# Biochemical, Molecular and Functional Analysis of Volatile Terpene Formation in *Arabidopsis* Roots

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## ABSTRACT

Plants produce secondary (or specialized) metabolites to respond to a variety of environmental changes and threats. Especially, volatile compounds released by plants facilitate short and long distance interaction with both beneficial and harmful organisms. Comparatively little is known about the organization and role of specialized metabolism in root tissues. In this study, we have investigated the root-specific formation and function of volatile terpenes in the model plant *Arabidopsis*.

As one objective, we have characterized the two root-specific terpene synthases, TPS22 and TPS25. Both enzymes catalyze the formation of several volatile sesquiterpenes with (*E*)-β-farnesene as the major product. *TPS22* and *TPS25* are expressed in the root in distinct different cell type-specific patterns and both genes are induced by jasmonic acid. Unexpectedly, both TPS proteins are localized to mitochondria, demonstrating a subcellular localization of terpene specialized metabolism in compartments other than the cytosol and plastids. (*E*)-β-Farnesene is produced at low concentrations suggesting posttranslational modifications of the TPS proteins and/or limited substrate availability in mitochondria. We hypothesize that the mitochondrial localization of TPS22 and TPS25 reflects evolutionary plasticity in subcellular compartmentation of TPS proteins with emerging or declining activity. Since (*E*)-β-farnesene inhibits *Arabidopsis* root growth *in vitro*, mitochondrial targeting of both proteins may fine tune

(*E*)-β-farnesene concentrations to prevent possible autotoxic or inhibitory effects of this terpene *in vivo*.

We further investigated the role of volatile terpenes in *Arabidopsis* roots in interaction with the soil-borne oomycete, *Pythium irregulare*. Infection of roots with *P. irregulare* causes emission of the $C_{11}$-homoterpene (or better called $C_4$-norterpene) 4,8-dimethylnona-1,3,7-triene (DMNT), which is a common volatile induced by biotic stress in aerial parts of plants but was not previously known to be produced in plant roots. We demonstrate that DMNT is synthesized by a novel, root-specific pathway via oxidative degradation of the $C_{30}$-triterpene, arabidiol. DMNT exhibits inhibitory effects on *P. irregulare* mycelium growth and oospore germination *in vitro*. Moreover, arabidiol and DMNT biosynthetic mutants were found to be more susceptible to *P. irregulare* infection and showed higher rates of *Pythium* colonization in comparison to wild type plants. Together, our studies demonstrate differences and plasticity in the metabolic organization and function of terpenes in roots in comparison to aboveground plant tissues.

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# TABLE OF CONTENTS

**CHAPTER** **PAGE**

**ABSTRACT**....ii
**ACKNOWLEDGEMENTS** .... iv
**TABLE OF CONTENTS** .... vi
**TABLE OF FIGURES AND TABLES** .... x

**CHAPTER I. General Introduction and Overview of Research** .... 1
**Chemical communication in belowground plant–organism interactions** .... 2
**Volatile terpene metabolites in plant-environment interactions** .... 4
**Terpene biosynthesis in plants** .... 8
*The terpene precursor IPP, terpene synthases, and their substrates* .... 8
*Spatial and temporal regulation of terpene biosynthesis* .... 11
*Subcellular localization of terpene biosynthesis* .... 12
*Function and formation of the common volatile homoterpene DMNT* .... 14
***Arabidopsis* as a model for studying the biochemistry and function of volatile terpenes** .. 16
**The *Arabidopsis-Pythium* pathosystem** .... 20
**Overview of research** .... 23
**Literature Cited** .... 26

**CHAPTER II. The Two Root-Specific and Jasmonate-Induced *Arabidopsis* (*E*)-β-Farnesene Synthases, TPS22 and TPS25, are Targeted to Mitochondria** .... 35
**Abstract** .... 36
**Introduction** .... 37
**Results** .... 39
Identification of *Arabidopsis* TPS22 and TPS25 as putative mitochondrial proteins .... 39
Biochemical characterization of TPS22 and TPS25 .... 41
Subcellular localization of TPS22 and TPS25 .... 46
Sesquiterpene product and expression analysis of *TPS22* and *TPS25* in *Arabidopsis* roots . 48
Organ- and tissue-specific expression of *TPS22* and *TPS25* .... 50
Volatile analysis in loss-of-function mutants of *TPS22* and *TPS25* .... 51
Ectopic expression of *TPS22* and *TPS25* .... 54

The effect of (*E*)-β-farnesene on plant growth *in vitro* ..... 54
**Discussion** ..... 57
The two root-specific sesquiterpene synthases TPS22 and TPS25 produce multiple sesquiterpenes ..... 57
*TPS22* and *TPS25* are expressed in different root tissues and growth zones ..... 59
TPS22 and TPS25 are located in mitochondria ..... 61
(*E*)-β-Farnesene inhibits root growth *in vitro* ..... 64
**Materials and Methods** ..... 66
Plant materials and growth conditions ..... 66
Reagents and radiochemicals ..... 67
Volatile collection and analysis ..... 67
Genotyping of plant materials ..... 69
Determination of *TPS* gene expressions by semi-quantitative RT-PCR and quantitative RT-PCR ..... 69
Heterologous protein expression and purification of TPS22 and TPS25 in *Escherichia coli* 71
TPS enzyme assay and kinetic characterization ..... 72
Complementation analysis ..... 73
*TPS22* and *TPS25* promoter-GUS reporter gene fusion constructs ..... 74
Subcellular localization of TPS22 and TPS25 proteins ..... 75
*In vitro* plant root growth analysis ..... 76
**Supplemental Materials** ..... 77
**Literature Cited** ..... 78

**CHAPTER III. The Volatile $C_{11}$-Homoterpene DMNT Synthesized from the Triterpene Arabidiol is Emitted from *Arabidopsis* Roots in a Defense Response against *Pythium irregulare*** ..... 89
**Abstract** ..... 90
**Introduction** ..... 91
**Results** ..... 93
Volatile emission in *Arabidopsis* roots treated with *Pythium irregulare* 110305 and jasmonic acid ..... 93
Microscopy analysis of *Arabidopsis* root infection by *P. irregulare* ..... 96
Analysis of the role of JA, salicylic acid, and ethylene in *Pythium*-induced DMNT emission ..... 98
Identification of the biosynthetic steps in DMNT biosynthesis ..... 99

Transcript analysis of *AtPEN1* and *CYP705A1* genes in *Pythium*-infected *Arabidopsis* roots ..... 104
The effect of DMNT, arabidiol and arabidonol on *Pythium* mycelium growth *in vitro* ..... 105
The effect of DMNT on the germination of *Pythium* oospores ..... 106
*Pythium* root colonization of wild type and DMNT biosynthesis mutants ..... 108
Analysis of disease progression in wild type and arabidiol biosynthesis and breakdown mutants ..... 109
**Discussion** ..... 113
DMNT is produced as a triterpene breakdown product in *Arabidopsis* roots. ..... 113
DMNT formation occurs transiently at the early stage of infection by *P. irregulare* ..... 115
Arabidiol breakdown products are involved in the defense against *P. irregulare* ..... 117
**Materials and Methods** ..... 119
Plant material and growth conditions ..... 119
Volatile collection and analysis ..... 120
Yeast expression and enzyme assay ..... 121
Genotyping of plant material ..... 122
Growth conditions of *P. irregulare* and inoculation of *Arabidopsis* ..... 122
Microscopy analysis of *Arabidopsis* root infection ..... 123
Transcript analysis of *AtPEN1 and CYP705A1* in response to *P. irregulare* by semi-quantitative RT-PCR ..... 123
Growth inhibition assay *in vitro* ..... 124
Disease assessment of soil-grown plants ..... 124
Oospore isolation and germination of *P. irregulare* 110305 ..... 125
Evaluation of colonization on root tissues ..... 126
**Literature Cited** ..... 128

**CHAPTER IV. Final Discussion and Future Perspectives** ..... 133
**The importance of root phytochemicals: regulation and function** ..... 134
**Cell-type or tissue specificity of root chemical defense** ..... 138
**Subcellular organization of specialized metabolism** ..... 141
**Literature Cited** ..... 144

**APPENDIX A** ..... 148
**APPENDIX B** ..... 150
**APPENDIX C** ..... 152

**APPENDIX D** ..... 154

# TABLE OF FIGURES AND TABLES

**FIGURE/TABLE** **PAGE**

**CHAPTER I**

**Figure 1.1** Simplified scheme and examples for the chemical communication between plant roots and other organisms in the rhizosphere. ..... 4
**Figure 1.2** Biosynthetic pathways of terpenes in plants..... 9
**Figure 1.3** Enzymatic steps for the formation of DMNT in plant leaves..... 16
**Figure 1.4** Phylogenetic relationship of terpene synthases in *Arabidopsis*..... 17
**Figure 1.5** General depiction of the *Pythium* life cycle ..... 22

**Table1.1** Properties of *Arabidopsis TPS* genes and proteins and TPS enzymatic products……19

**CHAPTER II**

**Figure 2.1** Protein sequence alignment of *Arabidopsis* TPS22 and TPS25 with the sesquiterpene synthases TPS12, TPS13, TPS11, and TPS21..... 40
**Figure 2.2** Analysis of products formed by recombinant TPS22 and TPS25 enzymes from farnesyl diphosphate (FPP) using SPME-GC/MS ..... 43
**Figure 2.3** Enantiomer (*S*)/(*R*) determination of the nerolidol product formed by TPS22 and TPS25..... 44
**Figure 2.4** The effect of divalent metal ion cofactors on enzyme activities of TPS22 and TPS25 ..... 45
**Figure 2.5** pH Optima of TPS22 and TPS25..... 46
**Figure 2.6** Subcellular localization of TPS22 and TPS25-eGFP fusion proteins in *Arabidopsis* root hairs. ..... 48

**Figure 2.7** JA-induced (*E*)-β-farnesene emission and expression of *TPS22* and *TPS25* in *Arabidopsis* roots ..... 49
**Figure 2.8** Histochemical analysis of *TPS22* and *TPS25* promoter activities..... 51
**Figure 2.9** Genomic structure and gene expression of *TPS22* and *TPS25*..... 52
**Figure 2.10** Quantitative analysis of (*E*)-β-farnesene emission in wild-type (Col-0) and *tps* mutants..... 53
**Figure 2.11** Gene transcript and volatile analysis from leaves of *TPS22* and *TPS25* overexpression plants.. ..... 55
**Figure 2.12** Plant growth analysis of *Arabidopsis* (Col-0) in the absence and presence of sesquiterpenes. ..... 56

**Table 2.1** Kinetic parameters of recombinant TPS22 and TPS25 enzymes..... 44

**Figure 2.S.1** Subcellular localization of plastidial ferredoxin NADP(H) oxidoreductase-eGFP fusion protein in *Arabidopsis* roots..... 77
**Figure 2.S.2** Gene transcript analysis from leaves of transgenic plants transformed with cytosolic *TPS22* and *TPS25* ..... 77

**CHAPTER III**

**Figure 3.1** GC-MS analysis of volatiles emitted from *Arabidopsis* roots challenged with *Pythium irregulare* 110305..... 95
**Figure 3.2** Quantitative analysis of DMNT emission in *Arabidopsis* roots infected with *P. irregulare*. ..... 96
**Figure 3.3** Infection of *Arabidopsis* roots with *P. irregulare*. ..... 97
**Figure 3.4** DMNT emission in wild-type (Col-0) and JA-, ET-, and SA-biosynthesis or signaling mutants 3 h after infection of roots with *P. irregulare*..... 99
**Figure 3.5** Transcript analysis of P450 candidate genes by semi-quantitative RT-PCR..... 100
**Figure 3.6** Quantitative analysis of DMNT emission in wild-type and DMNT biosynthesis mutants (*cyp705a1-1*, salk_090621C, *atpen1-1*, and *atpen1-2*)..... 101

**Figure 3.7** Biosynthetic pathway for the formation of DMNT via degradation of the triterpene arabidiol in *Arabidopsis* roots. ..... 103
**Figure 3.8** Co-expression of *CYP705A1* and *AtPEN1* in WAT11 yeast cells..... 103
**Figure 3.9** DMNT emission and expression of *CYP705A1* and *AtPEN1* in *Arabidopsis* roots at the early stage of infection with *P. irregulare*..... 105
**Figure 3.10** Effect of arabidiol, arabidonol and DMNT on the growth of *P. irregulare* 110305106
**Figure 3.11** Effect of DMNT on oospore germination of *P. irregulare* 110305..... 107
**Figure 3.12** Colonization of *P. irregulare* 110305 on roots of wild-type plants and the mutants *jar1-1*, *cyp705a1*, and salk_090621C ..... 109
**Figure 3.13** Susceptibility of DMNT biosynthetic mutants to infection by *P. irregulare* 110305. ..... 111
**Figure 3.14** Root infection assay of *P. irregulare* in wild-type and DMNT biosynthetic mutants (*atpen1-1, atpen1-2, cyp705a1* and salk_090621C). ..... 112

# CHAPTER I

## General Introduction and Overview of Research

## Chemical communication in belowground plant–organism interactions

Many plant secondary (or specialized) metabolites have been investigated as compounds that are especially active against either plant pathogens or herbivores and as communication signals for plant interaction with their environment (McGarvey and Croteau, 1995; Pichersky and Gershenzon, 2002; Taiz and Zeiger, 2006). In general, secondary/specialized metabolites, including terpenoids, alkaloids, glucosinolates, and phenolics, are implicated in the adaptation of plants to environmental stress, but are generally not considered to be directly involved in normal plant growth, development or reproduction. Many studies demonstrate the role of specialized metabolites in aboveground tissues. For example, flavonoids are color pigments and function in the recruitment of pollinators and seed dispersers, and can furthermore serve as defense compounds by their antimicrobial activities (Weidenbörner et al., 1990; Sundaresan et al., 1993; Winkel-Shirley, 2001). Glucosinolates and their breakdown products that are specifically found in Brassicaceous plants, have strong cytotoxic activities and have been found to efficiently control fungi, nematodes and some soil-borne pathogens (Lazzeri et al., 1993; Mari et al., 1993; Manici et al., 1997). Volatile specialized metabolites such as volatile terpenes (see below) are common constituents of floral scent and act as signals for the attraction of pollinators. Moreover, volatile terpenes are involved not only in direct plant defense by their toxic, repelling, or deterring properties (Bernasconi et al., 1998; De Moraes et al., 2001; Kessler and Baldwin, 2001; Vancanneyt et al., 2001), but also in indirect plant defense by attracting natural predators of attacking herbivores (Vet and Dicke, 1992; Drukker et al., 2000; Kessler and Baldwin, 2001).

Similar to aboveground plant tissues, roots are exposed to numerous beneficial and harmful organisms and interact with these organisms largely by the release of chemical

compounds, both of primary and specialized metabolic origin (Figure 1.1). To attract beneficial microbes, roots secrete carbohydrates and organic acids, including amino acids and fatty acids (Jaeger et al., 1999). For example, malic acid released by *Arabidopsis* roots helps recruit plant growth promoting rhizobacteria (*Bacillus subtilis*) that trigger an induced systemic resistance (ISR) against the attack of bacterial pathogens above ground (Rudrappa et al., 2008). Besides primary metabolites, specialized metabolites play an important role as signaling molecules to initiate interactions with mycorrhizal fungi and nitrogen-fixing rhizobia. A study showed that flavonoids can promote spore germination, hyphal branching and the formation of auxiliary cells of the arbuscular mycorrhizal (AM) fungi, *Gigaspora rosea, G. margarita, Glomus mosseae,* and *G. intraradices*, in a genus- or species-specific manner (Scervino et al., 2005). In addition, strigolactones, a group of sesquiterpene lactones induce hyphal branching in AM fungi, a pre-requisite for successful root colonization (Akiyama et al., 2005).

There is evidence for the role of specialized metabolites in root chemical defenses especially as phytoalexins, which are defined as bacterial- or fungal- induced compounds with antimicrobial activity. For example, pigmented naphthoquinones with antimicrobial activity are induced by the soil-borne pathogens, *Rhizoctonia solani*, *Pythium aphanidermatum*, and *Nectria hematococca*, in *Lithospermum erythrorhizon* hairy roots (Brigham et al., 1999). Antimicrobial triterpenoid glycosides known as avenacins are synthesized in oat (*Avena*) roots and confer resistance to a range of soil-borne pathogens (Papadopoulou et al., 1999). Furthermore, the flavone-C-glycosides, schaftoside and isoschaftoside, extracted from *Arisaema erubescens* (Wall.) Schott tubers exhibit nematicidal properties against the root-knot nematode *Meloidogyne incognita* (Du et al., 2011). Phytochemicals are also involved in allelopathy below ground. One of the best known compounds with allelopathic activity is juglone, found in the black walnut

*Juglans spp.*, which inhibits plant growth by disrupting the root plasma membrane (Hejl and Koster, 2004). Taken together, the described examples demonstrate dynamic heterospecific interactions of plants below ground and emphasize the importance of specialized root chemicals in such interactions.

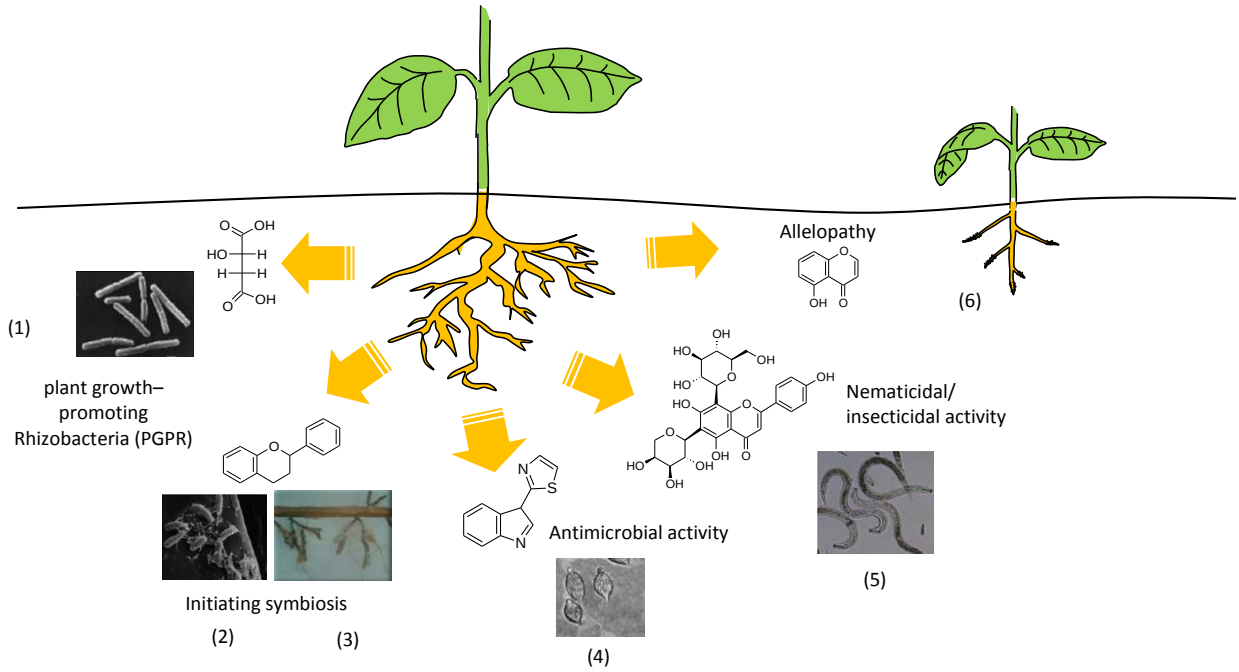


**Figure 1.1 Simplified scheme and examples for the chemical communication between plant roots and other organisms in the rhizosphere according to Badri et al., 2009.** (1), recruitment of rhizobacteria by organic acids; (2, 3), interactions with mycorrhiza (AM fungi) and rhizobia, initiated by the secretion of flavonoids; (4), antimicrobial defense mediated by phytoalexins such as camalexin; (5), direct anti-herbivore defense, e.g. by the release of flavonoid glycosides; (6), allelopathic interactions e.g. by secretion of phenolics.

## Volatile terpene metabolites in plant-environment interactions

Volatile organic compounds emitted by plants are known to serve as signals in interaction with the surrounding environment (Dudareva et al., 2004; Dudareva et al., 2006; Knudsen and Gershenzon, 2006). One of the largest groups of plant volatile compounds is represented by

terpenes (isoprenoids), which include $C_5$-hemiterpenes such as isoprene, $C_{10}$-monoterpenes, $C_{15}$-sesquiterpenes, and semi-volatile $C_{20}$-diterpenes (Dudareva et al., 2006; Baldwin, 2010). Terpenes are major contributors to the chemical repertoire used for plant-organism interactions such as the defense against enemies, the attraction of pollinators and signaling to other plants (Kessler and Baldwin, 2001; Pichersky and Gershenzon, 2002; Unsicker et al., 2009).

Studies in recent years indicate that plant volatile compounds, in particular volatile terpenes, can serve multiple roles. For example, isoprene is implicated in the protection of plants against sunlight-induced rapid heating or oxidative stress (Sharkey and Singsaas, 1995; Loreto and Velikova, 2001; Sharkey et al., 2008) but also deters attacking insect herbivores (Laothawornkitkul et al., 2008). Furthermore, volatile terpenes from floral tissues are not only involved in the attraction of pollinating insects (e.g. linalool from *CIarkia breweri* (Onagraceae); Pichersky et al., 1994; Pichersky and Gershenzon, 2002), but also appear to have defensive activities against pathogens as has been shown by recent studies in the Tholl lab on the role of β-caryophyllene in *Arabidopsis* flowers (Huang et al., under review).

Volatile terpenes are often synthesized and stored in specialized secretory cells and structures (e.g. resin ducts or glandular trichomes), which are frequently found in species in the Coniferae or Lamiaceae family such as rosemary, oregano, and peppermint. They provide a constitutive chemical barrier against herbivores or pathogens. The rupture of trichomes by feeding of a herbivore results in the release of volatile terpenes, which provides protection from the attacking herbivore by the toxic effects of the volatile compounds (Pichersky and Gershenzon, 2002). Furthermore, antimicrobial properties of volatile terpene constituents of plant essential oils have been intensively studied. Volatile oils analyzed from various plants have shown antibacterial activity against 25 different genera, including animal and plant pathogens

(Dorman and Deans, 2000). As another example, all components extracted from *Melaleuca alternifolia* (tea tree) oil, including monoterpene and sesquiterpene hydrocarbons and their related alcohols, except β-myrcene, have been reported for their activities against a wide range of fungi. Major components of this oil that exhibit antifungal activity are carvacrol, thymol, β-cymene and 1,8-cineole (Hammer et al., 2003). The exact mechanism by which terpenes confer antimicrobial activity is not fully understood, but it has been proposed that terpenoids affect cell membrane structure and function (Mendoza et al., 1997; Inouye et al., 2001; Trombetta et al., 2005; Pérez-Fons et al., 2006). The lipophilic property of terpenes allows these compounds to be introduced into membranes, resulting in increased membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration, and alteration of ion transport processes.

While terpenes accumulate in secretory structures or hair cells (trichomes) of many plants as components of essential oils, the same compounds can be released by other plants without prior storage in a constitutive manner or as the consequence of *de novo* biosynthesis induced by pathogen or herbivore attack. For example, *Medicago truncatula* challenged with pathogen-derived β-glucans (pathogen elicitors) emit a mixture of sesquiterpenes including (*E*)-nerolidol, α-copaene, cyclosativene, α-ylangene, and α-humulene (Leitner et al., 2008). Similarly, *Zea mays* (maize) leaves release a blend of volatiles including (*E*)-α-bergamotene, (*E*)-β-farnesene, and (*E*)-nerolidol in response to feeding damage by lepidopteran larvae (Turlings et al., 1990). And when lima bean (*Phaseolus lunatus* L.) plants are attacked by two-spotted spider mites (*Tetranychus urticae*), a mixture of volatiles is emitted, which consists of monoterpenes and the volatile homoterpenes ($C_4$-norterpenes), (3*E*)-4,8-dimethyl-l,3,7-nonatriene (DMNT) and (3*E*,7*E*)-4,8,12-trimethyl-l,3,7,11-tridecatetraene (TMTT) (Dicke et al., 1990). It has been

proposed that these volatiles are involved in the attraction of natural enemies of herbivores as an indirect defense strategy (Dicke, 1990). In many cases, the specific role of terpenes in the attraction of parasitoids or predators has not yet been established, but a study by Schnee et al. (2006) showing that genetically engineered *Arabidopsis* plants that produce volatile compounds from maize are attractive to parasitic wasps underscores the importance of such compounds in tritrophic interactions. Insect-induced volatiles can also function as signals of stress responses in the same plant and between neighboring plants (Bruin et al., 1992; Röse et al., 1996; Arimura et al., 2000). For example, lima bean plants exposed to the terpene volatile blend of neighboring plants infested by the spider mite *Tetranychus urticae* show induced defense gene expression and emission of volatiles and, as a consequence, become less susceptible to spider mites (Arimura et al., 2000; Choh et al., 2004).

The role of volatile terpenes in plant defense has been investigated mainly in aerial parts of the plant. However, Rasmann and co-workers (2005) have shown that emission of the sesquiterpene (*E*)-β-caryophyllene was induced in maize roots attacked by larvae of the western corn rootworm, *Diabrotica virgifera*, and could attract entomopathogenic nematodes that prey on the attacking insect larvae. This finding emphasizes the importance of root volatiles in plant defense, and it is therefore desirable to gain further insight into the biochemistry and defensive roles of volatile compounds in belowground interactions.

## Terpene biosynthesis in plants

### *The terpene precursor IPP, terpene synthases, and their substrates*

Terpene specialized metabolic pathways have been extensively studied over the past 30 years with a primary focus on aerial plant organs. Terpenes are derived from the five-carbon building block isopentenyl diphosphate (IPP), which is synthesized in plants by two independent pathways, the cytosolic mevalonic acid (MVA) pathway and the plastidial 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Figure 1.2) (Laule et al., 2003). The MVA pathway starts with the condensation of three molecules of acetyl-CoA and proceeds to form the intermediate mevalonate (MVA), followed by phosphorylation and decarboxylation of MVA to yield isopentenyl diphosphate (IPP) (Disch et al., 1998). IPP is then transformed into its allylic isomer, dimethylallyl diphosphate (DMAPP) by IPP isomerase.

The plastidial MEP pathway begins with a condensation of pyruvate and glyceraldehyde-3-phosphate to form the intermediate 1-deoxy-D-xylulose-5-phosphate (DOXP). DOXP is then converted into IPP and DMAPP by six enzymatic steps (Arigoni et al., 1997; Lichtenthaler et al., 1997; Lange and Ghassemian, 2003). Units of isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP), are subsequently condensed by enzymes called prenyltransferases to form the linear prenyldiphosphates, $C_{10}$-geranyl diphosphate (GPP), $C_{15}$-farnesyl diphosphate (FPP) and $C_{20}$-geranylgeranyl diphosphate (GGPP). These intermediates serve as substrates in the biosynthesis of monoterpenes ($C_{10}$), sesquiterpenes ($C_{15}$) and diterpenes ($C_{20}$), reactions that are catalyzed by terpene synthases (TPSs) (Bohlmann et al., 1998; Lange et al., 2000; Lange and Ghassemian, 2003; Fridman and Pichersky, 2005; Chen et al., 2011).

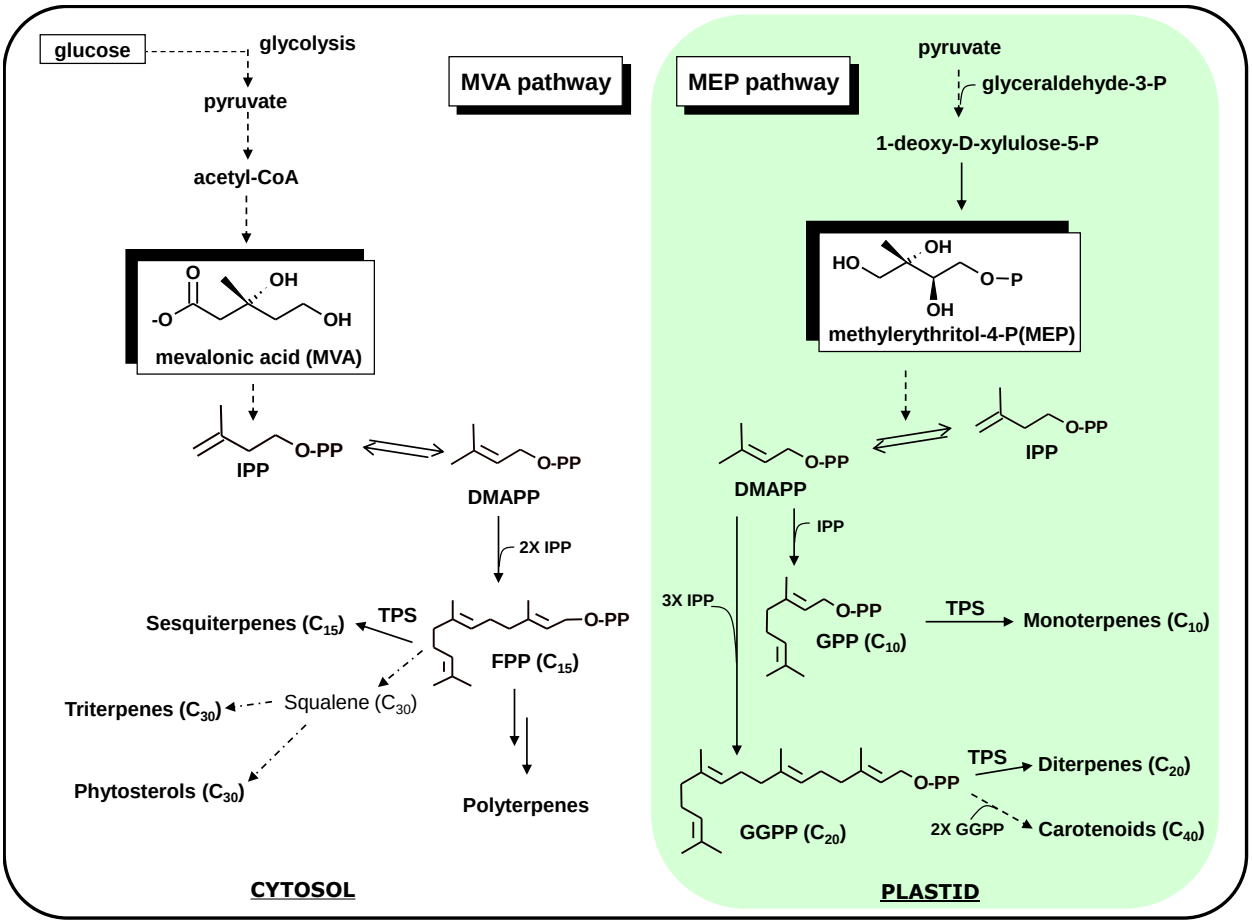


**Figure 1.2 Biosynthetic pathways of terpenes in plants (Laule et al., 2003).** IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyldiphosphate; FPP, farnesyl diphosphate and GGPP, geranylgeranyl diphosphate. TPS, terpene synthase, Dashed arrows indicate more than a single enzymatic step.

TPSs often produce more than one product from a single substrate. According to the reaction mechanism and products formed, they can be grouped as class I- and class II-type TPSs (Tholl, 2006; Chen et al., 2011). Most plant TPSs belong to the class I-type enzymes, which initiate the reaction by dephosphorylation of the prenyl diphosphate substrate resulting in the formation of an unstable carbocation intermediate. Structural rearrangements of the carbocation, which include cyclization, hydride shifts, and methyl migrations prior to a deprotonation reaction or capture of a water molecule as the final step, lead to the biosynthesis of multiple products

(Christianson, 2006; Degenhardt et al., 2009). By contrast, class II TPSs produce carbocations via protonation of the terminal double bond of the substrate such as in the formation of the gibberellin precursor, copalyl diphosphate (CPP) from GGPP (Gershenzon and Kreis, 1999).

Besides the ability of TPS enzymes to form multiple products, the large diversity of terpene metabolites is the result of a rapid evolution of TPSs, which often leads to the emergence of species-specific paralogous *TPS* gene clusters. Sesquiterpene and putative diterpene synthases in *Arabidopsis* are more closely related to each other than to those from other angiosperms (Aubourg et al., 2002; Tholl et al., 2005). Moreover, convergent evolution of *TPS* gene function is frequently observed. For example, isoprene synthases have evolved independently from monoterpene synthases in both angiosperms and gymnosperms by corresponding changes at the catalytic site of the enzymes (Sharkey et al., 2005).

