; Genschik et al., 1992; Nelson et al., 1994; Stermer et al., 1994; Weissenborn et al., 1995; Kondo et al., 2003). Arabidopsis *HMG1* is expressed throughout the plant and is induced in darkness in aerial organs, whereas *HMG2* seems to be expressed only in meristematic and floral tissues (Enjuto et al., 1994; Enjuto et al., 1995). Consistent with an expected housekeeping function of HMG1, *hmg1* null mutants show a pleiotropic phenotype of early senescence, sterility, and dwarfing due to the suppression of cell elongation (Suzuki et al., 2004). Sterol levels in *hmg1* were found to be reduced and the mutant phenotype could be rescued by the application of the sterol precursor squalene (Suzuki et al., 2004). Double *hmg1 hmg2* mutants are not viable because of the requirement of both genes for gametophyte development (Suzuki et al., 2009). *HMG1* and *HMG2* also play a major role in the biosynthesis of triterpene specialized metabolites (Fig. 1) since levels of triterpenes are reduced by 65 % and 25% in *hmg1* and *hmg2* mutants, respectively, in comparison to those in wild type plants (Ohyama et al., 2007). In contrast to other plant *HMGR* genes, neither of the Arabidopsis *HMGRs* appears to show major stress-induced responses.

It is generally accepted that HMGR catalyzes the main rate-limiting step in the MVA pathway and downstream terpene biosynthetic pathways. This is evident from reduced sterol and triterpene levels in the Arabidopsis *hmg* mutants and an increase in sterol levels in transgenic Arabidopsis overexpressing HMGR (Manzano et al., 2004). Moreover, positive correlations between *HMGR* gene expression and enzyme activity and the induced production of sesquiterpene phytoalexins have been reported in many plants although HMGR activity might not always be the sole rate-controlling step (e.g., Stermer and Bostock, 1987; Chappell et al., 1991; Choi et al., 1994; Chappell et al., 1995).

Besides the regulation of plant HMGRs at the transcriptional level in response to external cues and by feedback regulation from downstream metabolites such as sterols (Jelesko et al., 1999; Holmberg et al., 2002; Wentzinger et al., 2002), a developmental and light-regulated post-translational control of HMGR

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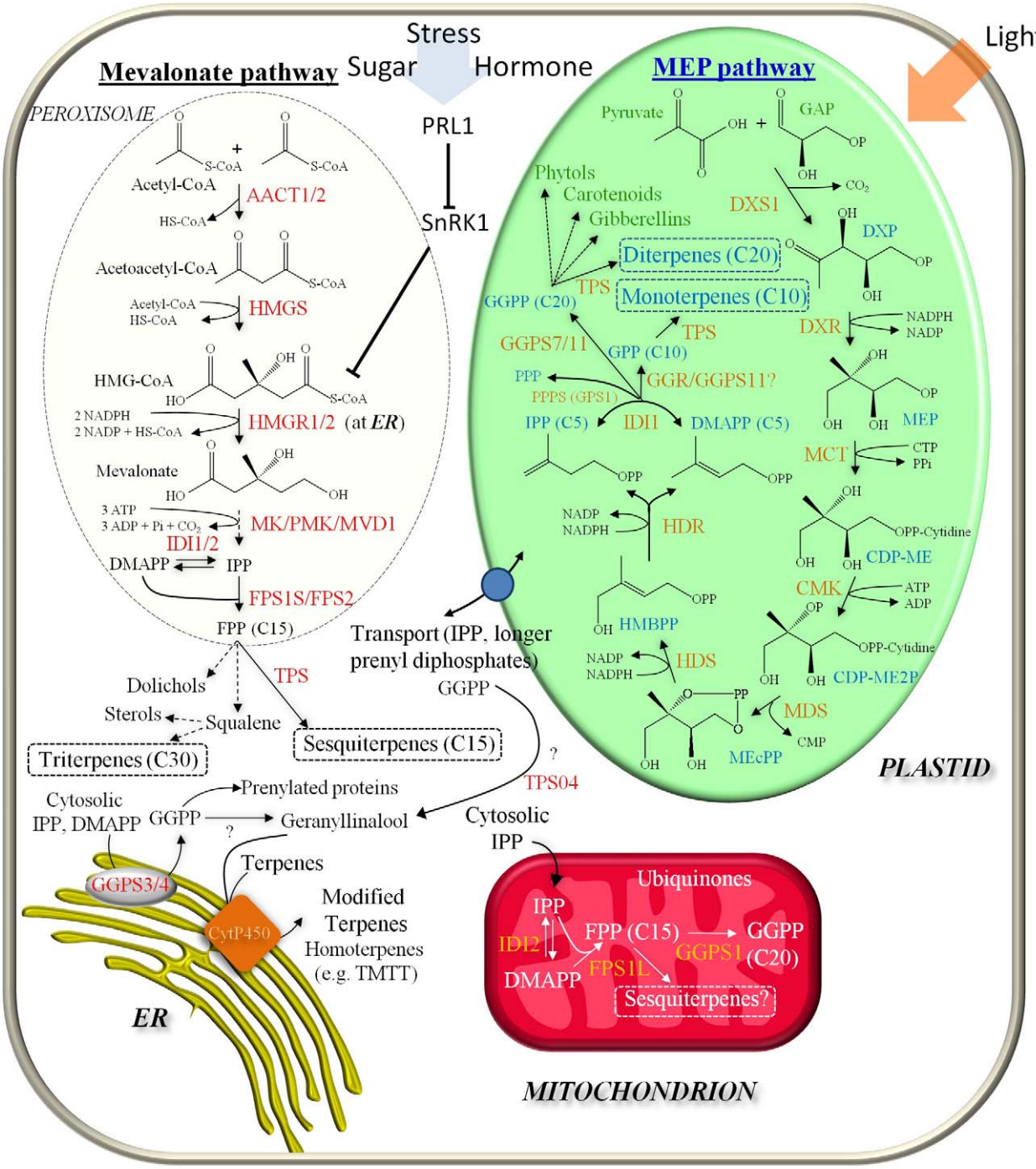


Figure 1. Terpene biosynthesis pathways and their subcellular organization in Arabidopsis.

The dashed line indicates a possible partial or complete location of MVA pathway enzymes in peroxisomes as predicted by Sapir-Mir et al. (2008). Dashed arrows indicate more than one enzymatic step. Enzymes are in red (cytosol/peroxisome), orange (plastid), or yellow (mitochondrion). Abbreviations for enzymes and metabolites are as described in the text.

protein levels has been demonstrated (Korth et al., 2000). In Arabidopsis, metabolic perturbation by enhancing or depleting the flux through the sterol pathway causes posttranslational down- or upregulation of HMGR activity, respectively, without changes in transcript or protein levels (Nieto et al., 2009). In addition, the inhibition of Arabidopsis HMGR activity by the SNF1 (sucrose nonfermenting)-related protein kinase 1 (SnRK1) was recently supported in mutants of *PRL1* (Pleiotropic Regulatory Locus 1), a global regulator of sugar, stress, and hormone responses, which inhibits SnRK1 (Flores-Perez et al., 2010).

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In the last three steps of the MVA pathway, mevalonic acid is converted into isopentenyl diphosphate (IPP) by two sequential ATP-dependent phosphorylation steps, catalyzed by the enzymes mevalonate kinase (MK) and phosphomevalonate kinase (PMK), and a third ATP-driven decarboxylative elimination catalyzed by mevalonate diphosphate decarboxylase (MVD). While crystal structures of mevalonate kinase and phosphomevalonate kinase have been solved from yeast and bacteria (Bonanno et al., 2001; Romanowski et al., 2002), comparatively little is known about these enzymes in plants. A single copy of the *MK* gene with relatively high homology to genes from yeast and mammals was characterized from Arabidopsis (Riou et al., 1994; Lluch et al., 2000) (Table 1). The *MK* gene is transcriptionally expressed in all parts of the plant with highest expression in root meristematic and floral tissue. Interestingly, Arabidopsis *HMG2* shows a similar expression pattern, which suggests a common regulatory mechanism of these genes in tissues with high requirements for mevalonate-derived terpenoid products (Lluch et al., 2000). Similar to yeast and mammals, plant MKs respond to feed-back inhibition by prenyl diphosphates like geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), which act as competitive inhibitors of ATP (Schulte et al., 2000). In contrast to MK and PMK, Arabidopsis MVD is encoded by two loci, of which *MVD1* was functionally characterized by complementation in yeast (Cordier et al., 1999) (Table 1). Indications that the last three enzymatic steps of the mevalonate pathway may not be important control points in plant terpene biosynthesis, came from earlier studies showing that these enzyme activities were higher or similar to that of HMGR or they did not correlate with the rate of terpene formation (Ji et al., 1993; Sandmann and Albrecht, 1994; Bianchini et al., 1996).

The Methylerythritol Phosphate (MEP) Pathway

Enzymatic steps of the pathway

In all plants, IPP and DMAPP are synthesized by a second alternative pathway, the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, which is located entirely in the plastid and was named originally after the first committed precursor of the corresponding bacterial pathway in *Escherichia coli*. Later studies showed that in plants the precursor of MEP, 1-deoxy-D-xylulose 5-phosphate (DXP), is considered the true committed intermediate of the pathway since, in contrast to bacteria, DXP does not give rise to precursors in thiamine and pyridoxine biosynthesis (Tambasco-Studart et al., 2005). The MEP pathway provides the C_5-building blocks for the biosynthesis of carotenoids, chlorophyll, gibberellins, and monoterpene and diterpene specialized metabolites, which are exclusively or primarily produced in plastids (Lichtenthaler, 1999) (Fig. 1). A combination of classical biochemical approaches and the use of genomics and bioinformatics tools in microbes and plants led to the identification of all enzymatic steps in the MEP pathway. The pathway occurs in apicomplexan protozoa, cyanobacteria, and all photosynthetic eukaryotes (Lichtenthaler, 1998) and is present in most eubacteria either alone or together with all or some elements of the MVA pathway (Rohmer, 1999; Boucher and Doolittle, 2000; Lange et al., 2000). It is absent in archaea, fungi, and animals.

Prior to their identification in Arabidopsis, the genes encoding the MEP pathway enzymes were first identified in *E. coli*. The MEP pathway consists of a total of seven enzymatic steps (Fig. 1). The first reaction is a Mg^{2+}-dependent transketolase-like condensation of (hydroxyethyl) thiamine diphosphate, derived from pyruvate, with glyceraldehyde-3-phosphate (GAP) to form DXP. The reaction is catalyzed by the enzyme DXP synthase (DXS, EC 2.2.1.7), which was identified in Arabidopsis by the albino phenotype of the corresponding *cla1* mutant (Mandel et al., 1996; Estevez et al., 2000). The crystal structures of bacterial DXS enzymes have recently been reported from *E. coli* and *Deinococcus radiodurans* (Xiang et al., 2007). All plant DXS sequences show a highly conserved thiamine phosphate binding domain, but are distinct from transketolases in the pentose phosphate pathway. The Arabidopsis genome contains a single functional *DXS* gene (*DXS1*) (Table 1), which is essential for plastidial terpene biosynthesis, while two homologous *DXS*-like genes are functionally inactive (Phillips et al., 2008a). The *chs5* mutant of the *DXS1* gene has a pale or albino phenotype only when exposed to lower temperatures indicating a temperature sensitivity of the mutated DXS protein (Araki et al., 2000). Arabidopsis DXS1 specifically belongs to the class I-type DXS enzymes, which are mainly expressed in photosynthetic tissues and floral organs and typically involved in primary terpene biosynthesis. By contrast, class II enzymes, which are absent in Arabidopsis, have been found to be involved in specialized terpene metabolism in glandular trichomes or in plant roots upon mycorrhizal fungal colonization inducing apocarotenoid formation (Walter et al., 2002; Paetzold et al., 2010). It is possible that the absence of a class II-type DXS enzyme in Arabidopsis compromises the plastidial formation of monoterpene and diterpene specialized metabolites since these terpenes are found at relatively low concentrations in Arabidopsis tissues under constitutive conditions (Chen et al., 2003; Vaughan, Tholl et al., unpublished results). Nevertheless, several Arabidopsis accessions emit the monoterpene (*E*)-β-ocimene at larger amounts (see below) in a response induced by herbivory and elicitor treatment indicating an increased metabolite flow toward specialized terpene metabolites under these conditions.

Several experimental studies have shown that DXS functions as an important regulation point in the supply of isoprenoid units for terpene biosynthesis (Bouvier et al., 1998; Lange et al., 1998; Chahed et al., 2000; Lois et al., 2000; Walter et al., 2000). Indications for DXS as a rate limiting enzyme in the MEP pathway came from transgenic, DXS over- or underexpressing Arabidopsis plants that showed positive correlation between RNA and protein levels of DXS1 and levels of plastidial terpenoids (Estevez et al., 2001).

