# Molecular and Functional Characterization of Terpene Chemical Defense in *Arabidopsis* Roots in Interaction with the Herbivore *Bradysia* spp. (fungus gnat)

Martha Marie Vaughan

Dissertation submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biological Sciences

Dorothea Tholl, Committee Chair

Brenda Winkel
Jill Sible
John Jelesko
Scott Salom

May 13th 2010
Blacksburg Virginia

Keywords: terpene synthase, diterpene, secondary metabolism, root defense, root herbivory

**Molecular and Functional Characterization of Terpene Chemical Defense in *Arabidopsis* Roots in Interaction with the Herbivore *Bradysia* spp. (fungus gnat)**

Martha Marie Vaughan

ABSTRACT

Roots and leaves are integrated structural elements that together sustain plant growth and development. Insect herbivores pose a constant threat to both above- and belowground plant tissues. To ward off herbivorous insects, plants have developed different strategies such as direct and indirect chemical defense mechanisms. Research has primarily focused on visible aboveground interactions between plants and herbivores. Root-feeding insects, although often overlooked, play a major role in inducing physical and physiological changes in plants. However, little is known about how plants deploy chemical defense against root herbivores. We have developed an *Arabidopsis* aeroponic culture system based on clay granulate, which provides access to root tissue and accommodates subterranean insect herbivores. Using this system, feeding performance and plant tissue damage by the root herbivore *Bradysia* (fungus gnat) were evaluated. Larval feeding was found to reduce *Arabidopsis* root biomass and water uptake.

Furthermore, we have characterized a root-specific terpene synthase AtTPS08, which is responsible for the constitutive formation of the novel volatile diterpene compound, rhizathalene, in *Arabidopsis* roots. Rhizathalene synthase is a class I diterpene synthase that has high affinity for the substrate geranylgeranyl diphosphate (GGPP) and is targeted to the root leucoplast. Expression of the β-glucuronidase (GUS) reporter gene fused to the upstream genomic region of *AtTPS08* demonstrated constitutive promoter activity in the root vascular tissue and root tips.

Using the established bioassay with *Arabidopsis* and *Bradysia* larvae, in aeroponic culture we could show that roots deficient in rhizathalene synthesis were more susceptible to herbivory. Our work provides *in vivo*-evidence that diterpene compounds are involved in belowground direct defense against root-feeding insects.

Future work is still required to improve our understanding of plant root defense. This study has provided a basis for future investigations on the biochemistry, molecular regulation and defensive function of *Arabidopsis* root chemicals in interaction with both above- and belowground herbivores (and pathogens).

# DEDICATION

I dedicate this dissertation to my Mother, Kyriaki Kafadari.

# ACKNOWLEDGEMENTS

I could never have reached the point I am at today without the encouragement, support and help of the community here at Virginia Tech. First and foremost, I must express my sincere gratitude to Dr. Dorothea Tholl. It has been a privilege to work with her. She has been an exceptional mentor and advocate. I owe her a great deal for the solid education and immense opportunities she has provided.

I must also thank my committee members, Dr. Brenda Winkel, Dr. Jill Sible, Dr. John Jelesko, and Dr. Scott Salom, for their continuous support and guidance. I would like to especially thank Dr. Brenda Winkel, who encouraged me to join the Molecular Plant Sciences program. This program was tremendously beneficial. It helped me establish a network of friends amongst my colleagues and allowed me to interact with many different brilliant professors.

I owe a special thanks to Dr. Argelia Lorance who believed in me and changed my graduate school application from a Master to a PhD program. In addition, I would like to thank Dr. Craig Nessler for giving me an undergraduate position in his lab that allowed me to learn the fundamentals of research. I extend my gratitude to Amy Vance and Jeff Stroud who taught me to pipette and make media.

A special recognition must also be made to Dr. Jim Tokuhisa who was always there when I needed advice and encouragement. I want to thank all of the members of the Tholl and Tokuhisa Lab whom I have loved working with: Whitnee Askew, Alice Mweetwa, Mengsu Huang, Reza Sohrabi, Jung-Hyun Huh, Sungbeom Lee, Will Wadlington and the many undergraduates that have not only been co-worker but also friends. I would also like to thank Kristi DeCourcy and Sheena Friend who greatly aided me in my teaching commitments.

I must acknowledge Shimadzu technical support, especially Paul Macek. I thank him for his patience and persistence in helping me with GC-MS problems.

I must also thank all of the people who have made this research possible. I am grateful for the help I received from Bhadra Gunesekera for the Southern blot and Janet Donahue with the radioactive assays. I want to thank Dr. Eric Day (Virginia Tech, Entomology Department) and Dr. Raymond J. Gragné (USDA Systematic Entomology Laboratory, Washington, DC) for the identification of the *Bradysia* species and Dr. Shawn Askew for letting me rear gnats in his greenhouse. I express my gratitude to Dr. Mehdi Ashraf-Khorassani (Virginia Tech, Department of Chemistry) for aiding in the purification of the diterpene compound, Dr. Reuben Peters for all the diterpene standards and Dr. Francis Webster for the NMR analysis.

Lastly, I need to thank my family and friends. First I must thank my Dad for supporting me throughout my education. When he made this promise to my Mother as she left this world, I don't think he ever imagined I would pursue a PhD. I would like to especially thank my brothers, Howard Cochran and Kristos Vaughan who taught me to love playing in the dirt with bugs. I don't know how I would have made it through the stressful moments of graduate school without my sister Josie Brown and my true friend Whitnee Askew.

This dissertation would not have been possible without my editors Emily Grant, Angela Heath, Whitnee Askew, Peter Bowerman and Robert Vaughan. This was a busy semester for Dr. Tholl, so I am very grateful to her for taking the time to edit my writing.

# ABBREVIATIONS

AtTPS - *Arabidopsis* terpene synthase

*CaMV* - Cauliflower mosaic virus

CPP - copalyl diphosphate

CytP450 - cytochrome P450

DOXP - 1-deoxy-D-xylulose-5-phosphate

DIG - digoxigenin

DMAPP - dimethylallyl diphosphate

*E. coli* - *Escherichia coli*

FPP - farnesyl diphosphate

FID - flame ionization detection

GC-MS - gas chromatography-mass spectrometry

GPP - geranyl diphosphate

GGPP - geranylgeranyl diphosphate

GUS - β-glucuronidase

GFP - green fluorescent protein

HMG-CoA - 3-hydroxy-3-methylglutaryl-CoA

IPP - isopentenyl diphosphate

IPTG - isopropyl *β-D*-1-thiogalactopyranoside

JA - jasmonic acid

KO - knockout

LC-MS - liquid chromatography-mass spectrometry

MEP - methylerythritol phosphate

MS - Mursashing and Skoog

NMR - nuclear magnetic resonance

*Pro35S* - *35S* promoter

RT-PCR - reverse transcriptase-polymerase chain reaction

SPME - solid phase microextraction

TPS - terpene synthase

UTR - untranslated region

# TABLE OF CONTENTS

# LIST OF FIGURES AND TABLES

# Chapter I

# General Introduction and Overview of Research

# Chapter I

# General Introduction and Overview of Research

## Belowground herbivory

Roots constitute 50% to 90% of plant biomass (Blossey and Hunt-Joshi 2003), and consequently a large number of diverse organisms exploit belowground plant tissues for their carbon and nutrient supply (van Dam 2009). For example, plant parasitic nematodes such as *Meloidogyne incognita*, which feed on the cytoplasm of living root cells (Davis *et al.* 2004), cause substantial loss of many crop plants throughout the world (Barker and Koenning 1998). Besides plant parasites, insects feeding on roots are among the most harmful herbivores that infest crop plants (Blossey and Hunt-Joshi 2003). Particularly detrimental are chewing insects that sever vascular connections between roots and shoots. Root herbivory by the lucerne weevil (*Sitona discoideus*), for example, causes drought stress and dormancy on alfalfa plants (Goldson *et al.* 1987). Larvae of the cabbage root fly, *Delia radicum*, reduce sugar content and root biomass by as much as 47% in swede, kale, and rape (Hopkins *et al.* 2009). As another example, the western corn rootworm (*Diabrotica spp*.), whose larvae are a common pest feeding on maize roots, reduces plant growth by affecting $CO_2$ assimilation, stomatal conductance, and net photosynthetic rates (Dunn and Frommelt 1998a, Dunn and Frommelt 1998b, Godfrey *et al.* 1993).

Recent research suggests that the physical and physiological changes caused by root herbivores have the potential to influence the diversity of plant communities (De Deyn and Van

der Putten 2005, Kaplan *et al.* 2008a, Van Der Putten 2003). Moreover, root herbivory has been shown to alter the rate and direction of succession, and the susceptibility of plants to other herbivores and pathogens both above- and belowground. In tobacco, root herbivory interferes with aboveground defenses and benefits foliar herbivores by reducing plant resistance (Kaplan, *et al.* 2008a). Thus, belowground root foragers are capable of shaping agricultural and natural ecosystems.

The most common practice employed to reduce root herbivore damage has been the use of chemical pesticides. Methyl bromide was applied as a soil fumigant to control nematode infestations prior to its phase-out as an ozone-depleting chemical. Also, chlorpyrifos was one of the most commonly-used insecticides until it was linked to neurological disorders (Hunter 2001). Imidacloprid is now frequently applied as a systemic insecticide (Rogers and Potter 2003). There are valid health concerns with the application of toxic chemicals directly into the soil (Hunter 2001), and several insects are developing resistance to the insecticides leading to increased chemical applications (Wright *et al.* 2000).

Besides the development of transgenic crops expressing *Bacillus thuringiensis* (Bt) toxin, alternative biological control methods using entomopathogenic nematodes or carabid ground beetles are available and appear promising (Hunter 2001). Their impact on different pests varies, however, and further ecological studies on both the pest and predator are required. Knowledge of the natural defense potential of plants against root herbivores is increasingly being transferred to agricultural applications in an effort to control belowground herbivore damage. For example, the western corn rootworm (*Diabrotica spp.*) causes maize roots to emit the sesquiterpene, (*E*)-β-caryophyllene, which attracts entomopathogenic nematodes that subsequently infect and kill the herbivore. Most North American maize varieties have lost the ability to produce (*E*)-β-

caryophyllene (Koellner *et al.* 2008). Since this discovery, efforts have been made to reintroduce this indirect chemical defense into North American maize by genetic engineering (Degenhardt *et al.* 2009). Thus far, these efforts have been successful and transformed plants suffer significantly less root damage. Hence, deciphering the chemistry of root defense opens up the opportunity to develop alternative pest control strategies by engineering plants with enhanced levels of root defense compounds. However, such efforts have been hampered by the lack of knowledge concerning the intricacies of root defense mechanisms.

## The chemistry of root defense

Anchored in the soil, plants employ multiple defensive strategies including tolerance and both direct and indirect resistance (Van Der Putten 2003). Plants tolerate herbivory by increasing their growth rate or reallocating resources to undamaged tissues (e.g. roots) as storage for assimilates that can enable regrowth after herbivory (Erb *et al.* 2009, Van Der Putten 2003). In contrast to tolerance which allows the herbivore to develop and reproduce, resistance reduces the performance of the herbivore by deploying chemical defenses that directly deter or intoxicate the pest (De Moraes *et al.* 2001, Kessler and Baldwin 2001, Vancanneyt *et al.* 2001) or indirectly attract natural predators of the attacking herbivores (Arimura *et al.* 2004, Mercke *et al.* 2004, Rasmann *et al.* 2005).