Phylogenic analysis divides the *TPS* gene family into seven subfamilies (TPS-a, b, c, d, e/f, g and h) (Chen et al., 2011). Each subfamily shares a common evolutionary origin and a minimum of 40% amino acid identity among members. Based on the phylogeny and functions of known TPSs, three subfamilies of TPS-a, TPS-b, and TPS-g are angiosperm-specific and function as monoterpene (TPS-b, TPS-g), and sesquiterpene or diterpene synthases (TPS-a) while enzymes of the TPS-d subfamily only occur in gymnosperms. The TPS-c subfamily contains the most conserved TPSs among land plants and the closely related subfamily TPS-e/f is conserved among vascular plants. Both subfamilies contain diterpene synthases such as kaurene synthase (KS, TPS-c) and CPP synthase (TPS-e/f), respectively, producing the precursors for gibberellin and diterpene biosynthesis. The recently described TPS-h subfamily was found in *Selaginella moellendorffii* and appears to encode bifunctional diterpene synthases (Bohlmann et al., 1998; Chen et al., 2011; Tholl and Lee, 2011).

### *Spatial and temporal regulation of terpene biosynthesis*

Terpene specialized metabolism is regulated at different stages of growth and development, is often tissue-/cell-type specific, and is frequently induced under biotic or abiotic stress conditions. Numerous reports indicate that the biosynthesis of terpenes is mainly regulated at the *TPS* transcript level by showing that the spatial and temporal expression of *TPS* genes correlates with the biosynthesis and emission of terpenes (Bohlmann et al., 1998; Gershenzon and Kreis, 1999; Dudareva et al., 2004; Tholl et al., 2005). For example, expression of a linalool monoterpene synthase in *Clarkia breweri* flowers is restricted to the stigma, style, and flower petals where linalool emission is found (Dudareva et al., 1996). The formation of the monoterpene volatiles, myrcene and ocimene, in upper and lower petal lobes of snapdragon flowers is regulated by the expression of the corresponding monoterpene synthases, which is not only tissue-specific but also depends on the developmental stage of the flower and on the circadian clock (Dudareva et al., 2003). These spatial and temporal biosynthetic patterns are important in correlating volatile emissions with pollinator visits and pollination events (Dudareva et al., 2003). Similar correlations in gene expression levels were found during flowering and fruit ripening for sesquiterpene and monoterpene synthases in grapevine (Lücker et al., 2004).

As mentioned above, volatile terpenes are emitted from plant tissues with or without pre-accumulation. Besides their constitutive expression in species with glandular trichomes, the *TPSs* of many plants respond to herbivore or pathogen attack with induced transcript and protein levels as observed in leaves of cotton, maize, *Medicago*, *Brassica*, and *Arabidopsis* (Pare and Tumlinson, 1997; Schnee et al., 2006; Arimura et al., 2008; Herde et al., 2008). An induction of

TPS enzyme expression can occur locally around sites of feeding damage as demonstrated in *Arabidopsis* leaves (Huang et al., 2010). In addition, the formation of terpenes can be induced in a systemic response, as shown in experiments with insect-damaged cotton (Röse et al., 1996). The spatial regulation of terpenes in roots is still unknown. Recent fine scale transcript profiling of *TPS* genes in *Arabidopsis* roots has revealed distinct cell type-specific expression pattern indicating specific cell layers of terpene metabolites that may correlate with selective pressures imposed by different soil-borne root attackers (*see section about Arabidopsis TPS below*).

### *Subcellular localization of terpene biosynthesis*

The subcellular compartmentation of terpene biosynthetic pathways adds another layer of spatial regulation in terpene formation and contributes to the variation of terpene blends. In general, the enzymatic reactions in the MEP and MVA pathways occur in the plastids and cytosol, respectively. All enzymes of the MEP pathway are characterized and have been shown in various plant species to be localized to plastids (Bouvier et al., 2000; Lois et al., 2000; Suire et al., 2000; Hsieh et al., 2008; Kim et al., 2008). Most enzymes of the MVA pathway are thought to be located in the cytosol, with the exception of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) being targeted to the endoplasmic reticulum (ER) membrane (Merret et al., 2007), while acetoacetyl-CoA thiolase (AACT1) appears to be targeted to peroxisomes (Carrie et al., 2007).

IPP isomerases (IDIs), which are responsible for the isomerization of IPP to DMAPP in both MEP and MVA pathways (Figure 1.2) occur in plastidial and cytosolic isoforms. However, in *Arabidopsis*, the cytosolic isoenzyme IDI2 is co-localized in the cytosol and mitochondria

indicating that the enzyme also provides five-carbon building blocks for terpene biosynthesis in mitochondria (Phillips et al., 2008). Differential subcellular compartmentation is also apparent for prenyltransferases and terpene synthases. Most of the investigated GPP or GGPP synthases in plants are located in plastids (Bouvier et al., 2000; Okada et al., 2000), where they provide the substrates for monoterpene and diterpene synthases and the precursors for gibberellin and carotenoid formation. FPP synthases, on the contrary, are most often cytosolic enzymes and FPP produced in the cytosol is further converted into sesquiterpenes by cytosolic TPSs or channeled into the triterpene/phytosterol and polyisoprene pathways (McGarvey and Croteau, 1995). In *Arabidopsis*, the *FPS1* gene encodes two different isoforms, FPS1L and FPS1S, located in the cytosol and mitochondria, respectively suggesting the possible role of FPP in the mitochondrial formation of sesquiterpenes (Cunillera et al., 1996; Cunillera et al., 1997).

Plastidial monoterpene and diterpene synthases generally contain N-terminal transit peptides of about 50 amino acids, which are responsible for the targeting of these proteins to plastids (Bohlmann et al., 1998; Bouvier et al., 2000). By contrast, most sesquiterpene synthases lack a plastidial transit peptide and, therefore, remain in the cytosol (Nagegowda et al., 2008; Nieuwenhuizen et al., 2009). Depending on its location in the cytosol or the plastid, a bi-substrate TPS enzyme, which can convert either GPP or FPP, produces either monoterpenes or sesquiterpenes *in vivo*. Studies in snapdragon and *Arabidopsis* have shown that differential targeting of TPS isoforms leads to diversification and variation in the composition of terpene volatile blends (Nagegowda et al., 2008; Huang et al., 2010). Contrary to the general notion of a strict plastidial and cytosolic formation of monoterpenes and sesquiterpenes, respectively, recent studies indicate that some sesquiterpene synthases contain transit peptides for import into plastids. For example, a sesquiterpene synthase isolated from the wild tomato, *S.*

*habrochaites*, that catalyzes the formation of sesquiterpenes from *Z,Z*-FPP (instead of *E,E*-FPP) was found to be located in plastids (Sallaud et al., 2009). However, it has not been clearly shown yet that sesquiterpene synthases can be targeted to mitochondria, despite evidence for an FPP pool in mitochondria.

### *Function and formation of the common volatile homoterpene DMNT*

Volatiles released from floral and vegetative tissues are implicated in pollinator attraction or plant defense against herbivores. Among the most common plant volatiles are the homoterpenes ($C_4$-norterpenes) such as the $C_{11}$- homoterpene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and the $C_{16}$-homoterpene, (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), which are components of floral odors contributing to the "white-floral image" of night scented flowers (Kaiser, 1991). In addition, homoterpene emissions induced by herbivore attack play a role in plant defense in vegetative tissues (Bouwmeester et al., 1999; Degenhardt and Gershenzon, 2000; Van Poecke et al., 2001; Kappers et al., 2005; Arimura et al., 2008). For example, when cucumber and lima bean leaves are attacked by spider mites, DMNT emission is induced and correlated with the attraction of predatory mites (Bouwmeester et al., 1999). Moreover, a similar function has been reported in genetically engineered *Arabidopsis* plants that constitutively emit DMNT (Kappers et al., 2005). Homoterpenes also seem to play a role in plant-plant interaction since they have been shown to induce the expression of defense genes in non-wounded plants by exposure to volatiles from conspecific plants infested with spider mites (Arimura et al., 2000).

The ecological significance of homoterpenes has raised increasing interest in characterizing the enzymatic steps in the formation of these compounds in greater detail. In an early study using $^{13}CO_2$ pulse-labeling experiments, Pare and Tumlinson (1997) showed that DMNT is synthesized *de novo* in cotton plants in response to herbivory. Later, Donath and Boland (1994) demonstrated, by using $^{2}H$-labeled precursors, that DMNT is derived from the sesquiterpene (*E*)-nerolidol. In addition, studies in maize, cucumber and lima bean have shown that (*E*)-nerolidol is formed from FPP by a nerolidol synthase activity (Bouwmeester et al., 1999; Degenhardt and Gershenzon, 2000). It was therefore suggested that (*E*)-nerolidol is converted into DMNT via oxidative degradation (Figure 1.3). The type of oxidative C-C bond cleavage involved in this reaction is prevalent in secondary metabolic pathways and often catalyzed by enzymes of the cytochrome P450 monooxygenase (P450) family such as in dealkylation of steroids or as shown for the oxidative degradation of (+)-marmesin into the furanocoumarin psoralen (Larbat et al., 2007). Recently, Lee et al. (2010) reported that CYP82G1, a P450 of the CYP82 family, catalyzes the insect-induced oxidative degradation of the $C_{20}$-diterpene precursor (*E,E*)-geranyllinalool to TMTT in *Arabidopsis*. The enzyme also accepts (*E*)-nerolidol as a substrate and converts it to DMNT *in vitro*. Together, these results obtained in aboveground tissues indicate that P450s are responsible for the formation of homoterpenes from their tertiary alcohol precursors. Until this study, there were no reports about the presence and possible role of homoterpene volatiles in plant roots and about whether their root-specific biosynthesis is comparable to that found in leaves.

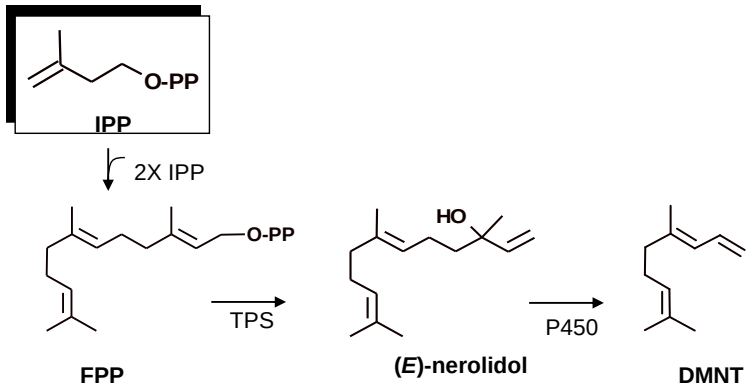


**Figure 1.3 Enzymatic steps for the formation of DMNT in plant leaves.** TPS, terpene synthase; P450, cytochrome P450.

## *Arabidopsis* as a model for studying the biochemistry and function of volatile terpenes

*Arabidopsis* has been shown to be a suitable model for studying the biosynthesis and regulation of terpene specialized metabolites and their role in plant-environmental interactions (Aharoni et al., 2003; Chen et al., 2003; Fäldt et al., 2003). The *Arabidopsis* genome contains 32 predicted *TPS* genes, which can be classified into six subfamilies at the amino acid sequence level (Figure 1.4).

The TPS-a subfamily comprises 22 *Arabidopsis* TPSs, which are all class I TPS proteins. Four *TPS* genes (*TPS11, 12, 13*, and *TPS21*) in this subfamily encode sesquiterpene synthases that do not carry transit peptides (Table1.2). Two of these sesquiterpene synthases (TPS11 and 21) are responsible for producing a mixture of sesquiterpenes emitted from *Arabidopsis* flowers (Tholl et al., 2005), whereas TPS12 and its closely related protein TPS13 are root-specific and make (*Z*)-γ-bisabolene as a main product (Ro et al., 2006). The other TPS proteins of the TPS-a subfamily either carry plastidial or mitochondrial transit peptides. Recently, Vaughan et al.

(submitted) characterized TPS08 as a root-specific diterpene synthase that is targeted to root leucoplasts, but the function and localization of the other TPS-a type enzymes have not been yet been determined.

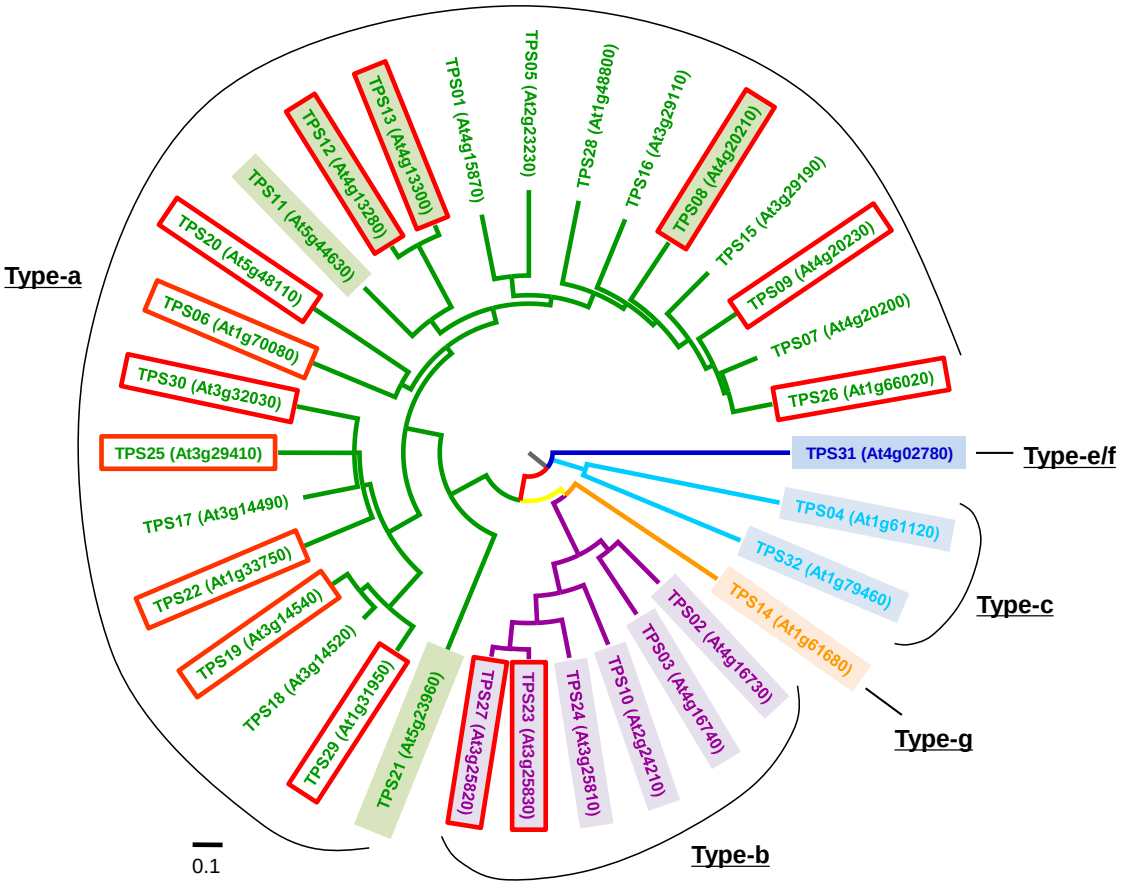


**Figure 1.4 Phylogenetic relationship of terpene synthases in *Arabidopsis* (Aubourg et al., 2002; Tholl and Lee, 2011).** TPS numbers are according to Aubourg et al. (2002). *Arabidopsis* terpene synthases (TPSs) belong to six subfamilies (TPS-a, b, c, d, e/f, and g). Colors indicate enzymes of TPS subfamilies a (green), b (purple), c (dark blue), e/f (light blue), and g (orange). Functionally characterized TPS proteins are shaded. All *TPS* genes with constitutive expression in roots are highlighted in red boxes.

The *Arabidopsis* TPS-b subfamily contains six plastidial monoterpene synthases with known function: 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 the identical enzymes, TPS23 and TPS27 (Chen et al., 2004) (Table1.2). The remaining four TPS proteins are all biochemically characterized and belong to three separate subfamilies: the (+)-*S*-linalool synthase TPS14, which is related to acyclic monoterpene synthases from snapdragon in the TPS-g subfamily (Dudareva et al., 2003), the class II-type CPP synthase TPS31 (TPSGA1, TPS-e/f subfamily), the class I-type kaurene synthase TPS32 (TPSGA2, TPS-c subfamily), which provide the precursors in gibberellin biosynthesis (Sun and Kamiya, 1994; Yamaguchi et al., 1998), and the class I-type geranyllinalool synthase TPS04 (TPS-c subfamily), which is involved in the synthesis of the volatile $C_{16}$-homoterpene TMTT (Herde et al., 2008).

*Arabidopsis TPS* gene expression profiles based on microarray and promoter-GUS analyses show that 15 genes are primarily expressed in *Arabidopsis* roots with different cell-type specific expression patterns (Chen et al., 2003; Chen et al., 2004; Ro et al., 2006) (Table1.2). Two of the root-specific TPSs, TPS23 and TPS27 (TPS-b subfamily), which are expressed in the epidermal layer of older root growth zones, catalyze the formation of the monoterpene 1,8-cineole (Chen et al., 2004). The emission of 1,8-cineole was shown to be induced by infection with the bacterium *Pseudomonas syringae* DC3000 or the fungus *Alternaria brassicola* in *Arabidopsis* hairy-root cultures (Steeghs et al., 2004). Specifically, 1,8-cineole has known activity against plant pathogens (Hammer et al., 2003), which indicates the involvement of root volatile terpenes in belowground defense. The other root-expressed TPSs belong to the TPS-a subfamily, of which two have been characterized as (*Z*)-γ-bisabolene synthases (TPS12, TPS13, see above), and the remaining enzymes have been predicted to either function as diterpene synthases targeted to plastids or possibly sesquiterpene synthases with location in mitochondria (Tholl and Lee, 2011). This study contributed to a better understanding of the function and the cellular and sub-cellular localization of this large group of TPSs in *Arabidopsis* roots.

**Table1.2 Properties of *Arabidopsis TPS* genes and proteins and TPS enzymatic products (according to Tholl and Lee, 2011).**

| TPS* | AGI No. | Subcellular protein localization[c] | Tissue-specific transcription/ promoter activity[d] | Enzyme products | References |
|---|---|---|---|---|---|
| 01 | At4g15870 | P | S | unknown | |
| 02 | At4g16730 | P | F;L-i | (*E*)-β-ocimene/myrcene (*E*,*E*)-α-farnesene | Huang et al., 2010 |
| 03 | At4g16749 | C | F;L-i | (*E*)-β-ocimene/myrcene (*E*,*E*)-α-farnesene | Fäldt et al., 2003 |
| 04 | At1g61120 | C | F;L-i | (*E*,*E*)-geranyllinalool | Herde et al., 2008 |
| 05 | At2g23230 | M/P/V | S | unknown | |
| 06 | At1g70080 | M/P | R;S | unknown | |
| 07 | At4g20200 | P | S | unknown | |
| 08 | At4g20210 | P | R | diterpene | Vaughan, et al., submitted |
| 09 | At4g20230 | M/P | R;F;S;Sh | unknown | |
| 10 | At2g24210 | P | F/L-i | (*E*)-β-ocimene/myrcene | Bohlmann et al., 2000 |
| 11 | At5g44630 | C | F | sesquiterpene blend | Tholl et al., 2005 |
| 12 | At4g13280 | P? | R;F,L-i | (*Z*)-*γ*-bisabolene | Ro et al., 2006 |
| 13 | At4g13300 | C | R;F,L-i | (*Z*)-*γ*-bisabolene | Ro et al., 2006 |
| 14 | At1g61680 | P | F | (+)-3S-linalool | Chen et al., 2003 |
| 15 | At3g29190 | C | S | unknown | |
| 16 | At3g29110 | M/P | S | unknown | |
| 17 | At3g14490 | P | S;R | unknown | |
| 18 | At3g14520 | P | F;S | unknown | |
| 19 | At3g14540 | P | R;F | unknown | |
| 20 | At5g48110 | P | R | putative diterpene | Vaughan, Tholl et al., unpublished |
| 21 | At5g23960 | C | F | (*E*)- β-caryophyllene | Chen et al., 2003 |
| 22 | At1g33750 | M | R | sesquiterpene blend | Huh, Tholl et al., unpublished |
| 23 | At3g25830 | M?/P | R | 1,8-cineole | Chen et al., 2004 |
| 24 | At3g25810 | P | F | monoterpene blend | Chen et al., 2003 |
| 25 | At3g29410 | M | R | sesquiterpene blend | Huh, Tholl et al., unpublished |
| 26 | At1g66020 | M/P | S;R;H | unknown | |
| 27 | At3g25820 | M?/P | R | 1,8-cineole | Chen et al., 2004 |
| 28 | At1g48800 | M/P | S;R | unknown | |
| 29 | At1g31950 | M/P | R | unknown | |
| 30 | At3g32030 | P | R | unknown | |

| | | | | | |
|---|---|---|---|---|---|
| 31 GA1 | At4g02780 | P | [#]S;R;Sh;L;F | *ent*-copalyl diphosphate | Sun and Kamiya, 1997 |
| 32 GA2 | At4g15901 | 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. [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.

## The *Arabidopsis-Pythium* pathosystem

Plant roots encounter numerous challenges by pathogenic organisms or herbivorous insects. In particular, destructive plant diseases caused by soil-borne pathogens such as *Pythium* and *Phytophthora* pose serious economic problems to modern agriculture (Kamoun et al., 1999; van West et al., 2003). Both *Pythium* and its closely related genus *Phytophthora* are difficult to control and cause damping off or root rot disease and vascular wilt disease, respectively, in various crops and ornamental plants throughout the world (Armstrong and Armstrong, 1981; Agrios, 1997). Despite the economic importance of *Pythium* species and other oomycetes, little is known about the metabolic defense responses of plants against these root pathogens.

The genus *Pythium* belongs to a group of filamentous eukaryotic pathogens called oomycetes and consists of about 120 species, some of which are highly virulent root pathogens and have a broad host range (Hawksworth et al., 1995). *Pythium* has two distinctive life cycles: an asexual and a sexual phase (Hendrix and Campbell, 1973; Agrios, 1997; van West et al., 2003)

(Figure 1.5). In the asexual life cycle, *Pythium* propagates by the formation of zoospores. A zoospore produced in a sporangium is a flagellated diploid cell and contains the full genome. Upon their release from sporangia in the presence of free water (rain, dew, or irrigation water), zoospores swim for a period of time before they eventually locate a host, encyst, and infect plant tissues. Asexually produced zoospores are well adapted to aquatic environments such as hydroponics because they are able to swim in the nutrient solution, which facilitates the spread of infection (Kucharek and Mitchell, 2000; Herrero et al., 2003). Zoospore movement can be stimulated by root exudates such as sugars, amino acids, calcium etc. (Royle and Hickman, 1964; Hardham and Gubler, 1990 ; Jones et al., 1991; Donaldson and Deacon, 1993). The spores accumulate and adhere on plant roots with precise orientation, such that their germ-tube outgrowth is located on roots and germination leads to host tissue penetration (Deacon, 1988; Donaldson and Deacon, 1992; Zhou and Paulitz, 1993).

In the sexual lifecycle, *Pythium* produces thick-walled oospores (Figure 1.5), which are produced by fertilization of oogonia with antheridia. Maturation of oospores leads to germination, mycelium production, and infection of plant tissues. Oospores are thought to be the main survival propagule of *Pythium* in soil (Ayers and Lumsden, 1975; Ruben and Stanghellini, 1978). Conditions that influence oospore development and subsequent germination in the soil are pH or temperature (Burr and ME, 1973; Ayers, 1975). Furthermore, numerous amino acids, carbohydrate and volatile compounds (such as ethanol or aldehydes) from root or seed exudates, plant debris, or organic matter can induce germination and germ tube growth (Barton, 1957; Stanghellini and Burr, 1973; Stanghellini and Burr, 1973; Nelson, 1990; Paulitz, 1991; Martin and Loper, 1999). A study with tomato roots showed that infection with purified oospores of *P. oligandrum* resulted in 90% root colonization by the pathogen (Vallance et al., 2009).

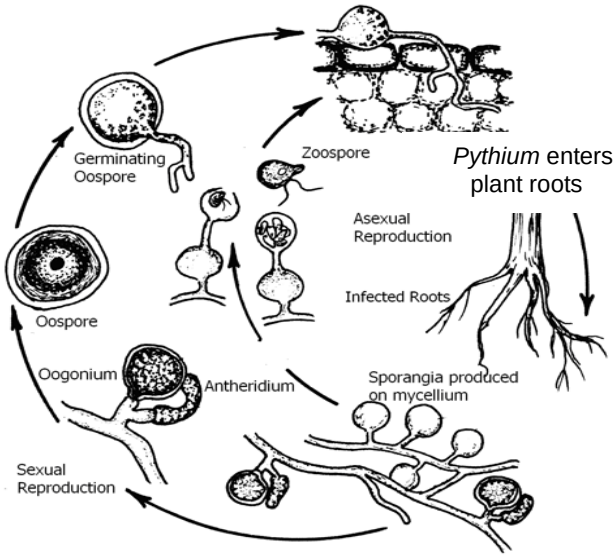


**Figure 1.5 General depiction of the *Pythium* life cycle. (**British Columbia: ministry of agriculture, http://www.gov.bc.ca/agri/)

*Pythium* spp. are considered as either necrotrophic or hemibiotrophic pathogens depending on the species (Latijnhouwers et al., 2003). For example, *P. ultimum* is classified as a necrotrophic pathogen since it produces lytic enzymes such as cellulases and pectinases or members of the Crinkler class of effectors that may trigger cell death (Haas et al., 2009; Levesque et al., 2010). *Pythium irregulare* can be categorized as a hemibiotrophic pathogen since it forms haustoria-like structures in the early stage of infection but also produces both lytic enzymes and phytotoxins that degrade plant tissue (Brandenburg, 1950; Deacon, 1979) and induces the jasmonate-defense pathway, which are typical features of necrotrophs (Oliver and Ipcho, 2004). *Pythium* spp. infect seeds or young seedlings and belowground tissues of several plants, including *Arabidopsis* (Agrios, 1997). Severe disease symptoms caused by *Pythium* are mainly found in seeds or young seedlings, while most mature plants reveal partial resistance to the pathogen (Kamoun et al., 1999). Studies of *Pythium* pathogenicity with *Arabidopsis*

demonstrate that plant defense responses involve the plant hormones, jasmonic acid (JA) and ethylene (ET) (Staswick et al., 1998; Vijayan et al., 1998; Geraats et al., 2002). Recently, Adie et al. (2007) reported that the resistance to *P. irregulare* is compromised in mutants that are deficient in JA/Salicylic acid (SA) and ET biosynthesis. *Pythium* infection increases JA levels in plants and induces the expression of JA-dependent defense genes such as *LOX1* or *PDF1.2*. ABA levels are also increased by *Pythium* infection and ABA activates defense genes and affects JA biosynthesis (Staswick et al., 1998; Vijayan et al., 1998; Adie et al., 2007). Despite the accumulated knowledge about pathogenicity and the defense mechanism of *Pythium* infection, the function of chemical defenses against *Pythium* is not well understood and was further addressed in this study.

## Overview of research

Volatile terpenes have been investigated as defense molecules in plant-insect and plant-pathogen interactions. So far, research on volatile terpenes has focused on aerial parts of plants, and to date only a few studies have been conducted to gain insights into the biochemistry and functions of volatile terpenes in plant roots. Preliminary experiments in the Tholl lab found root volatile terpenes to be induced by biotic stress such as root-herbivore or pathogen attack. Based on these findings, we hypothesized that root volatile terpenes have a role in plant chemical defense against root pathogens. We tested this hypothesis by identifying terpene synthases, which are responsible for the formation of root volatile terpenes and investigating the function of induced-volatile terpenes in belowground plant-pathogen interaction. Given the predictions of a possible mitochondrial localization of TPSs with expression in *Arabidopsis* roots (see above), we

further hypothesized that the formation of terpenes in roots might be organized differently at the subcellular level in comparison to that in leaves.

The specific objectives of this study were to:

I. characterize the cell-type specific expression and subcellular localization of terpene synthases in *Arabidopsis* roots

II. understand the formation and role of the $C_{11}$-homoterpene DMNT in *Arabidopsis* roots in response to infection by the root pathogen *Pythium irregulare*

**In Chapter II**, the biochemical and molecular characterization of two root-specific sesquiterpene synthases (TPS22 and TPS25) in *Arabidopsis* is investigated. The two *TPS* genes were originally selected as candidate genes in the formation of the sesquiterpene (*E*)-nerolidol as a precursor of the pathogen-induced $C_{11}$-homoterpene DMNT. However, preliminary results of volatile analysis in gene knock-out mutants for both genes showed that both TPSs are not responsible for DMNT synthesis in roots. Nevertheless, both genes were known to show high/constitutive expression in roots and the encoded proteins were predicted to be localized in mitochondria. Therefore, TPS22 and TPS25 were further studied to get a better understanding of their role in volatile sesquiterpene formation, their cell-type specific expression pattern and subcellular compartmentation in roots. The study shows a surprising localization of both TPS22 and 25 in mitochondria in correlation with low sesquiterpene emission levels and a differential cell-type specific expression pattern of both *TPS* genes in *Arabidopsis* roots.

**Chapter III** presents a study on the biosynthesis and role of the $C_{11}$-homoterpene DMNT in root chemical defense against *P. irregulare*. *Pythium*-induced DMNT is synthesized via a novel pathway as an oxidative degradation product of the root specific $C_{30}$-triterpene arabidiol. *In vitro* and *in vivo* functional analyses of DMNT reveal its involvement in root chemical defense.

A final discussion and future perspectives of the described research are presented in **Chapter IV**.

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# CHAPTER II

## The Two Root-Specific and Jasmonate-Induced *Arabidopsis* (*E*)-β-Farnesene Synthases, TPS22 and TPS25, are Targeted to Mitochondria

(Targeted for publication in **Plant Physiology**)

**Other Contributors:**

Titi-Mary Omotade, undergraduate research assistant, helped with the identification of T-DNA position in T-DNA insertion mutant lines.

## Abstract

Terpenes represent the largest class of plant secondary (specialized) metabolites and serve a variety of functions in the interaction of plants with other organisms. Terpene biosynthesis has been shown to occur in two distinct subcellular compartments, plastid and cytosol. It is generally accepted that monoterpenes ($C_{10}$) and diterpenes ($C_{20}$) are synthesized in plastids while sesquiterpenes ($C_{15}$) are produced primarily in the cytosol. However, there is limited knowledge as to what extent other organelles such as mitochondria might contribute to the compartmentation of terpene metabolism.

We have identified two sesquiterpene synthases, TPS 22 and TPS25, in *Arabidopsis* that are expressed primarily in the roots and moderately induced by the defense hormone, jasmonic acid. Biochemical analysis showed that both TPS22 and TPS25 convert only (*E*,*E*)-farnesyl diphosphate (FPP) as a substrate to a mixture of sesquiterpenes, with (*E*)-β-farnesene as the major volatile product. Analysis of TPS22 and TPS25 loss-of-function mutants and overexpression lines demonstrated that both enzymes contribute to the formation of (*E*)-β-farnesene *in vivo*. Expression of β-glucuronidase (GUS) fused to the TPS22 or TPS25 promoter indicated constitutive expression of both genes in the roots, but in different root growth zones and cell types. Surprisingly, subcellular localization of TPS22 and TPS25 fused to enhanced green fluorescent protein (eGFP) revealed that both proteins are targeted to the mitochondria.

Despite substantial *TPS22* and *TPS25* transcript accumulation in wild type and transgenic lines, the emission rate of (*E*)-β-farnesene is low, suggesting posttranslational modifications of the TPS proteins and/or limited substrate availability that keep biosynthetic rates low in mitochondria. *In planta* expression of *TPS22* and *TPS25* cDNAs lacking the predicted

mitochondrial targeting sequence resulted in stable gene transcripts but no measurable (*E*)-β-farnesene emission, indicating possible instability of the TPS proteins in the cytosol or perhaps limited access to substrates due to channeling in the cytosolic pathway. The mitochondrial localization of TPS22 and TPS25 may reflect evolutionary plasticity in subcellular compartmentation of TPS proteins with emerging or declining activity or may fine tune (*E*)-β-farnesene concentrations to prevent possible autotoxic or inhibitory effects of this terpene in *Arabidopsis* roots.