In the second step of the MEP pathway, DXP is converted into 2-*C*-methyl-D-erythritol 4-phosphate (MEP) through an intramolecular rearrangement of DXP into the intermediate 2-*C*-methyl-D-erythrose 4-phosphate, followed by an NADPH-dependent reduction. The reaction, which is reversible, is catalyzed by the enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR, EC 1.1.1.267) (Schwender et al., 1999; Carretero-Paulet et al., 2002) (Fig. 1). Deeper insight into the reaction and catalytic properties of DXR has been obtained primarily from bacterial recombinant enzyme (Kuzuyama et al., 2000; Hoeffler et al., 2002; Koppisch et al., 2002; Reuter et al., 2002; Yajima et al., 2002; Steinbacher et al., 2003). *E. coli* DXR has been characterized

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as a class B dehydrogenase with a homodimer architecture and a strict requirement for divalent metal ions and NADPH as cofactors (Takahashi et al., 1998; Radykewicz et al., 2000). DXR can be specifically inhibited by the antibiotic compound fosmidomycin, which functions as a structure analogue of the DXR substrate (Kuzuyama et al., 1998; Jomaa et al., 1999; Steinbacher et al., 2003) and efficiently blocks the biosynthesis of plastidial terpenes including monoterpene specialized metabolites (Zeidler et al., 1998; Rodriguez-Concepcion et al., 2001; Huang et al., 2010). Compared to DXS, the role of plant DXR as a rate limiting enzyme seems to be less clear and varies depending on the species, tissue, and developmental stage. Arabidopsis *DXR1* (Table 1), similar to *DXS1*, is widely expressed in different plant organs with highest levels in seedlings and flowers, and is inducible by light (Carretero-Paulet et al., 2002). Arabidopsis mutants carrying a disrupted *DXR* gene have an albino phenotype and show deficiencies in gibberellin and abscisic acid (ABA) biosynthesis, and defects in trichome initiation and stomata closure (Xing et al., 2010).

In the third step of the MEP pathway, MEP is converted into 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME) in a CTP-dependent reaction catalyzed by the enzyme 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase (MCT or IpsD, EC 2.7.7.60) (Rohdich et al., 1999; Rohdich et al., 2000a) (Fig. 1). T-DNA insertion mutants (*mct-1* [*ispD-1*] and *mct-2* [*ispD-2*]) of the Arabidopsis *MCT* gene (Table 1) show an albino phenotype with mutant chloroplasts filled with large vesicles (Hsieh et al., 2008). Nucleus- and chloroplast-encoded photosynthetic genes are downregulated in these mutants while transcripts of genes encoding subunits of the mitochondrial electron transport chain are in part upregulated (Hsieh et al., 2008).

The hydroxyl group in the C_2-position of the MCT enzyme product CDP-ME is further phosphorylated at the expense of one molecule of ATP by the enzyme 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase (CMK, IspE, EC 2.7.1.148) (Lange and Croteau, 1999; Luttgen et al., 2000; Rohdich et al., 2000b) (Fig. 1). CMK is a member of the GHMP kinase superfamily (Bork et al., 1993). The product of the CMK enzyme, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate (CDP-ME2P), is subsequently converted into 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) upon loss of CMP, catalyzed by 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS, IspF, EC 4.6.1.12) (Fig. 1). The enzyme was characterized in *E. coli* (Herz et al., 2000; Takagi et al., 2000) and a plant MDS was cloned and characterized from gingko (Gao et al., 2006). Immunoblot analysis demonstrated that Arabidopsis MDS (Table 1) is a chloroplast stromal protein. Arabidopsis *mds-1* (*ispF-1*) and likewise *cmk-1* (*ispE-1*) mutants show phenotypes similar to those described for the *mct* mutants (Hsieh and Goodman, 2006; Hsieh et al., 2008). Crystal structures of *E.coli* MCT, CMK, and MDS have been solved, which showed homodimer (MCT, CMK) or homotrimer (MDS) organization (Kemp et al., 2001; Richard et al., 2001; Kemp et al., 2002; Richard et al., 2002; Steinbacher et al., 2002; Miallau et al., 2003). Arabidopsis MCT exhibits a distinct quarternary structure and is possibly feedback regulated by CMP (Gabrielsen et al., 2006).

In the last two steps of the MEP pathway, MEcPP is converted into 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) in a two-electron reduction by the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS, IspG, EC 1.17.4.3) (Fig. 1, Table 1). The product HMBPP subsequently serves as substrate for the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR, IspH, EC 1.17.1.2) (Fig. 1), which finally converts it into a mixture of IPP and DMAPP with a ratio of 5 to 6:1, as determined by *in vitro* and *in vivo* experiments for the *E. coli* and tobacco enzymes (Rohdich et al., 2002; Rohdich et al., 2003; Tritsch et al., 2010). Both enzymes from *E. coli* carry Fe/S clusters as prosthetic groups which are involved in a two step electron transfer from a reducing equivalent to the substrate (Seemann et al., 2002; Rohdich et al., 2003; Wolff et al., 2003). In Arabidopsis and other plants, the HDS protein contains a 30 kDa central domain, which is absent from the bacterial protein (Querol et al., 2002; Gutierrez-Nava et al., 2004; Kim and Kim, 2010). Arabidopsis *hds* (*clb4-1, clb4-2*) and *hdr-1* (*ispH-1*) mutants show defects in chloroplast development (Gutierrez-Nava et al., 2004; Hsieh and Goodman, 2005). The partial loss-of-function *hds-3* (*csb3*) mutant revealed an unexpected link between the MEP pathway and plant defense responses since the mutation caused a high level of resistance to biotrophic pathogens (Gil et al., 2005).

Regulation of the MEP pathway and crosstalk with the MVA pathway

The expression of MEP pathway genes is regulated by several external factors. In Arabidopsis, transcription of all genes is induced in a coordinated manner upon exposure to light (Carretero-Paulet et al., 2002; Botella-Pavia et al., 2004; Hsieh and Goodman, 2005; Hsieh et al., 2008), although the expression of HDR has also been reported to be constitutive (Hsieh and Goodman, 2005). All gene transcripts appear to be regulated by a circadian rhythm (Cordoba et al., 2009). Besides the effect of light, the presence of sucrose has been reported to increase transcript levels of DXS, DXR, MCT, and CMK in dark-grown plants (Hsieh and Goodman, 2005). Biotic factors such as mycorrhizal colonization or fungal elicitors induce the transcription of class II-type *DXS* genes (Walter et al., 2002; Phillips et al., 2007). These results indicate functional diversification of MEP pathway genes for providing terpenoid precursors in chemical defense responses to pathogen or herbivore attack.

Posttranscriptional regulation has been demonstrated to uncouple transcript and protein levels of MEP pathway enzymes (Guevara-Garcia et al., 2005) and might operate by metabolic feedback regulation depending on the metabolic flux through the pathway. Posttranscriptionally regulated accumulation of DXS, DXR, and HDR proteins has also been demonstrated in *rif1* and *rif10* mutants, which are resistant to the DXR inhibitor fosmidomycin. Characterization of these mutants indicated that the levels of MEP pathway enzymes appear to be negatively correlated with the activity of Clp, a major plastid stromal protease (Flores-Perez et al., 2008).

All plant MEP pathway enzymes carry N-terminal transit peptides for import into plastids. Despite the strict compartmentalization of the MEP and MVA pathways in plant cells, studies on the incorporation of stable-isotope precursors in primary and specialized terpene metabolites such as floral monoterpene and sesquiterpene volatiles demonstrated some degree of exchange of isoprenoid intermediates between plastids and the cytosol (e.g., Lichtenthaler, 1999; Hemmerlin et al., 2003; Laule et al., 2003; Dudareva et al., 2005; Bartram et al., 2006). In several cases, as

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in experiments with light-grown Arabidopsis seedlings, evidence for the trafficking of isoprenoid intermediates from the plastid to the cytosol has been provided (Laule et al., 2003). No specific transporters that mediate the exchange of isoprenoid precursors have so far been identified. Bick and Lange (2003) suggested that the export of IPP from plastids to the cytosol proceeds by a plastidial proton symport system. Recent studies by Flügge and Gao (2005) indicated that the transport of IPP does not proceed via plastidic phosphate translocators but is dependent on phosphorylated counter-substrates.

In contrast to the simple unidirectional transport of terpene intermediates from plastids to the cytosol, recent studies indicate that the metabolite exchange depends on a more complex regulation of the MEP and MVA pathways by light and by metabolic and developmental factors (Kasahara et al., 2002; Rodriguez-Concepcion et al., 2004). For example, it has been suggested that during germination of Arabidopsis seedlings in the dark, prenyl diphosphates derived from the MVA pathway are transported into etioplasts for the synthesis of gibberellins and carotenoids prior to the induction of MEP pathway enzymes upon illumination (Rodriguez-Concepcion et al., 2004). A similar light/organ-specific regulation of precursor allocation might occur in Arabidopsis roots where extremely low transcript and protein levels of MEP pathway enzymes have been observed (Estevez et al., 2001). While the exchange of intermediates between the cytosol and plastids is usually not sufficient to rescue mutants impaired in the biosynthetic steps of one or the other pathway (e.g., Estevez et al., 2001; Suzuki et al., 2009; Xing et al., 2010), a recent study by Paetzold et al. (2010) on *dxs2* mutants in tomato indicated that both pathways can, to some extent, compensate each other. Similarly, Gaussian modeling approaches constructing an Arabidopsis isoprenoid gene network predicted a negatively correlated expression of MEP and MVA pathway genes (Wille et al., 2004). Both MVA and MEP pathways also have been shown to be coordinated with sugar and hormone responses via the global regulator *PRL1* (*Pleiotropic Regulatory Locus 1*) (Flores-Perez et al., 2010). Thus, research determining how both pathways and the allocation of terpene precursors are controlled by complex regulatory networks will require further attention.

ISOMERIZATION OF IPP

The formation of higher isoprenoids requires the condensation of IPP with its isomer DMAPP. IPP is isomerized to DMAPP by the enzyme IPP isomerase (IDI, EC 5.3.3.2) (Fig. 1). The Arabidopsis genome contains two type I IPP isomerase genes (*IDI1* and *IDI2*) (Campbell et al., 1998) (Table 1), whose corresponding enzymes require divalent metals for their activity. Arabidopsis IDI1 and IDI2 are localized to multiple subcellular compartments. While IDI1 is targeted to plastids, IDI2 is transported to the mitochondria (Phillips et al., 2008b). C-terminal fusions of IDI proteins to green fluorescent protein (GFP) suggested that both proteins also occur in the cytosol as a result of their translation from shorter transcripts (Phillips et al., 2008b). However, recent internal GFP fusions to the short IDI isoforms indicate localization of both proteins to peroxisomes supporting the hypothesis for a possible functional isoprenoid pathway in peroxisomes (Sapir-Mir et al., 2008). IPP isomerase activity in the cytosol, peroxisomes, and mitochondria allows the formation of DMAPP from MVA-derived IPP in these compartments. Although the MEP pathway produces both IPP and DMAPP (at a ratio of 5:1), IPP isomerase activity might still be necessary in this compartment to generate an optimal ratio of the C_5-precursors for plastidial terpene biosynthesis and for a possible transport to the cytosol. *IDI1* and *IDI2* are both expressed in all plant organs with highest levels of IDI1 protein in flowers and IDI2 protein in roots and flowers (Okada et al., 2008; Phillips et al., 2008b). *IDI1* and *IDI2* are only partially redundant since *IDI2* is required for proper development of flower sepals and petals (Phillips et al., 2008b). Double-mutants of both *IDI* genes were shown to be nonviable (Phillips et al., 2008b) or exhibit dwarfism and male sterility under long-day conditions with severely reduced levels of sterols and ubiquinone (Okada et al., 2008).

FORMATION OF THE PRENYL DIPHOSPHATES GPP, FPP, AND GGPP

In the second stage of terpene biosynthesis, prenyltransferases or isoprenyl diphosphate synthases (IDS, EC 2.5.1.1) catalyze the fusion of the C_5-units of IPP and DMAPP to produce the linear precursors of all isoprenoids. The reaction starts with an initial head-to tail (1'-4) condensation of IPP with the allylic co-substrate DMAPP following an ionization-condensation-elimination mechanism to produce a C_{10}-allylic diphosphate. Additional head-to tail condensations with IPP result in short chain (C_{15}-C_{25}), medium-chain (C_{30}-C_{35}), and long chain (C_{40}-C_n) prenyl diphosphates. Depending on the stereochemistry of the double bonds in the enzyme product, prenyltransferases are classified as *trans*-prenyltransferases or *cis*-prenyltransferases, which form families of structurally unrelated enzymes (Kharel and Koyama, 2003). Here, we focus primarily on short chain *trans*-prenyltransferases, which synthesize C_{10}-geranyl diphosphate (GPP), C_{15}-farnesyl diphosphate (FPP) or C_{20}-geranylgeranyl diphosphate (GGPP) as the main precursors in terpene specialized metabolism (Fig. 1, Table 1).

Geranyl Diphosphate Synthase

Geranyl diphosphate (GPP) synthases (GPSs, EC 2.5.1.1) produce GPP (Fig. 2a), which is generally considered to be the precursor of most C_{10} monoterpenes (Fig. 1). In plants, different classes of homodimeric and heterodimeric GPSs have been identified (Burke and Croteau, 2002; Schmidt and Gershenzon, 2008; Hsieh et al., 2011). In Arabidopsis, the single gene *GPS1* (Table 1) was originally characterized by Bouvier et al. (2000) to encode a homodimeric GPS. However, a recent study by Hsieh et al. (2011) questioned this finding since the enzyme appears to have a multi-product medium/long chain prenyl diphosphate synthase activity when IPP is supplied in excess to the allylic substrates DMAPP, GPP and FPP. The result was further supported by a structural analysis of the GPS1 protein (renamed by Hsieh et al. as polyprenyl di(pyro)phosphate synthase, PPPS), which indicated an active-site cavity sufficient to accommodate the medium/long-chain products (Hsieh et al., 2011). Since the

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Table 1. Arabidopsis genes encoding enzymes of the mevalonic acid and MEP pathways, IPP isomerases, and prenyltransferases.