Plant defense theories, such as the Green World Hypothesis and the Optimal Defense Theory, have been developed almost exclusively based on aboveground interactions. The Green World Hypothesis suggests that plant communities survive (the world remains green) because herbivore consumption is limited by plant defensive strategies and predators (Hairston *et al.*

1960). The Optimal Defense Theory states that defenses are costly to plants and are therefore allocated to tissues of greatest value and risk of attack (Zangerl and Rutledge 1996). However, researchers have begun to realize that such theories need to include empirical data on the distribution of root defenses because root-feeding insects play a major role in driving the composition of natural plant communities (van Dam 2009, Van Der Putten 2003).

Many phytochemical studies describing the diversity of specialized metabolites produced in plant roots, such as alkaloids and terpenes, support the notion that phytochemical investments are similarly apportioned to above and belowground plant tissues (Kaplan *et al.* 2008b, Rasmann and Agrawal 2008). The formation of pyridine alkaloids such as nicotine in tobacco (*Nicotiana tabacum*) and pyrrolizidine alkaloids in *Senecio* species are well-studied examples (Baldwin 1999, Ober and Hartmann 2000). Furthermore, the biosynthesis of terpene phytoalexins such as the sesquiterpene aldehyde gossypol and triterpene saponins has been investigated in roots of cotton (*Gossypium hirsutum*) and oat (*Avena sativa*) (Hunter *et al.* 1978, Osbourn *et al.* 2003, Senalik and Simon 1987). However, knowledge of the role of these and other root metabolites in defense against root herbivores has been limited. One of the more recent efforts to gain insight into root defense mechanisms has been made with brassicaceous plants. Herbivore damage caused by the cabbage and turnip root maggot (*Delia radicum* and *Delia floralis*) was found to induce the production of glucosinolate defense metabolites in the roots of several *Brassica* species (Hopkins*, et al.* 2009, van Dam and Raaijmakers 2006). However, it is not always clear whether these induced compounds have a direct biological effect on the herbivore (Hopkins*, et al.* 2009). The most direct way to test the defensive function of an individual compound is to isolate it from the plant and incorporate it in an artificial diet (van Dam 2009). In this manner, Schmelz et al. (2002) demonstrated that phytoecdysteroids, which are induced in spinach roots

by feeding of *Bradysia* (fungus gnat) larvae, have toxic effects on the root herbivore (Schmelz *et al.* 2002). Methods such as these can only be useful if the defense compound of interest can be isolated and if it functions independent of other plant constituents. Diets incorporating defense compounds do not represent the versatile interaction between herbivores and the live host plant. Artificial conditions may also result in altered feeding behavior. Furthermore, the results from such experiments can be deceiving if the concentration of the compound is not comparable to the amount produced by the live root tissue. A promising alternative and less artificial approach is to evaluate insect performance on mutant plants lacking a specific secondary metabolite thought to be involved in defense (Gigolashvili *et al.* 2007).

## Volatile terpene defense metabolites

Terpenes represent the largest and most diverse class of plant secondary or specialized metabolites. Terpene products of low molecular weight such as monoterpenes ($C_{10}$), sesquiterpenes ($C_{15}$) and diterpene hydrocarbons ($C_{20}$) have high vapor pressure at room temperature and can be released into the atmosphere or rhizosphere. The chemical and physical properties of volatile and semi-volatile terpenes make these compounds extremely useful tools for mediating ecological interactions of plants with their environment (Pichersky and Gershenzon 2002). Based on aboveground interactions, volatile terpenes can act from a distance and directly deter feeding or ovipositioning insects (De Moraes*, et al.* 2001, Kessler and Baldwin 2001). In response to herbivore damage, these chemicals can be mobilized as direct intoxicants or indirect signals to attract parasitoids and predators. For example, induced volatile blends deter *Manduca sexta* and *Manduca quinquemaculata* (Lepidoptera, Sphingidae) from ovipositioning

and feeding on *Nicotiana attenuata* plants (Kessler and Baldwin 2001). *Arabidopsis thaliana* leaves infested with *Pieris rapae* (cabbage white butterfly) emit a blend of volatile terpenes that attract *Cotesia rubecula*, a specialist parasitoid of *P. rapae* (Van Poecke *et al.* 2001).

There is evidence that volatile terpenes produced in roots have similar defensive functions in the rhizosphere (Rasmann*, et al.* 2005). *Arabidopsis* roots infested with the Russian wheat aphid (*Diuraphis noxia*) emit the monoterpene, 1,8-cineole (Steeghs *et al.* 2004), which has been implicated in direct defense because of its toxic and deterrent effects on certain insects. The sesquiterpene volatile terpene, (*E*)-β-caryophyllene, released from maize roots in response to *Diabrotica* feeding, functions as an indirect defense compound that attracts insect-parasitizing nematodes (Rasmann*, et al.* 2005). Volatile diterpene compounds have yet to be associated with belowground defense. Although many diterpenes are produced in roots, only nonvolatile diterpene compounds have been functionally characterized and are primarily considered antimicrobial substances. These include momilactones A & B, oryzalexins A-F, and phytocassanes A-E, labdane-related diterpene phytoalexins that are exuded from rice roots in response to the rice blast pathogen *Megneporthe grisea* (Peters 2006). The defensive function of diterpenes against root herbivores is currently not known.

## Biosynthesis of Terpenes

Terpenes are the most diverse class of secondary metabolites; however, they have a common biosynthetic origin based on the fusion of $C_5$-isoprenoid units. Terpene biosynthesis can be divided into three main phases: (1) the biosynthesis of the $C_5$-precursor isopentenyl

diphosphate (IPP), (2) the condensation of $C_5$-units to prenyl diphosphates, and (3) the conversion of prenyl diphosphates to terpenes (Gershenzon and Kreis 1999).

***Biosynthesis of the terpene precursor IPP***

In plants, the precursor IPP is synthesized via two alternate pathways: the mevalonate pathway located in the cytosol and the methylerythritol phosphate (MEP) pathway located in plastids (Figure 1). In the mevalonate pathway, the synthesis of IPP is initiated by the condensation of three molecules of acetyl coenzyme A to form a $C_6$ compound, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is then reduced to mevalonic acid, phosphorylated twice and subsequently decarboxylated to yield IPP. IPP is converted to its allylic isomer, dimethylallyl diphosphate (DMAPP), by the activity of an IPP isomerase. In the MEP pathway, IPP is formed from pyruvate and glyceraldehyde-3-phosphate. A $C_2$-unit derived from pyruvate is combined with glyceraldehyde-3-phosphate to form 1-deoxy-D-xylulose-5-phosphate (DOXP). DOXP is then transformed into IPP and DMAPP by a series of six enzymatic steps (Phillips *et al.* 2008).

***Terpene synthases and their prenyl diphosphate substrates***

The second phase of terpene biosynthesis involves the fusion of $C_5$-units to form prenyl diphosphates catalyzed by prenyltransferases. The enzymatic reaction starts with the binding of the more reactive $C_5$-precursor DMAPP, which is derived from IPP by the action of IPP isomerase (Figure 1.1).

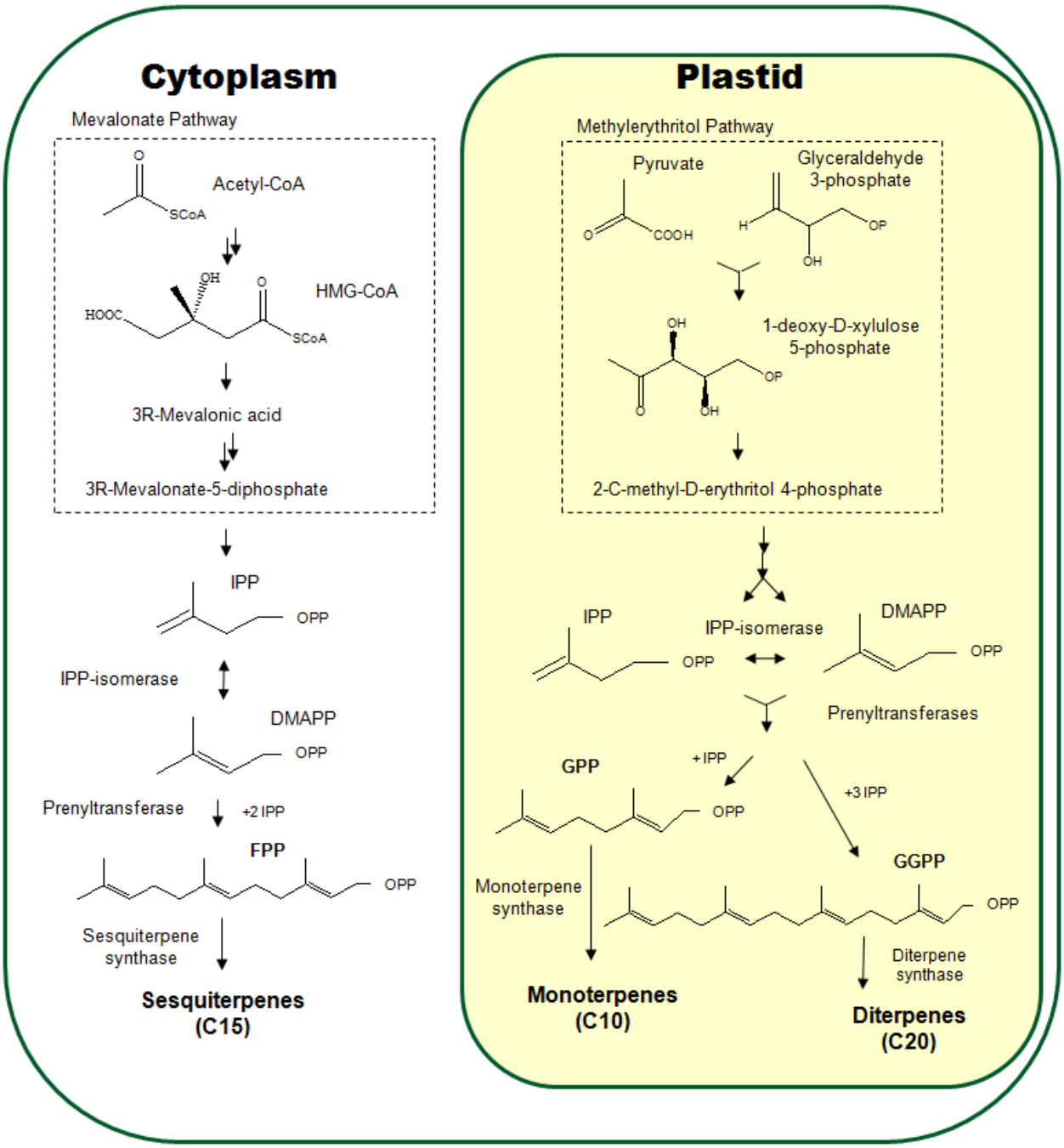


**Figure 1.1. Terpene biosynthesis in plants**

DMAPP is then condensed with multiple IPP units in a head-to-tail orientation to form the prenyl diphosphates of different chain length. Fusion of two, three, or four $C_5$-units yields geranyl diphosphate (GPP, $C_{10}$), farnesyl diphosphate (FPP, $C_{15}$) and geranylgeranyl diphosphate

(GGPP, $C_{20}$), respectively, which are the universal precursors of monoterpenes ($C_{10}$), sesquiterpenes ($C_{15}$) and diterpenes ($C_{20}$) (Bohlmann *et al.* 1998, Davis and Croteau 2000, Gershenzon and Kreis 1999).