## Introduction

Volatile organic compounds participate in many different interactions between plants and other organisms. In particular, plant-emitted volatile terpenes are major contributors to the chemical repertoire used for plant defense against insects and microbial pathogens, the attraction of pollinators and signaling to other plants (Loreto and Velikova, 2001; Pichersky and Gershenzon, 2002; Baldwin et al., 2006; Dudareva et al., 2006; Tholl, 2006).

The formation of terpenes, which represent the largest and most diverse class of specialized (secondary) metabolites, is regulated at different enzymatic steps of the early and late terpene biosynthetic pathways and by segregation of these pathways in different subcellular compartments. Terpenes are derived from the five-carbon building block, isopentenyl diphosphate (IPP), via the cytosolic mevalonate (MVA) or the plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathways (Arigoni et al., 1997; Lichtenthaler et al., 1997; Disch et al., 1998; Tholl, 2006). Condensation of IPP and its isomer, dimethylallyl diphosphate (DMAPP), leads to the formation of $C_{10}$-geranyl diphosphate (GPP), $C_{15}$-farnesyl diphosphate (FPP), and $C_{20}$-

geranylgeranyl diphosphate (GGPP), which is catalyzed by prenyltransferases (GPP, FPP and GGPP synthases, respectively). The prenyl diphosphates are then converted into monoterpenes ($C_{10}$), sesquiterpenes ($C_{15}$), or diterpenes ($C_{20}$) by enzymes called terpene synthases (TPS) (Bohlmann et al., 1998; Gershenzon and Kreis, 1999; Davis and Croteau, 2000).

Multiple studies have shown that monoterpenes and diterpenes are formed to the most part from GPP and GGPP, respectively, in plastids, while sesquiterpenes are largely synthesized from FPP in the cytosol (Bohlmann et al., 1998; Gershenzon and Kreis, 1999; Nagegowda, 2010). Depending on their subcellular location, multifunctional TPS enzymes may form either monoterpenes and diterpenes or sesquiterpenes (Nagegowda et al., 2008; Huang et al., 2010). Only a single study to date, on the formation for terpene aroma compounds in wild and cultivated strawberry, has suggested a possible natural formation of sesquiterpenes from FPP in mitochondria (Aharoni et al., 2004). The biosynthesis of sesquiterpenes in mitochondria seems plausible since studies in *Arabidopsis* have demonstrated the expression of mitochondrial isoforms of IPP isomerase (IDI2) and FPP synthase (FPS1L) (Cunillera et al., 1997; Phillips et al., 2008). Moreover, among the 32 terpene synthases in the *Arabidopsis* TPS family, 12 proteins of the TPS-a type clade have been predicted to carry putative mitochondrial or plastidial targeting sequences (Tholl and Lee, 2011). Only four TPSs (TPS11 – At5g44630, TPS21 – At5g23960, TPS12 - At4g13280, TPS13 - At4g13300) in this clade lack a transit peptide and these enzymes have been characterized as cytosolic sesquiterpene synthases (Chen et al., 2003; Tholl et al., 2005; Ro et al., 2006). Interestingly, two thirds of the type-a *TPS* genes are expressed in roots, of which TPS12 and TPS13 have been identified as (*Z*)-γ-bisabolene synthases and TPS08 was found to function as a plastidial diterpene synthase (Ro et al., 2006; Tholl and Lee, 2011).

Here we show that two terpene synthases, TPS22 and TPS25, with primary expression in *Arabidopsis* roots catalyze the formation of multiple sesquiterpene products with (*E*)-β-farnesene as the major product. We demonstrate that both enzymes reside in the mitochondria and contribute to some extent to constitutive and jasmonate-induced formation of (*E*)-β-farnesene *in vivo*. We show that, despite the accumulation of substantial transcript levels of *TPS 22* and *TPS25*, (*E*)-β-farnesene is produced at low concentrations, which may prevent this sesquiterpene from having growth inhibitory effects in *Arabidopsis* roots.

## Results

### Identification of *Arabidopsis* TPS22 and TPS25 as putative mitochondrial proteins

Among the 22 TPS-a type enzymes of the *Arabidopsis* TPS family, 12 have been previously predicted to contain putative plastidial or mitochondrial transit peptide sequences (Tholl and Lee, 2011). Two of these proteins are encoded by the root-specific *TPS* genes, *TPS22* (At1g33750) and *TPS25* (At3g29410), which share 56.5% amino acid sequence identity (Figure 2.1). An alignment with the sesquiterpene synthases TPS12 (At4g13280)/13(At4g13300), TPS11 (At5g44630), and TPS21 (At5g23960), show that TPS22 and TPS25 carry an approximately 30 amino acid N-terminal extension (Figure 2.1).

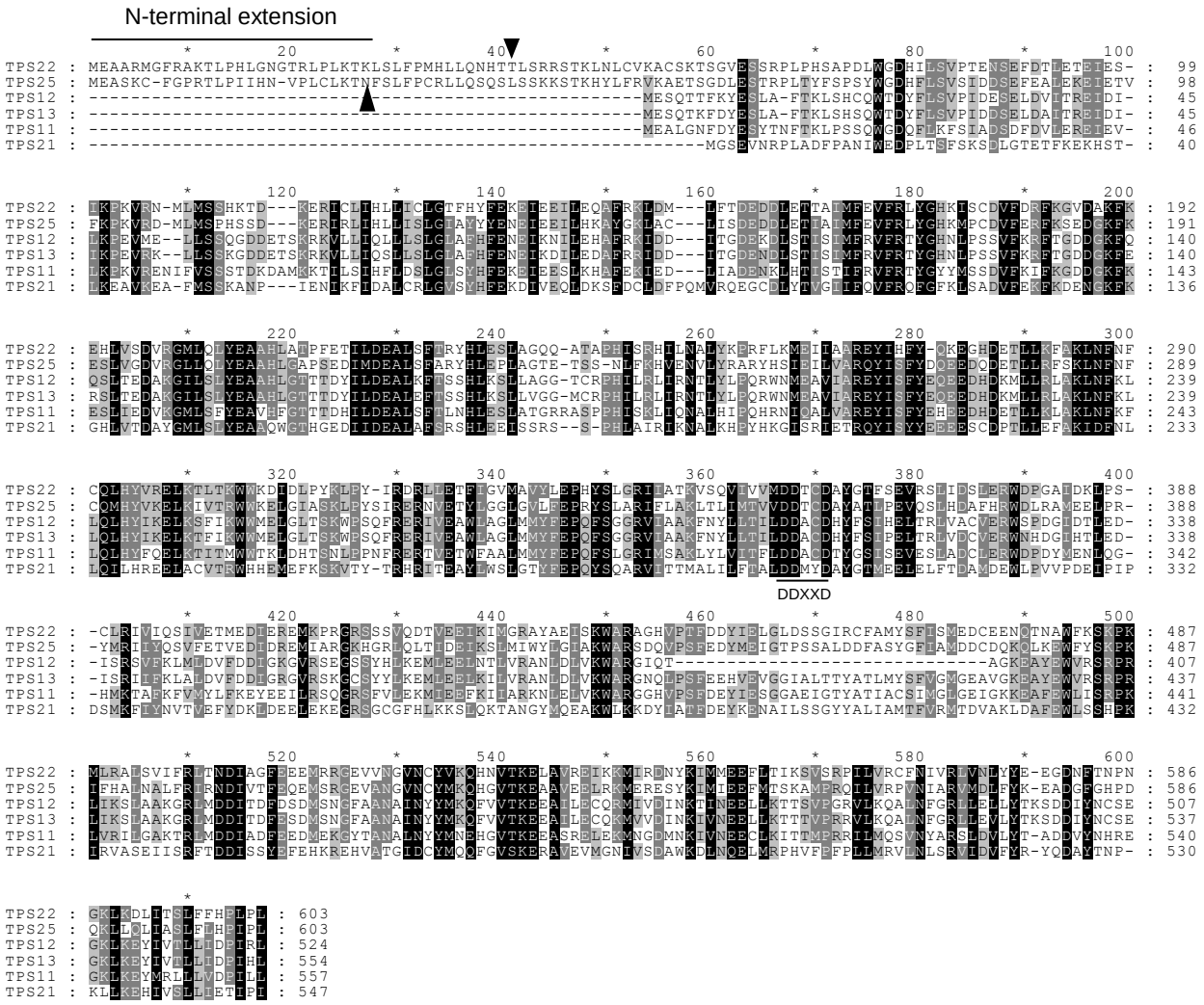


**Figure 2.1 Protein sequence alignment of *Arabidopsis* TPS22 and TPS25 with the sesquiterpene synthases TPS12, TPS13, TPS11, and TPS21.** The alignment was performed using CLUSTALW, and the sequences were edited in GeneDoc 2.7 (http://www.nrbsc.org/gfx/genedoc/). Black, gray, and light gray shading represent 100, 80, and 60% conserved identity between residues, respectively. The highly conserved metal cofactor binding region (DDxxD) is underlined. The horizontal line indicates the predicted mitochondrial or plastidial N-terminal transit peptides. Arrowheads indicate positions of Thr-41 in TPS22 and Asn-25 in TPS25 where truncations were made.

Analysis of these peptide sequences using the public servers ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) (Emanuelsson et al., 1999), TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al., 2000), Predotar

(http://urgi.versailles.inra.fr/predotar/predotar.html), MitoProt (http://mips.gsf.de/cgi-bin/proj/medgen/mitofilter) (Claros and Vincens, 1996), and PSORT (http://wolfpsort.org/) (Nakai and Horton, 1999), suggested that TPS22 is located in mitochondria and TPS25 may be targeted to mitochondria or plastids.

**Biochemical characterization of TPS22 and TPS25**

To characterize the biochemical activities of TPS22 and TPS25 proteins, 1812 bp cDNAs encoding 603 amino acids (aa) was amplified by RT-PCR from *Arabidopsis* root RNA using gene-specific primer pairs for *TPS22* and *TPS25*, respectively, and cloned into a bacterial expression vector (Topo-pET102, Sigma). The recombinant proteins were heterologously expressed in *Escherichia coli* and analyzed for TPS activity. Crude bacterial extracts containing the TPS22 or TPS25 proteins with their putative N-terminal signaling peptides showed no enzymatic activities (data not shown). Inclusion bodies were observed, which may be due to the presence of the transit peptide, a typical feature of monoterpene or diterpene synthases (Bohlmann et al., 1998; Williams et al., 1998; Williams et al., 2000). We then prepared corresponding expression constructs with N-terminal truncations of 40 and 24 amino acids for TPS22 and TPS25, respectively, which were based on the *in silico* analysis of the transit peptide sequences.

Recombinant Met-41-TPS22 and Met-25-TPS25 proteins partially purified by $Ni^{2+}$ affinity chromatography were further tested for their ability to convert geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) into terpene products. The enzymatic products from the recombinant enzymes were analyzed by gas

chromatography-mass spectrometry (GC/MS) and identified by a combination of retention time and mass spectral analysis in comparison with authentic standards. Both Met-41-TPS22 and Met-25-TPS25 proteins catalyze the formation of the same complement of sesquiterpene products by utilizing FPP, although in distinctly different proportions (Figure 2.2 a, b), whereas no enzymatic products were detected for either recombinant protein using GPP and GGPP as the substrate (data not shown). The enzymatic products of Met-41-TPS22 were (*E*)-β-farnesene (46.6%), α-farnesene isomers (34.6%), (*E*)-β-bisabolene (1.2 %), bisabolene isomers (4.4 %), and (*E*)-nerolidol (13.1 %) (Figure 2.2 a), and the reaction products of Met-25-TPS25 were (*E*)-β-farnesene (37.2 %), (*E*)-α-farnesene (5.2 %), (*E*)-β-bisabolene (18.2%), bisabolene isomers (5.9 %), and (*E*)-nerolidol (33.5 %) (Figure 2.2 b). The enzymatic products were not detected in reactions with denatured proteins (Figure 2.2 c, d) or vector controls (Figure 2.2 e). To determine the absolute configuration of (*E*)-nerolidol produced by TPS22 and TPS25 proteins, enantio-selective GC/MS analysis was performed. Both enzymes synthesized (*R*) and (*S*)-(*E*)-nerolidol from FPP in an equal ratio (Figure 2.3).

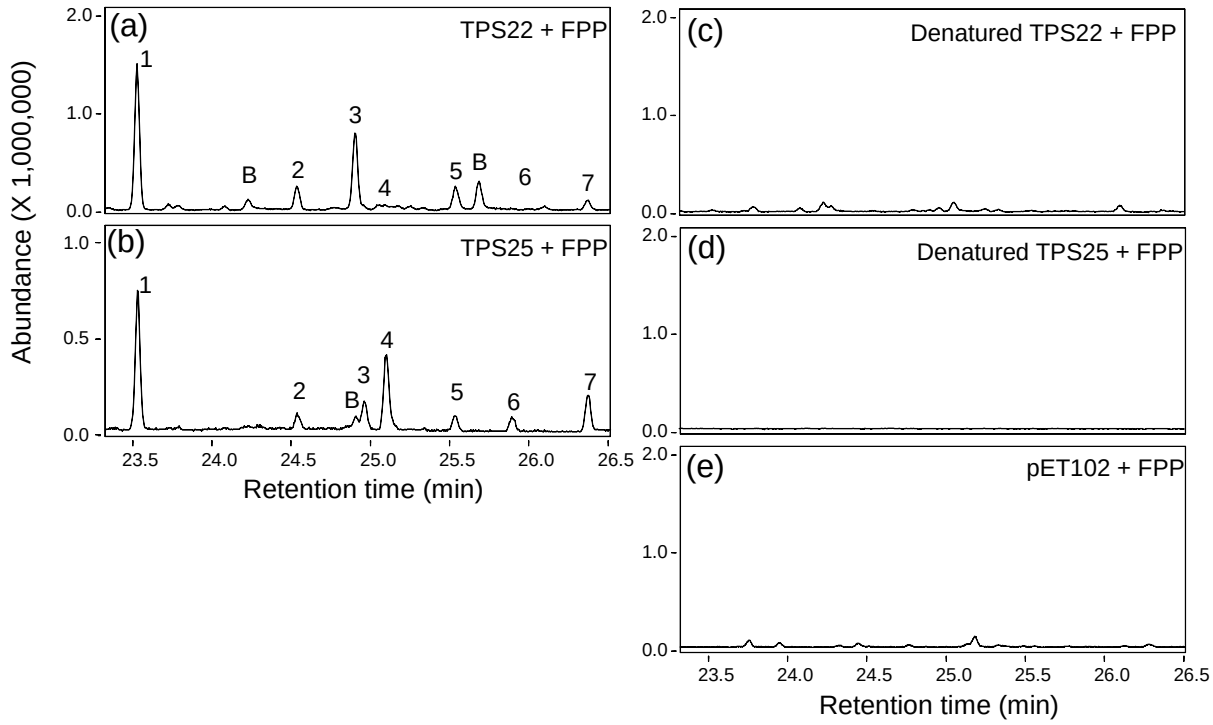


**Figure 2.2 Analysis of products formed from farnesyl diphosphate (FPP) by recombinant TPS22 and TPS25 enzymes using SPME-GC/MS.** TPS22 and TPS25 proteins without the N-terminal transit peptide were used for enzyme activity assays. (a, b) GC/MS chromatogram of enzymatic products formed by recombinant TPS22 and TPS25. Sesquiterpene products were identified by comparison with authentic standards (1 and 7) or by library suggestion (for 2, 3, 4, 5, and 6). 1, (*E*)-β-farnesene; 2 and 3, α-farnesene isomers; 4, (*E*)-β-bisabolene; 5 and 6, bisabolene isomers; 7, (*E*)-nerolidol; B: background. (c-e) GC/MS analysis of products formed by denatured TPS22 and TPS25 (c, d) and extracts (e) of *E. coli* expressing the pET102 vector without an insert.

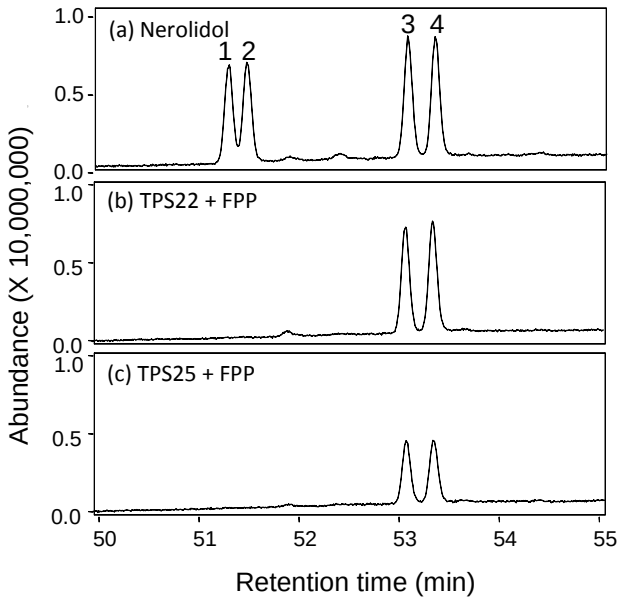


**Figure 2.3 Enantiomer (*S*)/(*R*) determination of the nerolidol product formed by TPS22 and TPS25.** (a) Racemic mixtures of nerolidol, (b, c) (3*R*)/(3*S*) (*E*)-nerolidol formed by TPS22 and TPS25, respectively, from FPP, 1, (3*R*) (*Z*)-nerolidol; 2, (3*S*) (*Z*)-nerolidol; 3, (3*R*) (*E*)-nerolidol; 4, (3*S*) (*E*)-nerolidol.

To further determine the biochemical properties of the two TPS proteins, we analyzed their catalytic activities with the substrate FPP. Although TPS25 exhibited about 6.0-fold higher $V_{max}$ and $K_{cat}$ values for FPP in comparison with TPS22, both enzymes showed similar $K_{cat}/K_m$ values because TPS22 has a 7.9-fold lower $K_m$ value for FPP than does TPS25 (Table 2.1).

**Table 2.1 Kinetic parameters of recombinant TPS22 and TPS25 enzymes.**

| Enzyme | Substrate | $K_m$ (μM) | $V_{max}$ (pkat mg$^{-1}$) | $K_{cat}$ ($s^{-1}$) | $K_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|---|---|
| TPS22 | FPP[a] | 0.55 ±0.10 | 4.41 ± 0.21 | 0.35 X 10$^{-3}$ ± 0.01 X 10$^{-3}$ | 0.64 ± 0.16 |
| TPS25 | FPP[a] | 4.36 ± 2.82 | 24.98 ± 5.74 | 2.08 X10$^{-3}$ ± 0.48 X 10$^{-3}$ | 0.47 ± 0.17 |

Each value represents the mean ± SE of three replicates. [a] at 20 mM $Mg^{2+}$

We then analyzed the effects of different divalent metals and pH conditions on the catalytic activities of the recombinant proteins. Both enzymes were dependent on the co-factors $Mg^{2+}$ or $Mn^{2+}$ (Figure 2.4) with no distinct preference for one or the other cation. A concentration of 30 mM $Mg^{2+}$ or $Mn^{2+}$ caused inhibition of the catalytic activities relative to enzyme activities at 5 mM. Both recombinant proteins were active over a broad range of pH. TPS22 was active from pH 6.0 to 11.0 with highest activity between pH 7.0 and pH 10.0, and TPS25 showed activity from pH 5.0 to 9.0 with maximum activity between pH 7.0 and pH 9.0 (Figure 2.5).

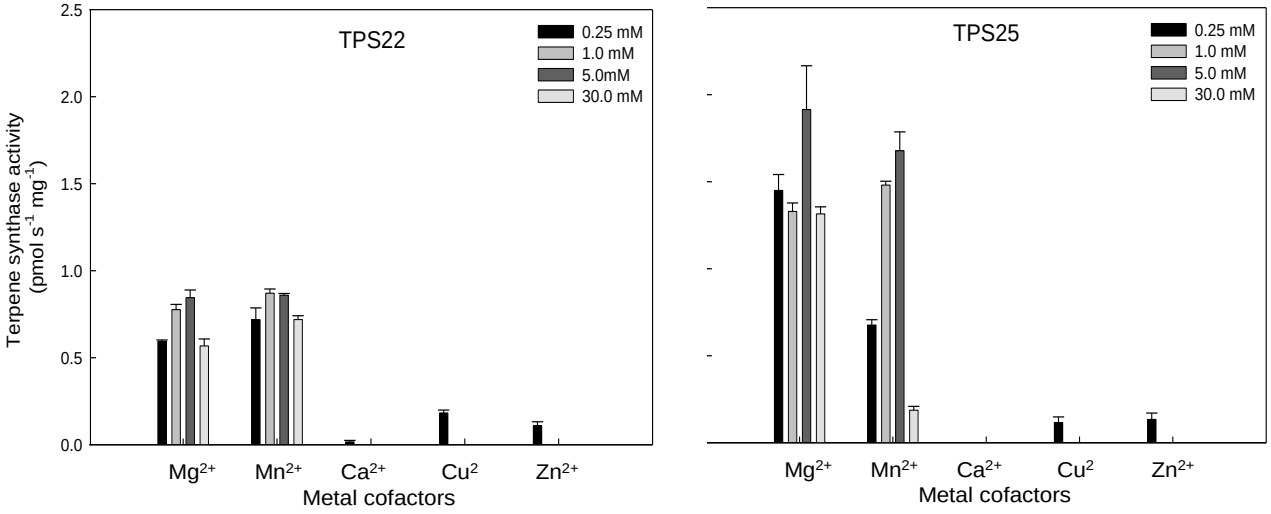


**Figure 2.4 The effect of divalent metal ion cofactors on enzyme activities of TPS22 and TPS25.** The catalytic activity of two recombinant enzymes was determined in the presence of various divalent metal ions. Means ± SEM of triplicate assays are shown. Linearity of enzyme concentration and reaction time was determined previously.

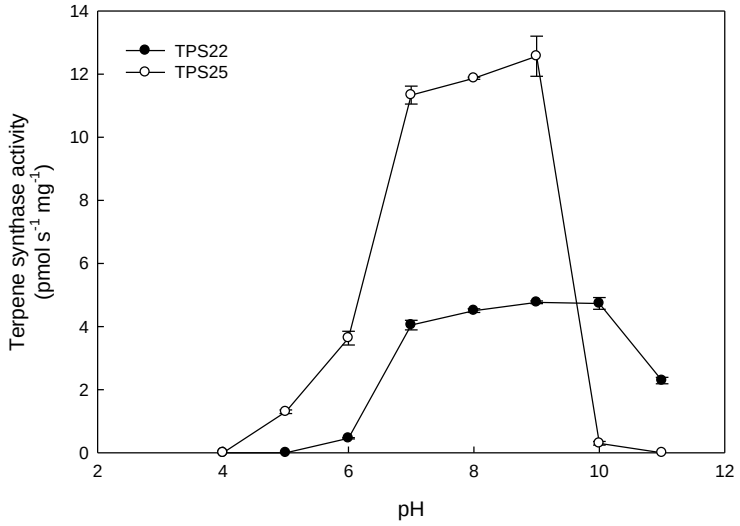


**Figure 2.5 pH Optima of TPS22 and TPS25.** The catalytic activity of two recombinant enzymes were based on the conversion of [1-$^{3}$H] FPP to sesquiterpene products at different pH of the assay buffer. Each value represents the average ± SEM of three replicates.

**Subcellular localization of TPS22 and TPS25**

In order to determine the subcellular compartments, in which TPS22 and TPS25 are located *Arabidopsis* was transformed with constructs, in which the coding region of each gene had been fused at its respective C-terminus with enhanced green fluorescent protein (eGFP) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Transgenic plants expressing eGFP without any fusion (vector control, pK7WG2) were used as controls for cytosolic localization (Figure 2.6 g). CoxIV, a cytochrome oxidase subunit IV involved in the respiratory electron transport chain of mitochondria and fused to red fluorescence protein (RFP) under control of the 35S promoter served as a marker for mitochondrial localization (Figure 2.6 d) (Nelson et al., 2007).

Fluorescence images from root hairs of non-transformed control plants showed no detectable green fluorescence (Figure 2.6 a), and no red autofluorescence derived from chlorophyll was detectable in root hairs of untransformed or transformed lines (data not shown). The absence of plastids in root hairs was confirmed by the lack of fluorescence from a plastidial ferredoxin NADP(H) oxidoreductase-eGFP fusion protein (Marques et al., 2004) (Figure 2.S.1).

Analysis of root hair tissues from at least four independent transgenic lines expressing either 35S::TPS22::eGFP (Figure 2.6 b) or 35S::TPS25::eGFP (Figure 2.6 c) revealed a pattern of GFP accumulation similar to that of RFP fused to the transit peptide of the mitochondrial CoxIV protein (35S::CoxIV::RFP) (Figure 2.6 d). Fluorescence was localized evenly throughout the mitochondria (Figure 2.6 b-d). To determine if the N-terminal transit peptides of both TPS proteins are essential for mitochondrial localization, similar C-terminal eGFP fusion constructs of TPS22 and TPS25 were generated but without the first 40 or 24 aa, respectively, and the proteins were stably expressed in *Arabidopsis*. In contrast to 35S::TPS22::eGFP and 35S::TPS25::eGFP, the N-terminal truncated proteins (35S::Met-41-TPS22::eGFP) (Figure 2.6 e) and TPS25 (35S::Met-25-TPS25::eGFP) (Figure 2.6 f) were localized in the cytosol. These results show that the TPS22 and TPS25 proteins contain a transit peptide that is essential for their mitochondrial localization.

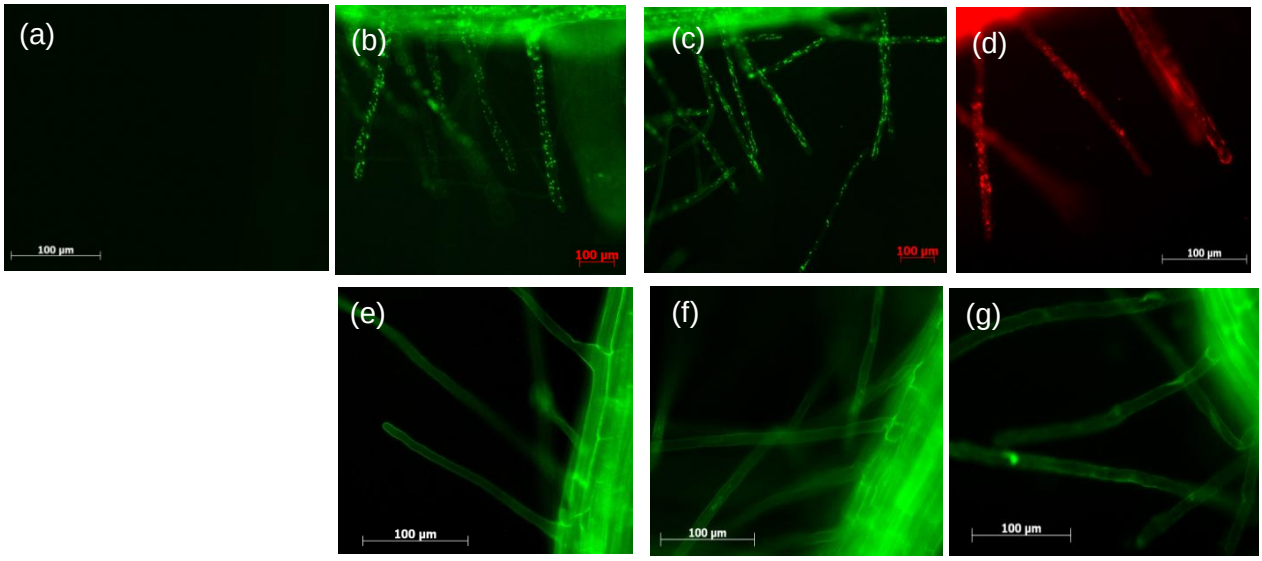


**Figure 2.6 Subcellular localization of TPS22 and TPS25-eGFP fusion proteins in *Arabidopsis* root hairs.** All fluorescence images were taken in a similar setting. Images are as follows: non-transformed *Arabidopsis* (a), 35S::TPS22::eGFP (b), 35S::TPS25::eGFP (c), 35S::CoxIV::RFP, mitochondrial control CoxIV (cytochrome oxidase subunit IV) (Nelson et al., 2007) (d), 35S::Met-41-TPS22::eGFP (e), 35S::Met-25-TPS25::eGFP (f), and cytosolic eGFP (empty vector control) (g).

**Sesquiterpene product and expression analysis of *TPS22* and *TPS25* in *Arabidopsis* roots**

We examined whether the sesquiterpenes produced by the recombinant TPS22 and TPS25 proteins could be found *in planta* by analyzing the profile of volatile compounds emitted from *Arabidopsis* roots grown in hydroponic culture by solid phase microextraction (SPME)-GC/MS. Of the sesquiterpenes synthesized by TPS22 and TPS25, we detected only (*E*)-β-farnesene, the major product of both enzymes. Since the formation of volatile terpenes can be induced by application of the defense hormone jasmonic acid (JA) (Arimura et al., 2008), we treated hydroponically-grown roots with 100 µM JA. The emission rate of (*E*)-β-farnesene in roots increased approximately 2-fold at 24 h after application (Figure 2.7 a).

We then tested whether the increase in (*E*)-β-farnesene emission correlates with transcript levels of the *TPS22* and *TPS25* genes by performing quantitative RT-PCR. Transcripts of *TPS22* and *TPS25* increased about 2.9-fold and 2.1-fold, respectively, in response to JA treatment indicating that both genes could be responsible for the induced (*E*)-β-farnesene emission (Figure 2.7 b). Under normal conditions, expression of *TPS25* was about 6-fold higher than that of *TPS22*. We were unable to detect the other sesquiterpene products of TPS22 and TPS25, most likely because the concentrations of these compounds were below detectable levels *in planta* keeping in mind that a conversion into non-volatile products cannot be totally excluded.

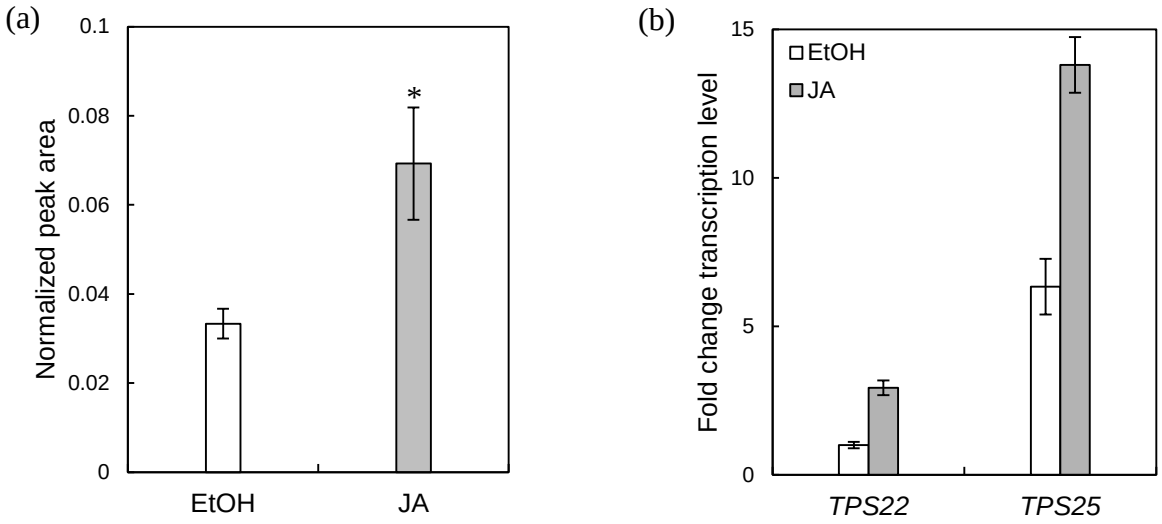


**Figure 2.7 JA-induced (*E*)-β-farnesene emission and expression of *TPS22* and *TPS25* in *Arabidopsis* roots.** (a) (*E*)-β-farnesene emission in *Arabidopsis* roots. Five-week-old *Arabidopsis* roots grown in hydroponic culture were used for volatile collection. Emission of (*E*)-β-farnesene was measured 24 h after application of 100 µM JA. Results show normalized peak areas for (*E*)-β-farnesene (quantification method as described in "Materials and methods"). Data represent the mean ± standard error mean (SEM) (n = 3), *, P< 0.05 (Student t-test), (b) Relative transcript levels of *TPS22* and *TPS25* genes in response to treatment with 100 µM JA analyzed by quantitative RT-PCR (means ± SEM, *n* = 3). The transcript level of *TPS22* was set to 1.0.