Gene	AGI No.	Subcellular Protein Localization[a]	Enzyme Products	Reference
Mevalonic acid pathway				
ACT1	At5g47720	PX	acetoacetyl-CoA	Ahumada et al., 2008
ACT2	At5g48230	C	acetoacetyl-CoA	Ahumada et al., 2008
HMGS	At4g11820	C	*S*-HMG-CoA	Montamat et al., 1995
HMG1	At1g76490	C/ER	*R*-mevalonate	Caelles et al., 1989
HMG2	At2g17370	C/ER	*R*-mevalonate	Caelles et al., 1989
MK	At5g27450	C	phosphomevalonate	Riou et al., 1994
PMK	At1g31910	C	mevalonate diphosphate	
MVD1	At2g38700	C	IPP	Cordier et al., 1999
MVD2	At3g54250	C	IPP	
MEP pathway				
DXS1	At4g15560	P	1-deoxy-D-xylulose-5-phosphate	Mandel et al., 1996 Estevez et al., 2000
DXR	At5g62790	P	2-*C*-methyl-D-erythritol 4-phosphate	Schwender et al., 1999 Carretero-Paulet et al., 2002
MCT	At2g02500	P	4-diphosphocytidyl-2-*C*-methyl-D-erythritol	Rohdich et al., 2000a
CMK	At2g26930	P	4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate	Hsieh et al., 2008
MDS	At1g63970	P	2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate	Hsieh and Goodman, 2006
HDS	At5g60600	P	4-hydroxy-3-methylbut-2-enyl diphosphate	Querol et al., 2002
HDR	At4g34350	P	IPP/DMAPP	Hsieh and Goodman, 2005
IPP isomerases				
IDI1	At3g02780	P/C/PX	DMAPP	Campbell et al., 1998
IDI2	At5g16440	M/C/PX	DMAPP	Campbell et al., 1998
Prenyltransferases				
GPS1 (PPPS)	At2g34630	P	Polyprenyl diphosphate (GGPP)	Bouvier et al., 2000/ Hsieh et al., 2011
GGR	At4g38460	P?	GPP (catalyzed by GGR/ GGPS11 *in vitro*)	Wang and Dixon, 2009
FPS1	At4g17190	C/M	FPP	Cunillera et al., 1997/2000a
FPS2	At5g47770	C	FPP	Cunillera et al., 1997/2000a
GGPS1	At1g49530	M	GGPP	Zhu et al., 1997b
GGPS2	At2g18620	P	GGPP	Wang and Dixon, 2009
GGPS3	At2g18640	C/ER	GGPP	Okada et al., 2000
GGPS4	At2g23800	C/ER	GGPP	Zhu et al., 1997a/ Okada et al., 2000
GGPS5	At3g14510	P	putative	
GGPS6	At3g14530	P	Polyprenyl diphosphate	Wang and Dixon, 2009
GGPS7	At3g14550	P	GGPP	Okada et al., 2000
GGPS8	At3g20160	P	putative	
GGPS9	At3g29430	P	putative	
GGPS10	At3g32040	P	putative	
GGPS11	At4g36810	P	GGPP	Okada et al., 2000

[a] C, cytosol; ER, endoplasmic reticulum; P, plastid; PX, peroxisome; M, mitochondria. Underlined letters indicate subcellular localizations confirmed by experimental evidence; other localizations were predicted by the Predotar, TargetP, and WoLFPSORT algorithms.
For tissue-specific or stress-induced transcript profiles of the listed genes, the reader is referred to The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/) and corresponding gene-specific links to the eFP and Expression Browser (http://bar.utoronto.ca/) and Genevestigator (https://www.genevestigator.com/gv/index.jsp).

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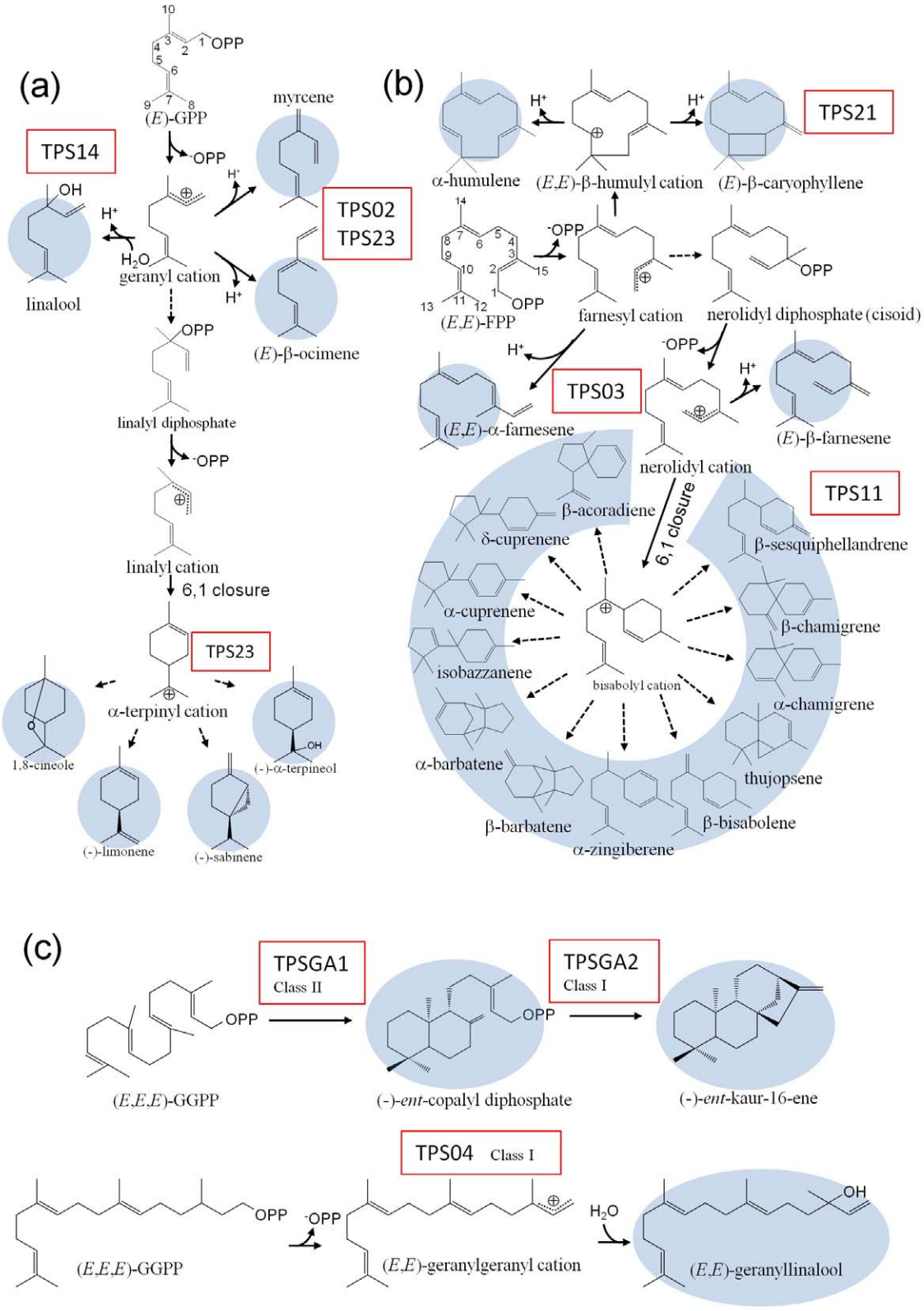


Figure 2. Reaction mechanisms and enzyme products of selected Arabidopsis monoterpene synthases **(a)**, sesquiterpene synthases **(b)**, and diterpene synthases **(c)**. TPS enzymes are marked by red boxes.

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PPPS protein is targeted to plastids (Bouvier et al., 2000) where IPP and DMAPP are produced at ratios of approximately 5:1 by the MEP pathway, it is possible that this enzyme indeed exhibits a polyprenyl diphosphate synthase activity *in vivo*. Van Schie et al. (2007) demonstrated that RNAi-silencing of *PPPS* (*GPS1*) resulted in a dwarfed phenotype and a *PPPS* knock-out line appeared to be embryo-lethal. While the exact function of PPPS remains to be determined, the enzyme might play a role in the biosynthesis of plastoquinones and possibly gibberellins since it produces some GGPP *in vitro* (Hsieh et al., 2011). Several proteins with homology to Arabidopsis PPPS/GPS1 have been found in other plants and designated as homodimeric GPSs. Van Schie demonstrated GPS activity for a protein in tomato, although at a low IPP/DMAPP ratio; thus, it remains to be determined whether the proteins in this clade, which are closer related to long chain prenyltransferases (Hsieh et al., 2011), may also have polyprenyl diphosphate synthase activity. In gymnosperms, homodimeric prenyl diphosphate synthases of a separate clade were found to have GPS activity (Schmidt and Gershenzon, 2008; Schmidt et al., 2010). These enzymes produce substantial amounts of the longer prenyl diphosphates FPP and/or GGPP, indicating their possible role in other terpene biosynthesis pathways. Interestingly, a flower-specific homodimeric GPS has recently been reported from orchids, which is closer related to the small subunit of heterodimeric GPSs (see below) (Hsiao et al., 2008).

Since the presence of a homodimeric GPS in Arabidopsis is questionable, GPP might instead be produced by a heterodimeric type GPS. Heterodimeric or heterotetrameric GPSs identified from *Mentha* x *piperita*, *Anthirrinum majus*, *Clarkia breweri*, and *Humulus lupulus* (Burke et al., 1999; Tholl et al., 2004; Wang and Dixon, 2009) are composed of a large and a small subunit. The large subunit (LSU) has significant homology (~50%) to GGPP synthases (see below) and can exhibit GGPP synthase activity as a recombinant protein, while the small subunit (SSU) shares only ~20% sequence similarity with homomeric prenyltransferases and is functionally inactive. Based on structural evidence obtained from heterotetrameric GPS from peppermint (Chang et al., 2010), physical interaction of both subunits is necessary to produce GPP. In Arabidopsis, a gene encoding a GGPP synthase-related protein (GGR) was identified (Table 1), which contains 2 conserved CxxxC motifs found to be essential for the interaction of GPS.SSU with GPS.LSU (Wang and Dixon, 2009). GGR modifies Arabidopsis GGPP synthase11 (see below) *in vitro* by increasing its GPS activity. Phylogenetically, GGR belongs to a separate lineage of GPS.SSU (SSU II). Whether proteins in this subfamily are essential for monoterpene biosynthesis is not clear since their expression is not tightly correlated with the formation of monoterpenes in contrast to SSU I proteins in monoterpene-rich plants such as snapdragon and hops (Tholl et al., 2004; Wang and Dixon, 2009).

In summary, it remains somewhat unclear which enzymes specifically contribute to the formation of GPP in Arabidopsis. The biosynthesis of GPP seems to be restricted to plastids and the presence of a substantial cytosolic GPP pool is unlikely as shown at the example of the bifunctional Arabidopsis monoterpene/sesquiterpene synthase TPS02, which is located in the cytosol and only produces sesquiterpenes but no monoterpenes *in vivo* (see below).

Farnesyl Diphosphate Synthase

(*E*,*E*)-Farnesyl diphosphate (FPP) (Fig. 2b) synthesized by FPP synthases (FPS, EC 2.5.1.10) is a central precursor in primary terpene metabolism (biosynthesis of sterols, brassinosteroids, dolichols, and ubiquinones; protein prenylation) and specialized metabolism in the formation of sesquiterpenes and triterpenes (Fig. 1). Plant *trans*-FPSs are homodimeric enzymes, which belong to type I (eukaryotic) FPSs. Three Arabidopsis FPS isoforms can be distinguished, which are encoded by two genes of high sequence similarity, *FPS1* and *FPS2* (Table 1). *FPS1* is expressed in all tissues; however, differential transcription of *FPS1* leads to the formation of a long version of *FPS1* mRNA (*FPS1L*) with primary accumulation in flowers and a short *FPS1* transcript (*FPS1S*) having highest expression in flowers and roots (Cunillera et al., 1997, 2000a). The isoform derived from *FPS1L*, which carries an N-terminal extension of 41 amino acids, is targeted to mitochondria, while FPS1S most likely remains in the cytosol (Fig. 1). FPP synthesized in mitochondria may function as a precursor of sesquiterpenes. A plastidial pool of FPP has been identified based on the formation of sesquiterpenes by bi-functional monoterpene/sesquiterpene synthases targeted to plastids (Aharoni et al., 2003; Huang et al., 2010). However, plastidial FPP is most likely a by-product of other prenyltransferases such as GGPP synthase located in plastids.

Expression of *FPS2*, which encodes the third cytosolic FPS isoform (Fig. 1), occurs primarily in floral organs, specifically in pollen grains, at the sites of lateral root initiation, and at the junctions between primary and secondary roots and stems (Cunillera et al., 2000a). Despite these reported expression patterns, studies with *FPS1* and *FPS2* knock-out lines demonstrated that a single functional FPS isozyme is sufficient to support growth and development (Closa et al., 2010). However, the functions of both enzymes are not completely redundant: FPS1 is required during several stages of plant development, while FPS2 plays a major role during embryo and seedling development. Double mutations of *FPS1* and *FPS2* severely impair male genetic transmission and cause an arrest of early embryo development (Closa et al., 2010).