In the third phase of terpene biosynthesis, terpene synthases (TPSs) catalyze the conversion of the prenyl diphosphate substrates into the different classes of terpene products. TPSs are dependent on a divalent metal ion cofactor such as $Mg^{2+}$ for catalysis. The reaction of most TPSs (Class I, Figure 1.2a) starts with the cleavage of the diphosphate moiety resulting in a carbocation intermediate. The carbocation can undergo a series of structural rearrangements including cyclization, hydride shifts and methyl migrations prior to a deprotonation reaction or capture of a water molecule as a final step. Class II TPSs, however, initiate the reaction by the protonation of the double bond at the opposite end of the molecule from the diphosphate moiety. This mechanism is characteristic of several diterpene synthases, such as those synthesizing copalyl diphosphate (CPP), a precursor in gibberellin biosynthesis (Gershenzon and Kreis 1999, Tholl 2006).

Labdane-related tricyclic diterpenes are synthesized by two sequential cyclization steps. Initially, a class II diterpene synthase catalyzes the reaction of GGPP to produce CPP, which can then be further converted by class I TPSs into the final diterpene product(s). For example, in rice, pimaradiene (Figure 1.2b) and other labdane-related diterpenes are synthesized via *syn*- or *ent*-CPP as a free intermediate. Finally, some diterpene synthases are bifunctional and can catalyze both cyclization steps autonomously such as abietadiene synthase from grand fir, which is considered both a class I and class II enzyme (Figure 1.2c) (Peters *et al.* 2003, Peters and Croteau 2002).

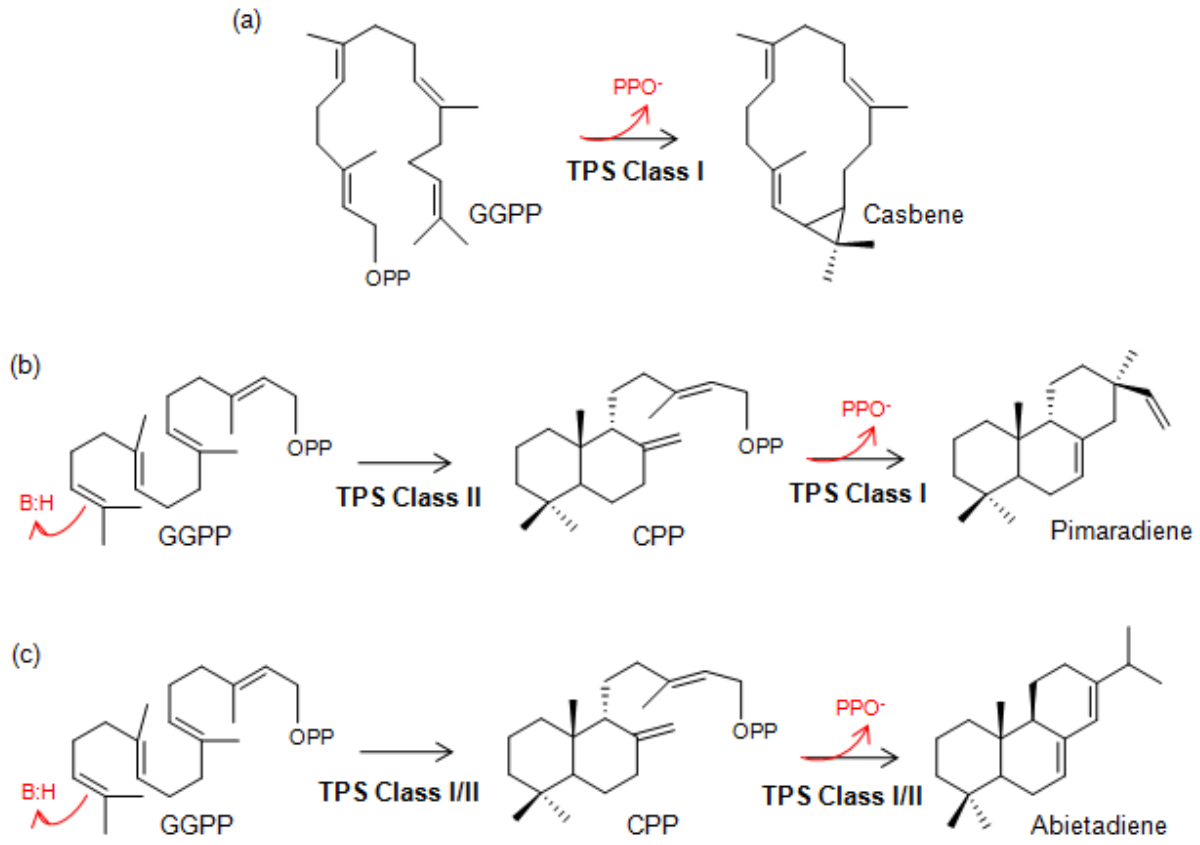


**Figure 1.2. Class I and class II diterpene synthases.** (a) Casbene synthase is a class I enzyme that directly converts GGPP into a macrocyclic diterpene hydrocarbon. The reaction is initiated by the cleavage of the diphosphate moiety. (b) Two enzymes are required for pimaradiene formation. A class II CPP synthase must first catalyze reaction of GGPP to CPP via the protonation of the double bond and then the diphosphate is cleaved from CPP by the class I synthase to form pimaradiene. (c) Abietadiene synthase is a bifunctional enzyme (class I and class II). First GGPP is converted into the CPP intermediate via the protonation of the double bond. Subsequently the diphosphate moiety is cleaved and the tricyclic abietadiene is formed.

A single TPS commonly produces multiple products. Most of the structural variation of terpenes arise from the diverse carbon backbones formed by TPSs (Davis and Croteau 2000). Many terpene volatiles are direct products of terpene synthases but others are formed through transformation of the initial products by oxidation, dehydrogenation, acylation, and other reactions (Gershenzon and Kreis 1999). For example, the $C_{16}$-homoterpene, TMTT (4,8,12-trmethyltrideca-1,3,7,11-tetraene) emitted from *Arabidopsis* leaves in response to *Pieris rapae* feeding is synthesized from GGPP via the diterpene intermediate (*E,E*)-geranyllinalool followed by an oxidative degradation step catalyzed by a single cytochrome P450 enzyme (Lee and Tholl, personal communication, Herde et al. 2008).

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

The biosynthesis of the different classes of terpenes is regulated at the subcellular level by the targeting of prenyltransferases and TPS enzymes to different cellular compartments. In general, the formation of monoterpenes and diterpenes from GPP and GGPP, respectively, occurs in plastids while sesquiterpenes are synthesized from FPP in the cytosol (Tholl, 2006). However, some studies including recent experiments in *Arabidopsis* suggest that small pools of FPP might also exist in plastids, and the mitochondria might be a natural subcellular compartment of volatile terpene biosynthesis (Aharoni *et al.* 2003).

Given the potential cytotoxic activities of many terpenes, these compounds are often synthesized and stored in specialized secretory cells and structures. For example, many plant species in the Lamiaceae family, such as peppermint and basil, store volatile terpenes in glandular trichomes located on the surface of leaves (Pichersky and Gershenzon 2002). When

these trichomes are disrupted (e.g. by feeding of an insect), the volatiles are immediately released. Other secretory structures include resin ducts in conifers and scent glands in flowers (Dudareva *et al.* 2004, Pichersky and Gershenzon 2002).

Terpene volatiles are also emitted from plant tissues without prior accumulation. The *de novo* biosynthesis and emission of terpenes is observed in particular in response to the damage of plant tissues by herbivores or microbial pathogens (Pare & Tumlinson, 1997). Volatile terpenes formed in *Arabidopsis* are typically emitted from tissues without accumulation.

Both environmental conditions and the developmental stage of plants influence the production of volatile terpenes. For example, floral volatile emissions are dependent on the developmental stage of the flower and peak a few days after anthesis to ensure successful pollination (Dudareva *et al.* 2003). Volatile emissions from flowers usually follow diurnal or circadian rhythms, as has been described for terpene emissions from snapdragon (*Antirrhinum majus*) flowers (Dudareva*, et al.* 2004). In addition to changes in light conditions, changes in temperature usually influence volatile emissions with the greatest release typically occurring under conditions of high light and high temperature (Dudareva*, et al.* 2003).

Many studies of spatial and temporal changes in terpene emissions have shown that the biosynthesis of terpene volatiles is regulated largely at the level of *TPS* gene expression (Bohlmann*, et al.* 1998, Dudareva*, et al.* 2004, Gershenzon and Kreis 1999). Since *TPS* genes represent important regulatory steps in terpene biosynthesis, they are of primary interest in studying the formation and function of terpene volatiles (Dudareva*, et al.* 2004).

## *Arabidopsis* as a model for studying volatile terpene root defense mechanisms against herbivores

*Arabidopsis* provides an ideal model system to investigate the biochemistry and biological function of root terpene secondary metabolism. *Arabidopsis* contains more than a hundred specialized metabolites spanning seven different classes of compounds (D'Auria and Gershenzon 2005). Terpenes constitute the largest class of *Arabidopsis* specialized metabolites. The *Arabidopsis* genome contains a large gene family of 32 terpene synthase genes. Based on sequence similarities, the *Arabidopsis* TPSs (AtTPSs) cluster into three main clades: clade I containing six monoterpene synthases, clade II containing two enzymes catalyzing initial steps in gibberellin biosynthesis and geranyllinallol synthase (AtTPS04), and clade III containing the remaining AtTPSs (Figure 1.3). Of the TPSs in clade III, 16 enzymes carry a putative plastid-targeting sequence and are predicted monoterpene or diterpene synthases. Six enzymes (AtTPS11, 12, 13, 21, 22, 25) lack a transit peptide and are all sesquiterpene synthases (Aubourg *et al.* 2002). According to *AtTPS* gene expression profiles obtained by RT-PCR analysis from soil-grown roots (Chen et al., 2003; Ro et al., 2006), 15 *AtTPS* genes are expressed primarily in *Arabidopsis* roots. Most of the root-specific AtTPSs belong to clade III, with the exception of the monoterpene synthases AtTPS23/24 in clade I (Aubourg*, et al.* 2002, Chen *et al.* 2004). Five of the root-expressed AtTPSs were predicted to function as diterpene synthases (AtTPS08, 17, 20, 26, 29) because of their similarity to the class I-type casbene synthase (Figure 1.3) (Aubourg*, et al.* 2002). According to gene expression profiles, all of these predicted diterpene synthases are constitutively expressed (Birnbaum *et al.* 2003, Ro *et al.* 2006).