**Organ- and tissue-specific expression of *TPS22* and *TPS25***

To understand the organ-/tissue-specific expression of *TPS22* and *TPS25*, we examined the promoter activities of both genes using plants stably transformed with promoter::β-glucuronidase (GUS) fusion constructs. Ten to fifteen day-old seedlings of at least four independent T2 lines were examined. For both genes GUS staining was detected mainly in primary and lateral roots and to some extent in leaves and flowers (Figure 2.8). Pro$_{TPS22}$::GUS staining was found in the root tip (Figure 2.8 d) while Pro$_{TPS25}$::GUS activity appeared in the cortex, epidermal, and possibly vascular tissues in the root elongation zone, and in epidermal tissues in the differentiation zone but not at the root tip (Figure 2.8 i and j). In addition, Pro$_{TPS22}$::GUS activity was detected in the vascular tissue of true leaves, at the base of leaf trichomes, and in anther filaments, whereas Pro$_{TPS25}$:GUS activity was only found in leaf trichomes (Figure 2.8).

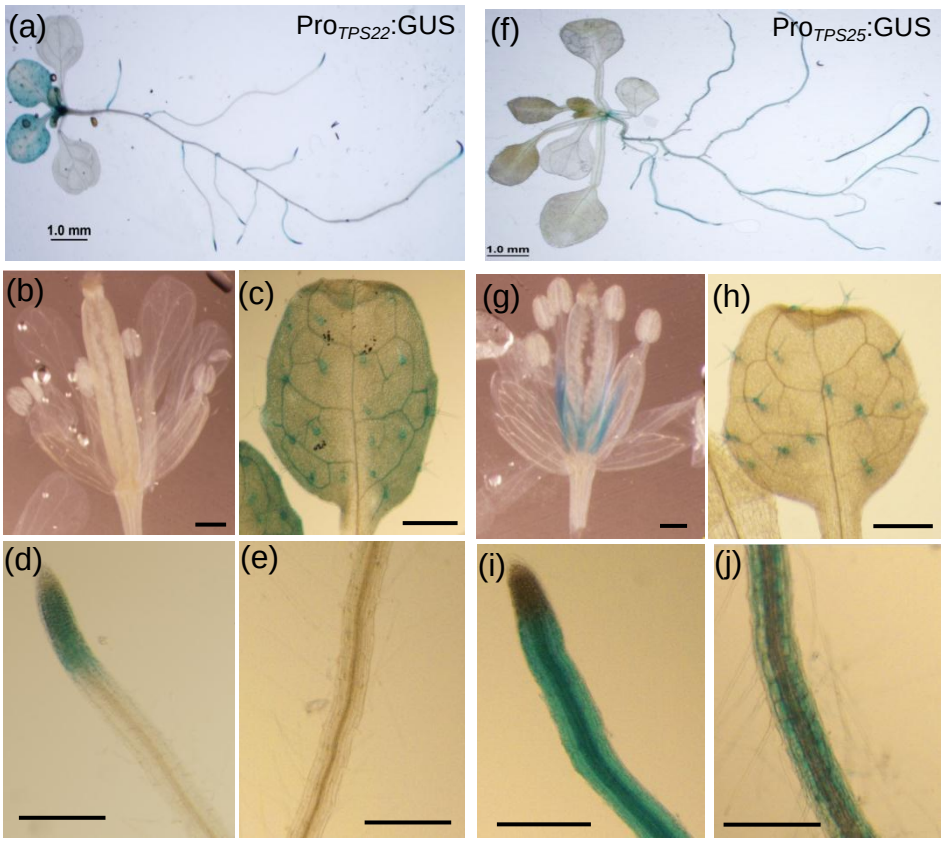


**Figure 2.8 Histochemical analysis of *TPS22* and *TPS25* promoter activities.** Organ- and tissue-specific *TPS22* and *TPS25* promoter activities were observed in transgenic *Arabidopsis* stably transformed with GUS fusion constructs. (a-e) Pro$_{TPS22}$::GUS staining in primary and lateral root tips (a, d), the vascular tissue of true leaves, and the base of trichomes (c). (f-j) Pro$_{TPS25}$::GUS activity associated with anther filaments (g), trichomes (h), epidermis, cortex and possibly vascular tissues in root elongation zone (i), and epidermal tissues in differentiation zone (j) of primary and lateral roots, except for root tips (f, i). Control lines carrying an empty vector without insert showed no GUS staining (not shown). Bars are 500 μm, if not indicated.

**Volatile analysis in loss-of-function mutants of *TPS22* and *TPS25***

To further correlate the activities of TPS22 and TPS25 with the formation of (*E*)-β-farnesene *in planta*, loss-of-function mutants for each gene were analyzed. For the *TPS22* gene, one *Arabidopsis* line with a T-DNA insertion in exon 4 (*tps22-1*, Salk_067831) and for *TPS25*,

two independent lines with T-DNA insertions in intron 3 and exon 4 (*tps25-1*; Salk_020266 and *tps 25-2*; Salk_123505) were used (Figure 2.9 a). The presence of the T-DNA insertion was confirmed by sequence analysis. Since both TPS enzymes make (*E*)-β-farnesene as the primary product, we also established homozygous double mutants by crossing *tps22-1* with *tps25-1* (Figure 2.9 a). Transcript analysis of each gene in axenically-grown roots treated with JA showed no functional full-length transcripts of *TPS22* and *TPS25* in each mutant and the double knock-out line (Figure 2.9 b). In each of the single gene knockout lines, the remaining functional *TPS* gene was still expressed (Figure 2.9 c).

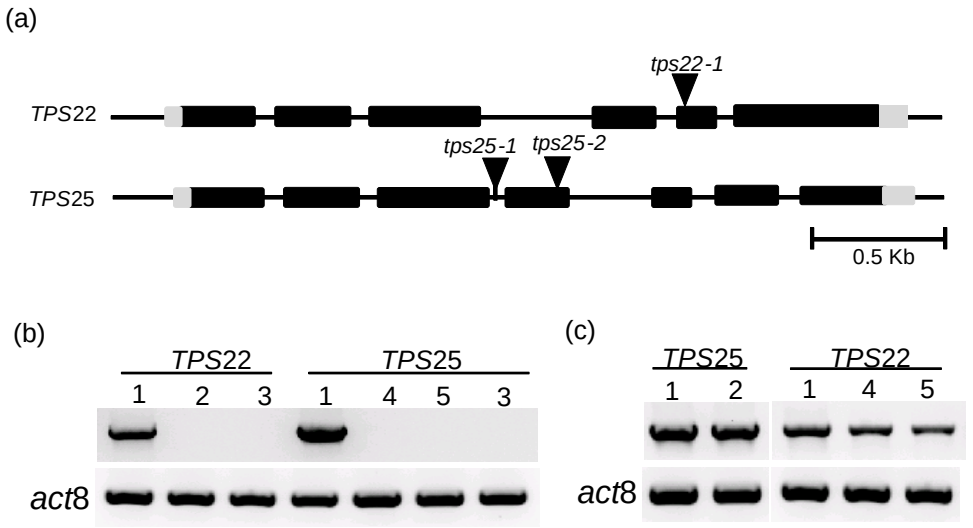


**Figure 2.9 Genomic structure and gene expression of *TPS22* and *TPS25*.** (a) Genomic structure of *TPS22* and *TPS25*. Black boxes indicate exons, while the thin lines in between represent introns. The grey boxes show the 5'- and 3'- untranslated regions. The insertion sites of the T-DNA insertion mutants used in this study are marked with black inverted triangles. (b) Semi-quantitative RT-PCR analysis of *TPS22* and *TPS25* transcripts in jasmonate-treated roots of wild-type (Col) and *tps* mutant lines. (c) Expression of the remaining functional *TPS* gene in wild type and *tps* mutant lines. *Actin8* (*act8*) was used as a control for an endogenous gene expression. 1, wild-type (Col); 2, *tps22-1*; 3, *tps22-1/tps25-1*; 4, *tps25-1*; 5, *tps25-2*.

Next, we analyzed the emission of (*E*)-β-farnesene in roots of the *tps* mutants treated with 100 µM jasmonic acid using SPME-GC/MS (Figure 2.10). (*E*)-β-Farnesene emission from roots of untreated single and double mutants was approximately 1.5- to 2-fold lower than that of wild type roots, although this decline was not statistically significant and no additive effect was observed in the double mutant. This finding suggests redundancies in constitutive (*E*)-β-farnesene formation by other as yet uncharacterized enzymes. JA-treated *tps25-1*, *tps25-2* and *tps22-1/tps25-1* mutants showed no increase in (*E*)-β-farnesene emission. In contrast, (*E*)-β-farnesene levels in *tps22-1* were comparable to those of JA-treated wild type roots. These results indicate that *TPS25* is primarily responsible for the JA-induced formation of (*E*)-β-farnesene *in vivo*.

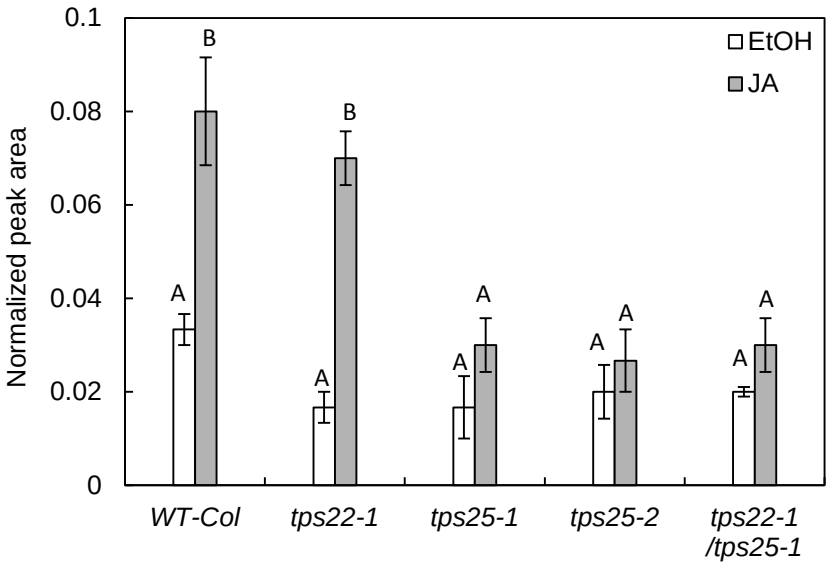


**Figure 2.10 Quantitative analysis of (*E*)-β-farnesene emission in wild-type (Col-0) and *tps* mutants.** (*E*)-β-farnesene emission from hydroponically grown roots was measured 24 h after treatment with 100 µM JA or EtOH. Normalized peak areas are shown as analyzed by SPME-GC/MS (see "Materials and Methods"). Data represent the mean ± SEM (n = 3). P<0.05, Tukey-Kramer HSD test.

**Ectopic expression of *TPS22* and *TPS25***

To further understand the function of *TPS22* and *TPS25* in (*E*)-β-farnesene formation *in planta*, each full length gene was expressed under the control of the CaMV 35S promoter in the background of the corresponding loss-of-function mutant for each gene (*tps22-1* and *tps25-2*). Functional full-length transcripts of *TPS22* and *TPS25* accumulated in the respective transgenic lines (Figure 2.11 a). GC/MS analysis of volatiles collected from leaves of 35S::*TPS25* lines showed a mixture of (*E*)-β-farnesene, (*E*)-β-bisabolene, and (*E*)-nerolidol, while 35S::*TPS22* plants released (*E*)-β-farnesene only (Figure 2.11 b and c). A comparison of emission rates for each volatile in each transgenic line showed that the emission of (*E*)-β-farnesene in 35S::*TPS25* lines was about twice that of the 35S::*TPS22* line. Although the total amount of sesquiterpenes emitted by these lines was low (≈ 2 ng collected from 8 plants for 24 h) (Figure 2.11 b), the results clearly showed that both TPS22 and TPS25 activities can produce the same sesquiterpene products at essentially the same relative levels as those observed *in vitro.*

**The effect of (*E*)-β-farnesene on plant growth *in vitro***

Since we found (*E*)-β-farnesene to be emitted at low rates from roots and foliage of wild type and transgenic lines, we reasoned that this sesquiterpene could exhibit inhibitory or autotoxic effects in *Arabidopsis* when produced at higher concentrations. We therefore investigated the possible root growth inhibitory effects of (*E*)-β-farnesene and (*E*)-nerolidol, both of which are enzymatic products of TPS22 and TPS25, in comparison to (*E*)-β-caryophyllene, a volatile sesquiterpene produced in *Arabidopsis* flowers. Root growth was inhibited by 50% in the

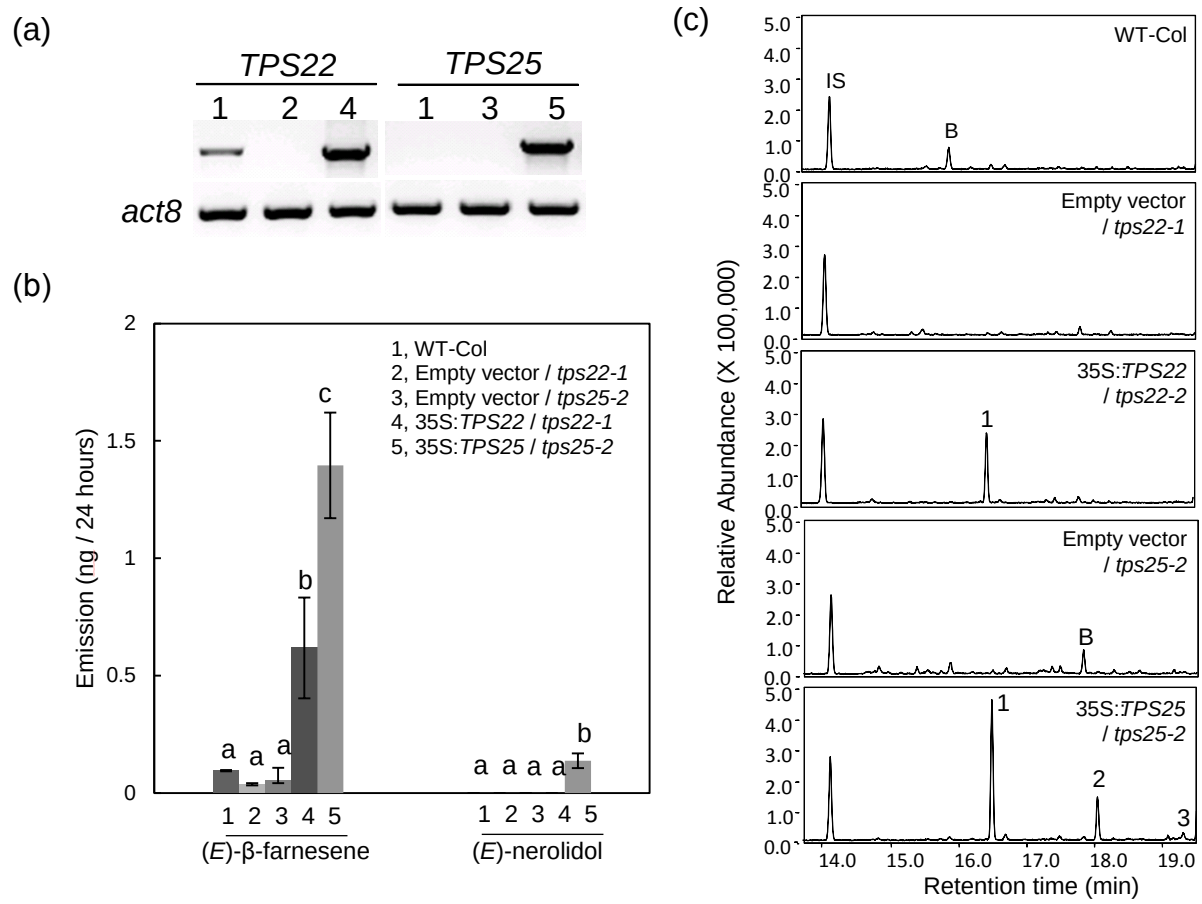


**Figure 2.11 Gene transcript and volatile analysis from leaves of *TPS22* and *TPS25* overexpression plants.** (a) Semi-quantitative RT-PCR analysis of *TPS22* and *TPS25* in wild-type (Col) and overexpression lines. *Actin8* (*act8*) was used as a control for endogenous gene expression. 1, wild-type (Col); 2, empty vector control/*tps22-1*; 3, empty vector control/*tps25-2*; 4, 35S::*TPS22*/*tps22-1*; 5, 35S::*TPS25*/*tps25-2*. (b) Quantification of sesquiterpene emissions from wild-type and overexpression lines of *TPS22* and *TPS25*. Release rates of volatile terpenes from eight plants of five-week-old *Arabidopsis* grown in soil were determined by closed-loop-stripping method. Volatiles were collected from leaves rather than roots of the transgenic plants to avoid measuring background (*E*)-β-farnesene emissions as they occur in roots. Transgenic lines carrying the empty vector were used as controls. Data represent the means ± SEM from three individual measurements. One-way ANOVA, $P<0.05$, Tukey-Kramer HSD test, (c) Gas chromatography (69 selected ion) of sesquiterpenes collected from eight plants of wild type or overexpression lines during 24 h (10h-light/14h-dark) of closed-loop stripping. 1, (*E*)-β-farnesene; 2, (*E*)-β-bisabolene; 3, (*E*)-nerolidol; IS, internal standard (1-bromo-decane); B, background.

presence of 2.5 to 3 μM of each compound (Figure 2.12). However, a similar inhibitory effect was already observed at a 10-fold lower concentration (0.3 μM) of (*E*)-β-farnesene, whereas (*E*)-nerolidol and (*E*)-β-caryophyllene did not show an effect at this concentration (Figure 2.12). Although no dose-dependent effect of (*E*)-β-farnesene was observed, the results indicate possible inhibitory effects of this sesquiterpene compound on *Arabidopsis* root growth.

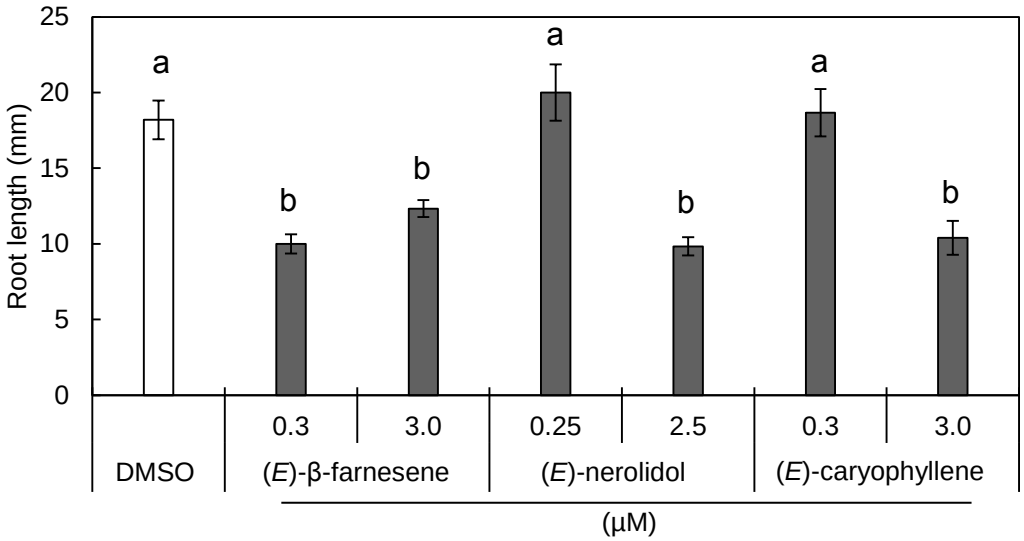


**Figure 2.12 Plant growth analysis of *Arabidopsis* (Col-0) in the absence and presence of sesquiterpenes.** Plants were grown vertically on ½ X MS media containing the different sesquiterpene compounds. All authentic standards were dissolved in DMSO, which was used as a control. The root length was measured three weeks after germination. Data represent the mean ± SEM of six plants, $P<0.05$, Tukey-Kramer HSD test.

## Discussion

### The two root-specific sesquiterpene synthases TPS22 and TPS25 produce multiple sesquiterpenes

We identified two *Arabidopsis* terpene synthases, TPS22 and TPS25, which are primarily expressed in the roots and catalyze the formation of multiple sesquiterpenes. Both enzymes belong to a clade of *Arabidopsis* type-a TPSs that can be distinguished from a group of type-b monoterpene synthases, as well as one type-c, and two type e/f enzymes of the *Arabidopsis* TPS family. TPSs of the type-a subfamily have been largely characterized as sesquiterpene synthases (Tholl et al., 2005; Ro et al., 2006). These TPSs do not contain transit peptide sequences, which is consistent with the general feature of sesquiterpene synthases (Aharoni et al., 2005). Most of the other TPSs in this clade were originally thought to be diterpene synthases because of the presence of predicted plastidial transit peptide sequences (Tholl and Lee, 2011). One root-specific diterpene synthase (TPS08) has recently been characterized (Vaughan et al., submitted) and there are evidences that other TPSs in this group have diterpene synthase activity, as well (Qiang, unpublished results).

Surprisingly, the TPS22 and TPS25 recombinant proteins, which share approximately 74% amino acid sequence similarity and 56% identity, neither converted GPP into monoterpene or GGPP into diterpene products *in vitro*. Instead, both proteins produce a mixture of multiple sesquiterpenes including (*E*)-β-farnesene, α-farnesene isomers, bisabolene isomers and (*E*)-nerolidol, but in different proportions (Figure 2.2). These proteins share about 30% identity with TPS1, TPS4, and TPS5 from *Zea mays*, which are most closely related to TPS22 and TPS25 at

the amino acid level and are all sesquiterpene synthases (Schnee et al., 2002; Köllner et al., 2004). In particular, TPS1 produces similar sesquiterpene products as TPS22 and TPS25 including (*E*)-β-farnesene, (*E*)-nerolidol, and (*E,E*)-farnesol (Schnee et al., 2002). Although TPS22 and TPS25 encode sesquiterpene synthase involved in multiple sesquiterpenes, the N-terminal extension of these proteins predicted their localization in mitochondria or plastids.

Both enzymes share very similar catalytic properties that are within the range of those reported for other sesquiterpene synthases ($K_m$, 0.1–10 µM, $K_{cat}$, 0.003–0.5 $sec^{-1}$) (Cane, 1999; Nagegowda et al., 2008; Huang et al., 2010). The cofactors $Mg^{2+}$ and $Mn^{2+}$ were found to be equally efficient at concentrations between 0.25 to 5 mM in contrast to reports of several other terpene synthases showing that these enzymes are inhibited at $Mn^{2+}$ but not $Mg^{2+}$concentrations higher than 20 mM (Bohlmann et al., 1998; Tholl et al., 2005; Picaud et al., 2006; Nagegowda et al., 2008).

The formation of terpenes is usually tightly regulated by the expression of the corresponding terpene synthases (Tholl et al., 2005; Attaran et al., 2008; Herde et al., 2008). We were able to correlate root-specific constitutive and jasmonate-induced transcript levels of *TPS22* and *TPS25* with the emission of the primary enzyme product (*E*)-β-farnesene. The other sesquiterpene products, except for small amounts of (*E*)-nerolidol, which showed sporadic emission, were not detected in wild type plants presumably because of the low enzyme activity *in vivo*. Volatile analysis of *TPS22* and *TPS25* loss of function mutants indicated that both enzymes have a small though not significant contribution to the constitutive formation of (*E*)-β-farnesene, while TPS25 but not TPS22 appears to be primarily responsible for the biosynthesis of (*E*)-β-farnesene in response to treatment of roots with jasmonic acid. The observation of (*E*)-β-farnesene emissions in all T-DNA mutant lines as well as wild-type plants suggests the

presence of other terpene synthases in *Arabidopsis* roots with redundant function in the formation of (*E*)-β-farnesene. The *in vivo* activity of the TPS22 and TPS25 proteins was confirmed by the accumulation of (*E*)-β-farnesene in leaves of transgenic lines ectopically expressing *TPS22* or *TPS25* in the double knockout mutant background (Figure 2.10). Although both genes appear to be transcribed at similar levels (Figure 2.11), the emission rate of (*E*)-β-farnesene in the lines expressing *TPS25* was higher than in *TPS22* expressing plants (Figure 2.11), however, the overall emission rates from both transgenic lines was low compared to previous reports on the quantity of total sesquiterpenes from transgenic *Arabidopsis* plants by introduction of maize sesquiterpene synthases (≈ 2.1 μg $g^{-1}$ leaf) (Schnee et al., 2006). In addition, (*E*)-β-bisabolene and (*E*)-nerolidol emissions were found in overexpression lines for *TPS25* in the same proportion as that observed *in vitro* (Figure 2.2). Since both enzymes showed similar catalytic efficiencies, the higher sesquiterpene emission levels in the *TPS25* expressing lines might be caused by a higher stability of the TPS25 enzyme than the TPS22 protein *in planta*.

### *TPS22* and *TPS25* are expressed in different root tissues and growth zones

The promoter activities of both *TPS22* and *TPS25* were detected primarily in root tissues, however, they indicated distinct, complementing tissue/cell type-specific gene expression patterns (Figure 2.8). While *TPS22* is primarily expressed in the root tip, expression of *TPS25* occurs in the epidermis and cortex but not in the root tip. The promoter-GUS patterns are largely in agreement with results from high resolution gene expression maps of *Arabidopsis* roots indicating high expression of *TPS22* in the root tip, especially in the columella, the lateral root

cap and epidermis, and the primary expression of *TPS25* in the cortex and endodermis of the differentiation zone (Birnbaum et al., 2003; Brady et al., 2007). Recent findings by Schiefelbein and coworkers (personal communication) based on transcript profiles of epidermal hair- (H) and non-hair (N)-forming cells separated by fluorescence activated cell sorting (FACS) revealed a differential expression of *TPS25* in N-cells but not in H-cells. This cell-type specific expression pattern is somewhat supported by the absence of *TPS25* promoter-GUS activity in root hairs (Figure 2.8). Why *TPS25* is specifically expressed in N-cells and whether TPS25 activity is absent in H-cells because its sesquiterpene products could interfere with hair cell formation is subject to further investigation. A recent study supports this idea by showing stunted growth and root hair deficiency in oat mutants that accumulate triterpene intermediates in the epidermis (Mylona et al., 2008).

Cell-type specific expression patterns have been demonstrated for other TPSs in *Arabidopsis* roots. For example, the identical genes *TPS23* and *TPS27* encoding 1,8-cineole synthases are mainly expressed in epidermal cells, whereas the related genes *TPS12* and *TPS13* encoding (*Z*)-γ-bisabolene synthases are primarily expressed in the cortex. Interestingly, whereas each gene encoding (*Z*)-γ-bisabolene synthase showed the same cell type-specific pattern of promoter activities, TPS22 and TPS25 produce similar mixtures of multiple sesquiterpene products but have completely different expression profiles. The distinctive expression patterns of *TPS* genes in roots suggest the presence of different cell-type specific layers or gradients of terpenes. It is possible that these chemical profiles contribute to the defense against root attacking organisms with different invasion strategies. For example, triterpene saponins, which highly accumulate in epidermal tissues of oat roots (*Avena* spp.) have been shown to exhibit

potent antifungal activity against *Gaeumannomyces spp. and Fusarium spp.* (Papadopoulou et al., 1999; Haralampidis et al., 2001).

The expression of both *TPS22* and *TPS25* is induced in response to treatment with the defense hormone jasmonic acid although only TPS25 is largely responsible for the corresponding formation of (*E*)-β-farnesene. Additional promoter-GUS studies should reveal to what extent the induced expression of *TPS25* remains cell-specific or occurs in all root tissues. The jasmonate-induced expression of both *TPS* genes suggests that similar responses might occur upon attack by root pathogens that trigger jasmonate defense signaling pathways. In fact, the transcript level of *TPS22* and *TPS25* was found to be elevated about four-fold and two-fold, respectively, by inoculation with the soil-born root pathogen *Pythium irregulare* (data not shown).

**TPS22 and TPS25 are located in mitochondria**

Sesquiterpene synthases typically lack a transit peptide for subcellular targeting and are located in the cytosol (Nieuwenhuizen et al., 2009; Nagegowda, 2010). By contrast, our results demonstrate that the two root specific sesquiterpene synthases, TPS22 and TPS25, carry N-terminal peptides that target the proteins to the mitochondria (Figure 2.6). Interestingly, both enzymes exhibit maximum catalytic activity in the range between pH 7.0 and 9.0 (Figure 2.5), which encompasses the pH of 8 found in mitochondria. It has been suggested that mitochondria represent a natural compartment for the biosynthesis of FPP. A few studies support this suggestion: First, the *Arabidopsis* FPP synthase 1 (FPS1) occurs in two isoforms (FPS1L and S), one of which (FPS1L) is transported into mitochondria (Cunillera et al., 1997). Second, leaves of transgenic *Arabidopsis* produced the sesquiterpene (*E*)-nerolidol when a cytosolic nerolidol

synthase (FaNES1) from cultivated strawberry was targeted to mitochondria (Kappers et al., 2005). Third, a nerolidol synthase (FaNES2) from cultivated strawberry containing an N-terminal targeting peptide was shown to co-locate in mitochondria and plastids (Aharoni et al., 2004). Our results of the subcellular localization of TPS22 and TPS25 and sesquiterpene emission detected in overexpression lines for each gene further demonstrate that mitochondrial FPP can be converted into terpene products.

We observed a relatively low emission rate of the TPS22/25 sesquiterpene product (*E*)-β-farnesene in wild type and overexpression lines. Since both TPS enzymes exhibit catalytic efficiencies similar to those described from other plant TPSs, a limited formation of their sesquiterpene products *in vivo* could be caused by posttranslational modification in the mitochondrial compartment. Alternatively, we cannot entirely exclude that the sesquiterpene hydrocarbon products are further modified to non-volatile compounds although extraction of roots with different organic solvents have not provided evidence for the presence of such products. Even though previous studies with transgenic *Arabidopsis* expressing a nerolidol synthase targeted to mitochondria indicated that the mitochondrial FPP pool appears to be sufficient to support sesquiterpene accumulation, we tested whether exposure of TPS22 and TPS25 to cytosolic FPP concentrations could increase the rate of (*E*)-β-farnesene formation. To this end, we expressed N-terminal-truncated *TPS22* and *TPS25* genes encoding proteins without the targeting sequence in transgenic *Arabidopsis*. However, we failed to find sesquiterpene emissions from these plants even though transcripts of the transgenes (although at lower levels as in the lines shown in Figure 2.11a) were detected (Figure 2.S.2). Mitochondrial proteins typically go through a multitude of processes to activate their function, which include translocation across the mitochondrial membranes and assembly after removal of the transit peptide (Huang et al.,

2009). It is possible that in the absence of a transit peptide and the mitochondrial protein processing machinery the stability and integrity of both TPS proteins is severely affected in the cytosol.

Our findings on the subcellular localization of TPS22 and TPS25 demonstrate that mitochondria represent an additional natural compartment, besides plastids and the cytosol/ER, in the biosynthesis of terpene specialized metabolites. This plasticity in subcellular targeting appears to be prominent in the type-a clade of *Arabidopsis* TPSs, in which potential mitochondrial transit peptides have been predicted for 10 other proteins (Tholl and Lee, 2011). The evolutionary forces driving mitochondrial terpene biosynthesis are yet difficult to understand. Aharoni et al. (2004) reported that a nerolidol synthase (FaNES1) with cytosolic localization was highly expressed in cultivated strawberry, whereas an additional nerolidol synthase (FaNES2) with co-localization in mitochondria and plastids showed very low expression in cultivated strawberry. According to these studies, changes of the subcellular localization of nerolidol synthases from mitochondria/plastids to the cytosol with higher FPP substrate levels caused an increase in fruit aroma during the cultivation of strawberry. Given that TPS22 and TPS25 produce very little sesquiterpene *in vivo*, mitochondrial targeting of these proteins may indicate an event of emerging or declining enzyme function. Alternatively, the mitochondrial location of these enzymes might be a means to partially mute constitutive enzyme activity and reduce terpene concentrations to levels with limited phytotoxic activity.