Overexpression of FPS1 leads to a metabolic imbalance resulting in necrosis coupled with increased levels of H_2O_2 and premature senescence (Masferrer et al., 2002; Manzano et al., 2004). Moreover, increased FPS activity appears to reduce the availability of IPP and DMAPP for cytokinin biosynthesis (Masferrer et al., 2002). Overexpression of HMGR together with FPS1 can rescue the necrosis phenotype indicating an insufficient supply of metabolites derived from the MVA pathway in FPS1-expressing plants (Manzano et al., 2004).

Geranylgeranyl Diphosphate Synthase

Trans-geranylgeranyl diphosphate (GGPP) synthases (GGPS, EC 2.5.1.29) represent important branch point enzymes since their enzymatic product (*E*,*E*,*E*)-GGPP (Fig. 2c) is a central precursor for a diverse group of primary and specialized isoprenoid compounds: carotenoids and carotenoid breakdown products including the hormones ABA and strigolactones, chlorophylls, tocopherols, gibberellins, plastoquinones, and diterpenes (all

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synthesized in plastids), geranylgeranylated proteins and polyprenols (synthesized in the cytosol), and polyterpenes (synthesized in mitochondria) (Fig. 1). Consistent with the subcellular compartmentation of these diverse pathways, different GGPS isoforms are encoded by small gene families. The Arabidopsis genome contains a gene family of 11 predicted GGPSs (GGPS1-11, Lange and Ghassemian, 2003), of which six (GGPS1, 2, 3, 4, 7, 11) have been functionally characterized *in vitro* and/or by genetic complementation (Zhu et al., 1997a; Zhu et al., 1997b; Okada et al., 2000; Wang and Dixon, 2009) (Table 1). All characterized enzymes are most likely homodimers and produce GGPP as the primary or sole (GGPS2) product. GGPS1 synthesizes GGPP from IPP and FPP as the allylic substrate (Zhu et al., 1997b). Wang and Dixon (2009) recently demonstrated that GGPS6 is probably not a functional GGPS enzyme since the recombinant GGPS6 protein produces a polyprenyl diphosphate of more than 20 carbon atoms *in vitro*. GFP-fusion experiments indicated that GGPS7 and 11 are located in plastids, GGPS1 is targeted to mitochondria, and GGPS3 and 4 are located in the ER/cytosol (Okada et al., 2000) (Fig. 1). Further analysis of putative targeting sequences using different algorithms (Lange and Ghassemian, 2003) suggested five other GGPS isoforms to be plastidial proteins (GGPS2, 5, 8-10, 11). Besides the subcellular segregation of GGPSs, different spatial expression patterns have been observed. According to Northern and GUS reporter experiments, *GGPS11* is expressed throughout the plant, while *GGPS4* is expressed in flowers and root tips (Okada et al., 2000). *GGPS3* promoter activity was detected in the vascular tissue and in flowers, and *GGPS7* promoter activity was observed in the hypocotyl and the vascular tissue of roots (Okada et al., 2000). Microarray analyses further support different expression profiles of the other *GGPS* genes in the root (lateral root cap, epidermis and stele), the embryo, flowers and vascular tissue (The Bio-Array Resource for Plant Functional Genomics, http://www.bar.utoronto.ca/). In summary, differential subcellular and tissue-specific regulation of the various Arabidopsis GGPS isoforms suggest a functional specialization of GGPS enzymes by allocating GGPP precursors and controlling metabolic flux to distinct terpene biosynthetic pathways.

Chain Length Regulation of Prenyltransferases

The mechanism that regulates the length of short-chain prenyltransferase products has been investigated by a combination of structural analyses and random or site-directed mutagenesis (Vandermoten et al., 2009). Crystal structures of homodimeric FPSs and GGPSs reported from chicken, bacteria, and protozoa, yeast, and human (Tarshis et al., 1994; Koyama et al., 2000; Hosfield et al., 2004; Chang et al., 2006; Gabelli et al., 2006; Kavanagh et al., 2006) have shown that short chain prenyltransferases are principally composed of 13 helices, ten of which surround the active site cavity. Two highly conserved aspartate-rich regions, a first $DDx_{2-4}D$ motif (FARM) and a second DDxxD motif (SARM), which are essential for substrate binding and catalytic activity, are positioned on opposite walls of the cavity. Amino acid residues upstream of the FARM motif determine the chain length of the prenyl diphosphate product by changing the size of the hydrophobic binding pocket (Ohnuma et al., 1996a; Ohnuma et al., 1996b; Tarshis et al., 1996). “Bulkier” aromatic amino acid residues cause a smaller binding pocket in type I FPP synthases such as Arabidopsis FPS1 and 2 but are replaced by smaller residues (alanine, serine, methionine) in type II GGPSs, which comprise eubacterial and plant GGPSs including all Arabidopsis GGPSs. Termination of chain elongation at C_{20} seems to be dependent on residues located deeper in the catalytic cavity as demonstrated for GGPS from yeast (Chang et al., 2006). The recently renamed Arabidopsis PPPS (former GPS1) does not possess aromatic amino acids near the FARM supporting the observed formation of longer chain products. Chain length termination in prenyltransferases is not absolute since the reactions of several enzymes such as GGPS11 show promiscuity by synthesizing shorter products in small or sometimes equal amounts. These reactions may allow evolutionary switches or transition between different chain-length prenyltransferases. Modifications of prenyltransferase activities are also obvious in heterodimeric GPSs. The recently elucidated structure of peppermint heterodimeric GPS shows that interaction of the small subunit, which does not contain a FARM motif, with the large GGPS-type subunit limits access to an elongation cavity and restricts the enzyme’s specificity to the formation of a C_{10}-product (Chang et al., 2010).

Cis-Isoprenyl Diphosphate Synthases

Most *cis*-prenyltransferases generate prenyl diphosphate products longer than C_{50} such as dolichols by using all-*trans* short chain prenyl diphosphates as allylic primer substrates (Takahashi and Koyama, 2006). However, recently a (*Z*,*Z*)-FPP synthase was identified from wild tomato, which provides (*Z*,*Z*)-FPP as substrate for the trichome-specific formation of sesquiterpenes (Sallaud et al., 2009). Likewise, the *cis*-prenyl diphosphate neryl diphosphate (NPP) and not GPP was demonstrated to function specifically as the substrate for a tomato monoterpene synthase (Schilmiller et al., 2009). These studies indicate that the role of short chain (*Z*)-prenyl diphosphates in terpene specialized metabolism has been underestimated. The Arabidopsis genome contains a total of nine genes with sequence similarity to *cis*-prenyltransferases. With the exception of two genes involved in dolichol biosynthesis (Cunillera et al., 2000b; Zhang et al., 2008), the function of these enzymes is largely unknown.

BIOSYNTHESIS OF MONOTERPENES, SESQUITERPENES, AND DITERPENES

The acyclic C_5- to C_{20}-prenyl diphosphate intermediates produced by prenyltransferases are further converted by enzymes known as terpene synthases (TPS) (Fig. 1) into C_5-hemiterpenes such as isoprene (does not occur in Arabidopsis), C_{10}-monoterpenes, C_{15}-sesquiterpenes, or C_{20}-diterpenes. TPS enzymes form single or multiple cyclic and acyclic products and are the main cause of the diversity of terpene compounds found in nature (Degenhardt et al., 2009). The primary terpene synthase products can be modified by secondary reactions such as hydroxylation, peroxidation, methylation, acylation, glycosylation or cleavage, which further add to the compound diversity. TPS enzymes contribute to the variation and plasticity of terpene formation because they can

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easily acquire new catalytic properties by minor structural changes (Köllner et al., 2004; Xu et al., 2007; Keeling et al., 2008). Several reviews have focused on the reaction mechanisms, catalytic plasticity, and structure-function relationships of TPSs (Cane, 1999; MacMillan and Beale, 1999; Wise and Croteau, 1999; Davis and Croteau, 2000; Christianson, 2006; Greenhagen et al., 2006; Yoshikuni et al., 2006; O'Maille et al., 2008; Degenhardt et al., 2009), which will, therefore, only be summarized. The enzymatic reactions of all class I terpene synthases involve an initial, divalent cation-dependent step, in which the prenyl diphosphate substrate is ionized to a carbocation intermediate. This intermediate is subject to different reactions including cyclizations, hydride shifts and rearrangements with termination by proton loss or the addition of a nucleophile (water in most cases) (Davis and Croteau, 2000). TPS enzymes vary in their substrate specificity from using only a single prenyl diphosphate substrate to converting two or more substrates (e.g., GPP and FPP) *in vitro*. However, depending on their subcellular localization, TPS enzymes targeted to plastids generally produce monoterpenes or diterpenes from the predominantly plastidial pools of GPP and GGPP, respectively, while TPSs located in the cytosol primarily convert FPP to sesquiterpenes (or squalene in the biosynthesis of C_{30} terpenes).

Monoterpene formation is initiated by dephosphorylation and ionization of GPP to a geranyl carbocation. Acyclic monoterpenes such as (*E*)-β-ocimene, myrcene, and linalool, which are, for example, produced by the Arabidopsis terpene synthases TPS02 and TPS14 (Fig. 2a) (Chen et al., 2003; Huang et al., 2010) can be directly derived from this carbocation via proton loss or the addition of water, respectively. Alternatively, formation of these compounds might proceed via a linalyl cation resulting from isomerization of the geranyl cation. Cyclic monoterpenes are derived from the central cyclic α-terpinyl cation intermediate, which is formed by cyclization of the cisoid linalyl cation. Depending on the specific enzyme activity, the α-terpinyl cation can undergo various steps of deprotonation, cyclization, ring closure, and hydride and Wagner-Meerwein shifts leading to different cyclic end products. For example, the predicted reaction mechanism of the Arabidopsis 1,8-cineole synthase TPS23 producing several cyclic monoterpenes is presented in Fig. 2a (Chen et al., 2004).

Analogous to the biosynthesis of monoterpenes, the formation of sesquiterpenes starts with the ionization of FPP to form a farnesyl cation, which can be isomerized subsequently to a nerolidyl cation (Cane, 1999; Degenhardt et al., 2009). Acyclic sesquiterpenes such as (*E*,*E*)-α-farnesene are derived from one of these cations via deprotonation as shown in Fig. 2b for Arabidopsis TPS03 (Fäldt et al., 2003; Huang et al., 2010). The farnesyl cation can also be cyclized, for example, to an (*E*,*E*)-humulyl cation, which is further converted into the common cyclic sesquiterpenes α-humulene and (*E*)-β-caryophyllene (see Fig. 2b for Arabidopsis TPS21) (Tholl et al., 2005). Furthermore, several cyclic carbocations can be formed via different ring closures of the cisoid nerolidyl cation, which are then converted to a variety of monocyclic, bicyclic, and tricyclic sesquiterpene end products via various modifications. Arabidopsis TPS11 produces at least 12 sesquiterpenes, which are all derived from a central bisabolyl cation formed from the nerolidyl cation by a 6,1-ring closure (Fig. 2b) (Tholl et al., 2005).

Diterpene synthases initiate carbocation formation in two different ways: via ionization of the diphosphate, as catalyzed by class I enzymes, or via substrate protonation at the 14, 15-double bond of GGPP, a reaction catalyzed by class II enzymes (Tholl, 2006). In one of the simplest reactions, the Arabidopsis class I diterpene synthase TPS04 ionizes the substrate GGPP to yield a geranylgeranyl cation, which is further converted to geranyllinalool by the addition of water (Fig.2c) (Herde et al., 2008). Given the length of the GGPP substrate of 20 carbon atoms and the presence of four double bonds, various modifications of the initially formed carbocation including downstream proton-induced cyclizations can lead to a tremendous number of structurally diverse monocyclic and polycyclic diterpene products (MacMillan and Beale, 1999; Keeling and Bohlmann, 2006b; Peters, 2006; Toyomasu, 2008). In Arabidopsis, several putative class I diterpene synthases have been identified (see below), whose biochemical characterization is in progress (Vaughan, Tholl et al., unpublished results). Class II diterpene synthases such as *ent*-copalyl diphosphate (CPP) synthases (CPS), which are involved in gibberellin and phytoalexin biosynthesis (Peters, 2006), catalyze a protonation-induced cyclization of the substrate GGPP to *ent*-CPP. In the gibberellin biosynthetic pathway, *ent*-CPP is further converted to *ent*-kaur-16-ene by a class I *ent*-kaurene synthase (KS) via ionization of *ent*-CPP (Fig. 2c). A bifunctional classII/I CPS/KS, which catalyzes the formation of *ent*-kaurene (and 16-hydroxykaurene) via a CPP intermediate has been identified in the moss *Physcomitrella patens* (Hayashi et al., 2006; Anterola et al., 2009). Similar class II/I diterpene synthases occur in gymnosperms such as abietadiene synthase from *Abies grandis*, which forms enzyme-bound (+)-CPP from GGPP in an initial cyclization step followed by a typical ionization-initiated cyclization of (+)-CPP to the diterpene product (Peters et al., 2003).

The three-dimensional structures of three plant monoterpene synthases from *Salvia officinalis* (Whittington et al., 2002a; Whittington et al., 2002b), *Mentha spicata* (Hyatt et al., 2007), and *Salvia fructicosa* (Kampranis et al., 2007), two sesquiterpene synthases from *Nicotiana tabacum* (Starks et al., 1997) and cotton (Gennadios et al., 2009), and an isoprene synthase producing the C_5-hemiterpene isoprene from DMAPP (Köksal et al., 2010) have been elucidated. Structural analyses of these proteins revealed highly similar tertiary structures consisting of an N-terminal domain and a catalytically active C-terminal domain, both of which are formed entirely of α-helices with short connecting loops and turns. The C-terminal domain, also called the terpene synthase fold, class I fold, or "α-domain" (Cao et al., 2010) (Fig. 3) carries a hydrophobic pocket formed by six α-helices, which accommodates the hydrophobic moiety of the prenyl diphosphate substrate. A highly conserved aspartate-rich motif, DDxxD, and a less conserved NSE/DTE motif are located on opposite sides of the entrance of the catalytic side (Christianson, 2006). Both motifs bind a trinuclear magnesium cluster, which is involved in positioning the diphosphate substrate for ionization. The N-terminal domain (relictual β-domain, see below) of class I monoterpene and sesquiterpene synthases is catalytically inactive but appears to function as a scaffold for proper folding of the C-terminal domain (Köllner et al., 2004) and capping the active side of this domain (Whittington et al., 2002a) (Fig. 3).