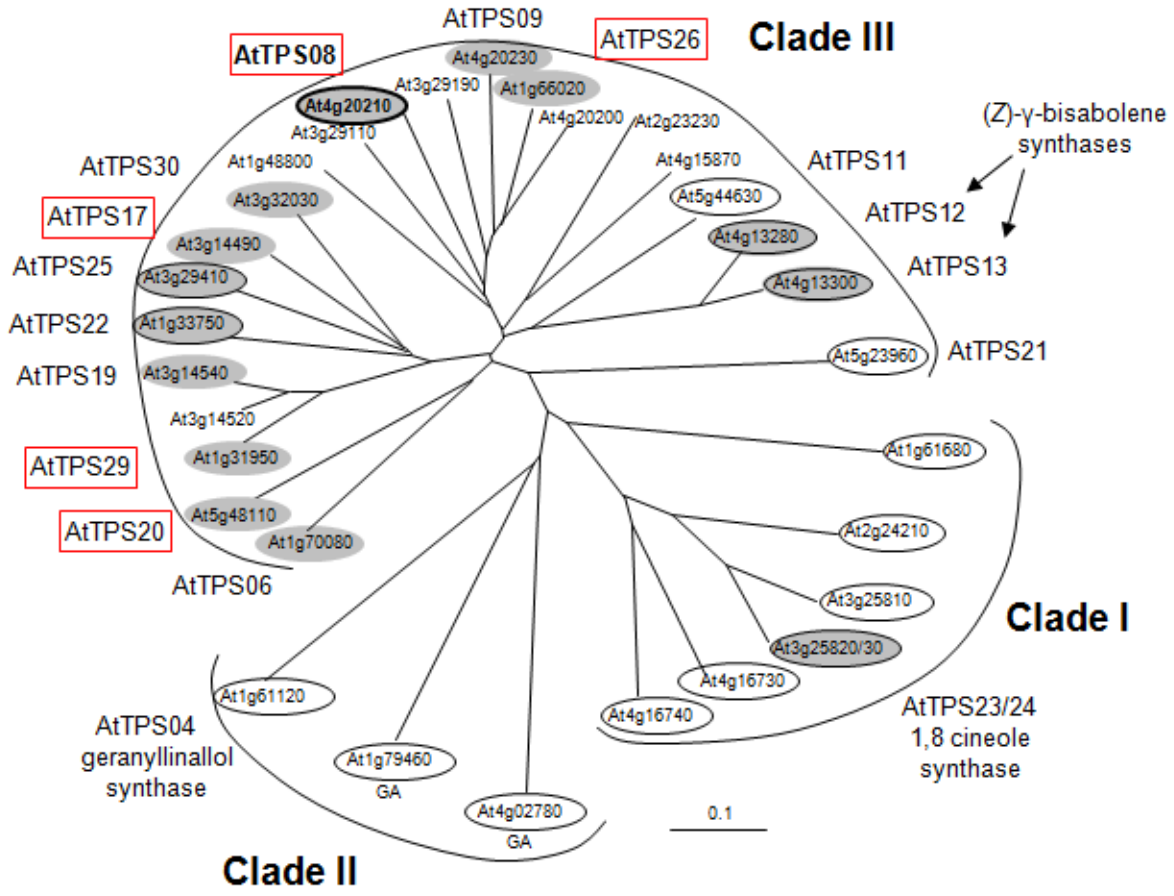


**Figure 1.3**. **Phylogenetic relationship of *Arabidopsis* terpene synthases**
*Arabidopsis* terpene synthases (AtTPSs) form three main clades. Biochemically characterized AtTPSs are circled. Characterized *TPS* genes in clade I are all monoterpene synthases. Clade II contains diterpene synthases involved in gibberellin (GA) biosynthesis and a geranyllinalool synthase (AtTPS04). The characterized TPSs in clade III are all sesquiterpene synthases (AtTPS11, 12, 13, 22, 25). The remaining *AtTPS* genes contain a putative plastid-targeting sequence and are presumably monoterpene and diterpene synthases. The putative diterpene synthases are in red boxes. *AtTPS* genes with primary expression in roots are indicated in gray. The root-expressed diterpene synthase (AtTPS08) that is characterized in this work is in bold and is discussed further in Chapter 3.

## Belowground herbivory on *Arabidopsis*

Prior to the current, a method for investigating belowground herbivory on *Arabidopsis* roots had not been established. The absence of such a bioassay was most likely due to difficulties in working with *Arabidopsis* root tissue embedded in soil and in selecting a herbivore that would be suitable for feeding experiments with *Arabidopsis* roots.

### Bradysia *larvae as a root herbivore*

Darkwinged fungus gnats are generalist herbivores that feed on the roots of a variety of plants including *Arabidopsis*. The two most common species, *Bradysia coprophila* (Lintner) and *Bradysia impatiens* (Johannsen), are both considered prominent greenhouse pests (Jagdale *et al.* 2007). The aphagous black gnats that commonly infest greenhouses were previously considered merely a nuisance as the larvae primarily feed on organic matter and fungi; however, when this food source is depleted, larvae will actively feed on root tissue. Larvae often chew and strip roots, negatively inpacting water and nutrient absorption. Extreme infestations can lead to loss of plant vigor and even mortality (Meers and Cloyd 2005). Seedlings are particularly more susceptible to larval feeding damage. For example, *Bradysia* larvae may pose a serious threat to legume seedling with a 3.3 ratio of seedling deaths to number of larvae (Springer and Carlton 1993). Adult female gnats lay between 250 and 1000 eggs in their lifetime of approximately 1 to 3 days. Larvae emerge within 4 to 6 days and feed in the soil for 12 to 14 days before pupating. The life cycle is completed within 20 to 25 days at 20 to 25°C (Figure 1.4) (Cloyd and Zaborski 2004).

*Bradysia* larvae have been used to investigate hormone-dependent defense responses in *Arabidopsis*. Larvae cause high mortality of soil-grown *Arabidopsis* mutants deficient in the biosynthesis of jasmonic acid (JA) (McConn *et al.* 1997). Large infestations also reduce wild-type *Arabidopsis* seedling establishment but do not appear to have any major deleterious effects on mature plants. However, these assessments were based on aboveground observations of foliage and survival. The severity of root damage caused by *Bradysia* larvae on mature wild type plants had, until this study, not been investigated.

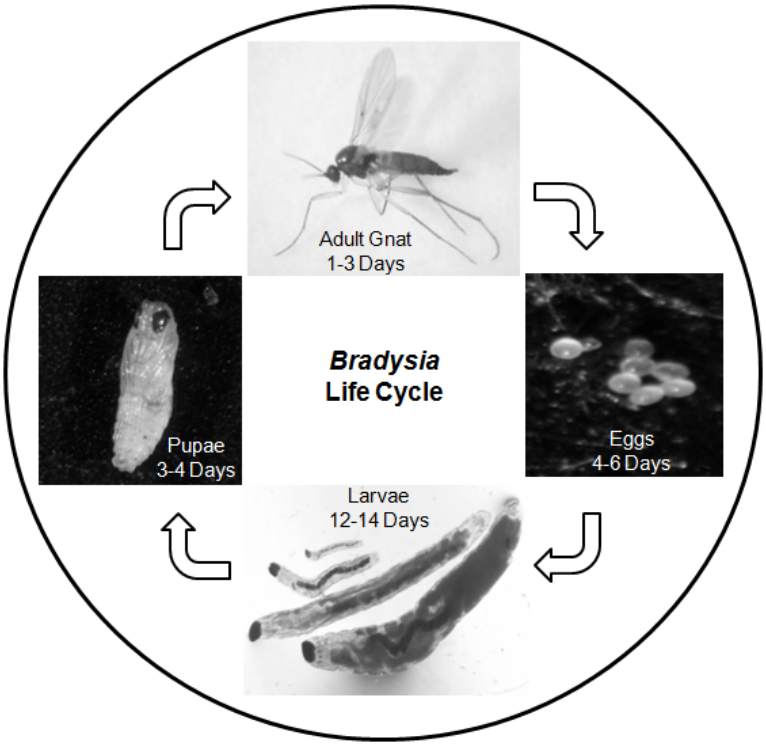


**Figure 1.4. *Bradysia* species life cycle.** Males of *Bradysia* species (spp.) emerge 1 day before females. There is a 24 hour preoviposition period following female emergence in which mating occurs. Males usually live longer than females, which die soon after oviposition. Eggs hatch in 4 to 6 days and larvae feed in the soil for 12 to 14 days while developing through 4 instars. Larvae then pupate for 3 to 4 days before subsequently emerging as adult gnats. The life cycle is largely dependent on temperature. At 20 to 25°C the cycle is usually completed in approximately 20 to 25 days (Cloyd and Zaborski 2004, Harris *et al.* 1996).

## Overview of research

Plants have evolved intricate mechanisms for protection against herbivores, including the formation of chemical defense metabolites. This study was guided by the hypothesis that root defense mechanisms are as chemically diverse as those produced aboveground and are capable of serving similar defensive functions in the rhizosphere. The potential of plants to influence the behavior of belowground pests using root chemical defenses has a major bearing on plant fitness. The overall goal of this work was to analyze root chemical defense mechanisms with a focus on volatile terpene metabolites in *Arabidopsis* for gaining a better understanding of the function of phytochemicals in belowground plant-herbivore interaction.

The specific objectives of the study were to:

I. Characterize the biosynthesis of volatile terpene metabolites in *Arabidopsis* roots.

II. Develop a bioassay for investigating the role of volatile root terpenes in the defense against the root herbivore *Bradysia*.

In Chapter 2, a root-herbivore system using aeroponic *Arabidopsis* and the root-feeding insect *Bradysia* larvae is presented; this system can be used as a bioassay to investigate the biological function of root defense metabolites. We describe an aeroponic clay pellet culture system for *Arabidopsis* that provides easy access to the root tissue while retaining a soil-like environment to accommodate herbivore feeding. Furthermore, the effects of *Bradysia* larvae on wild type plants in this system are evaluated.

In Chapter 3, the biochemical and molecular characterization of a root-specific *Arabidopsis* terpene synthase that catalyzes the formation of the volatile diterpene rhizathalene is reported. A functional analysis of rhizathalene was conducted revealing its involvement in direct defense against *Bradysia* herbivory.

Finally in Chapter 4, the significance of this study is discussed from a biochemical and ecological standpoint, including perspectives for using *Arabidopsis* and the developed bioassay in related future research.

## References


**Aharoni, A., Giri, A.P., Deuerlein, S., Griepink, F., de Kogel, W.J., Verstappen, F.W.A., Verhoeven, H.A., Jongsma, M.A., Schwab, W. and Bouwmeester, H.J.** (2003) Terpenoid metabolism in wild type and transgenic *Arabidopsis* plants. *Plant Cell*, **15**, 2866-2884.

**Arimura, G., Huber, D.P.W. and Bohlmann, J.** (2004) Forest tent caterpillars (*Malacosoma disstria*) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (*Populus trichocarpa x deltoides*): cDNA cloning, functional characterization, and patterns of gene expression of (-)-germacrene D synthase, PtdTPS1. *Plant Journal*, **37**, 603-616.

**Aubourg, S., Lecharny, A. and Bohlmann, J.** (2002) Genomic analysis of the terpenoid synthase (*AtTPS*) gene family of *Arabidopsis thaliana*. *Molecular Genetics and Genomics*, **267**, 730-745.

**Baldwin, I.T.** (1999) Inducible nicotine production in native *Nicotiana* as an example of adaptive phenotypic plasticity. *Journal of Chemical Ecology*, **25**, 3-30.

**Barker, K.R. and Koenning, S.R.** (1998) Developing sustainable systems for nematode management. *Annual Review of Phytopathology*, **36**, 165-205.

**Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W. and Benfey, P.N.** (2003) A gene expression map of the *Arabidopsis* root. *Science*, **302**, 1956-1960.

**Blossey, B. and Hunt-Joshi, T.R.** (2003) Belowground herbivory by insects: Influence on plants and aboveground herbivores. *Annual Review of Entomology*, **48**, 521-547.

**Bohlmann, J., Meyer-Gauen, G. and Croteau, R.** (1998) Plant terpenoid synthases: Molecular biology and phylogenetic analysis. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 4126-4133.

**Chen, F., Ro, D.K., Petri, J., Gershenzon, J., Bohlmann, J., Pichersky, E. and Tholl, D.** (2004) Characterization of a root-specific *Arabidopsis* terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole. *Plant Physiology*, **135**, 1956-1966.

**Cloyd, R.A. and Zaborski, E.R.** (2004) Fungus gnats, *Bradysia* spp. (Diptera : Sciaridae), and other arthropods in commercial bagged soilless growing media and rooted plant plugs. *Journal of Economic Entomology*, **97**, 503-510.

**D'Auria, J.C. and Gershenzon, J.** (2005) The secondary metabolism of *Arabidopsis thaliana*: growing like a weed. *Current Opinion in Plant Biology*, **8**, 308-316.