### *(E)*-β-Farnesene inhibits root growth *in vitro*

We demonstrated that *Arabidopsis* root growth *in vitro* is affected by the presence of volatile sesquiterpenes. (*E*)-β-Farnesene showed an inhibitory effect of approximately 50% at a ten-fold lower concentration (0.3 μM) than the sesquiterpene alcohol (*E*)-nerolidol and the sesquiterpene hydrocarbon (*E*)-β-caryophyllene (Figure 2.12). Phytotoxic or allelopathic activities have been described for many specialized metabolites including terpenes. For example, exposure to α-pinene results in the inhibition of radicle growth of several plant species by disrupting cell membrane integrity hence causing excessive ion leakage in the root cell (Singh et al., 2006). Volatile monoterpenes from *Salvia leucophylla* including eucalyptol, α- and β-pinene, camphene and camphor inhibit cell proliferation in roots of *Brassica campestris* by interfering with organelle and nuclear DNA synthesis within the root apical meristem (Nishida et al., 2005). In addition, sesquiterpenes from various plant species have been shown to exhibit cytotoxic activity against microbes or tumor cells (Wang et al., 2002; Hajdú et al., 2010). Attempts have been made to correlate the bioactivity or perception of terpenes with their diverse structures. For example, a combination of testing the antifeedant activity of terpenoid compounds containing a six-member-ring against the aphid, *Lipaphis erysimi* (Kalt.) with quantitative structure–activity relationship (QSAR) modeling led to the prediction of a possible mechanism of strong electrophilic interactions between terpenoid compounds and the aphid chemoreceptor (Wang et al., 2008). This modeling approach may also help predict possible interactions of volatile terpenes with mitochondrial proteins such as those involved in respiration.

Plants avoid autotoxic effects of specialized metabolites by accumulating and storing them in subcellular compartments such as the vacuole or in specialized cell structures such as

trichomes or by immediately releasing the compound into the environment to minimize internal pools (Pichersky and Gershenzon, 2002; Gopfert et al., 2009). In addition, in many cases, plants circumvent negative effects of specialized metabolites by releasing them only in a short time window under induced conditions. Despite the fact of being a volatile compound, (*E*)-β-farnesene exhibits growth inhibitory effects at surprisingly low concentrations, which are in the range of those observed for root growth inhibition by methyl jasmonate (0.1 μM), the volatile methylated form of jasmonic acid (Staswick et al., 1992). Since *Arabidopsis* does not have glandular trichomes to store terpenes, it seems plausible that (*E*)-β-farnesene is produced at concentrations that are below the threshold of inhibiting root growth. Transgenic approaches including those in *Arabidopsis* have shown consistently that the constitutive formation of terpenes leads to reduced growth or other stress symptoms (Aharoni et al., 2003; Herde et al., 2008).

Conclusively, transgenic plants expressing *TPS22* or *TPS25* did not show such effects because of the low sesquiterpene concentrations produced by the mitochondrial proteins. Both *TPS* genes show only a limited induction in response to treatment with jasmonic acid and the root rot pathogen *Pythium irregulare* (data not shown). By contrast, the formation of (*E*)-β-caryophyllene is strongly induced in maize roots upon herbivore attack, where it aids in attracting insect-parasitizing nematodes (Rasmann et al., 2005). The minimal induced formation of (*E*)-β-farnesene indicates that this compound might be of lower functional significance in induced *Arabidopsis* root defense or that it could be effective at low concentrations.

## Materials and Methods

### Plant material and growth conditions

*Arabidopsis thaliana* wild-type Columbia (Col) and mutants were grown under short day conditions (10-hours-light/14-hours-dark photoperiod in growth room (150 µmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation (PAR), 22 ℃, 55 % relative humidity (RH)). T-DNA insertion mutant lines (Salk_067831, Salk_020266 and Salk_123505) were obtained from the *Arabidopsis* Biological Resource Center stock center (ABRC) and were in the wild-type Columbia (Col) genetic background. Wild-type (Col) and transgenic/mutant plants were cultivated on soilless potting media supplemented with sand (Sunshine Growing Mix No.1: sand, 8:1) for 5 to 6 weeks. Transgenic plants carrying kanamycin or herbicide (basta) selection markers were pre-selected on half strength Murashige and Skoog (Duchefa) plates with 1% sucrose and 50 $\mu$g $mL^{-1}$ kanamycin or 10 $\mu$g $mL^{-1}$ glufosinate ammonium (Basta®/Liberty, AgrEvo Company, Wilmington, DE, USA) prior to being transferred to soil.

Protocols for a hydroponic system developed by Arteca and Arteca (2000) were followed with minor changes. Seeds were germinated on Jiffy peat pellets (Jiffy-7 pellet, Ferry-Morse Seed Company) by placing them into holes of a foam board sheet. Hydroponic plants were grown for four to five weeks under the same light and temperature conditions as described above. All plants were used at the pre-bolting rosette stage.

Axenic plants were maintained as described by Hetu et al (2005) with slight modification. Briefly, *Arabidopsis* seedlings were grown for 7 days on a nylon mesh on MS solid media containing 1% sucrose, then were transferred to MS liquid media containing 2% sucrose and

cultured for 13 days before changing the sucrose concentration to 0% for one day followed by a sucrose concentration of 1% for 2 days prior to treatment with jasmonic acid.

**Reagents and radiochemicals**

Unlabeled GPP, FPP and GGPP were obtained from Echelon Biosciences Incorporated (Salt Lake City, UT, USA). Tritium-labeled FPP ([1-$^{3}$H] FPP (35.2 MBq μmol$^{-1}$) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). All other reagents or solvents were obtained from Fisher Scientific, Sigma-Aldrich, Invitrogen, Promega and Fluka, unless otherwise stated.

**Volatile collection and analysis**

Volatiles from soil-grown plants were collected in 1-L glass jars using the closed-loop stripping method (Donath and Boland, 1995) under stable growth chamber conditions (22 ℃, 55% RH, and 150 μmol $\cdot$ m$^{-2}$ $\cdot$ s$^{-1}$ PAR). Root balls of individual plants were washed under a gentle stream of water and placed in glass jars, and volatile collection was performed for 24 h (10-hours light/14-hours dark) using 5 mg of activated charcoal and eluted with 40 μL of $CH_2Cl_2$ containing 3 ng of 1-bromodecane as an internal standard. The eluted samples were analyzed with a Shimadzu GC/MS QP2010S. Separation was performed on an Rxi-XLB column (Restek) of 30 m x 0.25 mm i.d. x 0.25 μm film thickness. Helium was the carrier gas (1.4 mL min$^{-1}$ flow rate), and a splitless injection mode (injection volume of 1 μL) was applied. A temperature gradient of 5 ℃ min$^{-1}$ from 70 ℃ (hold for 1 min) to 200 ℃ was used.

To collect volatiles from hydroponically grown roots, roots (1g of fresh weight) were detached from plants and were placed in 20 mL GC vials with 1 mL of distilled water containing 10 ng of 1-bromodecane as an internal standard. Root volatiles were adsorbed on a 100 μm polydimethylsiloxane fiber (Supelco) for 30 min at room temperature following incubation at 30 ℃ for 30 min in the headspace of a screw-capped vial (20 mL GC vial). Volatile compounds were desorbed from the fiber at 240 °C (4-min) with a 2:1 split injection and analyzed under the same conditions as described above but with a temperature gradient of 4 ℃ $min^{-1}$ from 40 ℃ (2-min hold) to 220 ℃ followed by a gradient of 5 ℃ $min^{-1}$ from 40 to 220 ℃ and 20 ℃ $min^{-1}$ from 220 to 240 ℃ (2-min hold). All volatile compounds were determined by comparison of their retention times and mass spectra with those of authentic standards with mass spectra in the National Institute of Standards and Technology and Wiley libraries (John Wiley & Sons, Inc., New York, NY).

For absolute quantification of (*E*)-β-farnesene and (*E*)-nerolidol collected by closed-loop stripping, the peak areas of each compound were integrated (total ion mode) and the amounts were calculated based on calibration curves established for each compound. For relative quantification of volatiles, peak areas [for (*E*)-β-bisabolene or (*E*)-β-farnesene] were integrated and normalized against 1-bromodecane.

The enantiomers of (*E*)-nerolidol were separated and identified by GC/MS using a hydrodex-B-3P column (Macherey-Nagel) of 25 m x 0.25 mm i.d. x 0.25 μm film thickness. The separation was operated with helium (1.13 mL $min^{-1}$ flow rate) as the carrier, split injection (2:1), and a temperature setting from 70 ℃ (1-min hold) at 2.3 ℃ $min^{-1}$ to 160 ℃ (10-min hold). Authentic standard (3*S*/3*R*) - (*E*/*Z*)-nerolidol (sigma) were used for identification.

**Genotyping of plant materials**

Homozygous knock-out mutants for *TPS22* and *TPS25* genes in the wild-type Col-0 background carrying a T-DNA insertion were determined in the insertional mutant population (Sessions et al., 2002). Genomic DNA was isolated using the method by Edwards et al., 1991. The T-DNA insertion in homozygous mutants was confirmed by PCR and sequencing of the right and left border PCR products, using the following primers; Salk_067831-LP, ACCTGTAGGATTGCCCATCTC; Salk_067831-RP, TCCTCTCCTCATCTCCTCCTC; Salk_020266-LP, GATGCCTTGCGGTATATTGAC; Salk_020266-RP, TCCCATCTGGACATTTGTTTC; Salk_123505-LP, TCATCCATTTGCTTATCAGCC; Salk_123505-RP, TTTTCTTTAAAGCTTTGGCCC, Left border primer of the T-DNA insertion, GCGTGGACCGCTTGCTGCAACT. In order to obtain plant mutant lines containing T-DNA insertion in both *TPS22* and *TPS25* genes, homozygous mutants of each gene were crossed in a reciprocal manner and analyzed in the self-fertilized $F_2$ for a double mutant genotype.

**Determination of *TPS* gene expressions by semi-quantitative RT-PCR and quantitative RT-PCR**

Total RNA from root or leaf tissues grown in either axenic or hydroponic culture was isolated using Trizol reagent according to the manufacturer's protocol. Two micrograms of total RNA was reverse-transcribed into cDNA by using SuperScript II reverse transcriptase (Invitrogen) in a 20 μL reaction according to the manufacturer's instructions using 50 pmol of an anchored poly (dT) primer [$dT_{20}$], and used for transcript analysis.

Semi-quantitative reverse-transcriptase (RT)-PCR was conducted to observe the presence of the transcript of *TPS22* and *TPS25* in wild-type Columbia and homozygous mutants grown in the axenic culture treated with 100 μM of jasmonic acid. PCRs were performed with 0.1 mM dNTP mixtures, 0.5 units of Taq polymerase (New England Biolabs) and 0.1 μM of the following gene-specific primers: TPS22-forward, ATGGAAGCAGCAAGAATGGG; TPS22-reverse, TCAAAGCGGAAGAGGATGGAAGA; TPS25-forward, ATGGAAGCATCAAAATG TTTTGG; TPS25- reverse, TCAAAGAGGTATTGGATGGAGGA; *Actin8*-forward, ATGAAGATTAAGGTCGTGGCAC; *Actin8*-reverse, GTTTTTATCCGAGTTTGAAGAGGC. PCR conditions were 95 ℃ for 3 min, followed by 28 cycles of 30 s at 95 ℃, 30 s at 58 ℃, and 2 min at 72 ℃. *Actin8* (*act8*) was used as the endogenous control.

The expression of *TPS22* and *TPS25* in response to the treatment with jasmonic acid was monitored by quantitative RT-PCR and SYBR Green assays. Quantitative RT-PCR was performed with a linear range of cDNA (1 ng) in a 96-well plate using the ABI PRISM 7700 Sequence Detection System with SYBR green fluorescent dye. About a 100-bp fragment in the 3' region of the coding sequence were amplified using the following gene-specific primers : *TPS22*-forward, TGTGCAAGACACCGTAGAAGAG; *TPS22*-reverse, ATATAGTCATCAAAGGTTG GAA; *TPS25*-forward, AAATGACATTGTCACGTTCGAG; *TPS25*- reverse, CGCTGCTTCTT TGGTGACACCA; *Ubc*-forward, AGTCCTGCTTGGACGCTTCA and *Ubc*-reverse, AACTGC GACTCAGGGAATCTTC. Quantitative RT-PCRs were conducted in 25 μL of SYBR Green I PCR Master Mix according to the manufacturer's protocol (Applied Biosystems), and conditions, which were as follows: 94 ℃ for 10 min for one cycle, followed by 40 cycles of 94 ℃ for 15 sec, 60 ℃ for 60 sec. Each real-time RT-PCR was run in three or four technical replicates per run. Ubiquitin conjugating enzyme 21 (*Ubc*) was used to normalize expression data. The efficiency of

amplification between target genes (*TPS22* and *TPS25*) and endogenous gene (*Ubc*) was approximately equal by running standard curves for each amplicon utilizing the same sample. For comparative quantification, the Comparative cycle threshold (Ct) method (Applied Biosystems) was performed.

**Heterologous protein expression and purification of TPS22 and TPS25 in *Escherichia coli***

Both full length and N-terminal truncated clones of *TPS22* and *TPS25* were amplified from wild-type Columbia cDNA with the following gene-specific primer pairs: forward-CACCATGGAAGCAGCAAGAATGG, reverse-TCCAAGCGGAAGAGGATGGAAG (full length of TPS22), forward-CACCATGGAAGCATCAAAATGTTTTGG, reverse-TCCAAGAG GTATTGGATGGAGG (full length of TPS25), forward–CACCACTCTTTCCCGGAGATCAA, reverse –TCCAAGCGGAAGAGGATGGAAG (truncated version of TPS22), forward-CACCAACTTCTCTCTCTTTCCTTGC, reverse – TCCAAGAGGTATTGGATGGAGG (truncated version of TPS25). The amplicons were cloned into the bacterial expression vector TOPO-pET102 (Invitrogen). Sequence analysis was confirmed that no errors had been introduced by DNA amplification. Each expression construct was transformed into *E. coli* BL21 DE3 competent cells (Invitrogen). Liquid cultures of transformed cells were grown at 37 ℃ until an optical density at 600 nm of 0.6 was reached. Protein expression was induced by the addition of 1 mM isopropyl-*β*-D-thiogalactopyranoside. After 16-24 h of additional incubation at 16 ℃, cells were harvested by centrifugation and lysed by sonication or freeze-thawing (manufacture's manual) in chilled lysis buffer (20 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.5 mM phenylmethylsulfonyl fluoride 5 mM DTT, and 10% [v/v] glycerol) containing

lysozyme (1 mg $mL^{-1}$). TPS protein was partially purified by affinity chromatography on nickel-nitrilotriacetic acid agarose columns (Invitrogen) according to the manufacturer's protocol. The purified proteins, judged to be > 70% pure by SDS-PAGE gel after Coomassie Brilliant blue staining, were subsequently desalted into TPS assay buffer (10 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, and 10% [v/v] glycerol) using column conditions according to the manufacturer's protocol (Invitrogen). The protein concentration was measured by the Bradford method (Bradford, 1976) and bovine serum albumin (BSA) was used as the calibration standard.

**TPS enzyme assay and kinetic characterization**

For identification of enzymatic reaction products, TPS activity assays were performed in a 500 µL assay buffer (10 mM Tris-Cl, pH 7.5, 20 mM $MgCl_2$, 0.2 mM $NaWO_4$, 0.1 mM NaF, 1 mM dithiothreitol, and 60 µM of GPP, FPP and GGPP) containing partially purified TPS22 and TPS25 proteins (~ 10 µg) and volatile products were adsorbed by solid-phase microextraction (SPME). After incubation for 1 h at 30 ℃ in screw-capped 10-mL glass vials in the presence of the polydimethylsiloxane fiber, enzymatic products were analyzed by GC/MS as described above.

For determination of enzymatic parameters, the assay was performed with [1-$^3$H] FPP (35.2 MBq $\mu mol^{-1}$; American Radiolabeled Chemicals, St. Louis, MO) under the same buffer conditions as described above. The appropriate enzyme concentration (1.3 µg for TPS22 and 4.1 µg for TPS25) and incubation time (20 min) were based on linear reaction velocity. The 50 µL of assay was overlaid with 250 µL of hexane for collection of volatile enzymatic products and incubated at 30 ℃, and then total radioactivity was determined by scintillation counting. Assays were carried out in three replicates, and kinetic parameters ($K_m$ and $V_{max}$ values)

were evaluated by Lineweaver–Burk, Eadie–Hofstee and Hanes plots using the Hyper 1.01 program (J.S. Easterby, University of Liverpool).

For pH optima and co-factor studies, each reaction was conducted with 1 µM or 3 µM [1-$^{3}$H] FPP (for TPS22 and TPS25, respectively) in three replicates under the same buffer conditions as described above. For determining enzyme activities under different pH conditions from pH 4.0 to pH 10, buffers consisting of 10 mM MES, MOPSO, and sodium carbonate were used.

**Complementation analysis**

The open reading frame of TPS22 (1812 bp), TPS25 (1812 bp), Met-41-TPS22 (1686 bp), and Met-25-TPS25 (1734 bp) was amplified by reverse-transcriptase PCR from total RNA extracted from roots using the following primer pairs: forward-ATGGAAGCAGCAAGAATGGG, reverse-TCAAAGCGGAAGAGGATGGAAGA (full length of TPS22), forward-CACCATGGAAGCATCAAAATGTTTTGG, reverse-TCAAAGAGGTAT TGGATGGAGGA (full length of TPS25), forward– ATGACTCTTTCCCGGAGATCAA, reverse –TCAAAGCGGAAGAGGATGGAAGA (truncated version of TPS22), forward-CACCATGAACTTCTCTCTCTTTCCTTGC, reverse –TCAAAGAGGTATTGGATGGAGGA (truncated version of TPS25). The amplicons were cloned into pGEM-T-easy vector (Promega). Using the *Eco*RI restriction enzyme site, the amplicons were ligated into the Gateway entry vector pENTR1A (Invitrogen, Carlsbad, CA), and then recombined into the binary vector pK7WG2 containing the constitutive CaMV 35S promoter (Karimi et al., 2002) according to the manufacturer's protocol (Invitrogen). The full length open reading frame of *TPS25* was cloned into Topo-pENTR (Invitrogen) and then recombined into pK7WG. Sequence analysis of each

clone showed that no errors were introduced and the direction of amplicons was correct. Plant transformation constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and used in floral dipping transformations with homozygous knock-out mutants (salk_067831 and salk_020266) (Zhang et al., 2006). Transformants were screened on half strength MS plates with 1% (w/v) sucrose and 50 μg $mL^{-1}$ kanamycin.

### *TPS22* and *TPS25* promoter-GUS reporter gene fusion constructs

Genomic DNA was extracted from 5 day-old *Arabidopsis* seedlings (ecotype Col) using the method of Edwards et al., 1991. A 1313-bp promoter region of *TPS22* was amplified from genomic DNA by PCR using primer pairs, forward-TTTACATGCTTTCGCACACTAG, reverse- TGCACGACGATGCCATTTAATT. A 1943-bp genomic fragment for the *TPS25* promoter region was isolated from genomic DNA with primer pairs, forward-CTGTGTGACGAATCTCCATGTT, reverse-TTTAAAACAGAGTAGGATGGGG. Promoter fragments of *TPS22* and *TPS25* were cloned into pGEM-T-easy vector (Promega). The promoter regions were then cut from the pGEM-T-easy vector by an *Eco*RI-digest, ligated into the Gateway entry vector pENTR1A (Invitrogen, Carlsbad, CA), and then recombined into the binary vector pKGWFS7 (Karimi, et al. 2002) with Gateway LR Clonase II (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. The constructs were introduced into *A. tumefaciens* strain GV3101 and used in floral dipping transformations with homozygous knock-out mutants (Salk_067831 and Salk_020266) (Zhang et al., 2006). Stably transformed *Arabidopsis* seedlings from $T_2$ generation (at least 5 independent lines for each construct) were analyzed after screening on half strength MS plates with 1% (w/v) sucrose and 50 μg $mL^{-1}$

kanamycin. Histochemical GUS assays were performed as previously described (Jefferson et al. 1987). GUS staining was observed with an Olympus SZX16 microscope.

**Subcellular localization of TPS22 and TPS25 proteins**

The open reading frames of *TPS22* (1812 bp), *TPS25* (1812 bp), Met-41-*TPS22* (1686 bp, N-terminal truncated (NTC)-*TPS22*), and Met-25-*TPS25* (1734 bp, NTC-*TPS25*) were amplified from wild-type Columbia cDNA with the following gene-specific primers: forward-AATGGAAGCAGCAAGAATGGG, reverse-TAAGCGGAAGAGGATGGAAGA (full length of TPS22), forward-AATGGAAGCATCAAAATGTTTTGG, reverse-TAAGAGGTATTGGAT GGAGGA (full length of TPS25), forward– ATGACTCTTTCCCGGAGATCAA, reverse – TAAGCGGAAGAGGATGGAAGA (truncated version of TPS22), forward-CACCATGAACTTCTCTCTCTTTCCTTGC, reverse– TCCAAGAGGTATTGGATGGAGGA (truncated version of TPS25). The coding regions were cloned into the binary vector pK7WG2 containing the constitutive cauliflower mosaic virus (CaMV*)* 35S promoter and C-terminal enhanced GFP (eGFP) fusion as describe above (Karimi et al., 2002). The stop codons were removed to produce a continuous open reading frame with eGFP fused to the C-terminus of proteins. Fully sequenced constructs that showed no errors and were in-frame with eGFP were introduced into *A. tumefaciens* GV3101 and used in the floral dipping method with homozygous knock-out mutants (salk_067831 and salk_123505) (Zhang et al., 2006). Transformants were screened on half strength MS plates with 1% (w/v) sucrose and 50 μg $mL^{-1}$ of kanamycin.

Two-week-old *Arabidopsis* seedlings of at least three independent lines from each construct were used for observation of eGFP fluorescence. Stably transformed *Arabidopsis*

seedlings in the $T_1$ or $T_2$ generation demonstrating four constructs (35S::TPS22::eGFP, 35S::TPS25::eGFP, 35S::NTC-TPS22::eGFP, and 35S::NTC-TPS25::eGFP) were grown on half strength MS media containing 1% sucrose and 50 μg $mL^{-1}$ of kanamycin. Root samples were mounted on a microscope slide with distilled water and visualized using a Zeiss Axiovert 200 inverted fluorescence microscope with FITC (*l*ex = 480 nm; *l*em = 535 nm), Texas Red (*l*ex = 570 nm; *l*em = 625 nm) fluorescent filter sets, an attached MRc5 Axiocamcolor digital camera, and an LD Achroplan 40X objective.

### *In vitro* plant root growth analysis

*Arabidopsis* wild-type Columbia was grown for three weeks on half strength MS plates containing different concentrations of chemical compounds ((*E*)-β-farnesene, (*E*)-nerolidol, (*E*)-caryophyllene). The plants were grown vertically in a growth chamber under the same conditions as described above. Root growth was determined three weeks after planting.

## Supplemental Materials

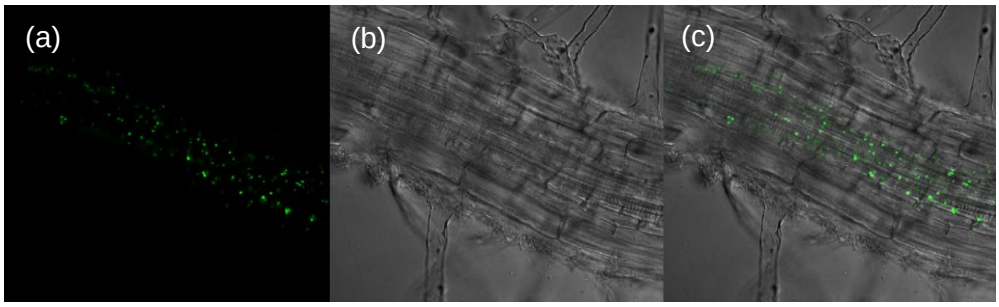


**Figure 2.S.1 Subcellular localization of plastidial ferredoxin NADP(H) oxidoreductase-eGFP fusion protein** (Marques et al., 2004) **in *Arabidopsis* roots.** Enhanced green fluorescence images from roots of *Arabidopsis* transformed with the ferredoxin NADP(H) oxidoreductase-eGFP construct are shown. Confocal images from the root are as follows: fluorescent image (a), bright-field image (b), and overlay image (c).

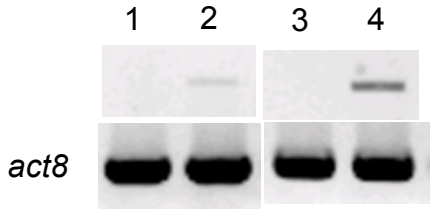


**Figure 2.S.2 Gene transcript analysis from leaves of transgenic plants transformed with cytosolic *TPS22* and *TPS25*.** *Actin8* (*act8*) was used as a control for endogenous gene expression. Cytosolic TPS22 and 25 constructs were produced by truncation of the N-terminus of TPS22 and 25 designated as Met-41-TPS22 and Met-25-TPS25. 1, empty vector control/*tps22-1*; 2, 35S::*Met-41-TPS22*/*tps22-1*; 3, empty vector control/*tps25-2*; 4, 35S::*Met-25-TPS25*/*tps25-2*.

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# CHAPTER III

## The Volatile $C_{11}$-Homoterpene DMNT Synthesized from the Triterpene Arabidiol is Emitted from *Arabidopsis* Roots in a Defense Response against *Pythium irregulare*

(Targeted for publication in **Plant Journal or Plant Cell**)

**Other Contributors:**

Reza Sohrabi, PhD candidate, identified the DMNT biosynthetic enzymatic steps and performed screening of CypP450 candidate genes, yeast co-expression assay, and the identification and characterization of CYP705A1 (salk_043195) and AtPEN1 (salk_018285 and salk_067736) T-DNA insertion lines.

Samuel Shon, undergraduate research assistant, helped with *in vitro* oospore germination rate assay

Brendan Karlstrand, undergraduate research assistant, assisted with the production and isolation of oospores.

## Abstract

Nearly all land plants emit volatile compounds, which have important functions in the interaction with insects and microbes. Recent studies have indicated that volatile terpenes released from plant roots play a role in belowground plant defense. We have used *Arabidopsis* as a model plant to further study the defensive activities and formation of terpene volatiles in interaction with the root-rot pathogen *Pythium irregulare*. Infection of *Arabidopsis* roots with *P. irregulare* causes a transient emission of the volatile $C_{11}$-homoterpene ($C_4$-norterpene) DMNT (4,8-dimethylnona-1,3,7-triene), which is normally emitted in stress-induced aboveground defense. The induced response depends on the jasmonate signaling pathway but is independent of ethylene and salicylic acid. In contrast to the biosynthesis of DMNT from (*E*)-nerolidol in plant leaves, *Arabidopsis* roots produce DMNT via a novel pathway by oxidative degradation of the root-specific $C_{30}$-triterpene alcohol arabidiol catalyzed by the cytochrome P450 monooxygenase CYP705A1. The *CYP705A1* gene is co-expressed and clusters on chromosome 4 with the gene coding for arabidiol synthase *AtPEN1*. A putative role of DMNT in belowground plant protection against soil-borne pathogens is supported by our results that DMNT reduces *P. irregulare* oospore germination rates and retards *Pythium* growth *in vitro*. Moreover, loss-of-function mutants that are impaired in the biosynthesis of arabidiol and/or DMNT showed more symptom severity and higher oospore counts than wild-type upon *P. irregulare* infection.

## Introduction

Plants release volatile organic compounds from flowers, leaves and roots in response to numerous pathogenic organisms and herbivores (Dudareva et al., 2006). These volatiles, especially volatile terpenes, have been shown to be involved in plant defense against herbivorous insects either by directly repelling or indirectly attracting predators of attacking herbivores (Turlings et al., 1995; Kessler and Baldwin, 2001; Pichersky and Gershenzon, 2002). In addition, volatile terpenes extracted from a wide variety of plants have been assessed for their antimicrobial properties (Dorman and Deans, 2000; Hammer et al., 2003).

The contribution of volatile compounds in belowground plant defense is by far less well understood. Studies on the defense of maize plants against root herbivores have shown that volatile terpenes released from roots in response to insect feeding can attract insect parasitizing nematodes (Rasmann et al., 2005). Besides attracting beneficial organisms by diffusion of root-specific volatiles into the soil, volatile compounds may also exhibit more direct, short range defensive activities against insect and microbes in the rhizosphere. Studies on *Arabidopsis* hairy roots demonstrated that treatment with the bacterial pathogen *Pseudomonas syringae* causes a rapid release of 1,8-cineole, a monoterpene with antimicrobial activity, against bacteria and fungi (Kalemba et al., 2002; Steeghs et al., 2004; Ben Marzoug et al., 2011). Despite these findings and a growing interest in understanding the interaction of plants with root pathogens such as filamentous oomycetes (Attard et al., 2010), knowledge of chemical defense mechanisms and the function of volatiles is extremely limited.

Among the most common plant volatile compounds released upon herbivory or microbial infection are the irregular volatile homoterpenes ($C_4$-norterpenes) DMNT [(3*E*)-4,8-dimethyl-

l,3,7-nonatriene] and TMTT [(3*E*,7*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene] (Tholl et al., 2011). *Arabidopsis* rosette leaves emit TMTT together with other volatiles in an induced response to insect feeding and the volatile blend aids in attracting insect parasitoids (van Loon et al., 2000; Van Poecke et al., 2001; Herde et al., 2008). The role of homoterpenes in indirect defense has also been supported by studies with transgenic *Arabidopsis* and other plant-herbivore systems (Dicke et al., 1990; De Boer et al., 2004; Kappers et al., 2005). TMTT is also released following infection of *Arabidopsis* with *P*. syringae, although its role in this response is not well understood (Attaran et al., 2008). Furthermore, homoterpenes have been implicated in the mediation of plant-plant interactions as described in lima bean (Arimura et al., 2000).

The biosynthesis of homoterpenes in aboveground tissues proceeds in two steps: First, the conventional prenyl diphosphate intermediates and terpene precursors, (*E*,*E*)-farnesyl diphosphate (FPP) and all-*trans* geranylgeranyl diphosphate (GGPP), are converted by enzymes of the terpene synthase family into the tertiary sesquiterpene alcohol (*E*)-nerolidol (Aharoni et al., 2004; Nagegowda et al., 2008) and the diterpene alcohol (*E*,*E*)-geranyllinalool, respectively (Ament et al., 2006; Herde et al., 2008). In the second step, the alcohol intermediate is degraded by an oxidative carbon-carbon cleavage reaction into the homoterpene end product (Boland et al., 1998). In *Arabidopsis*, the formation of (*E*,*E*)-geranyllinalool and its conversion into TMTT are catalyzed by the terpene synthase TPS04 and the cytochrome P450 monooxygenase CYP82G1, respectively (Herde et al., 2008; Lee et al., 2010). In contrast to other volatile terpenes, homoterpenes have only been reported to be produced in photosynthetically active tissues but not from plant roots.

Here we report that DMNT is emitted from *Arabidopsis* roots upon infection with the soil-born pathogen oomycete, *Pythium irregulare,* and upon treatment with the defense hormone

jasmonic acid (JA). *Pythium* is regarded as a soil-borne vascular pathogen causing seedling damping-off and root rot disease in various plant species. Unlike the biosynthesis of DMNT in leaves, our study shows that in *Arabidopsis* roots this compound is derived from the oxidative degradation of the root-specific $C_{30}$-triterpene, arabidiol. The reaction is catalyzed by the cytochrome P450 monooxygenase enzyme CYP705A1, whose encoding gene is co-expressed with the arabidiol synthase gene *AtPEN1*. We further demonstrate that DMNT inhibits oospore germination and retards growth of *P. irregulare in vitro*. We discuss a role of DMNT and arabidiol breakdown products in belowground plant defense against the soil-borne pathogen *P. irregulare* based on examining the susceptibility of DMNT biosynthetic mutants.

## Results

### Volatile emission in *Arabidopsis* roots treated with *Pythium irregulare* 110305 and jasmonic acid

To investigate whether *Arabidopsis* roots release volatile compounds when infected by a soil-borne root pathogen, plants were grown in axenic or hydroponic culture, and challenged with a uniform suspension of the oomycete root rot pathogen *P. irregulare* isolate 110305. Root tissue was detached at different time points after inoculation and root volatiles were analyzed by solid phase microextraction gas chromatography-mass spectrometry (SPME-GC/MS). We found that the emission of the $C_{11}$-homoterpene DMNT was transiently induced in response to *P. irregulare* 110305 treatment of axenically grown roots (Figure 3.1). Emission of DMNT was highest 3 h after inoculation with a 7-fold increase over constitutive background levels and then

decreased over the following 10 to 20 h (Figure 3.1). In addition to DMNT, we detected small amounts of the monoterpene 1,8-cineole, the recently reported volatile diterpene rhizathalene (Vaughan et al., submitted), and other aliphatics such as dodecane, but the emission of these compounds remained unchanged upon infection. The transient emission of DMNT was also observed in roots grown under non-sterile, hydroponic conditions (Figure 3.2 b).