The first crystal structure of a taxadiene diterpene synthase (TXS) has now been reported (Köksal et al., 2011). This structure and previously developed homology models of other diterpene synthases reveal significant insight in the structural evolution of

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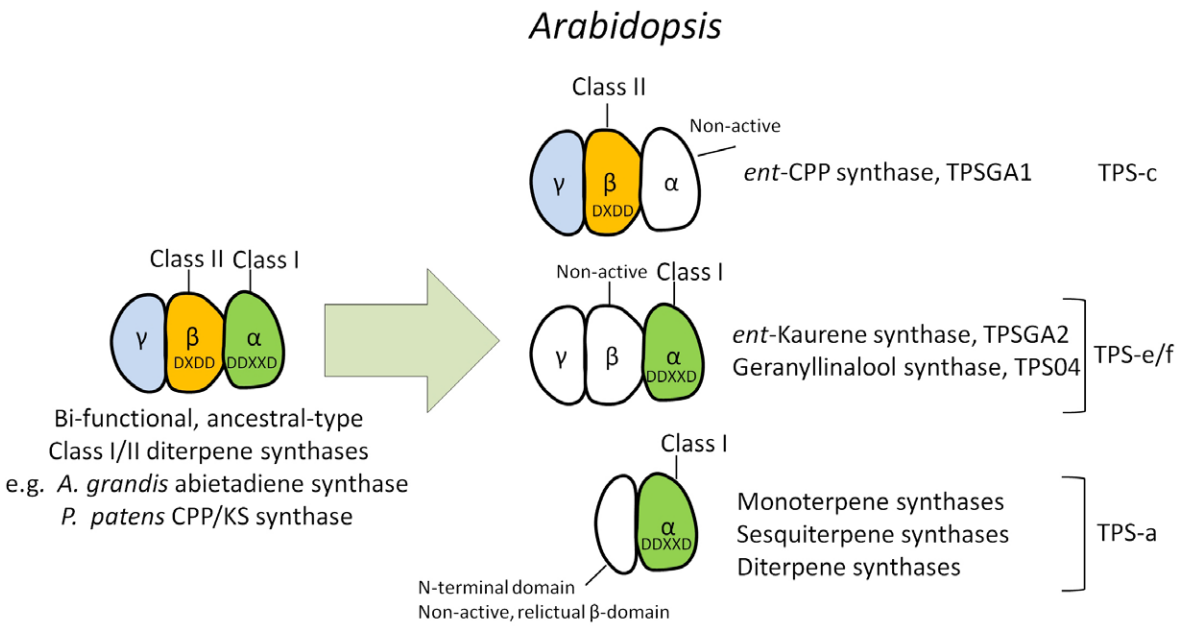


Figure 3. Protein domain structure of bi-functional class I/II TPSs and Arabidopsis class I and class II TPSs according to the structural evolutionary model by Cao et al. (2010).

DXDD and DDXXD motifs are conserved in functionally active class II (β) and class I (α) domains, respectively.

terpene synthases. Class I diterpene synthases such as TXS have a functionally active fold (α-domain) similar to that of other class I TPS. By contrast, class II enzymes such as CPSs possess a functionally active N-terminal class II fold (or β-domain, Cao et al., 2010) with a conserved DxDD motif, which is required for protonation-initiated carbocation formation; however, the α-domain of these enzymes lacks a functional DDxxD motif (Fig.3). A class II (β)-type domain can also be present in class I diterpene synthases (TXS, KSs) but it does not contain a functionally active DxDD motif (Fig.3). In many of the modern class I monoterpene and sesquiterpene synthases the class II (β)-type domain is relictual and has been substantially deleted (Fig.3).

Bifunctional (class I/II) diterpene synthases are equipped with both functional domains. These enzymes and class II type diterpene synthases possess an additional, amino-terminal 'insertional' or γ-domain of ancestral origin that is necessary for correct folding and carries an EDxxD-like motif with an apparent function in binding Mg^{2+} and diphosphate (Bohlmann et al., 1998; Cao et al., 2010) (Fig.3). According to the model developed by Cao et al. (2010), bifunctional diterpene synthases as they occur in gymnosperms (e.g. abietadiene synthase - similar to the CPS/KS protein of *P. patens*) resemble a three-domain ancestral TPS with origins in bacterial and fungal TPSs. A γ-type domain without an EDxxD-motif is also present in TXS, KSs, and other ancestral-type diterpene synthases such as Arabidopsis TPS04 (Fig. 3). The domain architecture of a non-functional β/γ (class II) fold and a functional class I fold, as it has been identified for the TXS enzyme, clearly demonstrates an evolutionary connection between class I and class II terpene synthases (Köksal et al., 2011). Further mechanistic and evolutionary aspects will be addressed below in the context of the Arabidopsis TPS family.

THE ARABIDOPSIS TPS FAMILY

Using standard homology search methods, Aubourg et al. (2002) were the first to show that the Arabidopsis genome contains a family of 32 full length *TPS* genes. Arabidopsis *TPS* genes are distributed over all five chromosomes with over two-thirds being located on chromosomes 1, 3, and 4. Similar to the *TPS* gene families of other angiosperms and gymnosperms such as maize, rice, tomato, and spruce, Arabidopsis *TPS* genes emanate from cycles of gene duplication, multiple mutations and functional divergence. Phylogenetic analysis of TPS protein sequences shows an association of Arabidopsis TPSs with several different subfamilies of the TPS superfamily (Fig. 4a). All functionally active AtTPS proteins characterized within these subfamilies depend on Mg^{2+} or Mn^{2+} as divalent cofactors and exhibit catalytic properties such as low K_m values, which are characteristic for most plant TPSs.

Arabidopsis TPSs of the TPS-a Family

Within the plant TPS superfamily, which is comprised of seven subfamilies (TPS-a, b, c, d, e/f, g, and h) (Chen et al., 2011), 22 Arabidopsis TPS proteins are most closely related to other angiosperm terpene synthases of the TPS-a subfamily (Aubourg et al., 2002; Tholl et al., 2005) (Fig. 4a). In this subfamily, Arabidopsis TPSs exhibit higher similarity to one another than to TPS proteins of other species, which indicates rapid evolution of a species-specific paralogous gene cluster, a common concept in plant *TPS* gene evolution. The cluster contains several "sub"-clusters of closely related TPSs (Fig. 4a), whose corresponding genes are not all located on the same chromosome indicating recombination and translocation events of these genes. The expansion of

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a TPS subfamily after speciation may be caused by ecological adaptations of the plant in attraction of or defense against other organisms. Because of this species-specific radiation, *TPS* genes of the same function can arise repeatedly in different plant species and families (Bohlmann et al., 1998; Martin et al., 2004).

All type-a Arabidopsis TPSs are class I proteins, which lack a γ-domain and carry a non-functional N-terminal domain (Fig. 3). Four type-a Arabidopsis *TPS* genes (*TPS11*, *TPS12*, *TPS13*, and *TPS 21*) encode proteins without plastidial transit peptides and have been characterized as sesquiterpene synthases. The compounds produced by the respective recombinant enzymes are listed in Table 2. TPS12 and its closely related enzyme TPS13 both catalyze the formation of (*Z*)-γ-bisabolene as a main product and α-bisabolol and (*E*)-nerolidol as minor products (Ro et al., 2006). The *TPS12* and *TPS13* genes are positioned in close proximity to one another on chromosome 4 and most likely emerged by recent tandem gene duplication (Fig. 4b). Of the remaining 18 type-a genes, six (*TPS01*, *TPS05*, *TPS07*, *TPS16*, *TPS17*, *TPS28*) were found not to be expressed in flowers, leaves, and roots of Arabidopsis accession Columbia-0 (Col-0) according to RT-PCR analysis in our laboratory. However, expression of these genes at mostly low transcript levels were reported in microarray datasets of siliques, seeds, embryo development, and roots (Table 2) (The Bio-Array Resource for Plant Functional Genomics, http://www.bar.utoronto.ca/). Proteins encoded by all 18 genes carry predicted plastidial or mitochondrial targeting sequences (Table 2) and are assumed to be diterpene synthases (or monoterpene synthases) located in plastids or sesquiterpene synthases possibly targeted to mitochondria. Biochemical characterization of TPS08 and TPS20 has revealed that these enzymes indeed function as diterpene synthases (Vaughan, Tholl et al., unpublished results), while characterization of TPS22 and its related protein TPS25 suggests that both enzymes are sesquiterpene synthases that appear to be localized to mitochondria (Huh, Tholl et al., unpublished results). Whether any of the other type-a *TPS* genes are functionally active *in vivo* remains to be determined. Recently, Shah has reported the detection of the diterpene aldehyde dehydroabietinal, which appears to function as a systemic signal at picomolar concentrations when induced by infection with an avirulent strain of *Pseudomonas syringae* (Shah, 2009). It is likely that the precursor of this diterpene is produced by a TPS in the type-a family. Hence, it is possible that some of the uncharacterized type-a TPSs produce volatile or non-volatile terpenes at very low amounts or concentrations below detectable levels. In addition, some type-a *TPS* genes might only be functionally active in specific accessions and inactive in others even though they are still transcribed.

Arabidopsis TPSs of the TPS-b Family

Phylogenetic amino acid sequence comparison places six Arabidopsis TPSs in the TPS-b subfamily, in which they form, similar to the Arabidopsis type-a TPSs, a species-specific clade (Aubourg et al., 2002; Tholl et al., 2005) (Fig 4a). The Arabidopsis TPS-b clade contains seven class I-type monoterpene synthases, all of which have been biochemically characterized: TPS02 (Huang et al., 2010), TPS03 (Fäldt et al., 2003; Huang et al., 2010), TPS10 (Bohlmann et al., 2000), TPS24 (Chen et al., 2003), and TPS-Cin encoded by the identical genes *TPS23* and *TPS27* (Chen et al., 2004). The terpene products synthesized by these enzymes *in vitro* are listed in Table 2. The genes *TPS23*, *TPS24*, and *TPS27* cluster together on chromosome 3 (Fig. 4b) and have emerged from a recent gene duplication event, which is particularly evident for *TPS23* and *TPS27*, whose coding sequences and promoter regions are 100% identical. The TPS-Cin protein encoded by *TPS23* and *TPS27* and the closely related TPS24 enzyme synthesize similar groups of monoterpene products but differ in their major products 1,8-cineole and (*E*)-β-ocimene/myrcene, respectively. Moreover, differences in the promoter sequences of the *TPS24* and *TPS23/27* genes lead to different expression patterns with *TPS-Cin* being expressed in roots and *TPS24* in flowers (Chen et al., 2003; Chen et al., 2004) (see below). The closely related genes *TPS02* and *TPS03* encoding bifunctional (*E*)-β-ocimene/(*E*,*E*)-α-farnesene synthases represent another gene pair as a result of gene duplication (Fig. 4b). In contrast to all the other monoterpene synthases of clade I (including TPS02), which contain predicted chloroplast transit peptide sequences and are assumed to be located in plastids (confirmed for TPS02), TPS03 lacks a functional plastidial transit peptide and is localized to the cytosol (Huang et al., 2010).

Monoterpene synthases in the type-b subfamily commonly contain a conserved $RR(x)_8W$ motif downstream of their 40 to 70 amino acid N-terminal plastidial transit peptide (Whittington et al., 2002b; Hyatt et al., 2007). Although the RR motif is thought to be required for the isomerization of GPP to a linalyl cation in the formation of cyclic monoterpenes (Williams et al., 1998), this motif is highly conserved in all TPS type-b Arabidopsis monoterpene synthases including those that produce acyclic terpenes (Aubourg et al., 2002). In comparison to the type-b enzymes, Arabidopsis type-a TPS proteins contain a modified $R(x)_9W$ motif, of which only the highly conserved W residue is present in the sesquiterpene synthases TPS11, TPS12, and TPS13. Interestingly, TPS06, TPS17, and TPS30 proteins carry modified versions of the highly conserved DDxxD motif (TPS06: DNTFD, TPS17: NDTCD, TPS30: NDVCD). To what extent these modifications interfere with substrate binding or impair enzyme activity is currently not known. However, it was demonstrated in goldenrod, that a naturally occurring variant of the DDxxD motif (NDxxD) in a germacrene D synthase had no impact on catalytic activity (Prosser et al., 2004).