**Davis, E.L., Hussey, R.S. and Baum, T.J.** (2004) Getting to the roots of parasitism by nematodes. *Trends in Parasitology*, **20**, 134-141.

**Davis, E.M. and Croteau, R.** (2000) Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. In *Biosynthesis: Aromatic Polyketides, Isoprenoids, Alkaloids*, pp. 53-95.

**De Deyn, G.B. and Van der Putten, W.H.** (2005) Linking aboveground and belowground diversity. *Trends Ecology and Evolution*, **20**, 625-633.

**De Moraes, C.M., Mescher, M.C. and Tumlinson, J.H.** (2001) Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature*, **410**, 577-580.

**Degenhardt, J., Hiltpold, I., Kollner, T.G., Frey, M., Gierl, A., Gershenzon, J., Hibbard, B.E., Ellersieck, M.R. and Turlings, T.C.J.** (2009) Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 13213-13218.

**Dudareva, N., Martin, D., Kish, C.M., Kolosova, N., Gorenstein, N., Faldt, J., Miller, B. and Bohlmann, J.** (2003) (*E*)-beta-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: Function and expression of three terpene synthase genes of a new terpene synthase subfamily. *Plant Cell*, **15**, 1227-1241.

**Dudareva, N., Pichersky, E. and Gershenzon, J.** (2004) Biochemistry of plant volatiles. *Plant Physiology*, **135**, 1893-1902.

**Dunn, J.P. and Frommelt, K.** (1998a) Effects of below-ground herbivory by *Diabrotica virgifera virgifera* (Col, Chrysomelidea) and soil moisture on leaf gas exchange of maize. *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie*, **122**, 179-183.

**Dunn, J.P. and Frommelt, K.** (1998b) Effects of below-ground herbivory by *Diabrotica virgifera virgifera* (Coleoptera) on biomass allocation and carbohydrate storage of maize. *Applied Soil Ecology*, **7**, 213-218.

**Erb, M., Lenk, C., Degenhardt, J. and Turlings, T.C.J.** (2009) The underestimated role of roots in defense against leaf attackers. *Trends in Plant Science*, **14**, 653-659.

**Gershenzon, J. and Kreis, W.** (1999) Biochemistry of terpenoids: monoterpenes, sesquiterpens, diterpenes, sterols, cardiac glycosides and steroid saponins. In M. Wink, ed, Biochemistry of Plant Secondary Metabolism. *Annual Plant Reviews*, **2**, 222-249.

**Gigolashvili, T., Berger, B., Mock, H.P., Muller, C., Weisshaar, B. and Fluegge, U.I.** (2007) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. *Plant Journal*, **50**, 886-901.

**Godfrey, L.D., Meinke, L.J. and Wright, R.J.** (1993) Vegetative and reproductive biomass accumulation in-field corn - response to root injury by western corn-rootworm (Coleoptera, chrysomelidae). *Journal of Economic Entomology*, **86**, 1557-1573.

**Goldson, S.L., Bourdot, G.W. and Proffitt, J.R.** (1987) A study of the effects of *sitona-discoideus* (Soleoptera, curculionidae) larval feeding on the growth and development of lucerne (*Medicago-sativa*). *Journal of Applied Ecology*, **24**, 153-161.

**Hairston, N.G., Smith, F.E. and Slobodkin, L.B.** (1960) Community structure, population control, and competition. *The American Naturalist*, **94**, 421-425.

**Hopkins, R.J., van Dam, N.M. and van Loon, J.J.A.** (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Annual Review of Entomology*, **54**, 57-83.

**Hunter, M.D.** (2001) Out of sight, out of mind: the impacts of root-feeding insects in natural and managed systems. *Agricultural and Forest Entomology*, **3**, 3-9.

**Hunter, R.E., Halloin, J.M., Veech, J.A. and Carter, W.W.** (1978) Exudation of terpenoids by cotton roots. *Plant and Soil*, **50**, 237-240.

**Jagdale, G.B., Casey, M.L., Canas, L. and Grewal, P.S.** (2007) Effect of entomopathogenic nematode species, split application and potting medium on the control of the fungus gnat, *Bradysia difformis* (Diptera : Sciaridae), in the greenhouse at alternating cold and warm temperatures. *Biological Control*, **43**, 23-30.

**Kaplan, I., Halitschke, R., Kessler, A., Rehill, B.J., Sardanelli, S. and Denno, R.F.** (2008a) Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecology Letters*, **11**, 841-851.

**Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S. and Denno, R.F.** (2008b) Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology*, **89**, 392-406.

**Kessler, A. and Baldwin, I.T.** (2001) Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, **291**, 2141-2144.

**Koellner, T.G., Held, M., Lenk, C., Hiltpold, I., Turlings, T.C.J., Gershenzon, J. and Degenhardt, J.** (2008) A maize (*E*)-beta-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. *Plant Cell*, **20**, 482-494.

**McConn, M., Creelman, R.A., Bell, E., Mullet, J.E. and Browse, J.** (1997) Jasmonate is essential for insect defense *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 5473-5477.

**Meers, T.L. and Cloyd, R.A.** (2005) Egg-laying preference of female fungus gnat *Bradysia sp nr. coprophila* (Diptera : Sciaridae) on three different soilless substrates. *Journal of Economic Entomology*, **98**, 1937-1942.

**Mercke, P., Kappers, I.F., Verstappen, F.W.A., Vorst, O., Dicke, M. and Bouwmeester, H.J.** (2004) Combined transcript and metabolite analysis reveals genes involved in spider mite induced volatile formation in cucumber plants. *Plant Physiology*, **135**, 2012-2024.

**Ober, D. and Hartmann, T.** (2000) Phylogenetic origin of a secondary pathway: the case of pyrrolizidine alkaloids. *Plant Molecular Biology*, **44**, 445-450.

**Osbourn, A.E., Qi, X.Q., Townsend, B. and Qin, B.** (2003) Dissecting plant secondary metabolism - constitutive chemical defences in cereals. *New Phytologist*, **159**, 101-108.

**Peters, R.J.** (2006) Uncovering the complex metabolic network underlying diterpenoid phytoalexin biosynthesis in rice and other cereal crop plants. *Phytochemistry*, **67**, 2307-2317.

**Peters, R.J., Carter, O.A., Zhang, Y., Matthews, B.W. and Croteau, R.B.** (2003) Bifunctional abietadiene synthase: Mutual structural dependence of the active sites for protonation-initiated and ionization-initiated cyclizations. *Biochemistry*, **42**, 2700-2707.

**Peters, R.J. and Croteau, R.B.** (2002) Abietadiene synthase catalysis: Conserved residues involved in protonation-initiated cyclization of geranylgeranyl diphosphate to (+)-copalyl diphosphate. *Biochemistry*, **41**, 1836-1842.

**Phillips, M.A., Leon, P., Boronat, A. and Rodriguez-Concepcion, M.** (2008) The plastidial MEP pathway: unified nomenclature and resources. *Trends in Plant Science*, **13**, 619-623.

**Pichersky, E. and Gershenzon, J.** (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Current Opinion in Plant Biology*, **5**, 237-243.

**Rasmann, S. and Agrawal, A.A.** (2008) In defense of roots: A research agenda for studying plant resistance to belowground herbivory. *Plant Physiology*, **146**, 875-880.

**Rasmann, S., Kollner, T.G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J. and Turlings, T.C.J.** (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature*, **434**, 732-737.

**Ro, D.K., Ehlting, J., Keeling, C.I., Lin, R., Mattheus, N. and Bohlmann, J.** (2006) Microarray expression profiling and functional characterization of AtTPS genes: Duplicated *Arabidopsis thaliana* sesquiterpene synthase genes At4g13280 and At4g13300 encode root-specific and wound-inducible (*Z*)-gamma-bisabolene synthases. *Archives of Biochemistry and Biophysics*, **448**, 104-116.

**Rogers, M.E. and Potter, D.A.** (2003) Effects of spring imidacloprid application for white grub control on parasitism of Japanese beetle (Coleoptera : Scarabaeidae) by *Tiphia vernalis* (Hymenoptera : Tiphiidae). *Journal of Economic Entomology*, **96**, 1412-1419.

**Schmelz, E.A., Grebenok, R.J., Ohnmeiss, T.E. and Bowers, W.S.** (2002) Interactions between *Spinacia oleracea* and *Bradysia impatiens*: A role for phytoecdysteroids. *Archives of Insect Biochemistry and Physiology*, **51**, 204-221.

**Senalik, D. and Simon, P.W.** (1987) Quantifying intraplant variation of volatile terpenoids in carrot. *Phytochemistry*, **26**, 1975-1979.

**Springer, T.L. and Carlton, C.E.** (1993) Oviposition preference of darkwinged fungus gnats (Diptera, Sciaridae) among trifolium species. *Journal of Economic Entomology*, **86**, 1420-1423.

**Steeghs, M., Bais, H.P., de Gouw, J., Goldan, P., Kuster, W., Northway, M., Fall, R. and Vivanco, J.M.** (2004) Proton-transfer-reaction mass spectrometry as a new tool for real time analysis of root-secreted volatile organic compounds in arabidopsis. *Plant Physiology*, **135**, 47-58.

**Tholl, D.** (2006) Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Current Opinion in Plant Biology*, **9**, 297-304.

**van Dam, N.M.** (2009) Belowground herbivory and plant defenses. *Annual Review of Ecology Evolution and Systematics*, **40**, 373-391.

**van Dam, N.M. and Raaijmakers, C.E.** (2006) Local and systemic induced responses to cabbage root fly larvae (*Delia radicum*) in *Brassica nigra* and *B. oleracea*. *Chemoecology*, **16**, 17-24.

**Van Der Putten, W.H.** (2003) Plant defense belowground and spatiotemporal processes in natural vegetation. *Ecology*, **84**, 2269-2280.

**Van Poecke, R.M.P., Posthumus, M.A. and Dicke, M.** (2001) Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: Chemical, behavioral, and gene-expression analysis. *Journal of Chemical Ecology*, **27**, 1911-1928.

**Vancanneyt, G., Sanz, C., Farmaki, T., Paneque, M., Ortego, F., Castanera, P. and Sanchez-Serrano, J.J.** (2001) Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 8139-8144.

**Wright, R.J., Scharf, M.E., Meinke, L.J., Zhou, X.G., Siegfried, B.D. and Chandler, L.D.** (2000) Larval susceptibility of an insecticide-resistant western corn rootworm (Coleoptera : Chrysomelidae) population to soil insecticides: Laboratory bioassays, assays of detoxification enzymes, and field performance. *Journal of Economic Entomology*, **93**, 7-13.

**Zangerl, A.R. and Rutledge, C.E.** (1996) The probability of attack and patterns of constitutive and induced defense: A test of optimal defense theory. *American Naturalist*, **147**, 599-608.

# Chapter II

## A novel bioassay for studying belowground herbivory: Analysis of *Bradysia* (fungus gnat) feeding on roots of *Arabidopsis thaliana* grown in potting substrate and aeroponic culture.

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

# Chapter II

# A novel bioassay for studying belowground herbivory: Analysis of *Bradysia* (fungus gnat) feeding on roots of *Arabidopsis thaliana* grown in potting substrate and aeroponic culture.


## Abstract

**Background:** Root-feeding herbivores have adverse effects on plants. To combat herbivore attack, plants have evolved complex chemical defensive strategies. Research investigating molecular and chemical defense responses belowground has been hindered by experimental difficulties associated with the accessibility of root tissue and the lack of bioassays using model plants with altered defense metabolite profiles.