Since *Pythium* produces elicitors such as lytic enzymes and phytotoxins (Brandenburg, 1950; Deacon, 1979), we tested the effect of a soluble *Pythium* filtrate on DMNT emission. Furthermore, we examined whether treatment with *Escherichia coli*, a non-plant pathogen, could cause induction of DMNT emission. As a result, transient DMNT emission was observed only following inoculation with a suspension of *P. irregulare* containing mycelium and oospores and neither inoculation with the soluble filtrate nor treatment with an *E. coli* suspension could mimic this response (Figure 3.2 a).

The importance of the defense hormone, jasmonic acid (JA) in plant defense against *Pythium* has been reported previously (Vijayan et al., 1998). To further understand the role of JA in DMNT formation in *Arabidopsis* roots, the axenically- and hydroponically-grown *Arabidopsis* were treated with 100 μM JA. The root was excised and emission of root volatiles was measured by SPME-GC/MS. DMNT was strongly induced 24 h after inoculation in both axenically- and hydroponically-grown roots (Figure 3.2 c, d).

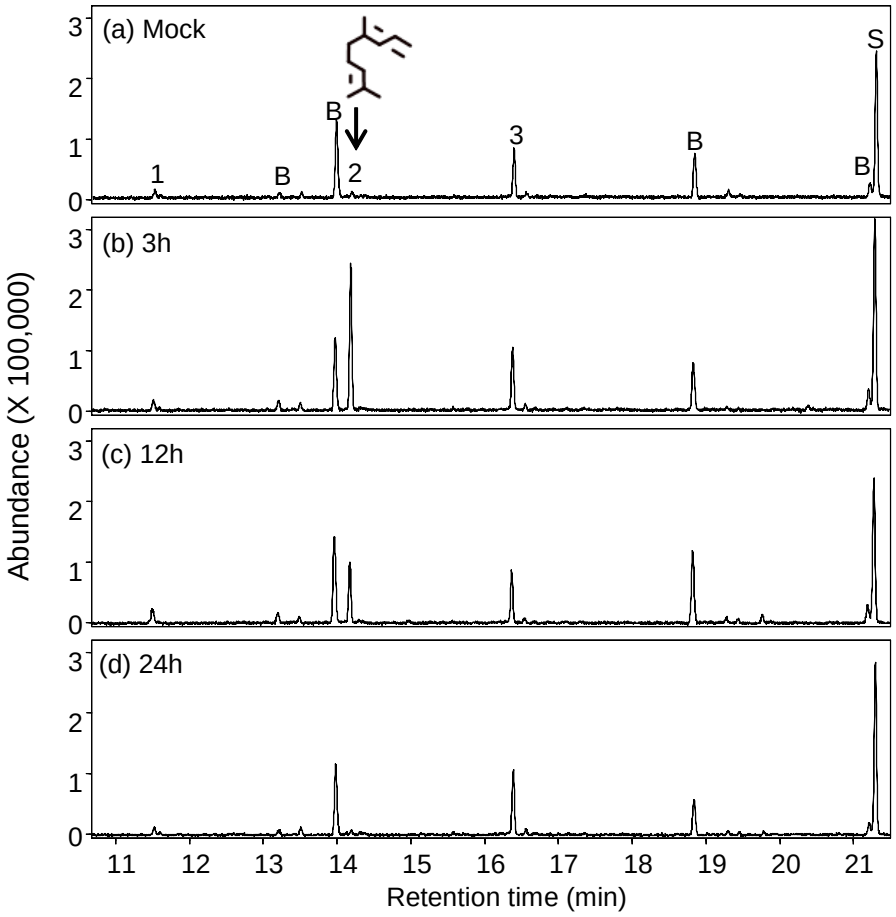


**Figure 3.1 GC-MS analysis of volatiles emitted from *Arabidopsis* roots challenged with *Pythium irregulare* 110305.** Three-week-old *Arabidopsis* grown in axenic culture were treated with a uniform *Pythium* suspension. Root volatiles were analyzed by SPME-GC/MS. Total ion GC/MS chromatograms are shown. 1-Bromodecane was used as the internal standard. 1, 1,8-cineole; 2, DMNT ((*E*)-4,8-dimethyl-1,3,7-nonatriene); 3, dodecane; B, Background; S, standard (1-bromodecane).

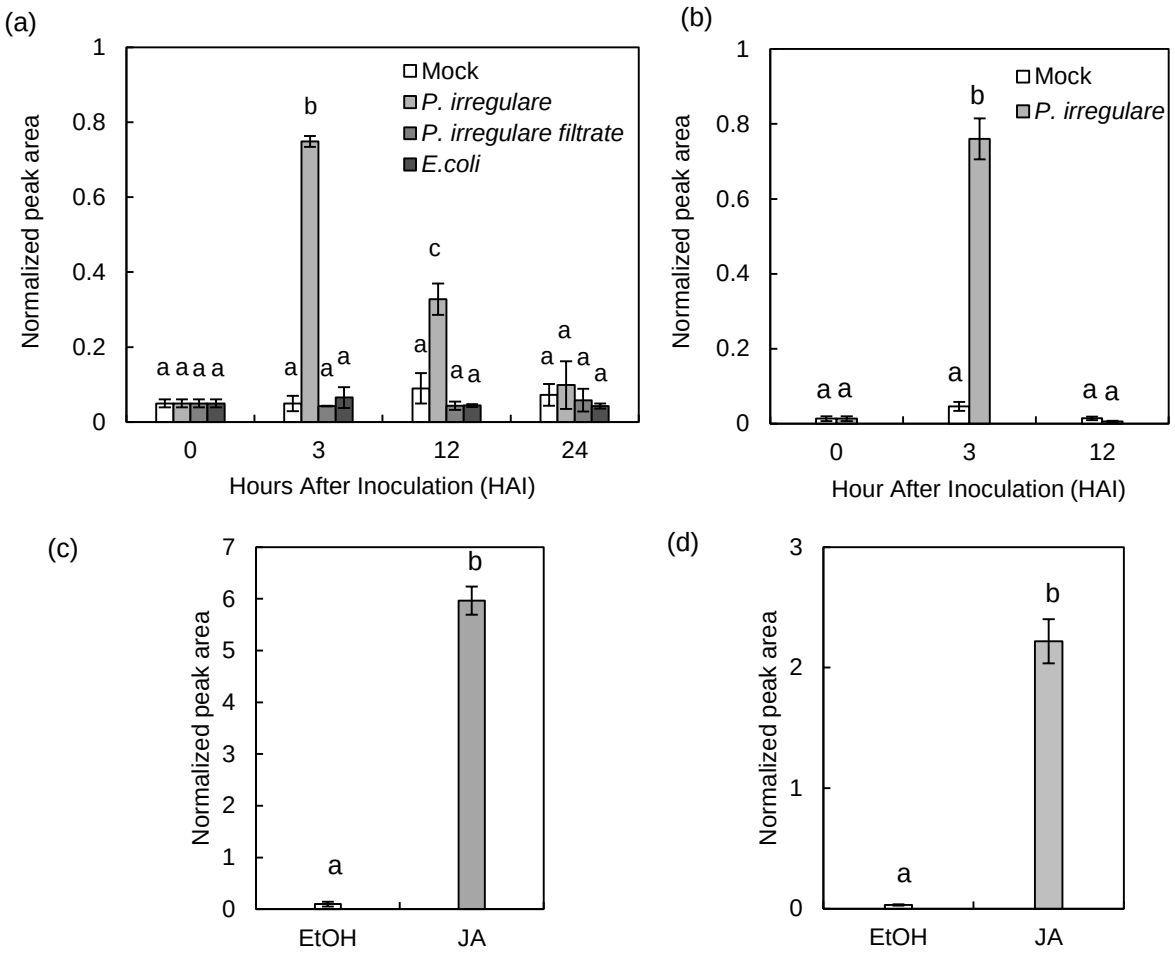


**Figure 3.2 Quantitative analysis of DMNT emission in *Arabidopsis* roots infected with *P. irregulare*.** Axenically (a, c) and hydroponically (b, d) grown *Arabidopsis* roots were infected with *P. irregulare* (a, b) or treated with 100 µM JA (c, d), and root volatiles were analyzed at different time points (3 h, 12 h and 24 h for *Pythium,* 24 h for JA) by SPME-GC/MS. Normalized peak areas are shown. Values represent the mean ± standard error mean (SEM) of three biological replicates. The experiment was repeated at least two times with similar results. P < 0.001 (a) and P<0.05 (b), One-way ANOVA, Tukey-Kramer HSD test for all comparisons of mock and treatments, P<0.01 (c,d), Student t-test.

**Microscopy analysis of *Arabidopsis* root infection by *P. irregulare***

To characterize the infection process of *P. irregulare* on *Arabidopsis* roots in correlation with the time course of induced DMNT emission, *Pythium* colonization of *Arabidopsis* root

tissues was monitored by lactophenol-trypan blue staining. Inoculation occurred with a suspension of ground hyphae and oospores of a 7 day-old *Pythium* colony. It should be mentioned that *P. irregulare* 110305 propagates by oospore but not zoospore formation as tested on *Arabidopsis* and spring wheat as its natural host. Oospores were attached to the surface of roots 1 h after inoculation (Figure 3.3 a) and germinated after 3 h when highest DMNT emission was observed (Figure 3.3 b). Furthermore, fragmented hyphae attached and formed appressoria on root epidermal tissues 3 h after inoculation. Hyphal growth inside root endodermis/vascular tissues was observed 24 h and 48 h post inoculations (Figure 3.3 c, d). Together the results demonstrate that *Pythium*-induced DMNT emission occurs at an early stage of colonization of the root tissue.

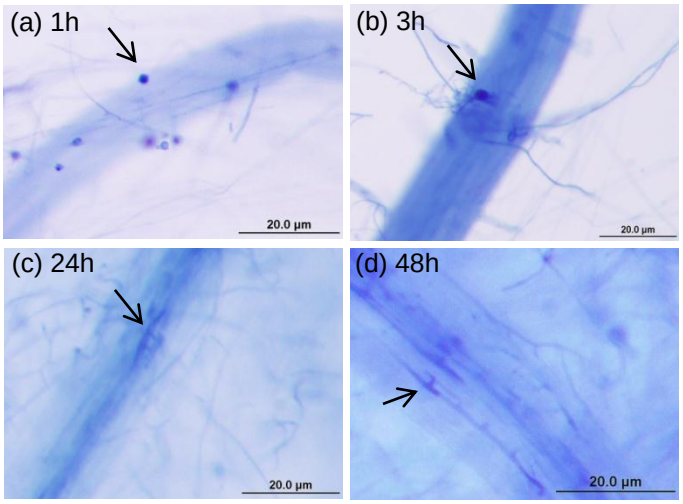


**Figure 3.3 Infection of *Arabidopsis* roots with *P. irregulare*.** Five-day-old *Arabidopsis* seedlings grown in ½ X MS liquid media were used to monitor the infection process. A *P. irregulare* colony grown on potato dextrose agar (PDA) was used for infection. For each time point, approximately ten seedlings were observed after staining with lactophenol-trypan blue. Arrows indicate oospores attached to roots (a, b) and *Pythium* hyphal growth inside roots (c, d).

**Analysis of the role of JA, salicylic acid, and ethylene in *Pythium*-induced DMNT emission**

Previous studies on the regulation of plant defense mechanisms against *Pythium* have shown that the phytohormones JA, abscisic acid (ABA), salicylic acid (SA), and ethylene (ET) are involved in resistance against *P. irregulare* (Staswick et al., 1998; Vijayan et al., 1998; Coego et al., 2005; Adie et al., 2007). In particular, transcriptome profile analysis of *Pythium*-infected *Arabidopsis* seedlings and susceptibility measurements of JA-signaling pathway mutants to *Pythium* demonstrated a primary role of the JA-signaling pathway in the defense of *Arabidopsis* against *Pythium* (Staswick et al., 1998; Vijayan et al., 1998).

To determine the function of JA and other hormonal regulators in the induced formation of DMNT, we observed DMNT emission from roots of wild-type plants and several signaling mutants including the JA-deficient mutant, *dde2-2* (*aos*) (von Malek et al., 2002), the ET-insensitive mutant, *ein2-1* (Guzman and Ecker, 1990), and the SA-deficient mutant, *sid2-1* (Wildermuth et al., 2001). DMNT emission was detected at similar levels in *Pythium*- and mock-treated roots of wild-type, and the *ein2-1* and *sid2-1* mutants (Figure 3.4), but no DMNT emission was found in *Pythium*-inoculated *dde2-2* plants. Interestingly, *Pythium* treatment of *dde2-2* mutants suppressed DMNT formation below constitutive levels of mock-treated plants indicating down-regulation of the biosynthetic steps in the formation of DMNT or its metabolic precursors. The results demonstrate that, among the hormones tested in this study, *Pythium*-induced DMNT emission is mainly regulated by the JA signaling pathway.

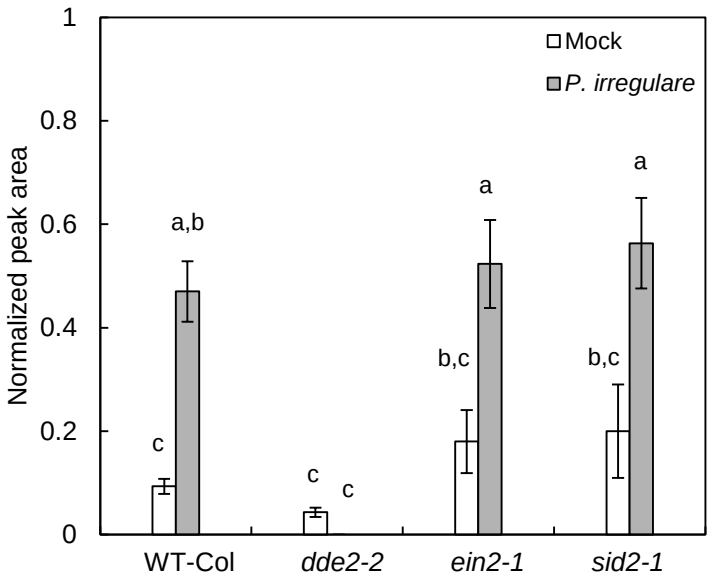


**Figure 3.4 DMNT emission in wild-type (Col-0) and JA-, ET-, and SA-biosynthesis or signaling mutants *dde2-2*, *ein2-1*, and *sid2*-1, respectively, 3 h after infection of roots with *P. irregulare*.** *Arabidopsis* was grown in axenic culture for three weeks prior to infection. Normalized peak areas are shown for DMNT emission. The values represent the mean ± SEM of three biological replicates. The experiment was repeated at two times with similar results for similar inoculum. $P < 0.001$, Tukey-Kramer HSD test for all comparison of mock and treatments.

**Identification of the biosynthetic steps in DMNT biosynthesis**

Previous studies on the formation of TMTT in *Arabidopsis* leaves elucidated a two-step biosynthetic pathway consisting of the formation of the tertiary alcohol (*E*,*E*)-geranyllinalool and its breakdown into TMTT catalyzed by the P450 CYP82G1 (Lee et al., 2010). We therefore assumed that DMNT found in roots could be produced by a similar P450 activity from the sesquiterpene alcohol (*E*)-nerolidol. Volatile analysis of gene knockout lines of several root-specific terpene synthases including TPS22 and TPS25, both of which produce (*E*)-nerolidol as a side product, did not identify *TPS* gene candidates that were specifically involved in DMNT biosynthesis. Therefore, we focused our analysis on screening P450 genes responsible for the

second step in DMNT formation. In order to narrow down the list of P450 candidates, a comprehensive analysis of publically available microarray data was conducted using genevestigator (https://www.genevestigator.com/gv/). We searched for induced expression of P450 genes under all experimental conditions reported to induce JA biosynthesis including treatment with methyl jasmonate (MeJA) and wounding, assuming that expression of the target P450 gene was induced in roots under these conditions. This approach excluded *CYP82G1* and resulted in a list of six candidate genes. We then conducted a comparative RT-PCR analysis of transcripts of the selected genes with and without JA-treatment in roots of wild type plants and the JA-insensitive mutant *coi1-1* (Figure 3.5).

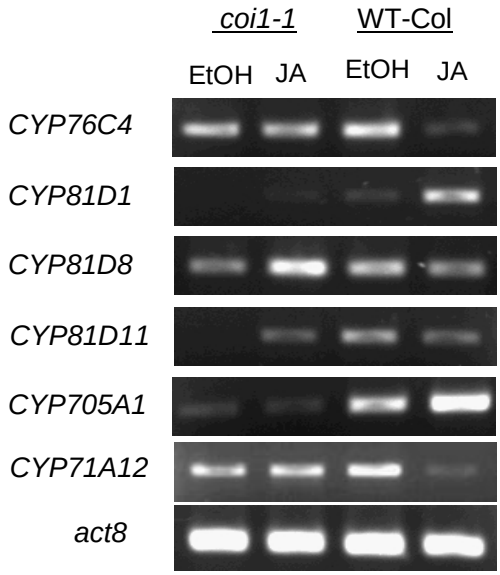


**Figure 3.5 Transcript analysis of P450 candidate genes using semi-quantitative RT-PCR.** Total RNA was extracted from roots of hydroponically grown *Arabidopsis* wild type and *coi1-1* plants, the latter of which showed no JA-induced DMNT formation. Semi-quantitative RT-PCR was performed using gene specific primers. Actin8 (*act8*) was used as a control for endogenous gene expression.

As a result, two P450 genes, *CYP705A1* and *CYP81D1*, were found, whose transcripts accumulated upon JA-treatment in wild type but not *coi1-1* roots. A following SPME-GC/MS analysis of volatiles emitted from roots of T-DNA insertion lines of each gene showed that only the *cyp705a1* knockout line (salk_043195) lacked emission of DMNT upon JA-treatment (Figure 3.6). This finding suggested that *CYP705A1* is involved in the final step of DMNT biosynthesis in *Arabidopsis* roots.

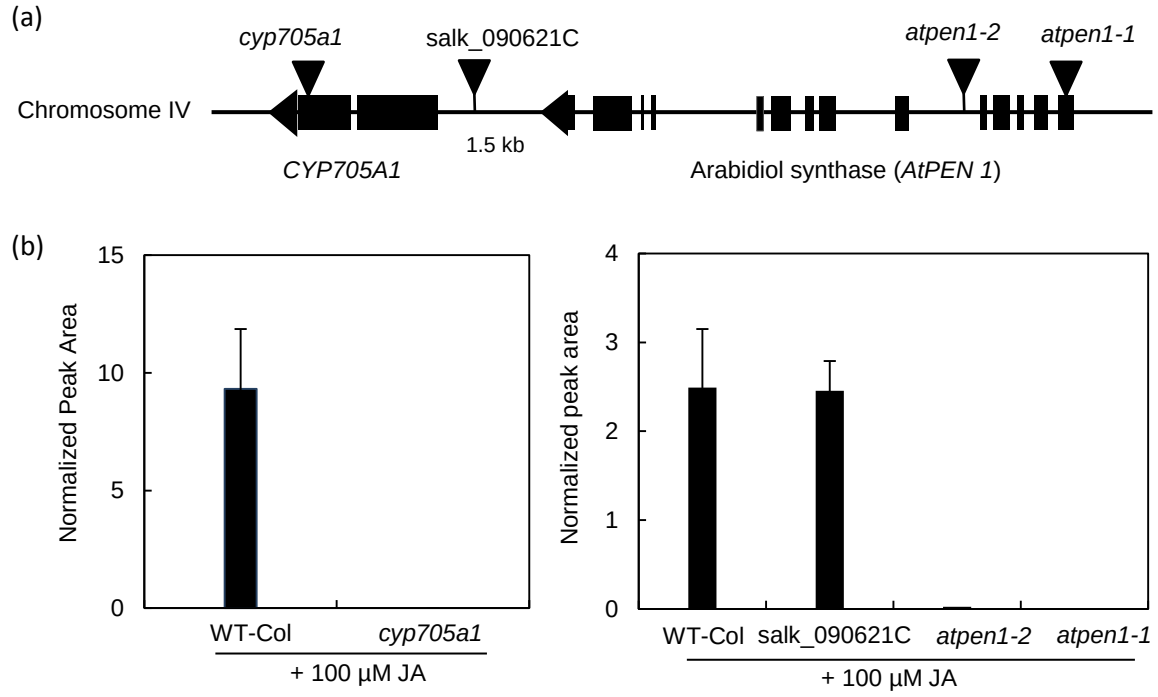


**Figure 3. 6 Quantitative analysis of DMNT emission in wild-type and DMNT biosynthesis mutants (*cyp705a1-1*, salk_090621C, *atpen1-1*, and *atpen1-2*).** (a) Schematic genomic structures of *Arabidopsis CYP705A1* (At4g15330) and *AtPEN1* (Arabidiol synthase, At4g15340). Black boxes indicate exons, while the thin lines in between represent introns. The insertion sites of the T-DNA mutants used in this study are marked with black inverted triangles. They are *cyp705a1-1*, Salk insertion line_043195; salk_090621C, Salk insertion line_090621C; *atpen1-2*, Salk insertion line_067736C; *atpen1-1*, Salk insertion line_018285. (b) DMNT emission in roots of wild-type and DMNT biosynthetic mutants. Volatiles were collected from roots 24 h after treatment with 100 µM jasmonic acid and analyzed by SPME-GC/MS. Normalized peak areas are shown and the values represent the mean ± SEM of three biological replicates.

Since P450s are often co-expressed with genes of the same biosynthetic pathway (Field and Osbourn, 2008; Lee et al., 2010), we took an *in silico* gene co-expression approach using the ATTED II database (http://atted.jp/) to identify genes that may be responsible for the formation of the DMNT precursor. According to this analysis, the gene *AtPEN1* encoding an arabidiol triterpene synthase (Xiang et al., 2006) was highly co-expressed with the *CYP705A1* gene. Moreover, both genes clustered in tandem on chromosome 4 strongly suggesting a possible contribution to the same biosynthetic pathway (Figure 3.7). Arabidiol has a 6,6,5-tricyclic ring sy stem with two hydroxyl groups, whose tricyclic backbone is covalently linked to a $C_{13}$-prenylalcohol side chain (Figure 3.7). We predicted that the tertiary hydroxyl group of this side chain made arabidiol a suitable substrate for oxidative degradation by a P450 enzyme to produce a compound resembling DMNT and a second non-volatile degradation product. To test this hypothesis, we co-expressed the *CYP705A1* and *AtPEN1* genes in yeast WAT11 cells according to previous approaches studying triterpene modifying P450 genes (Seki et al., 2008). *CYP705A1* was expressed under a galactose-inducible promoter while *AtPEN1* was expressed constitutively under control of the constitutive *ADH1* promoter. In addition, we prepared a transgenic strain expressing a mutated form of the *CYP705A1* gene lacking 72 nucleotides from the first exon together with *AtPEN1* to control for any possible non-specific conversion of arabidiol to DMNT. Volatile headspace analysis of 4 ml of the respective yeast cultures demonstrated that DMNT was detected only after induction with galactose and when a functional copy of both genes, *CYP705A1* and *AtPEN*1, was expressed (Figure 3.8). Therefore, both *CYP705A1* and *AtPEN1* are necessary and sufficient for the biosynthesis of DMNT. Subsequent extraction of the DMNT-producing yeast cultures with organic solvent, compound purification, and NMR analysis

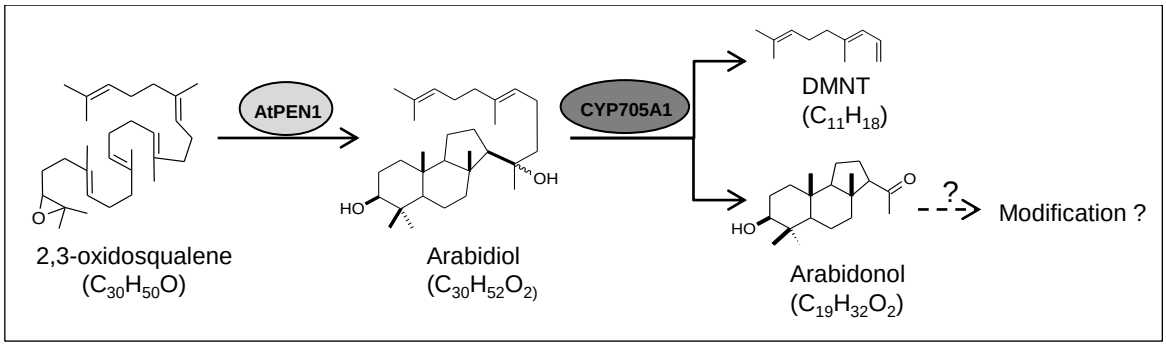


**Figure 3.7 Biosynthetic pathway for the formation of DMNT via degradation of the triterpene arabidiol in *Arabidopsis* roots.**

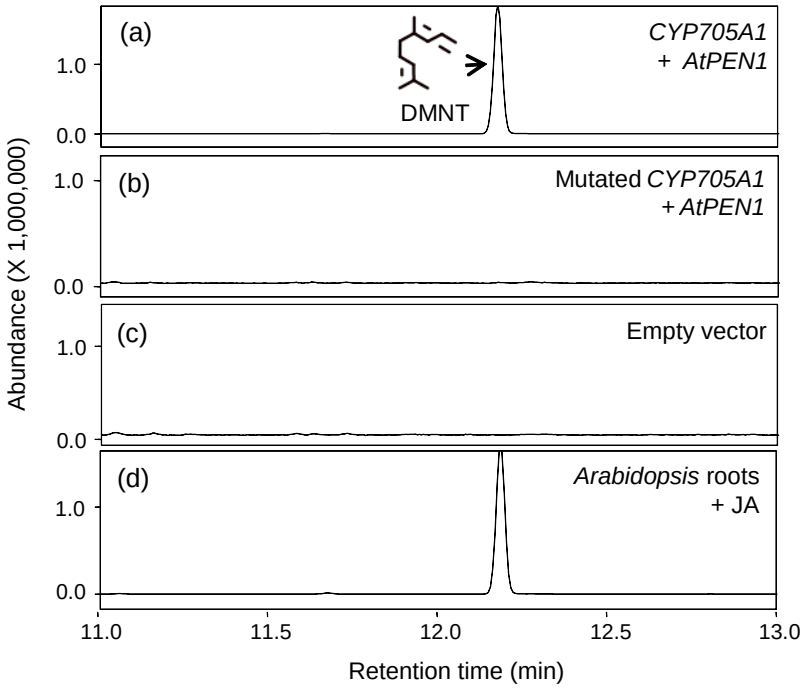


**Figure 3.8 Co-expression of CYP705A1 and AtPEN1 in WAT11 yeast cells.** Volatile enzymatic products were analyzed by SPME-GC/MS from yeast cultures co-transformed with the *AtPEN1* gene and the *CYP705A1* wild type or mutated gene. Expression of CYP705A1 was induced by galactose (2%) and volatiles were collected in the headspace of the 4 ml culture. Selected 69 *m*/*z* ion GC/MS chromatograms of DMNT are shown. (a) DMNT emitted from yeast co-expressing the recombinant *CYP705A1* and *AtPEN1* genes. (b, c) Volatile profiles of yeast carrying the mutated *CYP705A1* gene (b) or the empty galactose inducible expression vector (c). (d) DMNT emission from *Arabidopsis* roots treated with 100 μM JA.

revealed that the non-volatile breakdown product of arabidiol was a $C_{19}$-triterpene ketone, which we named arabidonol (mass spectra and NMR data not shown).

Next, we investigated whether T-DNA insertion lines with loss of function of the *AtPEN1* gene would not produce DMNT. Since the expression of *AtPEN1* gene was induced by treatment with 100 µM jasmonic acid (data not shown), the volatile was observed in JA-treated roots. SPME-GC/MS analysis of root volatiles from the two *atpen1* insertion mutants, salk_018285 (*atpen1-1*) and salk_067736 (*atpen1-2*), both of which lacked a full length *AtPEN1* transcript, showed no DMNT emission upon JA-treatment (Figure 3. 6). This finding demonstrated the role of arabidiol as a precursor for DMNT formation *in vivo*.

**Transcript analysis of *AtPEN1* and *CYP705A1* genes in *Pythium*-infected *Arabidopsis* roots**

To understand whether the biosynthesis of DMNT is regulated by the expression of *AtPEN1*and *CYP705A1*, transcript levels of both genes were monitored by semi-quantitative RT-PCR in axenically grown roots during the early stage of *Pythium* infection (1, 3, 12 h post-inoculation) (Figure 3.9). Constitutive levels of transcript for both genes were detectable in mock treated roots. While no changes in the transcript levels of *AtPEN1* were found within the observed time period, expression of *CYP705A1* was slightly induced one hour after inoculation prior its decline to constitutive levels. Since this transient increase of *CYP705A1* mRNA precedes the emission of DMNT, we conclude that the *Pythium*-induced formation of DMNT appears to be, at least in part, regulated by expression of *CYP705A1* but not *AtPEN1*.

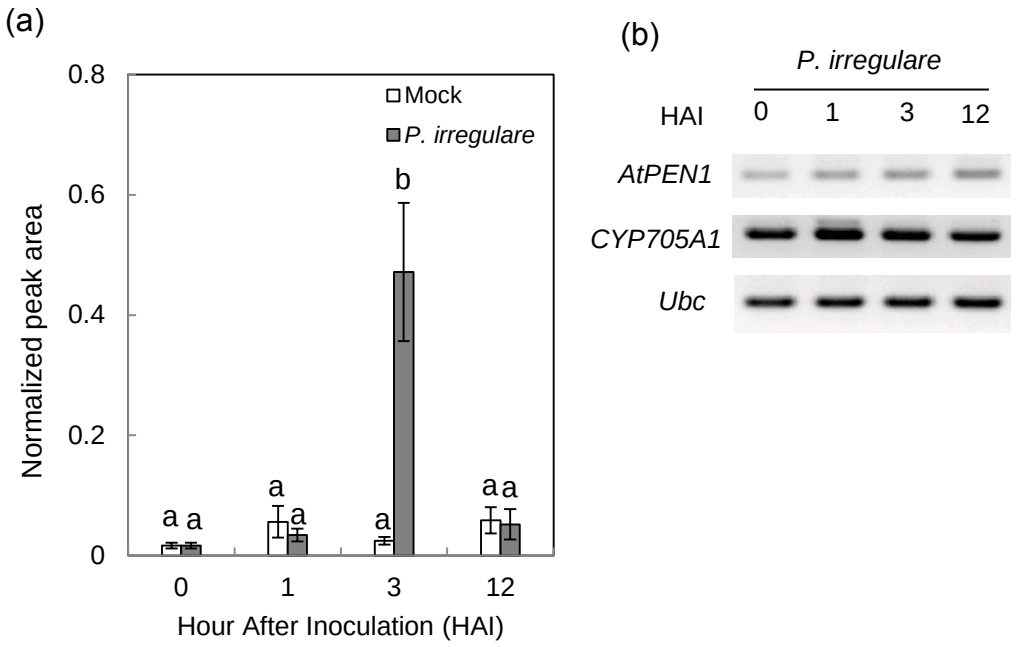


**Figure 3.9 DMNT emission and expression of *CYP705A1* and *AtPEN1* in *Arabidopsis* roots at the early stage of infection with *P. irregulare.*** (a) Quantitative analysis of DMNT emission in *Arabidopsis* roots. Three-week-old *Arabidopsis* was grown in axenic culture and infected with *Pythium*. Root volatiles were analyzed by SPME-GC/MS. Normalized peak areas are shown for DMNT emission. Values represent the mean ± SEM of three biological replicates. $P<0.05$, Tukey-Kramer HSD test. (b) Semi-quantitative RT-PCR analysis of *AtPEN1* and *CYP705A1* in axenically grown roots challenged with *P. irregulare*. Ubiquitin conjugating enzyme (*Ubc*) was used as a control for endogenous gene expression.