Enzymes of Other TPS Subfamilies

Four Arabidopsis *TPS* genes have not, in contrast to the type-a and type-b genes, undergone duplication and belong to different *TPS* subfamilies. TPS14 is a (+)-*S*-linalool synthase that lacks the $RR(x)_8W$ motif and is related to acyclic monoterpene synthases from snapdragon in the TPS-g subfamily (Dudareva et al., 2003) (Fig. 4a). The remaining three TPSs are all diterpene synthases and form a separate clade within the Arabidopsis TPS family (Fig. 4a): the class II-type CPP synthase, TPSGA1, the class I-type kaurene synthase, TPSGA2, both of which provide the precursors in gibberellins biosynthesis (Sun and Kamiya, 1994; Yamaguchi et al., 1998), and the class I-type geranyllinalool synthase TPS04, which produces the alcohol precursor in the biosynthesis of the volatile C_{16}-homoterpene 4,8,12-trimethyltridecatetra-1,3,7,11-

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ene (TMTT) (Herde et al., 2008). Recently, a cytochrome P450 monooxygenase (P450) of the CYP82 family has been identified, which converts (*E*,*E*)-geranyllinalool into TMTT via an oxidative C-C cleavage reaction (Lee et al., 2010) (Fig. 1). All three diterpene synthases contain the insertional or γ-domain of approximately 200 amino acids with an EDxxD-like motif (Fig. 3) in class II-type TPSGA1. TPSGA1 is highly similar to other CPSs in the plant TPS-c subfamily, while TPSGA2 is related to *ent*-KS in the recently merged TPS-e/f subfamily (Chen et al., 2011), and TPS04 shows highest similarity to *S*-linalool synthases from *Clarkia breweri and Clarkia concinna* in the TPS-e/f family (Herde et al., 2008).

The *TPSGA1*, *TPSGA2*, and *TPS04* genes contain 12 to 15 exons (Table 2) similarly to the gene structure of the presumed progenitor gene of all plant *TPS* (Bohlmann et al., 1998; Trapp

Table 2. Properties of *Arabidopsis TPS* genes and proteins and TPS enzymatic products.

TPS[a]	AGI No.	Exons[b]	Subcellular Protein Localization[c]	Tissue specific Transcription/ Promoter Activity[d]	Enzyme Products	Reference
01	At4g15870	7	P	S	unknown	
02	At4g16730	7	P	F; L-i	(*E*)-β-ocimene/myrcene (*E*,*E*)-α-farnesene	Huang et al., 2010
03	At4g16740	7	C	F; L-i	(*E*)-β-ocimene/myrcene (*E*,*E*)-α-farnesene	Fäldt et al., 2003
04	At1g61120	12	C	F; L-i	(*E*,*E*)-geranyllinalool	Herde et al., 2008
05	At2g23230	7	M/P/V	S	unknown	
06	At1g70080	7	M/P	R; S	unknown	
07	At4g20200	7	P	S	unknown	
08	At4g20210	7	P	R	diterpene	Vaughan, Tholl et al., unpublished
09	At4g20230	7	M/P	R; F; S; Sh	unknown	
10	At2g24210	7	P	F/L-i	(*E*)-β-ocimene/myrcene	Bohlmann et al., 2000
11	At5g44630	7	C	F	sesquiterpene blend	Tholl et al., 2005
12	At4g13280	7*	P?	R; F; L-i	(*Z*)-γ-bisabolene	Ro et al., 2006
13	At4g13300	7	C	R; F; L-i	(*Z*)-γ-bisabolene	Ro et al., 2006
14	At1g61680	7	P	F	(+)-3*S*-linalool	Chen et al., 2003
15	At3g29190	7	C	S	unknown	
16	At3g29110	7	M/P	S	unknown	
17	At3g14490	7	P	S; R	unknown	
18	At3g14520	7	P	F; S	unknown	
19	At3g14540	7	P	R; F	unknown	
20	At5g48110	7*	P	R	putative diterpene	Vaughan, Tholl et al., unpublished
21	At5g23960	7	C	F	(*E*)-β-caryophyllene	Chen et al., 2003
22	At1g33750	6	M	R	sesquiterpene blend	Huh, Tholl et al., unpublished
23	At3g25830	7	M?/P	R	1,8-cineole	Chen et al., 2004
24	At3g25810	7	P	F	monoterpene blend	Chen et al., 2003
25	At3g29410	7	M	R	sesquiterpene blend	Huh, Tholl et al., unpublished
26	At1g66020	7	M/P	S; R; H	unknown	
27	At3g25820	7	M?/P	R	1,8-cineole	Chen et al., 2004
28	At1g48800	7	M/P	S; R	unknown	
29	At1g31950	7	M/P	R	unknown	
30	At3g32030	7	M/P	R	unknown	
31 GA1	At4g02780	15	P	#S; R; Sh; L; F	*ent*-copalyl diphosphate	Sun and Kamiya, 1997
32 GA2	At1g79460	14	P	S; R; Sh; L; F	*ent*-kaurene	Yamaguchi et al., 1998

[a] *TPS* gene numbers are adopted from Aubourg et al. (2002). *TPS23* and *TPS27* are identical genes. [b] Predicted gene structure according to Aubourg et al. (2002) and The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/). Asterisks indicate genes, for which 8 exons are indicated by TAIR. [c] Subcellular localization predicted by the Predotar, TargetP, and WoLFPSORT algorithms. P, plastid; M, mitochondria; V, vacuole; C, cytosol. Underlined letters indicate subcellular localizations confirmed by GFP fusion analysis. [d] Information of tissue-specific *TPS* gene transcription and/or promoter activity in the Col-0 accession was retrieved from Affymetrix ATH1 GeneChip data of the eFP and Expression Browser (http://bar.utoronto.ca/), by RT-PCR, Northern blot, and/or promoter-GUS reporter gene analysis according to the indicated references. Only tissues with the highest expression levels of each gene are shown (F, flower; H; hypocotyl; L, leaf; R, root; S, seed/embryo; Sh, shoot. i, induced by insect feeding and/or mechanical wounding); # highest expression in rapidly growing tissues.

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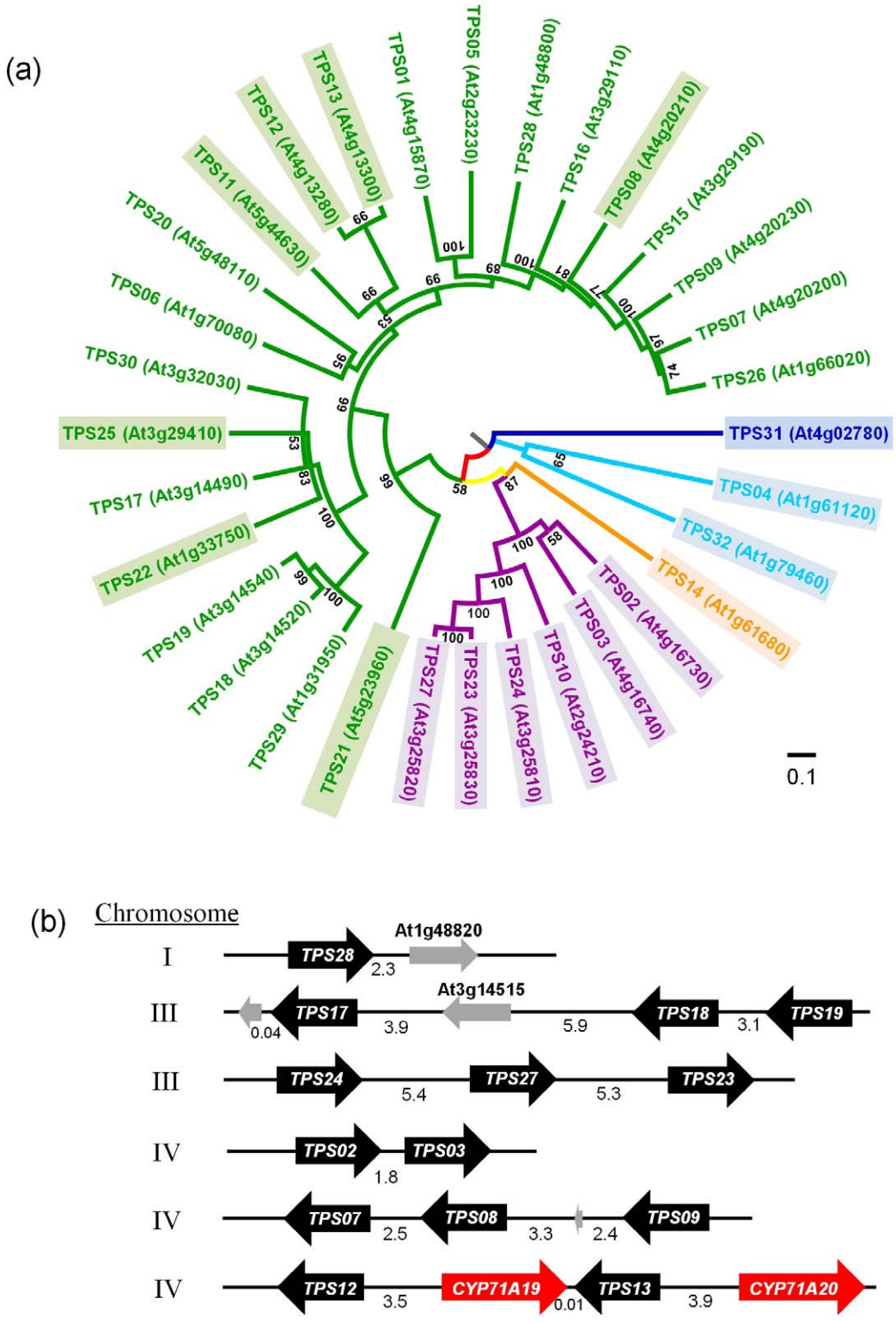


Figure 4. Clades of the Arabidopsis TPS family **(a)** and chromosomal Arabidopsis *TPS* gene clusters **(b)**.

(a) Arabidopsis TPS amino acid sequences were aligned by the MUSCLE algorithm and a tree was built with the GENEIOUS program (Biomatters Ltd.). Bootstrap values larger than 50% are shown. Colors indicate enzymes of TPS subfamilies a (green), b (purple), c (dark blue), e/f (light blue), and g (orange). Functionally characterized TPS proteins are shaded. **(b)** *TPS* pseudogenes (with AGI number) or gene fragments (non-annotated) are marked with grey arrows. Numbers indicate distances in kb between genes.

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and Croteau, 2001; Aubourg et al., 2002). Four exons interrupted by three introns code for the insertional (γ) domain. Consecutive loss of exons and introns including those of the insertional domain most likely gave rise to the emergence of modern class I type *TPSs*, which contain seven exons (all Arabidopsis type-a and type-b TPS except for *TPS22* with 6 exons) (Trapp and Croteau, 2001; Aubourg et al., 2002) (Table 2). Exon/intron positions and the order of intron phases are conserved among the paralogous type-a and type-b Arabidopsis genes. The larger gene sizes of *TPSGA1*, *TPSGA2*, and *TPS04* correspond to molecular weights between 90 and 100 kDa in contrast to type-a and type-b proteins with sizes between 60 and 70 kDa. The fact that all three diterpene synthase genes are single-copy genes and have not emerged from paralogous gene clusters indicates their function in evolutionary early terpene biosynthetic pathways, which is supported by the assumed emergence of angiosperm and gymnosperm *CPS* and *KS* from a bi-functional CPS/KS prototype as found in *P. patens* (Hayashi et al., 2006).

While TPSGA1, TPSGA2, and other diterpene synthases are targeted to the plastid, the TPS04 protein does not contain an obvious plastid transit peptide sequence. Fusion studies with yellow fluorescent protein (YFP) indicated that TPS04 is localized in the cytosol, where it uses GGPP either produced by a cytosolic GGPS or exported from chloroplasts as the substrate (Fig. 1) (Herde et al., 2008). The latter assumption is supported by the severely reduced formation of TMTT in the presence of the MEP pathway inhibitor fosmidomycin (Lee, Tholl et al., unpublished results).

Gene Clusters and Biosynthetic Modules

Repeated gene duplications in the Arabidopsis *TPS* gene family gave rise to the formation of six small gene clusters each containing two to five genes positioned head to tail to each other by a close distance (0.04 to 6 kb) (Fig 4b). Three of these clusters contain pseudogenes or *TPS* gene fragments (Fig 4b). A total of eight pseudogenes (plus the *TPS02* pseudogene in the Col-0 accession, see below) have been identified in the Arabidopsis genome (Aubourg et al., 2002), all of which exhibit disruptions of the coding sequence by deletions or insertions with four genes consisting of only a partial number of exons. Pseudogenes in the *TPS17*/*TPS18*/*TPS19* cluster are very similar in sequence to the intact *TPS* genes indicating their origin by gene duplication. Frequent pseudogenization of Arabidopsis *TPS* genes may have arisen by genomic instability caused by insertion of transposon-like elements, several of which are positioned in close proximity to *TPS* genes or occur in a gene cluster (Aubourg et al., 2002).

Besides their tendency to form *TPS* gene clusters, several *TPS* genes also appear to cluster with *GGPS* genes as well as genes encoding P450s or glycosyltransferases that may catalyze secondary transformations of the primary terpene compounds. For example, the (*Z*)-γ-bisabolene synthase genes *TPS12* and *TPS13* each cluster head-to-head with a P450 gene located on the opposite strand sharing the same promoter region (Fig 4b). Both P450 genes *CYP71A19* (At3g13290) and *CYP71A20* (At4g13310) share 88% amino acid sequence identity and were duplicated in pair with the respective *TPS* gene. According to GUS-reporter gene analyses and microarray expression profiles (Birnbaum et al., 2003), *CYP71A19* and *CYP71A20* are co-expressed with *TPS12* and *TPS13* in the root cortex/endodermis suggesting that the corresponding enzymes form modules in the biosynthesis and possible hydroxylation of (*Z*)-γ-bisabolene. Other functional *TPS*-P450 gene clusters have been suggested for genes *TPS05*, *TPS09*, *TPS11*, *TPS22* (Aubourg et al., 2002). The seed-specific transcription of *TPS05* correlates well with that of the clustering gene *CYP81D6* but is different from expression patterns of neighboring glucosyltransferase genes. Another gene pair with organ-specific co-expression in flower ovaries consists of the multi-sesquiterpene synthase gene *TPS11* and the clustering P450 gene *CYP706A3*, which may be involved in the oxidation of one or several of the TPS11 sesquiterpene products. The other P450 genes that cluster with the root-expressed genes *TPS22* and *TPS09* show no or only low expression in root tissue.