**Results:** We have developed an aeroponic culture system based on clay substrate that allows insect herbivores to feed on plant roots while providing easy access to the root tissue. The culture method was used to establish a root-herbivore system for *Arabidopsis thaliana* and larvae of the root herbivore *Bradysia* (fungus gnat). The amount of *Arabidopsis* root mass obtained from aeroponically grown plants was comparable to that in other culture systems, and roots were morphologically normal. *Bradysia* larvae caused considerable root damage resulting in reduced root biomass and water absorption. Similar to the root damage of mature plants cultivated in aeroponic substrate, *Arabidopsis* seedlings grown in potting mix were notably more susceptible to *Bradysia* feeding and showed decreased overall growth and survival rates.

**Conclusions:** A root-herbivore system consisting of *Arabidopsis thaliana* and larvae of the root herbivore *Bradysia* has been established that will allow profiling and *in vivo* functional analysis of root defenses such as chemical defense metabolites that are released in response to belowground herbivory.

## Background

Belowground herbivory affects plants in several ways. For example, insect-feeding on plant roots reduces uptake of water and nutrients, limits carbohydrate storage, and changes the production of phytohormones (Blossey and Hunt-Joshi 2003, van Dam 2009, Van Der Putten 2003). Such alterations in the physical and physiological state of plants can influence surrounding organismal communities both above- and belowground (De Deyn and Van der Putten 2005, Van Der Putten 2003).

Plants have developed multiple strategies such as tolerance and direct and indirect defense to cope with or defeat herbivore attack (Chen *et al.* 2005, Pechan *et al.* 2002, Rasmann*, et al.* 2005, Zhu-Salzman *et al.* 2008). Direct defense mechanisms usually involve the production of defense proteins and secondary (specialized) plant metabolites, which directly affect herbivores, while in indirect defense such metabolites help to attract natural enemies of herbivores (Chen*, et al.* 2005, Pechan*, et al.* 2002, Rasmann and Agrawal 2008). In contrast to the many plant defense responses investigated aboveground, few studies have focused on plant defenses against root-attacking herbivores. For example, feeding of the cabbage and turnip root maggot (*Delia radicum* and *Delia floralis*) was shown to induce the production of glucosinolate defense metabolites in the roots of several *Brassica* species (Birch *et al.* 1992, van Dam and Raaijmakers 2006). Schmelz et al. (2002) demonstrated that phytoecdysteroids in spinach roots serve as inducible defense compounds that decrease root feeding of *Bradysia* (fungus gnat) larvae. Moreover, indirect defense responses have been demonstrated belowground. When attacked by larvae of the western corn rootworm (*Diabrotica vigifera vigifera*), maize roots

release the sesquiterpene volatile (*E*)-β-caryophyllene, which attracts insect parasitizing nematodes (Rasmann*, et al.* 2005).

Detailed investigations of molecular and chemical defense responses in plant roots are still limited, which can be largely attributed to experimental shortfalls associated with the accessibility of root tissue or the interference of soil particles with root metabolite analysis (Hunter 2001, van Dam 2009) Moreover, research on belowground defenses has been hindered by the lack of bioassays using model plants deficient in the formation of defense metabolites. In response to these challenges, we have developed a root-herbivore system using *Arabidopsis* and larvae of the root herbivore *Bradysia*.

Darkwinged fungus gnats are generalist opportunistic herbivores whose larvae feed on organic matter and fungi, but upon depletion of this food source, larvae will actively feed on root tissue of a variety of plants including *Arabidopsis* (Harris *et al.* 1996, McConn*, et al.* 1997). Larvae often chew on roots and strip away the cortex, negatively impacting water and nutrient absorption (Harris*, et al.* 1996). The two most common species, *Bradysia coprophila* (Lintner) and *Bradysia impatiens* (Johannsen)*,* are both considered important greenhouse pests (Jagdale*, et al.* 2007), which, at extreme infestations, lead to loss of plant vigor and even mortality (Meers and Cloyd 2005). For example, 90% of alfalfa seedlings are killed at densities of less than one larva per seedling (Harris*, et al.* 1996). Young soybean plants have been shown to survive *Bradysia coprophila* feeding but produce less seed, which has repercussions on plant fitness (Harris*, et al.* 1996). These damaging effects of *Bradysia* species can be attributed to their short life cycle of 20 to 25 days with females laying between 250 and 1000 eggs in approximately 3 days. Emerging larvae feeding in the soil for 12 to 14 days prior to pupating (Jagdale*, et al.* 2007).

*Bradysia* larvae have been used to investigate hormone-dependent defense responses in *Arabidopsis*. Larvae caused high mortality of soil-grown *Arabidopsis* mutants deficient in the biosynthesis of jasmonic acid, but also affected the growth of wild type seedlings (McConn*, et al.* 1997). Effects on mature plants were described to be minor but this assessment was based only on observations of foliage and survival without investigating the severity of root damage (McConn*, et al.* 1997). *Bradysia* feeding was also employed to analyze root-specific defense activities of phytoectosteroids in spinach (Schmelz*, et al.* 2002). However, this study used *in vitro* feeding assays, instead of an *in vivo* approach, utilizing diets of powdered root tissue containing different phytoecdysteroid concentrations.

In this paper, we describe an aeroponic clay pellet (Seramis®) culture system that can easily be implemented to investigate belowground herbivory on *Arabidopsis* roots. The system maintains a soil-type environment for the herbivore while providing easy access to root tissue for analysis. Furthermore, we evaluate fungus gnat larval feeding on seedlings grown in soil in comparison to feeding on roots of mature plants in aeroponic culture. We propose this bioassay to be a useful tool for studying chemical and molecular defense responses of *Arabidopsis* to belowground herbivory.

## Methods

### *Plant material*

*Arabidopsis thaliana* ecotype Columbia (Col-0 6000) was grown under controlled conditions at 22 to 25°C, 150 μmol $m^{-2}$ $sec^{-1}$ photosynthetically active radiation (PAR) and a 10

h light – 14 h dark photoperiod. Seeds were vernalized for 24 h at 4˚C prior to planting in potting mix [90% Sunshine mix #1 (Sun Gro Horticulture, Bellevue, WA) and 10% sand].

***Arabidopsis aeroponic culture system***

Seramis® clay granules were purchased from www.seramisuk.co.uk. Fifty ml plastic conical tubes (Fisher scientific, Suwanee, GA) were prepared by drilling five 3.5 mm (9/64" drill bit) diameter holes around the bottom and a 25 mm hole in the cap (Figure 2.1).

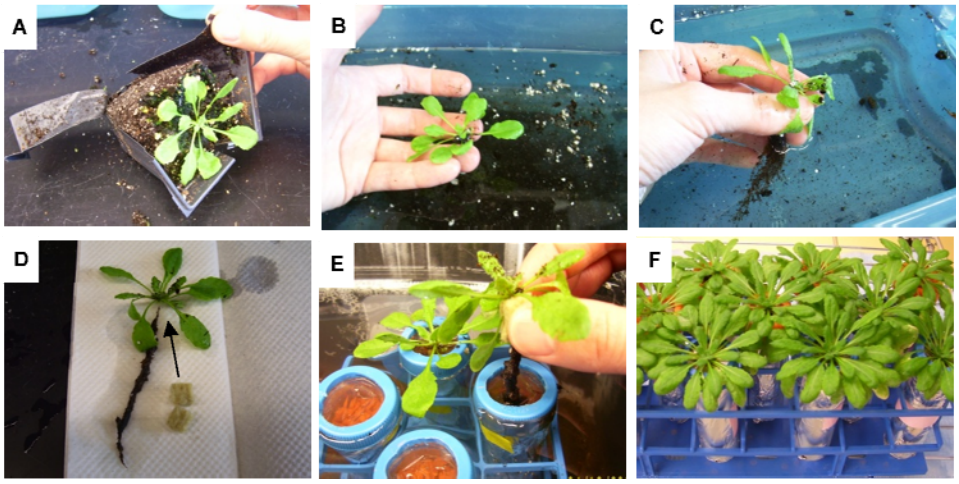


**Figure 2.1. Technique for growing *Arabidopsis* in aeroponic culture.** (A) Four-week-old *Arabidopsis* plants were removed from pots. (B,C) Roots were rinsed repeatedly in water to remove potting mix debris. (D) For transfer of plants into clay granulate, a small cube of rockwool (1.25 cm) was placed around the base of the rosette. (E) Plants were transferred to plastic tubes containing 50 ml of Seramis® clay granules saturated in Hoagland's solution. (F) Aeroponic *Arabidopsis* plants grown for four weeks at short day conditions after transfer to aeroponic culture tubes. At this stage, plants began to produce primary shoots.

Tubes were then filled with Seramis® clay granules and wrapped with aluminum foil to reduce growth of algae. The top of each tube was covered with plastic wrap and the caps were screwed on. A small hole large enough for the root mass to pass through was punctured with the tip of a

scalpel in the plastic wrap. The tubes were then placed in a rack and submerged in Hoagland's solution (Gibeaut *et al.* 1997) in order to moisten the Seramis® substrate. Four-week-old *Arabidopsis* plants grown on potting mix were carefully removed from their pots (Figure 2.1). Roots were repeatedly submerged in water to remove as much of the potting substrate as possible. A 1.25 cm cube of rockwool was divided and placed beneath the rosette, around the top of the roots. Plants were then transferred to Seramis®-containing tubes and allowed to grow. The rockwool and plastic wrap stabilized the plant until its roots had grown into the clay granulate. Clay granules were kept moist by submerging the culture tubes in Hoagland's solution for 10 to 15 min every other day. After 4 weeks, when plants started to produce primary shoots, roots had grown to the bottom of the falcon tube and were used for herbivore feeding assays. During the feeding experiments, plants were watered every other day by applying 10 ml of Hoagland's solution to the Seramis® through the top of the tubes with a Pasteur pipette**.**

***Bradysia (fungus gnat) culture***

Fungus gnat larvae were collected from the soil of contaminated pots in a greenhouse by the "potato disk" method (Harris *et al.* 1995) and subsequently used to establish a laboratory colony. The colony was maintained in 8 L plastic containers with screened openings for ventilation. The culture medium consisted of 4 L of moist Sunshine mix #1 enriched with 1.5 kg of shredded potato (Cloyd and Zaborski 2004). The cultures were kept at ambient greenhouse conditions (21 to 23°C; indirect sunlight). To maintain the colony, 0.5 L of medium (containing *Bradysia* larvae, pupae, adults and eggs) was transferred to a fresh container of soil and potato mix every 3 to 4 weeks. Specimens were identified by Dr. Raymond J. Gragné (USDA

Systematic Entomology Laboratory, Washington, DC) as a mixed colony of *Bradysia coprophila* (Lintner) and *Bradysia impatiens* (Johannsen).

***Isolation of Bradysia larvae***

Larvae were collected from the culture using a modified flotation/extraction method previously described by Zaborski and Cloyd (2004). Approximately 1 L culture medium containing *Bradysia* larvae was placed into a 2 L wide-mouth Erlenmeyer flask. The flask was filled with tap water and agitated to break up the culture substrate and release the larvae. The flask was then inverted and placed on top of a 2 L graduated cylinder filled with cold water so that the mouth of the flask opened just below the water surface in the cylinder (Figure 2.2A). In this position the flask remained filled with water with the graduated cylinder serving as both a container and a rack to hold the flask. Denser material including fungus gnat larvae sank from the flask into the cylinder while most of the potting mix remained floating in the flask. After approximately 5 min, the flask was removed, and its contents were discarded. The water, larvae and remaining organic matter were poured from the graduated cylinder into a plastic container. After the larvae had settled to the bottom of the container, the water was poured off and 1.5 M $MgSO_4$ solution was added. Larvae floated to the top because of their lower density compared to the $MgSO_4$ solution, while most of the remaining organic matter sank to the bottom. Larvae were then collected in a sieve, rinsed in water, and placed in a Petri dish on moist filter paper without food for 20 to 24 h prior to their application for feeding assays. The isolation method collects all four instars of *Bradysia* larvae (Figure 2.2B).