**The effect of DMNT, arabidiol and arabidonol on *Pythium* mycelium growth *in vitro***

We investigated whether arabidiol and its breakdown products, arabidonol and DMNT, are involved in the chemical defense against *Pythium* by observing the effect of the three compounds on the *in vitro* growth of *Pythium* mycelium. Inhibitory effects of each compound were measured by comparing the growth area of *Pythium* mycelium on potato dextrose agar (PDA) with and without the compound, including distilled water and organic solvent (ethanol, DMSO) controls. Of the compounds tested, arabidiol and arabidonol showed no effect. However,

*Pythium* growth was reduced by approximately 60% in the presence of 13 nM DMNT (Figure 3.10). A 10-fold higher DMNT concentration caused only a small further decrease in *Pythium* growth and may indicate dose-specific effects. Based on these results, we concluded that DMNT can retard *Pythium* growth *in vitro*.

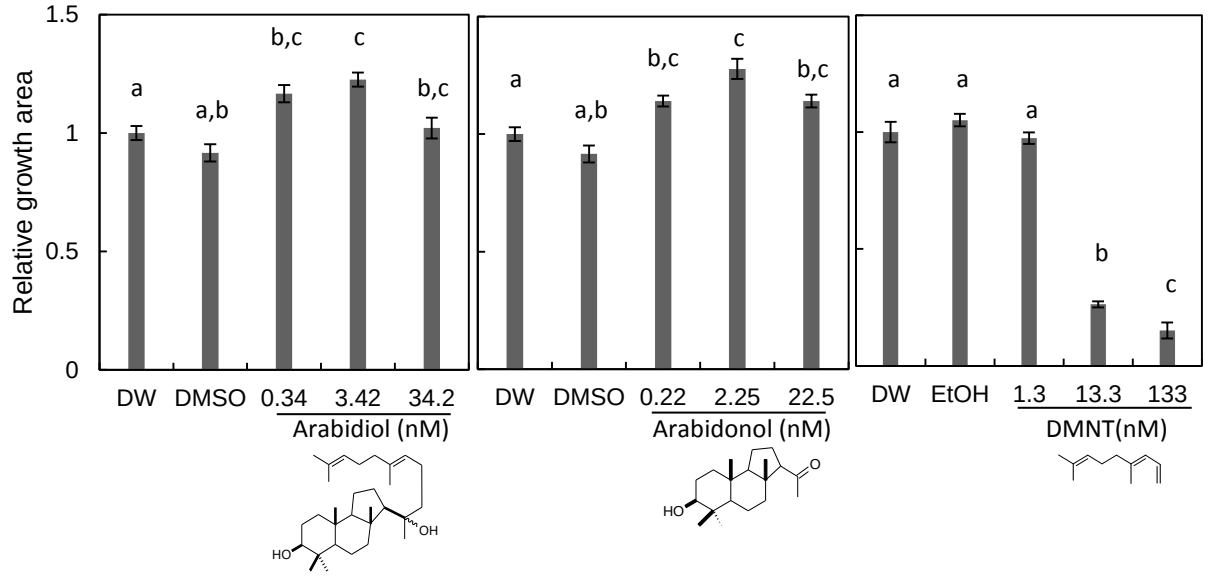


**Figure 3.10 Effect of arabidiol, arabidonol and DMNT on the growth of *P. irregulare* 110305.** Compounds were applied at different concentrations in plates containing 10 ml of fresh potato dextrose agar (PDA) plates. An agar plug with *Pythium* mycelium (2 x 2 mm) was positioned in the center of each plate and incubated in the dark at room temperature. The *Pythium* growth zone was determined 2 days after inoculation. The results were plotted relative to distilled water (DW). The growth zone area for DW was set to 1. Data represent the mean value ± SEM of at least three replicates. One-way ANOVA, $P<0.001$, Tukey-Kramer HSD test.

**The effect of DMNT on the germination of *Pythium* oospores**

Since we had observed highest emission of DMNT within hours of oospore germination and germ tube penetration, we examined the *in vitro* effect of DMNT on *Pythium* oospore germination rates. To measure the germination rate, oospores were germinated on corn meal agar

plates with and without DMNT including distilled water and organic solvent (ethanol) controls. Oospore germination was inhibited by about 50% at 50 ρM DMNT (Figure 3.11) and higher DMNT concentrations caused only a minor inhibitory effect, which may suggest dose-specific effects similar to the inhibitory effects of DMNT on *Pythium* mycelium growth although at about 200-fold lower concentrations. These results indicate that DMNT can also inhibit oospore germination *in vitro*.

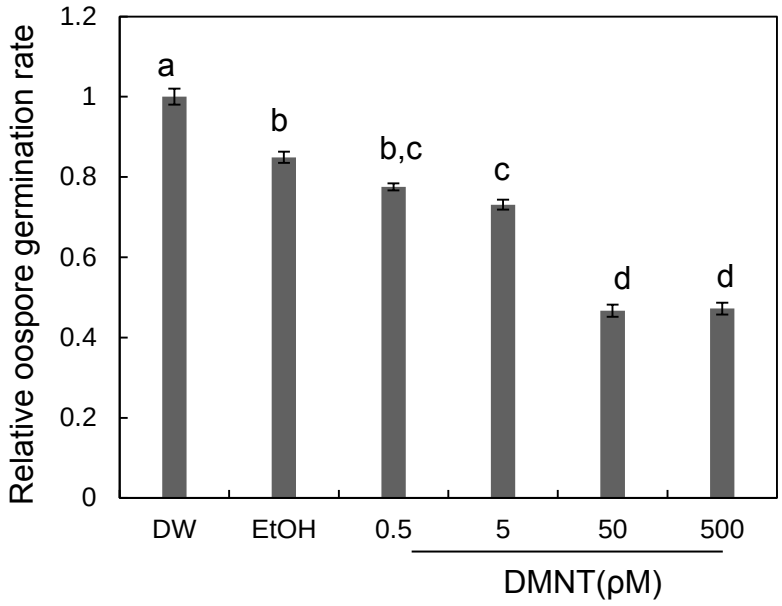


**Figure 3.11 Effect of DMNT on oospore germination of *P. irregulare* 110305.** DMNT was applied at different concentrations in 10 ml of corn meal agar containing streptomycin. Oospore suspensions were added into each plate and incubated at 27 ºC in the dark. Germination rates were determined 24 h after inoculation. Thirty percent of the oospores germinated in the control treatment. The results were plotted relative to distilled water (DW). The oospore germination rate for DW was set to 1. The value represents the mean ± SEM of 5 replicates, One-way ANOVA, $P<0.01$, Tukey-Kramer HSD test.

***Pythium* root colonization of wild type and DMNT biosynthesis mutants**

To further examine a possible defensive role of the breakdown products of arabidiol *in planta*, we assessed root colonization at the early stage of root infection. To this end, the extent of colonization of roots by *Pythium* was determined in wild type and the *cyp705a1* mutant at 3 h, 1 d, and 3 d post inoculation. *Pythium* colonization of root segments from inoculated wild type and mutant plants was assessed on *Pythium* selective medium ($P_5$ARP). As a positive control for susceptibility assessment, we used the jasmonate resistant mutant *jar1-1*, which has been shown to be highly susceptible to *P. irregulare* (Staswick et al., 1998). As a negative control, we used the line salk_090621C, which carries a T-DNA insertion in the *CYP705A1* promoter region but expresses CYP705A1 and produces DMNT at wild type levels. The percentage of colonized roots increased over time for all plants but at different rates between wild type and mutants (Figure 3.12). As expected, colonization of the *jar1-1* mutants was highest at all time points with 30% colonization at 3 h and 98% at 3 d post inoculation. By contrast, roots of wild type and salk_090621C plants were colonized to only 5-10% at 3 h and 1 d of infection with a 30% level on day 3 (Figure 3.12). In comparison to plants with wild type levels of DMNT, roots of the *cyp705a1* mutant exhibited a significantly higher rate of infection on day 1 and day 3 post-inoculation (Figure 3.12). The results indicate that DMNT and possibly derivatives of arabidonol have an effect on delaying colonization and subsequent disease progression.

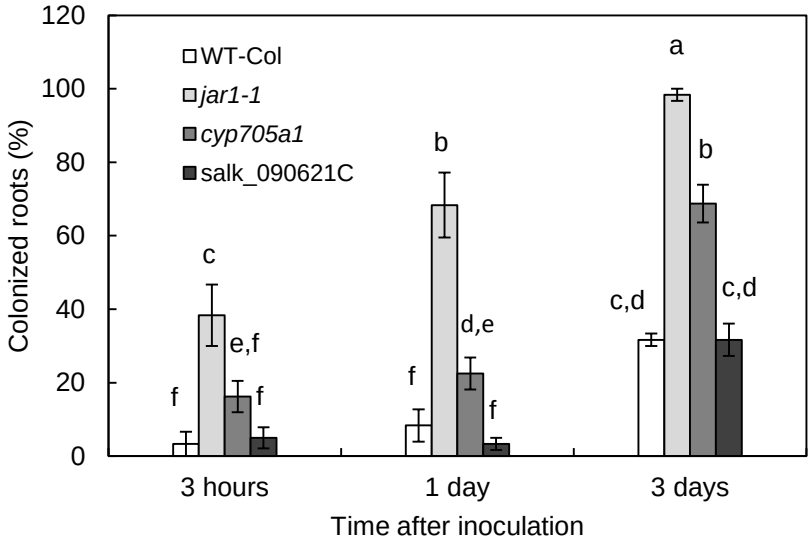


**Figure 3.12 Colonization of P. irregulare 110305 on roots of wild-type plants and the mutants jar1-1, cyp705a1, and salk_090621C.** Three-week-old *Arabidopsis* grown in hydroponic culture was infected with an oospore suspension of *P. irregulare* 110305. Roots colonized by the pathogen were observed on *Pythium* selective media ($P_5$ARP) over a period of three days. Data represent the mean ± SEM of at least three plants. $P<0.05$, Tukey-Kramer HSD test.

**Analysis of disease progression in wild type and arabidiol biosynthesis and breakdown mutants**

Although the degradation of arabidiol seems to occur only transiently at the early stage of infection, we asked whether the production of arabidiol and its breakdown products could have an effect on long-term disease progression. Recent studies by graduate student Reza Sohrabi indicate that arabidonol is most likely converted to at least two derivatives, one of which accumulates in the root tissue and the other is in part secreted.

Successful infection by *Pythium* generally causes symptoms of root rot as well as wilting or chlorosis on aerial parts of the plant and can lead to a severe reduction in root and shoot

biomass (Huang and Jarvis, 2002; Khan et al., 2003). Therefore, we observed disease progression in wild type and arabidiol biosynthesis and degradation mutants (*atpen1-1*, *atpen1-2*, *cyp705a1*) by determining the percentage of plants with wilted leaf symptoms over a time course of 18 days post inoculation and prior to bolting of the plants. Furthermore, we determined the abundance of oospores inside the infected root tissue three weeks after inoculation (Figure 3.13). As a positive control for disease assessment, we used *jar1-1* and as a wild type control, salk_090621C. For *Pythium* infection in plants, three-week-old *Arabidopsis* grown in jiffy pots (a soil-like substrate) were transplanted into *Pythium*-infested soil, and disease symptoms were observed over the time course.

Whereas *jar1-1* already showed severe wilting symptoms on day 6 after infection and the number of diseased plants increased to 90% after 18 days, only mild symptoms of wilting and yellowing symptoms were observed in wild-type plants at two weeks after inoculation and approximately 20% of the plants showed disease symptoms at day 18 (Figure 3.13). Disease progression in salk_090621C was similar to that of wild type plants (Figure 3.13). By contrast, disease symptoms, already appeared on day 12 post-inoculation in the *cyp705a1* mutant with approximately 60% diseased plants after 18 days. Disease progression in the two independent arabidiol synthase mutants, *atpen1-1* and *atpen1-2*, was in-between that of wild type and the *cyp705A1* mutant, showing the first symptoms on day 15 and approximately 10% more diseased plants compared to wild type (Figure 3.13 a).

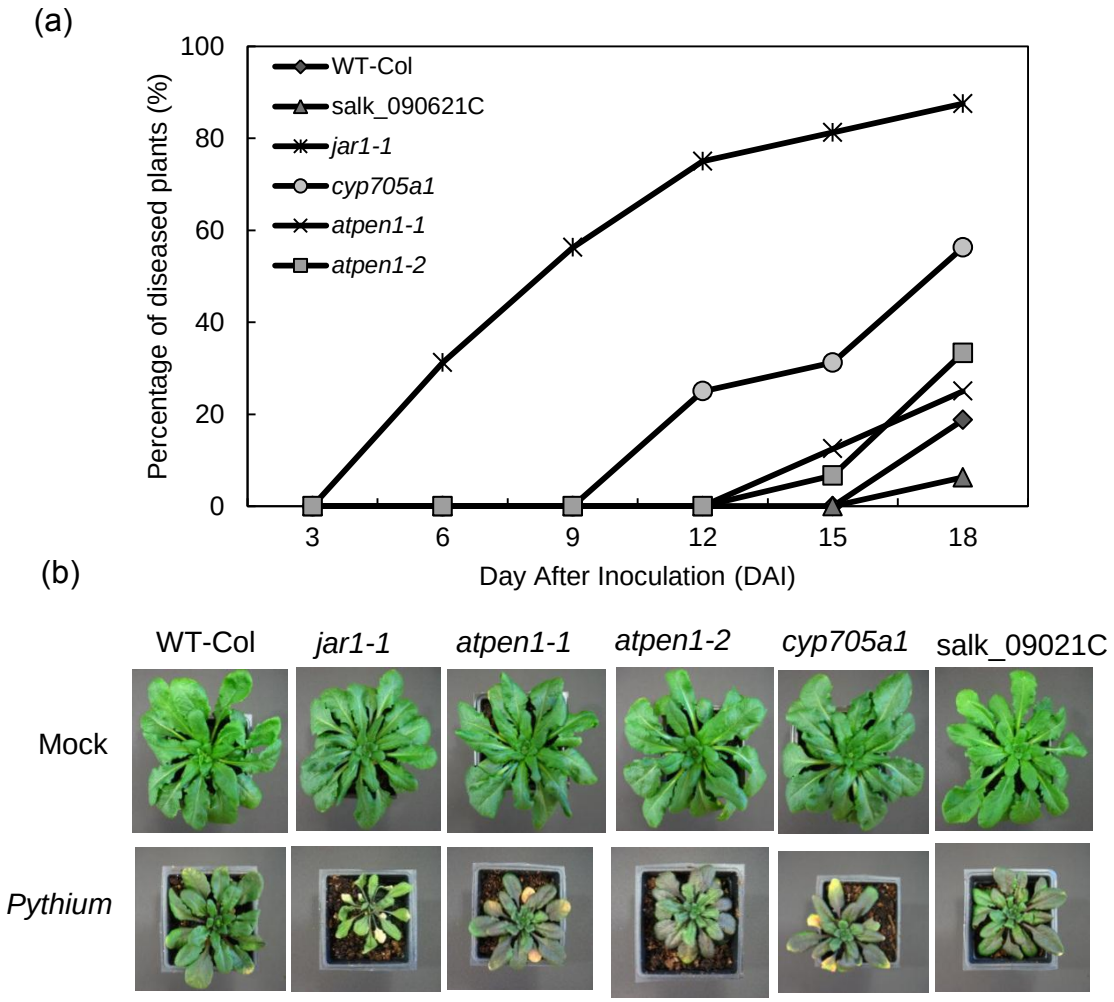


**Figure 3.13 Susceptibility of DMNT biosynthetic mutants to infection by *P. irregulare* 110305.** (a) Disease development in wild-type and DMNT biosynthetic mutants (*atpen1-1, atpen1-2, cyp705a1*, and salk_090621C). Plants with more than a single wilted rosette leaf were counted over 18 days after infection with *P. irregulare*110305. The experiment was repeated two times with similar results and results are from one representative experiment. (N=16 for each mock and *Pythium* treatment) (b) Disease symptoms of infected wild-type plants and DMNT biosynthetic mutants.

To further investigate the infection level in roots, root tissue was collected three weeks after infection, washed to remove soil particles, and stained with acid-fuchsin lactophenol. Oospores inside root tissues were observed and counted in a 10-mm zone behind the root tip (Figure 3.14). Whereas few oospores were found in wild-type and the salk_09021C line, both

*atpen1-1* mutants and *cyp705a1* exhibited higher levels of oospores inside infected root tissues (significant for *atpen1-1* and *cyp705a1*), though apparently to a lesser extent than *jar1-1*. In summary, these results indicated that DMNT biosynthetic mutants are more susceptible to *P. irregulare* than wild-type.

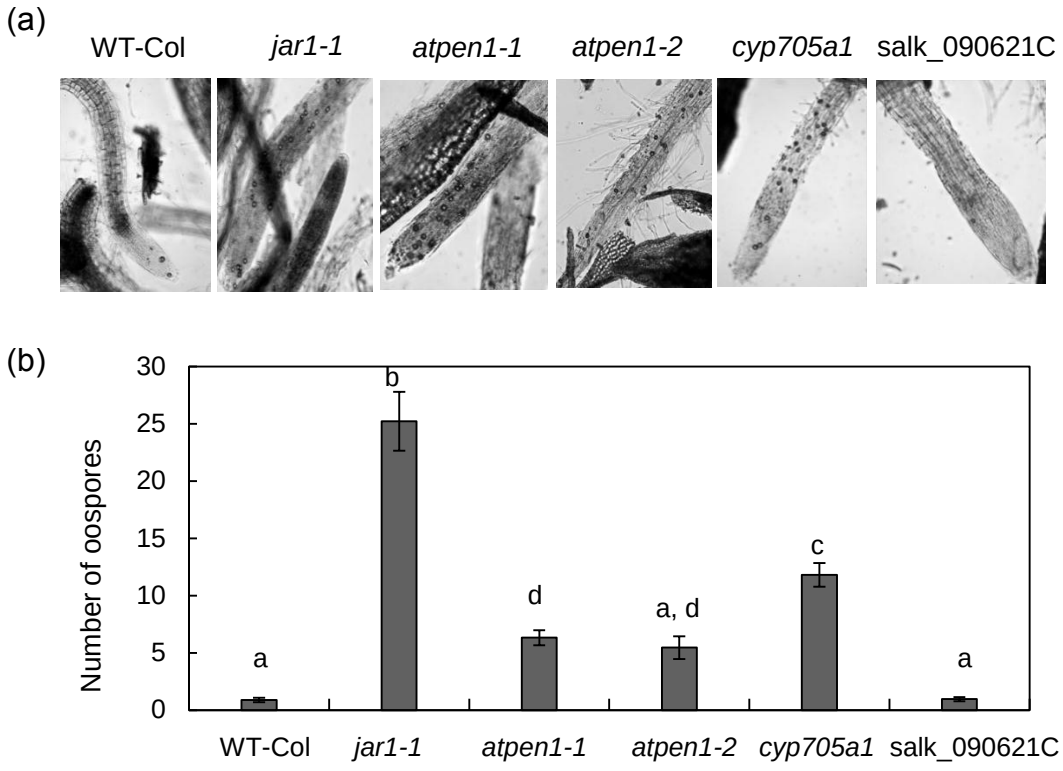


**Figure 3.14 Root infection assay of *P. irregulare* in wild-type and DMNT biosynthetic mutants (*atpen1-1, atpen1-2, cyp705a1* and salk_090621C).** Random samples were excised and oospores inside the infected root tissue were observed under light microscopy after staining with acid-fuchsin lactophenol. (a) Oospore formation by *P. irregulare* in infected roots. Representative root segments were taken three weeks after infection with the pathogen. (b) Oospores were counted about 10 mm behind the root tips. The values are shown mean ± SEM for 19-24 roots (at least six roots from each plant, N=3), One-way ANOVA, $P<0.05$, Tukey-Kramer HSD test.

## Discussion

### DMNT is produced as a triterpene breakdown product in *Arabidopsis* roots.

We have found that DMNT is synthesized via oxidative degradation of the triterpene alcohol, arabidiol, in *Arabidopsis* roots. This finding was rather unexpected since previous studies on the biosynthesis of DMNT in aerial parts of plants had demonstrated with enzyme activity assays and by administration of stable isotope precursors that DMNT was formed from the sesquiterpene alcohol (*E*)-nerolidol (Boland et al., 1998; Bouwmeester et al., 1999). These studies were further supported by the characterization of the leaf-specific and insect-induced *Arabidopsis* P450 enzyme CYP82G1, which converts both (*E*)-nerolidol and (*E*,*E*)-geranyllinalool into DMNT and TMTT, respectively, although only TMTT is produced *in vivo* because of the absence of a (*E*)-nerolidol synthase activity in leaf tissue (Lee et al., 2010). CYP82G1 catalyzes an oxidative C-C bond cleavage reaction of the alcohol substrates via syn-elimination of the polar head, together with an allylic C-5 hydrogen atom. Similar cleavage reactions of a tertiary alcohol catalyzed by P450s have been described for the dealkylation of 22-hydroxycholesterol into androstenolone (Mast et al., 2011) and the formation of the furanocoumarin psoralen from its precursor (+)-marmesin in plants of the Apiaceae (*Ammi majus*) (Hübner et al., 2003). We can now also apply this reaction to the biosynthesis of DMNT from the tricyclic triterpene arabidiol (Figure 3.7). The configuration of the arabidiol compound with a tertiary hydroxyl group at its prenyl side chain allows a C-C cleavage equivalent to that catalyzed by CYP82G1. However, the reaction is catalyzed by CYP705A1, a P450 enzyme of the Brassicaceae-specific CYP705 family, which shares only about 50% amino acid sequence similarity with CYP82G1. While it is not yet well understood whether CYP82G1 produces

DMNT or TMTT in a single cleavage step or in two sequential steps (Lee et al., 2010), CYP705A1 synthesizes DMNT by a one-step cleavage reaction yielding a second non-volatile ketone product that we have named arabidonol (Figure 3.7). A recent analysis of JA-treated roots of the *cyp705a1* mutant showed an accumulation of small amounts of arabidiol, but the compound was not detected in JA-treated wild type roots, suggesting a rapid breakdown of the triterpene precursor in wild type plants (Sohrabi, personal communication). Interestingly, while arabidonol could not be detected in roots of JA-treated wild type plants, two putative derivatives have been identified, which are most likely modification products of arabidonol (Sohrabi, personal communication).

The *CYP705A1* gene clusters with the arabidiol synthase gene, *AtPEN1* (Xiang et al., 2006), on chromosome 4. According to high resolution expression maps (Birnbaum et al., 2003; Brady et al., 2007), both genes are co-expressed under constitutive conditions in the vascular tissue of the root. While treatment with JA induces transcription of *AtPEN1* and to some extent of *CYP705A1* (Figure 3.5), inoculation with *P. irregulare* caused only a small transient increase in expression of *CYP705A1* but has no effect on AtPEN1 expression. While both genes are required for the production of DMNT (Figure 3. 6), it is obvious that the induced formation of this compound cannot be explained by the regulation of gene transcript levels alone. Besides post-translational modifications, an increase in metabolite flux toward squalene and 2,3-oxidosqualene (Fulton et al., 1994), the general precursor in triterpene biosynthesis, might contribute to the enhanced formation of arabidiol and its breakdown products. *CYP705A1* and *AtPEN1* form a putative gene cluster together with two members of the CYP702 family, two putative cellulose synthase like (AtCSL) genes, and three putative glucosyltransferase enzymes. A related, highly coordinated cluster containing genes of the same families as the “arabidiol

cluster" has recently been described for the formation and modification of the triterpene thalianol in *Arabidopsis* roots (Field and Osbourn, 2008). Thalianol is structurally very similar to arabidiol, however, its prenyl side chain does not carry a hydroxyl group and is therefore not cleaved by a P450 activity. Instead, thalianol undergoes hydroxylation and desaturation, catalyzed by two P450 enzymes, the latter of which belongs to the CYP705 family (Field and Osbourn, 2008). It is possible that the other genes of the putative arabidiol cluster are involved in downstream modifications of arabidonol such as hydroxylation and/or glycosylation, although these genes are not as tightly co-expressed with *CYP705A1* and *AtPEN1* as in the equivalent example of the thalianol cluster. Gene clusters such as those described for thalianol and arabidiol have been found in other terpene metabolic pathways such as the one responsible for the biosynthesis of diterpenes in rice (Shimura et al., 2007). The selective forces driving the evolution of gene cluster assembly in terpene metabolism are not well understood and require further attention. In summary, the formation of DMNT in *Arabidopsis* roots evolved as part of a triterpene biosynthesis gene cluster indicating plasticity in the biosynthesis of homoterpene volatiles and differences of specialized metabolic pathways above- and belowground.

**DMNT formation occurs transiently at the early stage of infection by *P. irregulare***

We observed a transient breakdown of arabidiol and formation of DMNT at the time when oospores make initial contact with the root tissue and germ tubes start to penetrate the epidermis. In addition, expression of *CYP705A1* was transiently induced prior to highest emission levels of DMNT 3 h post-inoculation. A similar transient accumulation of transcripts was found for marker genes of JA- and SA-dependent pathways (e.g. *PR1*, *PDF1.2*) within the first 5 h of inoculation of *Arabidopsis* roots with the oomycete, *Phytophthora parasitica* (Attard

et al., 2010). In accordance with these observations, the downregulaton of DMNT production might be caused by pathogen effectors that suppress host-specific defense responses within several hours after penetration of the pathogen. The recently sequenced genome of *Pythium ultimatum* was shown to contain several candidate effector genes with putative virulence function (Levesque et al., 2010). The suppression of host responses benefits a hemibiotrophic lifestyle, as described for *Phytophthora*-plant root pathosystems with biotrophic growth of the pathogen in the root at an early stage of infection and a later switch to necrotrophic growth causing root loss (Schlink, 2010). Previous studies on the infection of *Arabidopsis* seedlings with *P. irregulare* suggested an infection process similar to that of a hemibiotrophic pathogen with the formation of biotrophic appressoria and haustoria at the beginning of the infection followed by a nectrotrophic movement of hyphae through the vasculature and invasion of all tissues (Adie et al., 2007).

Adie et al. (2007) also reported that the onset of plant defense responses against *Pythium* is regulated by signaling pathways involving JA, ABA, ethylene (ET), and SA with JA as the primary defense regulator. In agreement with these findings, *Pythium*-induced emission of DMNT in roots is mainly dependent on the JA signaling pathway. In contrast to the general notion of an antagonistic relationship between JA/ET and SA signaling pathways, plant defense against *Pythium* is triggered by a cooperation of JA, SA, ET and ABA signaling. Our results support this finding since DMNT emission rates in *ein2-1* and *sid2-1* are similar to those in wild type (Figure 3.4). If DMNT biosynthesis is a marker for JA-induced responses, JA levels should decline at approximately 5 h post-inoculation. This assumption stands in contrast to an increase of JA in *Pythium*-infected seedlings (Adie et al., 2007). It is possible that the formation of DMNT is suppressed by factors independent of JA and/or that signaling responses in roots are

different from those in leaves or whole seedlings as described by Adie et al. (2007).

**Arabidiol breakdown products are involved in the defense against *P. irregulare***

Our results support a role of DMNT in chemical defense against *Pythium* showing that DMNT retards *Pythium* mycelium growth and oospore germination *in vitro*. Although it is somewhat difficult to compare the actual concentration of DMNT released at the root surface with those in the *in vitro* assays, the concentrations of DMNT with inhibitory effects were in the range of the amounts emitted per g fresh weight of hydroponically grown roots. Due to the highly lipophilic nature of terpenes, it is assumed that terpenes exhibit antimicrobial activity by interfering with the integrity of the cellular membrane (disruption/alteration), which leads to ion leakage, membrane potential reduction, proton pump dysfunction and ATP pool depletion (Mann et al., 2000; Kalemba et al., 2002; Bakkali et al., 2008; Field and Osbourn, 2008). Other more recently reported antimicrobial activities of terpenes include the disruption of cell microtubules (Chaimovitsh et al., 2010). We did not find a dose-response effect on *Pythium* growth within a 10-fold increase in DMNT concentration (Figure 3.10). Interestingly, Inoue et al. (2005) reported that the inhibitory effect of diterpenes including geranylgeraniol, teprenone, teprenone and phytol on *Staphylococcus aureus* was dependent on a defined concentration range and was even reduced and had growth accelerating effects above a certain threshold. Since volatile terpene hydrocarbons react with certain oxygen species in the gas phase (Atkinson and Arey, 2003), it is possible that DMNT is converted to a derivative with lower inhibitory activity. Moreover, higher concentrations of DMNT could induce detoxification mechanisms that counteract its inhibitory effects.

Despite the fact that the breakdown of arabidiol seems to occur only within the first hours

of infection, our comparative studies on root colonization at the early stage of infection and long term disease progression in wild type and arabidiol and DMNT biosynthesis mutants demonstrate that metabolites of the arabidiol biosynthetic pathway contribute from the onset of infection to slow down the infection process in roots, which also reduces disease in the aerial parts of the plant. The volatile and non-volatile breakdown products might exhibit different activities in this process. While DMNT appears to be primarily effective at the stage of oospore germination and penetration, it may also have signaling or priming effects. It has been shown that volatile blends including homoterpenes that are released from a site of wounding or infection can function as systemic signals by priming non-affected parts of the plant for defense responses (Frost et al., 2007).

Since arabidonol did not have inhibitory activity *in vitro*, we assume that its derivatives that are secreted or accumulate in the root tissue are functionally more important defense compounds. Strong antifungal activities have previously been described for root-produced triterpene saponins such as avenacin that is secreted from oat roots (Mary et al., 1986). Since the concentrations of arabidiol and other triterpenes in *Arabidopsis* are low compared to those in the roots of other plants such as oat, they represent only one component in the chemical defense machinery of *Arabidopsis* roots. Bednarek et al. (2005) reported that the infection of *Arabidopsis* roots with *P. sylvaticum* in axenic culture induced changes in the concentrations of secondary metabolite changes including indole glucosinolates and phenylpropanoids. Especially, glucosinolates, the primary defense metabolites in Brassicaceae, are important for *Arabidopsis* root defense as shown by preliminary results with mutants impaired in glucosinolate formation (data not shown). Together, these specialized metabolites combined with other chemical and physical responses such as the formation of ROS or the reinforcement of cell walls (Adie et al.,

2007; Oliver et al., 2009) may contribute to the comparatively mild pathogenicity of the *P. irregulare* strain investigated in this work on mature wild-type *Arabidopsis* plants. The partial resistance of many plants to *Pythium* infection at the mature stage (Kamoun et al., 1999) may also relate to the recently reported absence of RXLR virulence effectors that are common in other oomycete pathogens (Levesque et al., 2010).

## Materials and Methods

### Plant materials and growth conditions

*Arabidopsis* mutants (*atpen1-1, atpen1-2, cyp705a1-1,* salk_090621C*, dde2-2* (*aos*)*, jar1-1, ein2-1* and *sid2-1)* used in this study were from wild-type Col-0 genetic background. The signaling mutants, *dde2-2* (*aos*), *jar1-1*, *ein2-1, sid2-1,* and *coi-1*, were kindly provided by Dr. S. Gazzarrini, J. Browse, J. Glazebrook and C. Gatz (Guzman and Ecker, 1990; Xie et al., 1998; Wildermuth et al., 2001; von Malek et al., 2002). All plants were grown in short day (10-h light/14-h dark photoperiod) under standard growth conditions ($150\,\mu$mol $m^{-2}\,s^{-1}$ photosynthetically active radiation (PAR), 22°C, 55 % relative humidity (RH)).

Axenic plants were maintained as described by Hetu et al., (2005) with slight modification. Briefly, *Arabidopsis* seedlings were grown for 7 days on a mesh on MS solid medium containing 1% sucrose. Seedlings were then transferred to MS liquid medium containing 2% sucrose and cultured for 13 days before changing the sucrose concentration to 0% for one day followed by a sucrose concentration of 1% for 2 days prior to volatile analysis or treatment with *Pythium* or jasmonic acid (JA).

The hydroponic culture system was maintained according to Arteca and Arteca (2000) with minor modification. Seeds were germinated on Jiffy peat (Jiffy-7 pellet, company) by placing them into holes of a foam board sheet. Hydroponic plants were grown in 4L of 1/4X MS solution for four to five weeks under the same light and temperature conditions as described above.