The GGPP synthase genes *GGPS5*, *GGPS6*, and *GGPS7*, each of which has apparently been duplicated with genes *TPS22*, *TPS24*, and *TPS25*, respectively, share overlapping expression with these genes in siliques and developing seeds but have highest transcript levels in roots, where the clustering *TPS* genes are not expressed (The Bio-Array Resource for Plant Functional Genomics, http://www.bar.utoronto.ca/). Moreover, the recombinant TPS22 and 25 proteins have been shown to have no diterpene synthase activity *in vitro* (Huh, Tholl, unpublished results) indicating that these enzymes are most likely not part of a functional biosynthetic module. Finally, the root-specific expression of genes *GGPS9* and *GGPS10* correlates to some extent with that of their clustering genes *TPS19* and *TPS20* although expression of the genes of each pair does not synchronize in the same cell type (The Bio-Array Resource for Plant Functional Genomics, http://www.bar.utoronto.ca/).

TISSUE- AND CELL TYPE-SPECIFICITY OF TERPENE FORMATION

All genes of the Arabidopsis *TPS* family exhibit fairly specific constitutive expression patterns with limited overlap between different tissues and cell-types. For example, all of the flower-specific *TPS* genes are not transcribed in roots and, likewise, transcripts of only few root-expressed genes have been found in floral tissues (Table 2). It can be assumed that the spatial expression patterns of most Arabidopsis *TPS* evolved under selection pressures by herbivores and pathogens or in interaction with mutualistic organisms, evidence of which will be discussed below. In contrast to the high tissue- and cell-type specificity of constitutive expression, several *TPS* genes overlap in their transcriptional response to herbivore and pathogen attack in leaves resulting in the release of specific terpene mixtures with possible defensive activities against the attacking organisms.

Constitutive Formation of Volatile Terpenes in Flowers

By far, the largest number of terpene compounds produced by Arabidopsis has been detected in floral tissues. Flowers of the Col-0 accession emit a complex mixture of over 20 sesquiterpene hydrocarbons with (*E*)-β-caryophyllene as the predominant

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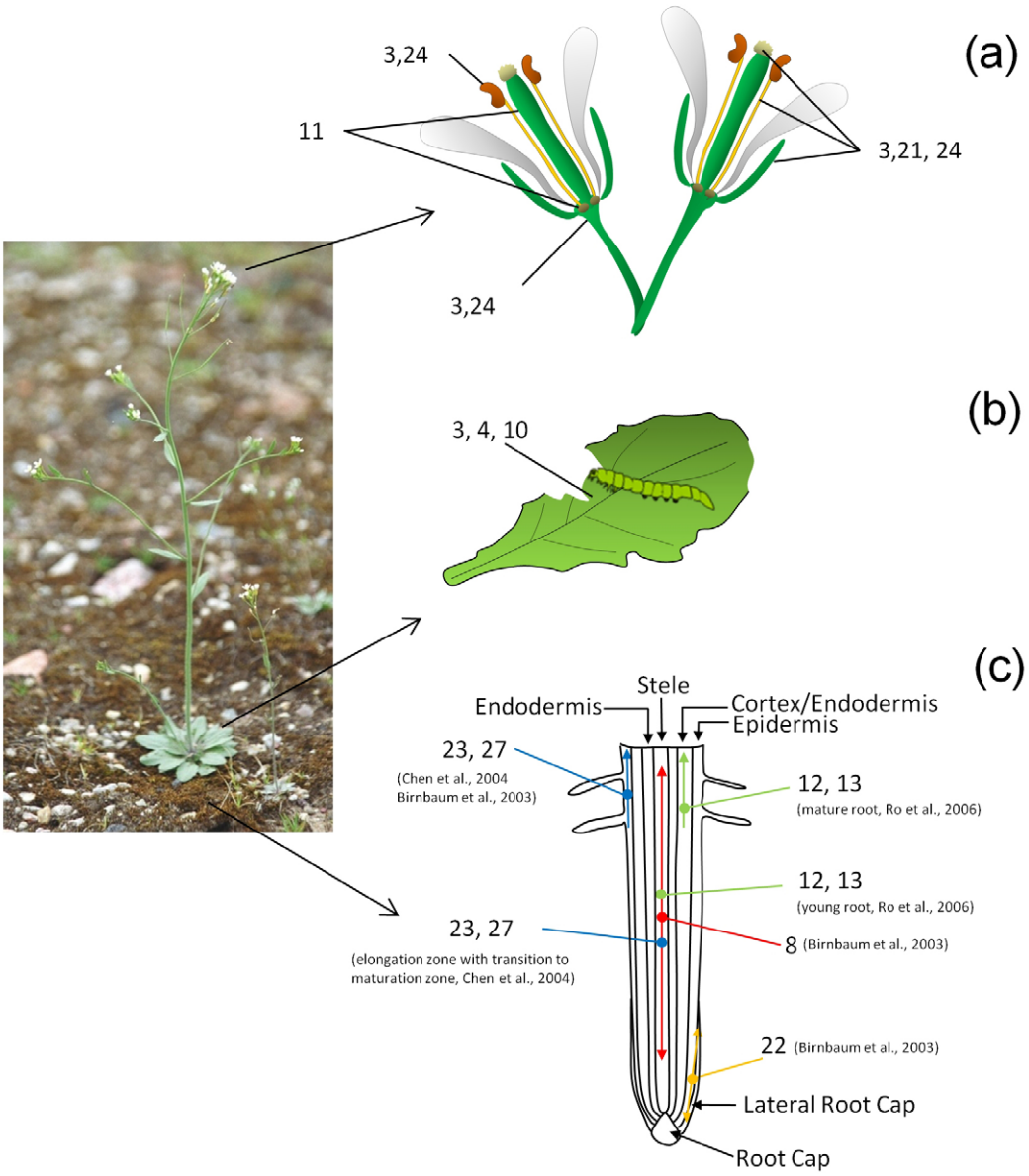


Figure 5. Tissue- and cell-type specific Arabidopsis *TPS* gene expression patters according to RT-PCR and promoter-GUS reporter gene analyses.

Spatial expression is shown for flowers **(a)**, insect-damaged leaves **(b)**, and roots **(c)**. Arrows in **(c)** mark gene expression in specific root cell types. Numbers indicate different *TPS* genes. Only those genes are shown, for which enzymatic products have been detected *in vivo*.

compound (Chen et al., 2003; Tholl et al., 2005). Two enzymes, TPS11 and TPS21, are responsible for the formation of nearly all of the flower-specific sesquiterpenes: TPS21 synthesizes primarily (*E*)-β-caryophyllene and α-humulene, and TPS11 catalyzes the formation of essentially all of the remaining sesquiterpenes (Tholl et al., 2005) (Table 2, Fig. 5a). In contrast to floral scent emissions from many other angiosperms, Arabidopsis floral volatiles are not produced in flower petals. Promoter-GUS reporter gene experiments and microarray datasets show that *TPS11* is primarily expressed in intrafloral nectaries and ovules, while expression of *TPS21* occurs largely in the stigma of open flowers and to some extent in sepals (Fig. 5a). Formation of volatile terpenes in these flower organs can principally serve two different functions: Volatiles may attract small pollinating insects although only at a short distance since the emission of the floral volatile blend is fairly low compared to highly scented flowers (Chen et al., 2003). Cross-fertilization leading to more than 1% outcrossing events in natural populations may stabilize populations by reducing inbreeding depression (Abbott and Gomes, 1989). Second, the specific expression pattern of both *TPS* genes indicates possible defensive activities of terpenes in these organs. Especially, nectaries and the stigma represent sugar-containing and/or moist areas that can promote bacterial or fungal growth (Buban et al., 2003). Evidence for the role of (*E*)-β-caryophyllene in inhibiting

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the growth and invasion of microbial pathogens in the floral stigma has been obtained by the analysis of *TPS21* gene knock-out plants impaired in (*E*)-β-caryophyllene emission (Huang, Tholl et al., unpublished results).

Besides the emission of sesquiterpenes, small amounts of the monoterpenes β-myrcene, limonene, and linalool have been detected in Col-0 flowers. Three monoterpene synthases, the multiproduct monoterpene synthase TPS24 (Fig. 5a), the β-myrcene/(*E*)-β-ocimene TPS10, and the linalool synthase TPS14, all of which are expressed in Arabidopsis flowers (Chen et al., 2003), contribute to some degree to the formation of these compounds (Table 2). Other terpene volatiles emitted by Col-0 flowers in low amounts are (*E*,*E*)-α-farnesene and the homoterpene TMTT (Tholl et al., 2005; Huang et al., 2010), which are produced by TPS03 (Fig. 5a) and via the homoterpene-specific pathway, respectively (see below). The low emission of these compounds and of the detected monoterpenes, in most cases, does not correlate with the transcript levels of the corresponding *TPS* genes and may be related to substrate limitation and/or posttranslational protein modifications.

Induced Formation of Terpenes in Leaves

Most of the terpenes detected in Arabidopsis leaves in response to biotic stress have been of volatile nature although recent findings by Shah indicate the formation of a diterpene aldehyde as a potential systemic signal in microbial pathogen infection (Shah, 2009). This finding resembles that of a diterpene alcohol as an endogenous defense signal in tobacco in response to infection by tobacco mosaic virus (Seo et al., 2003).

Under physiologically normal growth conditions, leaves of Arabidopsis Col-0 plants release only traces of volatile terpenes. However, treatment with the fungal peptide elicitor alamethicin, application of jasmonate and the jasmonate-isoleucine mimic coronalon, and feeding by the crucifer specialist insects *Pieris rapae* and *Plutella xylostella* induce the emission of a volatile blend consisting of green leaf volatiles, the benzenoid compound methyl salicylate, and the C_{16}-homoterpene TMTT and the sesquiterpene (*E*,*E*)-α-farnesene as the primary terpene constituents (Van Poecke et al., 2001; Herde et al., 2008; Snoeren et al., 2010). Snoeren et al. (2010) observed the additional release of five monoterpenes (3-carene, linalool, β-myrcene [also detected by Van Poecke et al., 2001], (*Z*)-β-ocimene, α-phellandrene), the sesquiterpene (*E*)-nerolidol, and the C_{11}-homoterpene 4,8-dimethylnona-1,3,7-triene (DMNT) from pools of Col-0 plants treated with *P. rapae* and/or jasmonate, although no specific emission rates of these compounds were reported. Also, the apocarotenoids β-ionone and cyclocitral were detected in response to insect feeding (Van Poecke et al., 2001). The observed composition of the induced volatile mixtures depends on the type of stressor (type of herbivore, chemical treatment) but can also vary with conditions of plant growth and volatile collection (Tholl, 2006). Furthermore, depending on the accession, volatile profiles vary qualitatively and quantitatively. For example, (*E*)-β-ocimene represents a common induced volatile in other accessions (see below). Van Poecke et al. (2001) and Loivamäki et al. (2008) demonstrated a possible role of herbivore-induced volatiles in plant indirect defense via the attraction of parasitoids such as *Cotesia rubecula* and *Diadegma semiclausum*, which parasitize *P. rapae* larvae. In the laboratory, attack by *C. rubecula* can increase plant fitness in terms of seed production (van Loon et al., 2000). A possible role of homoterpenes such as TMTT in the attraction of herbivore predators has been suggested by several studies (de Boer et al., 2004; Mumm et al., 2008). In lima bean, TMTT has been characterized for its ability to mediate plant-plant interactions by inducing the transcription of defense genes such as *LOX2* and *PDF2* in un-attacked neighboring plants (Arimura et al., 2000).

The first committed step in the biosynthesis of TMTT is catalyzed by the (*E*,*E*)-geranyllinalool synthase TPS04 (GES) of the TPS-f subfamily (Herde et al., 2008). Expression of the *TPS04* gene is induced locally by insect feeding (Fig. 5b) and elicitor treatment and its expression is dependent on the jasmonate signaling pathway but independent of salicylic acid and ethylene. *TPS04* is tightly co-expressed with the recently identified P450 gene *CYP82G1* (At3g25180) responsible for the oxidative breakdown of (*E*,*E*)-geranyllinalool (Lee et al., 2010) resulting in an efficient conversion of the alcohol intermediate to the homoterpene end product.

In the Col-0 accession, insect-induced formation of (*E*,*E*)-α-farnesene is catalyzed by the bi-functional mono-/sesquiterpene synthase TPS03 (see under variation of terpene biosynthesis) (Huang et al., 2010). *TPS03* is expressed locally at wound sites (Fig. 5b) but also constitutively in flowers, where it contributes to low emissions of (*E*,*E*)-α-farnesene. Expression of *TPS03* occurs in sepals, anthers (particularly in pollen), and in the stigma of immature and mature flowers (Fig. 5a) (Huang et al., 2010). The flower-specific expression profile of *TPS03* suggests a role of (*E*,*E*)-α-farnesene in pollinator attraction or florivore/antimicrobial defense similar to that discussed for the other flower-expressed genes *TPS21* and *TPS11*. (*E*)-β-Ocimene, the monoterpene analog of (*E*,*E*)-α-farnesene, which is emitted in response to herbivory by other Arabidopsis accessions such as Wassilewskija (Ws), was shown to be produced by TPS02, the enzyme closely related to TPS03 (see variation of terpene biosynthesis) (Huang et al., 2010). Similar to *TPS03*, the *TPS02* gene shows a dual expression pattern - insect-induced expression in leaves and constitutive expression in floral tissues - consistent with the induced formation and floral release of (*E*)-β-ocimene in accessions emitting this terpene.