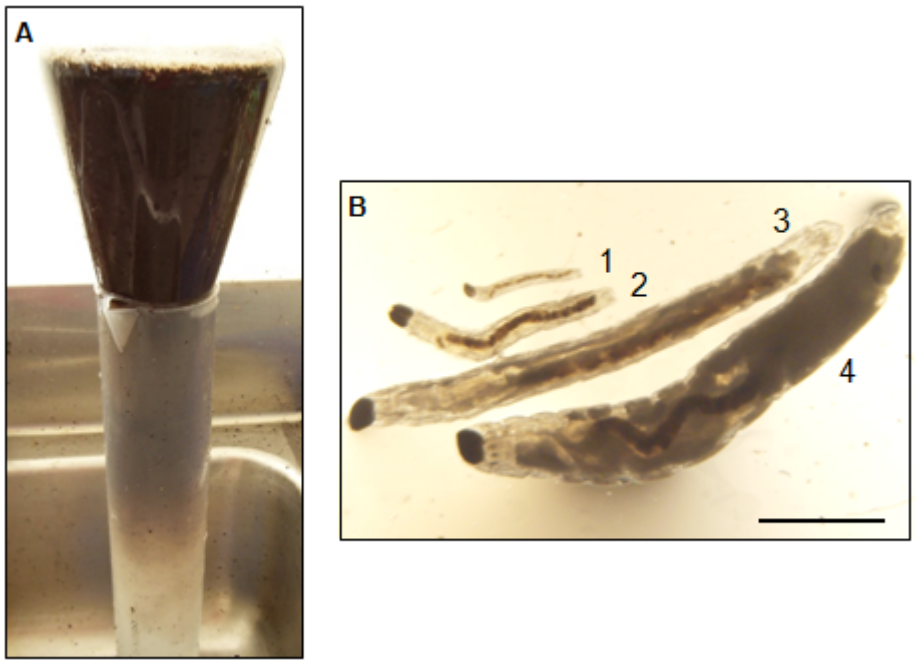


**Figure 2.2. Floatation method for the collection of *Bradysia* larvae.** (A) A 2 L wide-mouth Erlenmeyer flask containing larvae and larval culture substrate was filled with tap water and agitated to break up the substrate and release larvae. By placing the flask upside down on a cylinder filled with cold water, fungus gnat larvae were collected at the bottom of the cylinder while most of the culture substrate remained floating in the flask. (B) Instars of *Bradysia* larvae collected by this method. Numbers indicate the different instars. Scale bar: 1mm

***Bradysia feeding experiments in aeroponic culture***

Four weeks after their transfer to aeroponic culture tubes, *Arabidopsis* plants were challenged by fungus gnat larval feeding. To this end, the plastic tube caps were carefully opened to avoid leaf damage. Approximately 200 to 300 second- and third-instar larvae, collected as described above, were transferred to a single aeroponic culture tube. Fourth instar larvae were excluded from the feeding experiments since they would soon be pupating. First instar larvae (~ 1mm) were difficult to see let alone count and therefore, were excluded as well.

Larvae were submerged in the dish with 1 mL of Hoagland's solution (Hoagland's solution was used instead of water to avoid washing nutrients from the clay pellets) and then released with a pipette into the Seramis® substrate. Two and 4 days after larval feeding, roots were removed from the tubes by submerging the plant and the tube in water. Roots were then separated from the granulate by simply holding the plant rosette and moving it back and forth in the water. The heavier clay granules fell to the bottom of the container, while the roots stayed attached to the rosette. The shoots and roots were then dried with Kimwipes (Fisher Scientific, Suwanee, GA) and their fresh weights were recorded. After two days of air drying at room temperature, the dry weights were also measured. For data analysis, the weights performing a one-way analysis of variance (ANOVA) followed by Tukey-Kramer HSD, n=8. The shoot and root biomass and percentage of water weight for each tissue were calculated. For these measurements a T-test was preformed (n=12, $\alpha$=0.05) to test the null hypothesis of no biomass change for the variables.

***Bradysia feeding experiments with seedlings grown in potting mix***

In addition to analyzing feeding damage by *Bradysia* larvae on roots of mature *Arabidopsis* plants, we investigated the effect of larval feeding at the seedling stage. These experiments were performed in potting mix rather than in aeroponic culture because of inconsistent seed germination and seedling development on rockwool and clay substrate. Individual seedlings were grown in pots (8 cm x 8 cm x 10 cm) under the described conditions. *Bradysia* larvae (second and third instar, Figure 2.2B), collected as described above, were dislodged from the bottom of the Petri dish with 1 mL of water, and 10 larvae were transferred with a Pasteur pipette into each pot. A more moderate infestation of 10 larvae per plant was used

on seedlings because the young developing plants are more susceptible to larval damage. Larvae that did not move into the potting substrate within 5 minutes were removed from the experiment and replaced. Larvae were transferred to pots during germination and at 10 and 14 days after germination. Infested and control plants were kept within a net enclosure. The percentages of surviving seedlings from 3 independent experiments were determined 7 days after inoculation. Then, plants were removed from the pots, roots were rinsed in water, and the entire plant fresh and dry weight was recorded. For data analysis, the weights were log transformed before performing a one-way ANOVA and Tukey-Kramer HSD (n= 8-10).

### *Statistical Analysis*

Specific data analysis performed has been described with the individual experiments. Analysis of variance was accomplished by the JMP (SAS Institute Inc.) statistical software.

## Results

### *Optimization of an Arabidopsis aeroponic root culture system*

To study herbivory on *Arabidopsis* roots, it was necessary to establish a culture system that could accommodate belowground herbivores while still provided easy access to the root tissue for further molecular analysis. Initial attempts to grow *Arabidopsis* in sand and vermiculite resulted in poor plant growth. By contrast, optimal growth conditions were achieved by growing plants in nonsterile perlite or Seramis clay granules filled in 50 ml plastic tubes and moistened with Hoagland's mineral solution every two days. Seramis substrate was preferred over perlite

since roots could be easily removed from the clay granules by submersion in water without causing any substantial tissue damage (see below).

Plants were first grown on potting mix for approximately 4 weeks prior to their transfer to aeroponic culture tubes using rockwool supports. Transfer of younger plants was avoided due to inconsistent growth performance under aeroponic conditions. Likewise, seedlings were not grown directly on rockwool plugs placed on top of the aeroponic substrate because of low survival rates post germination. Within 4 weeks after transition to the clay granulate, roots had grown from the residual potting substrate particles to the bottom of the plastic tube. Plants appeared morphologically normal with primary and secondary root growth as well as normal formation of root hairs. When roots were removed from the tubes by submersion in water, the heavier clay pellets sank to the bottom while the intact root tissue remained attached to the floating rosette. The rockwool was removed without difficulty since roots had not grown into the rockwool substrate. Approximately 1 g of healthy root tissue was obtained from each individual plant (Table 2.1). The root-to-shoot ratio can be used to assess the overall health of a plant. An increase can indicate a healthier plant as long as the increase comes from increased root growth and not from decreased shoot growth. The root-to-shoot ratio for fresh and dry weight was 0.40 and 0.45, respectively (Figure 2.3C).

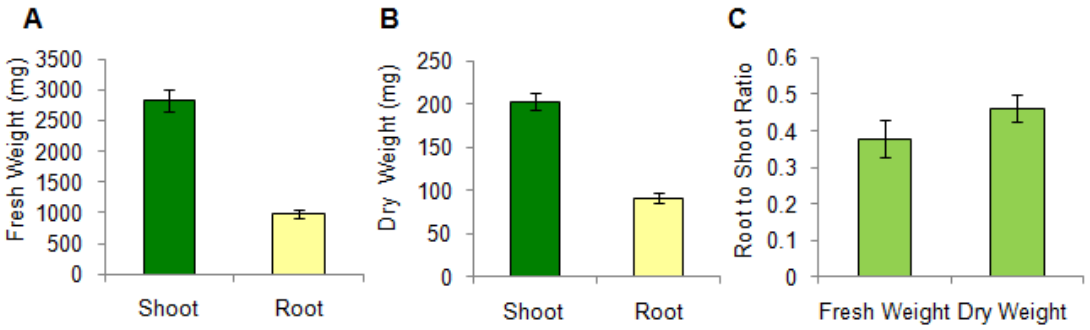


**Figure 2.3. Biomass analysis of *Arabidopsis* grown in aeroponic culture.** (A) Average shoot and root fresh weight (A) and dry weight (B) are shown. (C) Root-to-shoot ratios of fresh and dry weight. Values are the mean ± standard error (n=12). Shoots include leaves and stems.

### *Larval feeding behavior on aeroponically grown* Arabidopsis *roots*

Approximately, 200 to 300 second and third instar larvae were released into each aeroponic culture tube. Within 24 h, larvae were observed through the clear plastic tube and were actively feeding on the root tissue. Two and four days after the release of the larvae, severe feeding damage was visible on most roots (Figure 2.4A, B). The majority of root consumption was observed within the upper 5 cm of the root tissue (Figure 2.4B) indicating that most larvae fed from the top and gradually moved toward the bottom of the tube as the food source became depleted. Larvae stripped away the root epidermis and cortex, but generally avoided consuming the vascular tissue (Figure 5E-H). Some fine roots were completely severed. Feeding damage was most severe on root tips, young secondary roots, and root hairs suggesting a preference of larvae for these cells and tissues. Thick tap roots displayed minimal, if any, feeding damage.

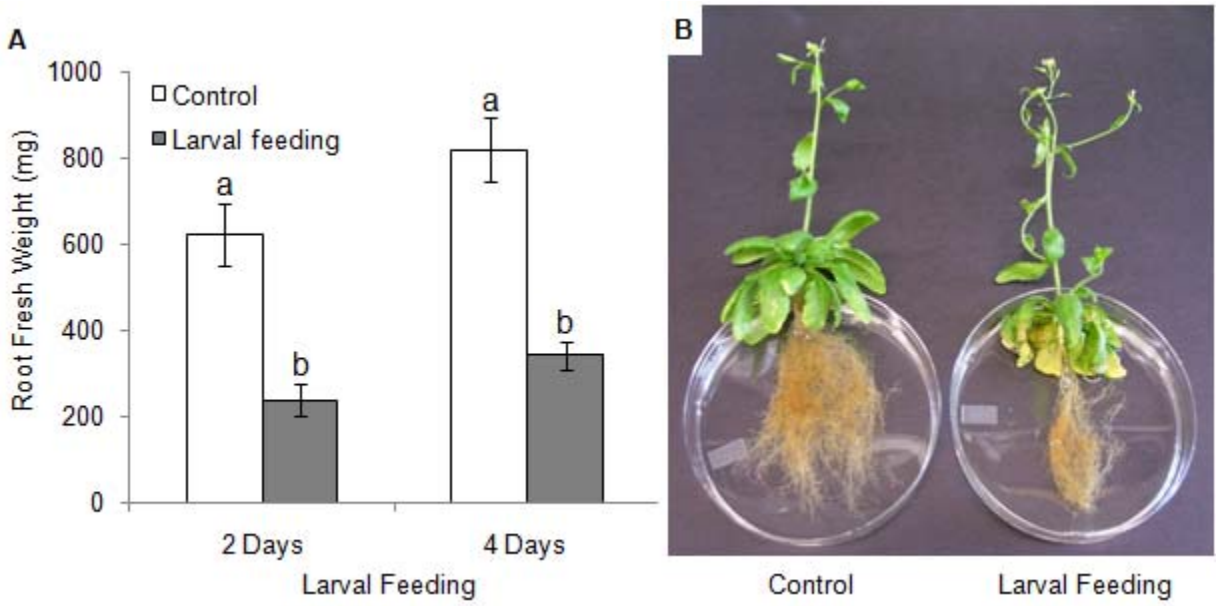


**Figure 2.4. Feeding damage and root consumption by *Bradysia* larvae on *Arabidopsis* grown in aeroponic culture.** (A) Root mass was significantly reduced after 2 and 4 days of root herbivory. Values represent averages ± standard error. Letters indicate significant differences between days and treatments (one-way ANOVA and Tukey-Kramer HSD, n=8, $P < 0.0001$). (B) *Arabidopsis* plants removed from clay substrate after 4 days of larval feeding in comparison to a non-feeding control. Root mass was visibly reduced and aerial tissues were beginning to wilt.