**Volatile collection and analysis**

Root volatiles from axenically or hydroponically grown plants were collected in the head space and analyzed by SPME-GC/MS. One gram of roots (fresh weight) were detached from plants and placed in screw-capped vials (20 mL) containing 1 mL of distilled water with 10 ng of 1-bromodecane as an internal standard. Root volatiles were adsorbed with a 100 μm polydimethylsiloxane fiber (Supelco) for 30 min at room temperature following incubation at 30ºC for 30 min in the headspace of a screw-capped vial (20-mL GC vial). Volatile compounds were desorbed from the fiber at 240°C (4-min) with a 2:1 split injection and analyzed with a Shimadzu GC/MS-QP2010S. Separation was performed on an Rxi-XLB column (Restek) of 30 m x 0.25 mm i.d. x 0.25 $\mu$m film thickness. Helium was the carrier gas (1.4 mL $min^{-1}$ flow rate), and a temperature gradient was applied at 4°C $min^{-1}$ from 40°C (2-min hold) to 220°C followed by a gradient of 5°C $min^{-1}$ from 40°C to 220°C and 20°C $min^{-1}$ from 220°C to 240°C (2-min hold). Identification of all volatile compounds was achieved by comparison of their retention times and mass spectra with those of authentic standards and with mass spectra of the National Institute of Standards and Technology and Wiley libraries (John Wiley & Sons, Inc., New York, NY).

Standardization of the SPME-based volatile analysis for $C_{11}$-homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT) was validated by performing the volatile analysis with and without root materials. Incubation was carried out in the presence of DMNT and 1-bromodecane standard with or without root material, and volatiles were analyzed as described above. Linear calibration was obtained between 5 and 50 ng (for 1-bromodecane, $R^2 = 0.99$; DMNT, $R^2 = 0.99$).

**Yeast expression and enzyme assay**

The full-length cDNAs of the *CYP705A1* and *AtPEN1* genes were cloned from roots of plants treated with JA for 24 h using the proofreading enzyme P*fx* Turbo Cx hot start (Staratagene, Neta Scientific). The amplified fragments were cloned into pENTR-TOPO (Invitrogen). Upon sequence verification, the *CYP705A1* gene was subcloned into the pESC-TRP galactose-inducible yeast expression vector. The *AtPEN1* gene was subcloned into the YEp-352-GW vector under control of a constitutive promoter ADH1 obtained from Dr. Joe Chappell (University of Kentucky). Both vectors were simultaneously transformed to yeast line WAT11 (obtained from Dr. Joe Chappell). Transgenic yeast strains were grown in yeast selective media and subsequently transferred to a galactose containing media for induction of *CYP705A1* gene expression. Then, 4 ml of galactose-induced yeast cells were transferred to a screw cap SPME vial and allowed to grow for another 4 h followed by direct headspace volatile analysis using SPME-GC/MS.

**Genotyping of plant materials**

A T-DNA insertion line of the *CYP705A1* gene (salk_043195) with an insertion in the second exon was obtained from ABRC (Alonso et al., 2003). Also two independent T-DNA insertion lines, salk_018285 and salk_067736, with insertions in exon 1 and intron 5, respectively were obtained for the *AtPEN1* gene. Homozygous mutants were identified using primers recommended by the T-DNA express website (http://signal.salk.edu/tdnaprimers.2.html). Absence of full-length transcript in these mutant lines was verified by RT-PCR.

**Growth conditions of *P. irregulare* and inoculation of *Arabidopsis***

*P. irregulare* 110305 was grown and maintained as described by Huffaker et al., (2006) and kindly provided by Dr. Clarence A. Ryan. The strain was originally isolated from spring wheat by Timothy Paulitz, Department of Plant Pathology, Washington State University. Briefly, the isolate was grown on water-agar (2%) plates for a week under dark conditions for maintenance and stored at 4°C. For infection assays, plugs taken from water agar were grown on ½ x potato dextrose agar (Difco). One-week-old *P. irregulare* cultures were collected from the plates into sterile water and lightly ground with a mortar and pestle to yield a uniform suspension. Aliquots (300 µL) of the suspension (≈ 2.475 X $10^3$ propagules) or water (used as a control) were added to the growth medium of axenically grown cultures containing approximately 20 plants per flask.

**Microscopy analysis of *Arabidopsis* root infection**

*Arabidopsis* seeds were grown on six-well plates containing 3 mL of ½ x MS media with 1% sucrose under short day conditions. Five-day-old *Arabidopsis* seedlings were used for infection with *P. irregulare* and a uniform suspension of *P. irregulare* was added and the infection was allowed to progress for 48 h. Seedlings were collected according to set time periods and immediately stained with lactophenol-trypan blue (Koch and Slusarenko, 1990) to observe *P. irregulare* on root tissues. The samples were mounted in 50% glycerol and observed under a Olympus SV-16 stereomicroscope.

**Transcript analysis of *AtPEN1 and CYP705A1* in response to *P. irregulare* by semi-quantitative RT-PCR**

The expression of *AtPEN1* and *CYP705A1* in root tissues was monitored by semi-quantitative RT-PCR. Total RNA was isolated from axenically grown root tissues infected with *P. irregulare* 110305. Two micrograms of total RNA was reverse-transcribed into cDNA by using SuperScript II (Invitrogen) in a 20 µL reaction according to the manufacturer's instructions using 50 pmol of an anchored poly(dT) primer [$dT_{20}$]. One µl of cDNA was used for PCR amplification with the gene specific primers: *AtPEN1*-forward, 5'-TCTACTTGCAGAGTGATAACGGA-3'; *AtPEN1*-reverse, 5'-CTCGATGACCGTGTCTTGAA CAA-3'; *CYP705A1*-forward, 5'-AGTTTATGGATGCCTTGTTGGC-3'; *CYP705A1*-reverse, 5'-ATTGCTATTGATGAGGCGTCAG-3'; *Ubc*-forward, 5'-AGTCCTGCTTGGACGCTTCA-3'; *Ubc*-reverse, 5'-AACTGCGACTCAGGGAATCTTC-3'. PCRs were performed with 0.1 mM

dNTP mixtures, 0.5 units of Taq polymerase (NewEngland Biolabs) and 0.1 μM of each gene-specific primer. PCR conditions were 95°C for 3 min, followed by 28 cycles of 30 s at 95°C, 30 s at 58°C and 2 min at 72°C. Ubiquitin conjugating enzyme (*ubc*) was used as the endogenous gene.

**Growth inhibition assay *in vitro***

*P. irregulare* was cultured on half strength potato dextrose agar (PDA) (Difco213400) containing different concentrations of each chemical. *P. irregulare* plugs taken from water agar were placed in the center of the plate and incubated at room temperature under dark conditions for two days. The growth area was measured and calculated according to Adie et al (2008) with minor modifications. Hyphal growth from a single inoculate establishes a distinct growth zone, therefore two diameters ($R_1$, $R_2$), perpendicular to each other, were measured for each growth zone and the mean was obtained. The growth area was calculated:

$$Area = \pi \left( \frac{R_1 + R_2}{2} \right)^2$$

The growth inhibition assay was repeated at least twice with more than three replicates and the inhibitory effect of chemicals on *Pythium* growth was determined by comparison with the control growth area.

**Disease assessment of soil-grown plants**

*Arabidopsis* wild-type (Col) and DMNT biosynthetic mutants (*atpen1-1, atpen1-2, cyp705a1*, and salk_090621C) were used to test their susceptibility to *Pythium irregulare*

110305. *Jar1-1* (jasmonate signaling mutant) was used as a positive control for disease assessment due to its high susceptibility to *Pythium* (Staswick et al., 1998).

Plants were grown in jiffy pots (Φ 5 cm, height 6 cm) for three weeks under short day conditions (10-h light/14-h dark). Randomly selected individual plants were then transplanted along with the jiffy pot into single pots (6x6x8 $cm^3$) containing *Pythium*-infested soil. *Pythium*-infested soil was prepared by slicing Potato Dextrose agar (PDA) containing one week-old *P. irregulare* mycelium mixed with the soil, and followed by further incubation for two days to produce uniformly infested soil. For mock treatments, sliced PDA pieces without *Pythium* were mixed with soil.

Root rot disease was monitored by tracking the wilting of rosette leaves over a period of 18 days (Staswick et al., 1998; Vijayan et al., 1998). Disease development was estimated every three days by counting the number of plants with more than one wilted rosette leaf. At the end of the time period, the abundance of oospores inside root tissues was determined by staining of roots with acid-fuchsin lactophenol (Vijayan et al., 1998).

**Oospore isolation and germination of *P. irregulare* 110305**

Oospores were prepared following previous studies with minor changes (Yuan and Crawford, 1995; Manici et al., 2000). Briefly, an agar plug from 4-day-old cultures on PDA was inoculated on a V8 juice (Campbell Juice co.) agar plate and incubated at room temperature under dark conditions. After 10 days, V8 agar plugs containing mycelium were transferred into distilled water and further incubated for 10 days under dark conditions. The cultures containing abundantly produced oospores were comminuted with distilled water by a Polytron tissue

homogenizer. The homogenized mycelial and oospore mixture was filtered through two layers of cheesecloth, and then the filtrate was subjected to centrifugation (4,500 x *g* for 10 min). The pellet was suspended in distilled water, and the concentration of oospores was determined using a hemacytometer.

To determine the germination rate of oospores according to chemicals, oospore germination conditions were applied as described by (Ruben and Stanghellini, 1978). Oospores were induced to germinate directly on corn meal agar (Difco) containing different concentrations of DMNT with 15 μg $mL^{-1}$ streptomycin. One hundred microliters of oospore suspension were applied to the surface of an agar plate (about 200 oospores per plate) and incubated for 24 h in an incubator at 27ºC under dark conditions. The oospore germination rate was measured by counting oospores with emerging germ tubes using light microscopy.

**Evaluation of colonization on root tissues**

Four-week-old *Arabidopsis* wild-type (Col), DMNT mutants (*cyp705a1* and salk_090621C), and *jar1-1* were used to determine root colonization by *P. irregulare* 110305. Plants were grown in hydroponic culture as describe above under short day conditions (10-h light/14-h dark). Hydroponic units (8x11.5 cm of foam board) holding five to six plants were positioned in a container (9x13 cm) containing an oospore suspension of *P. irregulare* 110305 ($2.8 \times 10^4$ oospores $mL^{-1}$), and the container was placed on a shaker (60 rpm) and incubated at room temperature over a period of three days. Distilled water was used for mock treatment.

Examination of root colonization by *Pythium* was performed as described by Johnstone et al. (2005). At each time point of sampling, each plant was surface-disinfected in 50% ethanol for

10 sec and in 1% NaOCl for 10 sec and then rinsed more than three times with sterile distilled water. To determine the percent of roots colonized by *Pythium*, ten 8 cm-root segments from the root tips were randomly removed from each surface-disinfected plant and further cut into 4 cm-root segments. Twenty root segments per plant were placed on *Pythium* selective media ($P_5$ARP) (Jeffers and Martin, 1986). Root segments with *Pythium* colonies were counted after 24 h.

## ACKNOWLEDGMENTS

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# CHAPTER IV

## Final Discussion and Future Perspectives

Plants rely on complex and diverse specialized metabolic pathways to respond to environmental changes and threats. Although many phytochemical studies clearly demonstrate the capacity of plant roots to produce specialized metabolites, there is little knowledge of the biological activity of root metabolites and to what extent plants coordinate their metabolic pathways in roots at cellular and subcellular levels. My study has focused on the investigation of terpene secondary metabolism and function in *Arabidopsis* roots. My work accomplished two goals. First, I characterized two root-specific terpene synthases (TPS22 and TPS25) with an unexpected localization in mitochondria and I investigated possible reasons for this unusual compartmentation of terpene metabolism. My second goal was to understand the formation and defensive function of the $C_{11}$-homoterpene DMNT in the interaction of *Arabidopsis* roots with the soil-borne oomycete pathogen, *Pythium irregulare*. In this chapter, I review the significance of my findings in a broader context and discuss possible future research directions.

## The importance of root phytochemicals: regulation and function

Plant roots are the targets of diverse organisms, including pathogens, herbivores and even parasitic plants. Root-attacking organisms, especially soil-borne pathogens contribute to economic losses of approximately US $4 billion annually (Lumsden et al., 1995). Root pest and pathogen control relies primarily on chemical treatment such as the use of methyl bromide (Martin and Bull, 2002; Martin, 2003). However, application of methyl bromide has been phased out since January 2005 because of its impact on the stratospheric ozone layer (http://www.epa.gov/ozone/mbr/) and the effectiveness of other chemical treatments may decline because of the emergence of pathogen/pest resistance. Hence, there is a growing necessity for

alternative approaches to control plant disease with more selective and environmentally-friendly disease management.

With the growing interest in rhizosphere biology (Bais et al., 2006; Badri et al., 2009; Lambers et al., 2009), an increased focus has been placed on establishing plant root-pathosystems in model plants as indicated by recent studies on *Arabidopsis*-oomycete interactions (Adie et al., 2007; Attard et al., 2010) and on improving knowledge of root chemical defenses in such systems. A future challenge of the *Arabidopsis-Pythium* pathosystem presented here and other systems will be to understand the complexity of constitutive and induced chemical defenses in roots and how they differ in organization and regulation from those found in aerial parts of the plant. *Pythium* does not only affect terpene metabolism, as shown in this study, but also induces the production of lignin, indole glucosinolates, phenylpropanoids, and nicotinamide within 48 h of infection (Bednarek et al., 2005). The question remains whether these metabolites act individually or synergistically as has been proposed for mixtures of specialized metabolites and other defense molecules. For example, it has been reported that terpene mixtures have higher toxic effects on larvae of the generalist lepidopteran *Spodoptera litura* than each individual compound (Hummelbrunner and Isman, 2001). Segura and co-workers demonstrated that Snakin-1, a peptide produced in potato plants acts synergistically with potato defensin PTH1 against *Clavibacter michiganensis subsp. sepedonicus* and both peptides show similar expression patterns in tubers and reproductive organs (Segura et al., 1999). Since many oomycetes change lifestyles from biotroph to necrotroph, studying the biological functions of these compounds in a time-sensitive manner should give insight into the interaction of the different defense-related phytochemicals.

Experiments using gene knockout plants that are impaired in the formation of one or more specialized metabolites will be essential in deciphering the contribution of the different metabolites to the chemical defense repertoire. A variety of *Arabidopsis* mutants targeting flavonoid (Winkel-Shirley, 2001) and glucosinolate biosynthetic enzymes (Kliebenstein et al., 2005) are available for such studies. In *Arabidopsis*-oomycete pathosystems, it will also be important to determine possible attractive or repellent effects of specialized metabolites on zoospore activity. While we used a *Pythium* strain that did not produce zoospores under our laboratory conditions, zoospores from other *P. irregulare* isolates or *Pythium* species as well as those from *Phytophthora parasitica* could be tested using *in vitro* and possibly *in situ* approaches with biosynthetic mutants. It is known that zoospores respond differentially to various root exudates such as sugars and amino acids. For example, L-glutamine attracts zoospores of *P. aphanidermatum*, but it does not induce cyst germination on the root; however, glucose has the converse effect on zoospores in the same species (Donaldson and Deacon, 1993; Donaldson, 1993). Although they do not directly affect zoospore activity, volatile seed exudates such as ethanol or acetylaldehyde were shown to have stimulatory effects on *Pythium* sporangium germination. For example, ethanol induced sporangium germination at low concentrations of 1-7 nmol/ml while higher concentrations exerted inhibitory effects (Nelson, 1987).

Future investigations may also consider the function and action mode of root phytochemicals in mutualistic interactions with microbes in the rhizosphere. The role and action mechanism of flavonoids in legume-rhizobia interactions is well known (Shaw et al., 2006). Flavonoids interact with rhizobial NodD proteins to initiate transcription of nodulation genes which results in deformation of plant root hairs and promotes rhizobial entry via infection threads (Broughton et al., 2000). More recently, it has shown that a novel class of plant

hormones, the strigolactones, which are produced by carotenoid breakdown and released into the soil (Xie et al., 2010), inhibit shoot branching by interfering with auxin transport (Beveridge et al., 2000). Strigolactones control the expression of genes involved in the biosynthesis and perception of the branch-inhibiting hormone via an F-box protein (Leyser, 2008; Dun et al., 2009; Beveridge and Kyozuka, 2010). Furthermore, they do not only promote branching of mycorrhizal fungi (Akiyama et al., 2005) but also induce seed germination of parasitic plants (Bouwmeester et al., 2003; Akiyama and Hayashi, 2006).

The role and mechanisms of volatile compounds in these various interactions is of particular interest. Volatile terpenes emitted from aerial tissues seem to exert multiple functions in attraction and defense and this might also be the case for volatiles released belowground. While the long distance mobility of volatiles in the soil is somewhat limited and depends on the physicochemical properties, it has been shown that volatile terpenes such as the sesquiterpene (*E*)-β-caryophyllene easily partition and diffuse into air pockets of the soil to attract insect-parasitizing nematodes (Rasmann et al., 2005). Besides these and possible allelopathic activities on competitive plants, volatile terpenes or other volatiles compounds released by roots may at the same time have direct short-range effects on microbial colonization at the root surface. Additional functions of stress-induced volatiles such as intra- or interplant signaling and defense priming are currently poorly understood.

From a metabolic/molecular point of view, studies on root-pathogen/herbivore interactions will require a better understanding of how specialized metabolism is integrated with primary metabolism and changes in C- and N- allocation. Recent studies on *Arabidopsis* provide interesting insights on the effect of root stress on aboveground C-metabolic changes (Ward et al., 2011). Moreover, regulatory networks coordinating chemical defenses against different root

attackers need to be investigated, which includes a more detailed analysis of hormonal responses and transcriptional regulators that respond to hormonal changes. Responses of JA, ET, and SA signaling pathways in *Arabidopsis* roots challenged with *Pythium* or members of the closely related genus, *Phytophthora* differ from those in leaves (Attard et al., 2010; this study) and demonstrate that findings from one organ cannot be extrapolated to another. Investigating signaling not only in roots but also between roots and shoots is crucial to understand the influence of underground tissues attacked by root pathogens on aboveground physiology and resistance and vice versa. Studies in maize have emphasized the role of ABA in root-shoot signaling (Erb et al., 2011) and similar analyses need to be performed in *Arabidopsis*. A major challenge for many of the described studies will be an easy access to clean root tissue grown in a soil-type substrate. While this study has analyzed root tissue grown primarily under liquid culture conditions, a transition should be made to soil-like culture systems. The Tholl lab has recently developed an aeroponic culture for insect feeding experiments on *Arabidopsis* roots (Vaughan et al., 2011), which also seems suitable for inoculation with root pathogens such as *Pythium*.

## Cell-type or tissue specificity of root chemical defense

Roots and aerial plant parts are challenged by selective pressures from different microbial and herbivore communities and, therefore, have evolved different chemical defense profiles. In *Arabidopsis*, root and aboveground terpene metabolism is largely separated with several terpene synthases (TPSs) being only expressed in roots (Tholl and Lee, 2011). Moreover, most of the root-specific TPSs, including the TPSs examined in this study, are constitutively expressed and

show some response to JA while the TPSs in leaves are mostly expressed in response to insect or pathogen attack (Tholl and Lee, 2011). Since roots are constantly exposed to a large number of soil-borne microbes and root-attacking organisms, a predominantly constitutive expression complies with an optimal defense strategy according to which plants tend to produce defense metabolic pathways in tissues and cell types that are likely to be under attack by pathogens or herbivores (Rhoades, 1979).

To understand how chemical defenses function in root tissues, one needs to gain insight into their tissue or cell type-specific organization. Roots are an ideal system to investigate the cell type-specificity of specialized metabolism because of their radial structure consisting of different cell types. Studies of morphine alkaloid biosynthesis in opium poppy showed that biosynthetic enzymes reside in three cell types (sieve elements, companion cells, and laticifers) and extended the fundamental physiological role of sieve elements beyond the transport of solutes (Bird et al., 2003).

*Arabidopsis* roots are a valuable model to examine the cell-specific formation of specialized metabolites because of the availability of fine-scale transcriptome maps. According to these transcript profiles, most root specific TPSs have distinct expression patterns in the root tip (sesquiterpene synthase - TPS22, rhizathalene synthase - TPS08), the epidermis (1,8-cineole synthase - TPS23/27, sesquiterpene synthase - TPS25), the cortex and endodermis ((*Z*)-γ-bisabolene synthases- TPS12/13), or the vascular tissue (TPS08). Genes that belong to triterpene biosynthesis clusters or modules such as *AtPEN1* and *CYP705A1* seem to especially adhere to cell-type specific expression profiles in the vascular tissue or epidermis (Field and Osbourn, 2008; this study). A cell-specific organization of defense metabolism in roots may be critical for warding off soil-borne, root-attacking organisms such as nematodes, microbial pathogens and

insect larvae with different invasion and feeding mechanisms (Maron, 1998; Wardle, 2006; Rasmann and Agrawal, 2008). Allocation of defense compounds to the epidermis or root tips appears to be important to combat penetration of pathogens at early stages of infection. Such cell type-specific profiles should be compared among all root-specific metabolites. Although somewhat difficult to apply to *Arabidopsis* roots, new analytical procedures such as matrix-free UV-laser desorption/ionization (LDI) mass spectrometric imaging allow *in situ* cell-specific location of specialized metabolites (Hölscher et al., 2009).

One way to understand the importance or stringency of the tissue and cell type-specific organization of specialized metabolism in roots is to modify the cell-specific compound or to switch its production to a different cell type. Selective changes in the cell-/tissue- type specificity of terpene synthases or biosynthesis may interfere with root development or growth. Mylona et al. (2008) showed that the loss of a glycosylation step in triterpene formation causes stunted growth and suppression of root hair formation. Experiments are currently underway to swap the expression of TPSs to other cell types where they are normally not active by using cell type-specific promoters. These studies include changing the expression of TPS25 from non-hair (N) to hair (H) epidermal cells under control of the *GL3*-promoter (Bernhardt et al., 2003) with higher activity in hair cells. The experiments should reveal whether a different cell type sustains the metabolic program to produce novel compounds and how tightly *TPS* expression is controlled at transcriptional and posttranscriptional levels.

## Subcellular organization of specialized metabolism

The subcellular compartmentation of biosynthetic enzymes and their metabolites is one way that the cell controls metabolite production as well as storage, and turnover (Srere and Knull, 1998). In plant specialized metabolism subcellular regulation is important to a) avoid substrate competition, b) ensure efficient substrate conversion by enzymes of the same pathway targeted to the same organelle, c) increase the variability in compound formation, and d) store potentially toxic compounds. Terpene specialized metabolism is highly compartmentalized and resides in the cytosol and the plastid. It is generally accepted that the formation of monoterpenes or diterpenes occurs in plastids and sesquiterpenes are mainly formed in the cytosol (Dudareva et al., 2004; Tholl, 2006; Cheng et al., 2007). Our studies on the localization of TPS22 and TPS25 now add mitochondria as another natural compartment for terpene biosynthesis. Moreover, partial localization of enzymes of the central mevalonate pathway in peroxisomes has been discussed (Sapir-Mir et al., 2008). Targeting multifunctional terpene synthases to organelles with different substrates results in the formation of different end products (Aharoni et al., 2004; Nagegowda et al., 2008). Differences in subcellular location of TPS enzymes among ecotypes can therefore lead to variation in terpene volatile blends (Huang et al., 2010). Metabolic engineering efforts have taken advantage of subcellular compartmentation to avoid substrate competition. For example, targeting a FPP synthase and a sesquiterpene synthase to plastids resulted in higher amounts of sesquiterpene products in comparison to plants with cytosolic expression of the enzymes because FPP pools are not shared with the sterol biosynthetic pathway (Wu et al., 2006). However, the compartmentation is not absolute because of some crosstalk between the compartments (Laule et al., 2003) and engineering approaches have provided

evidence for the presence of small pools of FPP in chloroplasts (Wu et al., 2006). For example, transgenic potato and *Arabidopsis* plants expressing a strawberry linalool/nerolidol synthase targeted to plastids, produced both linalool and nerolidol from GPP and FPP, respectively suggesting a small pool of FPP is present in plastids (Aharoni et al., 2003).

Why sesquiterpene synthases are targeted to mitochondria remains to be determined. Possible reasons are the avoidance of competition for FPP substrate in the cytosol or reducing the production of terpenes to non-inhibitory concentrations by exposing the enzyme to possibly lower FPP concentrations in the mitochondrion. Interestingly, several TPSs in the type-a clade of the *Arabidopsis* TPS family appear to carry mitochondrial targeting sequences and are expressed in roots. Since only a limited number of sesquiterpenes has been found in *Arabidopsis* roots to date, it is unclear whether these predictions are correct or the enzymes exhibit no activity *in vivo*. However, it is possible that non-volatile derivatives of the sesquiterpene products of these enzymes have not been detected so far. The expansion of the type-a clade, of which twelve TPSs are root-specific, suggests a divergent evolution of terpene formation in adaptation to different communities of soil organisms. In this process, targeting of TPSs to mitochondria might indicate an emergence of terpene biosynthetic pathways in these organelles.

Finally, the transport and storage of specialized metabolites to specific cellular compartments is important to avoid the accumulation of phytochemicals at toxic levels (Marrs, 1996; Coleman et al., 1997). Flavonoids and glucosinolates are synthesized in the cytosol and stored in the vacuole to avoid their toxic levels and to respond to environmental challenges (Kelly et al., 1998; Grotewold, 2004; Grubb and Abel, 2006; Pourcel et al., 2010). Hydroxylation followed by glycosylation are also common modifications in the biosynthesis of terpenes as has been, for example, demonstrated for the formation of hydroxygeranyllinalool glycosides with

antifeedant activity in leaves of *Nicotiana attenuata* and triterpene saponins with potent antifungal activity (Osbourn et al., 1991; Jassbi et al., 2008). Glycosylation of triterpenes is not only important for the bioactivity of the compound but also prevents autotoxic effects and developmental defects (Mylona et al., 2008). Interestingly, a recent metabolomics study in *Arabidopsis* in response to nitrate starvation showed high accumulation of $C_5$-(hemi) terpene glycosides in leaves and this response was also induced by wounding of roots (Ward et al., 2011). It is possible that triterpenes of the arabidiol biosynthesis pathway are glycosylated and reside in the vacuole. Butanol extractions of large amounts of hairy roots are currently being conducted (Reza Sohrabi, personal communication) to determine the presence of such compounds. Furthermore, at least one of the arabidonol derivatives seem to be secreted and it will be interesting to identify possible transporters that are involved in this process.

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# APPENDIX A

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

This work is published in **Phytochemistry (2011) vol. 72, pp. 1635-46.** My contribution to this review includes the development of figures in correlation with various topics of the article.

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**Summary**

Volatile organic compounds emitted by plants mediate a variety of interactions of plants with other organisms. The irregular acyclic homoterpenes, 4,8-dimethylnona-1,3,7-triene (DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), are among the most widespread volatiles in angiosperms with emissions from flowers and vegetative tissues upon herbivore feeding. Special attention has been placed on the role of homoterpenes in attracting parasitoids and predators of herbivores and has sparked interest in engineering homoterpene formation to improve biological pest control. The biosynthesis of DMNT and TMTT proceeds in two enzymatic steps: the formation of the tertiary $C_{15}$-, and $C_{-20}$ alcohols, (*E*)-nerolidol and (*E*,*E*)-

geranyl linalool, respectively, catalyzed by terpene synthase, and the subsequent oxidative degradation of both alcohols by a single cytochrome P450 monooxygenase (P450). In *Arabidopsis thaliana*, the herbivore-induced biosynthesis of TMTT is catalyzed by the concerted activities of the (*E*,*E*)-geranyllinalool synthase, AtGES, and CYP82G1, a P450 of the so far uncharacterized plant CYP82 family. TMTT formation is primarily controlled at the level of AtGES expression; however, both enzymatic steps are tightly co-expressed at wound sites allowing an efficient conversion of the alcohol precursor. The identified homoterpene biosynthesis genes in *Arabidopsis* and related genes from other plants provide gene tools to engineer homoterpene formation and to address questions of the regulation and specific activities of homoterpenes in plant-herbivore interactions.

# APPENDIX B

## Role of aromatic aldehyde synthase in wounding/herbivory response and flower scent production in different *Arabidopsis* ecotypes

This work was published in **The Plant Journal (2011) vol. 66, pp. 591–602.** My contribution to this work includes the transcript analysis of an aromatic aldehyde synthase (AtAAS) in different tissues of *Arabidopsis* ecotypes by using quantitative RT-PCR.

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### SUMMARY

Aromatic L-amino acid decarboxylases (AADCs) are key enzymes operating at the interface between primary and secondary metabolism. The *Arabidopsis thaliana* genome contains two genes, At2g20340 and At4g28680,encoding pyridoxal 5'-phosphate-dependent AADCs with high homology to the recently identified *Petunia hybrida* phenylacetaldehyde synthase involved in floral scent production. The At4g28680 gene product was recently biochemically characterized as an L-tyrosine decarboxylase (AtTYDC), whereas the function of the other gene

product remains unknown. The biochemical and functional characterization of the At2g20340 gene product revealed that it is an aromatic aldehyde synthase (AtAAS), which catalyzes the conversion of phenylalanine and 3,4-dihydroxy-L-phenylalanine to phenylacetaldehyde and dopaldehyde, respectively. AtAAS knock-down and transgenic AtAAS RNA interference (RNAi) lines show significant reduction in phenylacetaldehyde levels and an increase in phenylalanine, indicating that AtAAS is responsible for phenylacetaldehyde formation in planta. In A. thaliana ecotype Columbia (Col-0), AtAAS expression was highest in leaves, and was induced by methyl jasmonate treatment and wounding. *Pieris rapae* larvae feeding on Col-0 leaves resulted in increased phenylacetaldehyde emission, suggesting that the emitted aldehyde has a defensive activity against attacking herbivores. In the ecotypes Sei-0 and Di-G, which emit phenylacetaldehyde as a predominant flower volatile, the highest expression of AtAAS was found in flowers and RNAi AtAAS silencing led to a reduction of phenylacetaldehyde formation in this organ. In contrast to ecotype Col-0, no phenylacetaldehyde accumulation was observed in Sei-0 upon wounding, suggesting that AtAAS and subsequently phenylacetaldehyde contribute to pollinator attraction in this ecotype.

# APPENDIX C

## Cyclophilin mediates the effect of 12-oxo-phytodienoic acid during jasmonate-signaling

This work is targeted for publication in Nature**.** My contribution to this work includes susceptibility tests of cysteine (Cys) biosynthetic mutants in response to infection by *Pythium irregulare*.

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**Summary**

Jasmonic acid (JA) and related signaling molecules play essential roles in plant development and survival. However, our knowledge of architecture of their signaling circuits remains unclear. Here we report for the first time that 12-oxo-phytodienoic acid (OPDA, a JA precursor)-mediated signaling directly targets an amino-acid biosynthetic pathway. Our results demonstrate in *Arabidopsis* that OPDA stimulates cysteine (Cys) production by assisting a

cyclophilin protein ($K_d$ = 196 nM) to activate a plastid Cys synthase complex. This sulfur assimilation leads to an increased-level of thiols and the build-up of redox-potentials. Disrupting Cys biosynthesis impairs typical JA-responsiveness including defense responses toward necrotrophic pathogens. We conclude that plastid cyclophilin (20-3) is a key effector of OPDA-mediated signaling that operates downstream metabolic and molecular activity.

# APPENDIX D

## Potential function of a novel class of sugar transporters in feeding microbes in the rhizosphere

My contribution to this work includes the isolation of total RNA and preparation of cDNA in *Pythium*-infected *Arabidopsis* root tissues and susceptibility tests of sugar transporter mutants upon infection by *Pythium irregulare*.

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**Summary**

Most plants can efflux 20-30% of assimilated carbon into the rhizosphere as root exudates. The potential roles of root exudates are to feed beneficial microorganism or mediate the interactions between roots and microorganisms. In rice, sugars account for 25% of exudates. Within these exudates, monosaccharaides are the dominant sugars. Previous studies showed that root glucose efflux was not dependent on a proton gradient but was driven by the concentration gradient. There exists a new class of unknown glucose facilitators mediating sugar efflux from *Arabidopsis* roots. To understand the mechanisms of glucose efflux in the root, we identified a

novel glucose transporter family (Sweets), which consists of 17 genes, from the *Arabidopsis* root by using glucose FRET sensors. The cell-type specific and subcellular localization of glucose transporters was observed. Interestingly, two of glucose transporters (*sweet* b and f) were highly induced by to the soil-born root pathogen, *Pythium irregulare.* We are currently in the process of observing the disease severity of sweet mutants infected with *P. irregulare* to understand the physiological role of sweet genes in plant defense against root pathogens. We hypothesize that glucose transport into the rhizopshere upon *Pythium* infection might promote the growth of beneficial microbes that counteract *Pythium* attack. Preliminary results from sweet mutants with increased susceptibility to *P. irregulare* support this hypothesis.