The formation of β-myrcene induced by feeding of *P. rapae* (Van Poecke et al., 2001; Snoeren et al., 2010) can most likely be attributed to the β-myrcene/(*E*)-β-ocimene synthase TPS10, which is induced under these conditions (Van Poecke et al., 2001) (Fig. 5b). The other monoterpenes detected by Snoeren et al. (2010) might be produced by uncharacterized TPS enzymes of the TPS-a family or by TPS-b monoterpene synthases, although the reported compounds are not primary products of these enzymes and/or the respective genes have not been found to be induced by insect feeding.

Root-Specific Formation of Terpenes

RT-PCR expression profiles (Chen et al., 2003) and detailed root cell transcriptome analyses by Birnbaum et al. (2003) and Brady et al. (2007) revealed a considerable number of 14 *TPS* genes with primary or exclusive expression in Arabidopsis roots (Table 2). This group of root-specific genes includes the two identical 1,8-cineole synthase (*TPS-Cin*) genes *TPS23* and *TPS27* and 12 genes of the TPS-a family. The CPP synthase *TPSGA1* and the

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kaurene synthase *TPSGA2* of the gibberellin biosynthetic pathway are also expressed in roots with highest transcript signals in the quiescent center, but their expression is not limited to root tissues. According to high resolution spatio-temporal maps of the root transcriptome (Brady et al., 2007) supported by promoter-GUS reporter gene studies, several of the root-specific *TPS* genes exhibit cell type-specific expression patterns in root zones of different developmental stages. For example, the *TPS-Cin* genes *TPS23* and *TPS27* are expressed in the stele (vascular tissue) of the root elongation zone and in epidermal cells and root hairs of older root-growth zones suggesting a direct diffusion or secretion of the synthesized monoterpene into the rhizosphere (Chen et al., 2004) (Fig. 5c). A similar dual-expression pattern was observed in promoter-GUS analyses of the (*Z*)-γ-bisabolene synthase genes *TPS12* and *TPS13* with expression in the stele of younger roots and in the cortex and endodermis of mature roots (Ro et al., 2006) (Fig. 5c). Furthermore, transcripts of the predicted diterpene synthase gene *TPS08* were reported in the root vasculature, more specifically in the xylem pole pericycle and the procambium (Fig. 5c). Gene-coexpression profiles suggest that root-specific *TPS* genes form biosynthetic modules with up- or downstream enzymes expressed in the same cell type such as specific GGPS isoenzymes or P450 enzymes catalyzing secondary transformations. One such example is the duplicated gene clusters of *TPS12* and *TPS13* and the P450 genes *CYP71A19* and *CYP71A20* described above.

The primary products of the described root-specific TPSs have been detected in different culture systems (Steeghs et al., 2004; Vaughan, Tholl et al., unpublished results) and provide grounds for testing terpene chemical defenses in Arabidopsis roots. The cell-specific nature of terpene metabolism in Arabidopsis roots may emerge in defense against soil-borne, root-attacking organisms such as nematodes, microbial pathogens, and insect larvae, which have different feeding or infection strategies that target different cells and tissues. These constitutive defenses are enhanced or supported by additional induced responses depending on the root-attacker (Sohrabi and Tholl, unpublished results).

VARIATION OF TERPENE BIOSYNTHESIS IN ARABIDOPSIS ACCESSIONS (ECOTYPES)

Specialized metabolic profiles typically vary within and between plant species and are thought to arise under selection pressures by different communities of attacking and beneficial organisms. Arabidopsis accessions have been used to genetically dissect several traits including specialized metabolic profiles involved in plant ecological interactions (Kliebenstein et al., 2001a; Kliebenstein et al., 2001b; Lambrix et al., 2001). In this context, Arabidopsis has emerged as a suitable model to elucidate intra- and interspecific variation of terpene metabolism at molecular and genetic levels.

Transcript analysis of the *TPS* gene family from accession Landsberg *erecta* (L*er*) demonstrated constitutive expression profiles mostly similar to those of the Col-0 accession (Ro et al., 2006). However, a survey of floral terpene volatiles from 37 Arabidopsis accessions revealed qualitative and quantitative differences of the emitted monoterpene and sesquiterpene blends (Tholl et al., 2005). For example, (*E*)-β-ocimene was not emitted or only at low levels from 1/3 of the investigated accessions (including Col-0), while (*E*)-β-caryophyllene was absent or produced in traces in only two accessions (Oy-1, CVI), and the sesquiterpene products of enzyme TPS11 were absent in four accessions (CVI, Co-1, C-24, Lu-1). The lack of sesquiterpene volatiles in a small number of accessions was found to result from mutation or post-translational regulation of the *TPS11* and *TPS21* genes (Tholl et al., 2005).

Recent studies have investigated the variation of volatile terpene blends induced by *P. xylostella* feeding and jasmonate. Snoeren et al. (2010) found significant quantitative variation in the emission of monoterpenes, sesquiterpenes, and homoterpenes between nine accessions and treatments by insect feeding or jasmonic acid. The phenotypic variation of jasmonate-induced volatiles was correlated with differences in the attraction of the parasitic wasp *D. semiclausum*. Qualitative and quantitative differences in volatile terpene emission were also observed in a different set of 27 accessions in response to treatment with the jasmonate-isoleucine mimic coronalon (Huang et al., 2010). For example, quantitative variation of correlated emissions of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene were found in the majority of the investigated accessions, while four accessions such as Col-0 produced (*E*,*E*)-α-farnesene but none or only small amounts of (*E*)-β-ocimene (Fig. 6). The difference in terpene emission between Col-0 and the accession Ws, a high (*E*)-β-ocimene-emitter, is caused by allelic differences of the tandem-duplicated genes *TPS02* and *TPS03* and subcellular regulation of the corresponding bi-functional proteins. While the Col-0 *TPS02* allele and the Ws *TPS03* allele are pseudogenes, the enzymes encoded by each of the functional alleles operate in different cellular compartments with different product outputs: WsTPS02 is targeted to the chloroplast, where it produces (*E*)-β-ocimene from GPP and small amounts of (*E*,*E*)-α-farnesene from a plastidial FPP pool. By contrast, the TPS03 protein expressed in Col-0, lacks a plastidial transit peptide and makes only (*E*,*E*)-α-farnesene from cytosolic FPP. These molecular mechanisms controlling the natural variation of terpene metabolism are in agreement with those demonstrated among varieties of cultivated plants such as maize (Köllner et al., 2004).

Interspecific natural variation of floral and herbivore-induced volatiles has been investigated between *A. thaliana* and its perennial outcrossing relative *Arabidopsis lyrata* (Abel et al., 2009). In this species, floral volatiles are dominated by the aromatic compounds phenylacetaldehyde and benzaldehyde, which presumably replaced the emission of terpenes such as (*E*)-β-caryophyllene under pollinator selective pressures (Fig. 6). Instead, (*E*)-β-caryophyllene was found to be emitted from individuals in some *A. lyrata* populations upon herbivore damage. In these plants, (*E*)-β-caryophyllene is produced by an ortholog of the flower-specific TPS21 enzyme of *A. thaliana* (Abel et al., 2009) (Fig. 6). Herbivore-induced (*E*)-β-caryophyllene emissions may contribute to elevated levels of direct or indirect defense possibly shaped by the perennial nature of *A. lyrata*.

BIOSYNTHESIS OF TRITERPENES

Triterpenes represent an important group of non-volatile terpene specialized metabolites, which have gained increasing attention for the cluster-specific organization of their biosynthetic genes and their biological function in plant defense against pathogens

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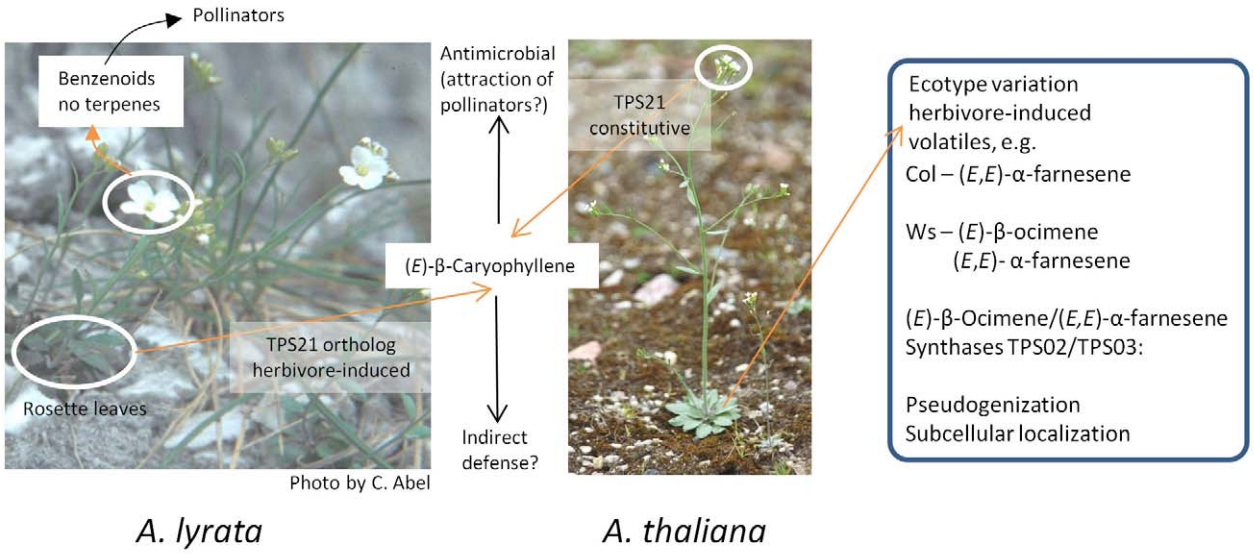


Figure 6. Inter- and intra-specific variation of terpene volatile emission and *TPS* gene expression and function in *Arabidopsis thaliana* and *Arabidopsis lyrata*.

and herbivores. Because of space limitations in this chapter, the reader is referred to several recent reviews and articles in the field (Jenner et al., 2005; Phillips et al., 2006; Zhao et al., 2010). Briefly, all triterpenes including sterols, steroid hormones, and nonsteroidal triterpenes such as triterpene saponins, are synthesized from FPP via the C_{30}-intermediate squalene. The biologically active nonsteroidal triterpenes are produced by conversion of squalene into oxidosqualene and cyclization via formation of a dammarenyl cation catalyzed by oxidosqualene cyclases (OSCs) (Segura et al., 2003; Phillips et al., 2006). The Arabidopsis genome encodes a gene family of 13 OSCs, of which, to the best of our knowledge, nine have been functionally characterized. Besides two OSCs that produce cycloartenol and lanosterol as precursors in sterol and steroid biosynthesis, the enzymes encoded by the other seven genes catalyze the formation of single triterpenes (arabidiol, thalianol, marneral) or multiple products including lupeol, β-amyrin, and taraxasterol (Phillips et al., 2006; Xiang et al., 2006).

Recently, the gene coding for thalianol synthase (*THAS*) was described to be organized in an operon-like cluster with four other genes that are highly co-expressed and responsible for consecutive derivatizations of the thalianol precursor (Field and Osbourn, 2008). Metabolite analysis of gene knock-out mutants of these genes provided evidence for a functional pathway *in planta*. Other Arabidopsis *OSC* genes appear to be part of gene clusters similar to that described for *THAS*. Diterpene biosynthesis pathways (Wilderman et al., 2004) have provided additional evidence for the general tendency of terpene biosynthesis gene cluster organization, which raises further questions about the evolutionary forces driving the assembly of these functional gene modules.

CONCLUSIONS AND OUTLOOK

The Arabidopsis genome allowed first insight into a plant's entire gene inventory of terpene primary and specialized metabolism. To date, most genes and enzymes of the early and intermediate biosynthetic steps leading to primary and specialized terpenes have been characterized and growing insight has been gained into the regulation of these steps at transcriptional and posttranscriptional/translational levels. At the same time, the enzymatic activities of more than 50% of the Arabidopsis terpene synthases have been described, which produce an astonishingly rich palette of specialized terpene compounds in tissues above- and belowground. Detailed profiles of the spatial organization and temporal regulation of *TPS* genes have been elucidated and correlations with their biological roles can be established in various loss-of-function mutants. Simple manipulations of terpene specialized metabolism in transgenic Arabidopsis lines by expressing *TPS* genes from other plants have become important tools for dissecting the function of individual terpene compounds or blends in the attraction of beneficial organisms or defense against herbivores and pathogens.

Open questions remain about the cell-type specific, developmental and stress-induced regulatory elements of terpene specialized metabolism as well as the networks regulating primary and specialized metabolism. Moreover, the localization of terpene biosynthesis enzymes in other compartments such as peroxisomes and the transport mechanisms of isoprenoids between organelles will require further attention. Finally, Arabidopsis represents one of the few wild species, whose accession- and genus-specific genomic resources provide fruitful ground for future studies on the molecular regulation of inter- and intra-specific variation in plant terpene specialized metabolism.

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