### *Effects of* Bradysia *larval feeding on roots of aeroponically grown* Arabidopsis *plants*

Larval root consumption significantly reduced *Arabidopsis* root biomass. On day 2 and 4 of larval feeding, average root fresh weight was reduced by 58% and 55%, respectively (Figure 2.4A). These values reflect the highest reduction observed for 10 different independent experiments; the average root reduction for all experiments was 37%. In addition to root consumption, stems had less vigor and rosette leaves were beginning to wilt (Figure 2.4B). To determine whether leaf wilting was caused by a decreased uptake of water, shoot fresh and dry

weight were measured and the percentage of weight attributed to water was determined (Figure 2.6). After 4 days of larval feeding, shoots showed significantly reduced water content, while shoot dry weight remained unaffected. By contrast, both root fresh and dry weight were significantly reduced with no significant change in the water content of roots due to feeding damage (Figure 2.6).

**Bradysia *larval feeding on seedlings grown in potting mix***

*Bradysia* larvae reduced *Arabidopsis* seedling establishment. Only 24 to 33% of seedlings survived when exposed to 10 *Bradysia* larvae during germination (Figure 2.7). When seedlings were treated with larvae 10 days after germination, survival rates increased slightly to 45 and 51%. However, more than 95% of seedlings survived when larval treatment started after the first pair of true leaves had developed 10 to 14 days after germination. Nevertheless, all of the surviving plants were considerably affected by herbivore damage since they had significantly less biomass than unchallenged seedlings and were noticeably stunted in growth (Figure 2.8).

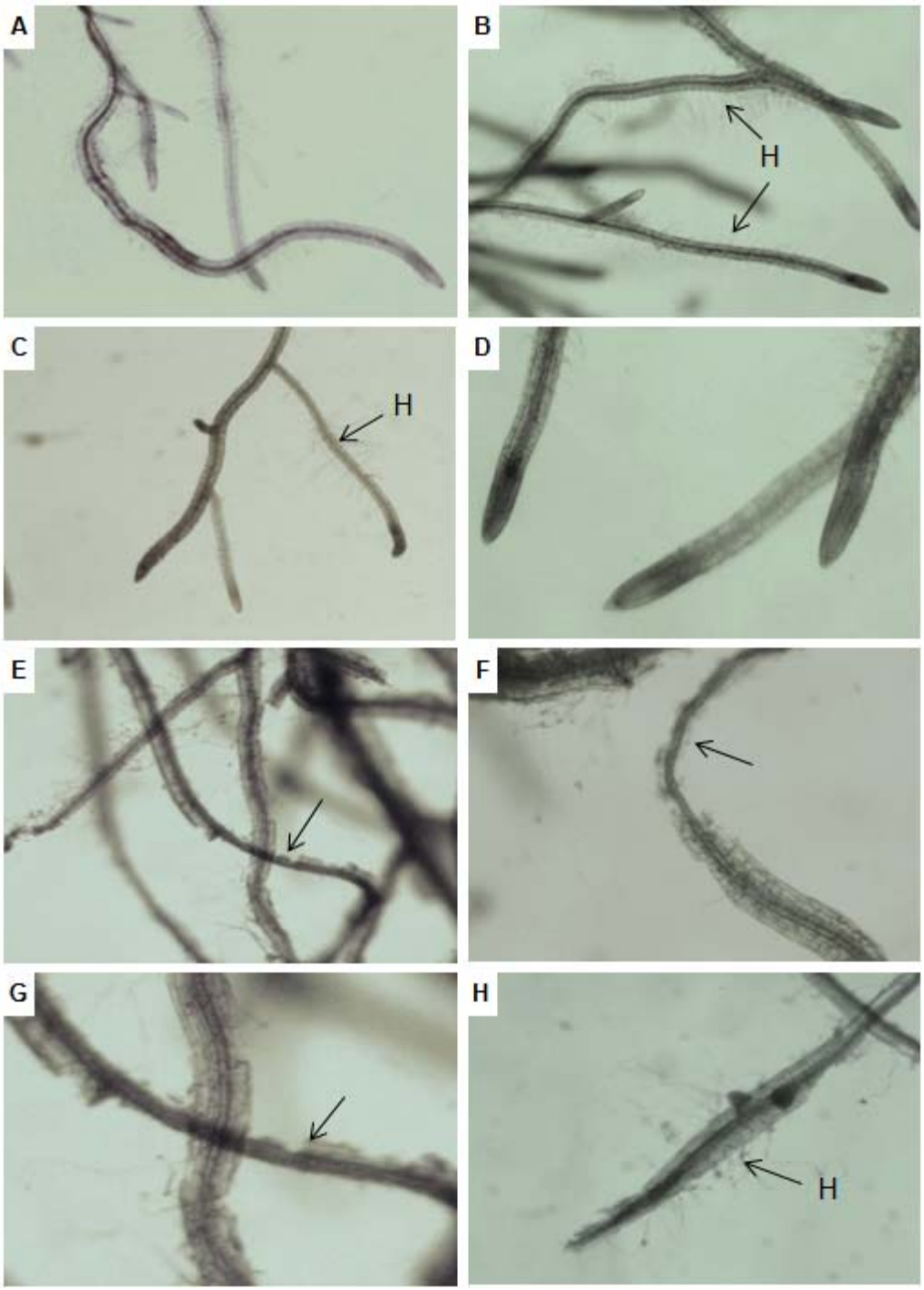


**Figure 2.5. Roots of aeroponically-grown *Arabidopsis* with and without feeding damage by *Bradysia* larvae.** (A-D) Undamaged roots of 8-week-old *Arabidopsis* grown in aeroponic culture. Primary and secondary roots and root tips (D) are shown. (E-H) Aeroponic roots damaged by the feeding of 200 to 300 *Bradysia* larvae over 4 days. (E-G) Larvae chewed and stripped away the root epidermis and the cortex but avoided feeding on the vascular tissue (arrows). (H) Feeding damage of root hairs and root tips. H, root hairs.

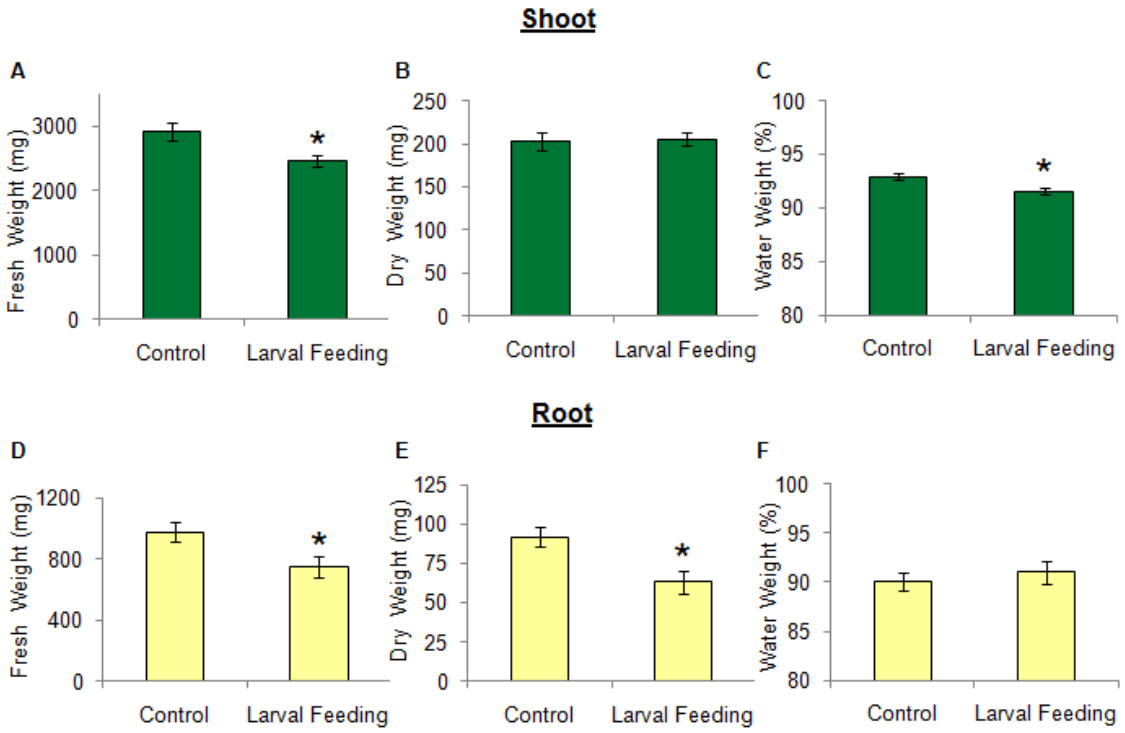


**Figure 2.6. Shoot and root biomass and water content in response to larval feeding in aeroponic culture.** Shoot fresh weight (A) and dry weight (B) upon 4 days of larval feeding in comparison to control plants. (C) The percentage of water in the shoot tissue was significantly reduced by root herbivore damage. (D,E) Change in fresh and dry weight of roots in response to larval feeding. (F) The percentage of water weight remained the same. Values represent averages ± standard error. Asterisks above bars indicate significant differences (t-test, n=12, $P < 0.02$).

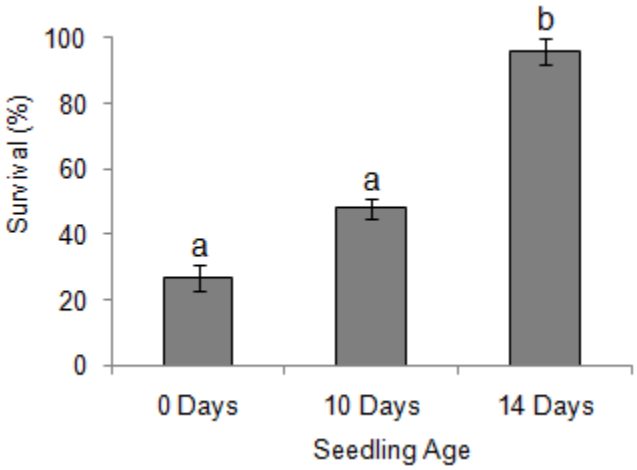


**Figure 2.7. Survival of *Arabidopsis* seedlings challenged by *Bradysia* larval feeding increased with age.** *Arabidopsis* seedlings were challenged with 10 *Bradysia* larvae at germination (0 days), 10 days and 14 days after germination. The average (± standard error) percent of seedling survival was estimated from 3 independent experiments (n=15) after 7 days of larval feeding. Letters indicate significant differences (one-way ANOVA and Tukey-Kramer HSD, $P < 0.0001$).

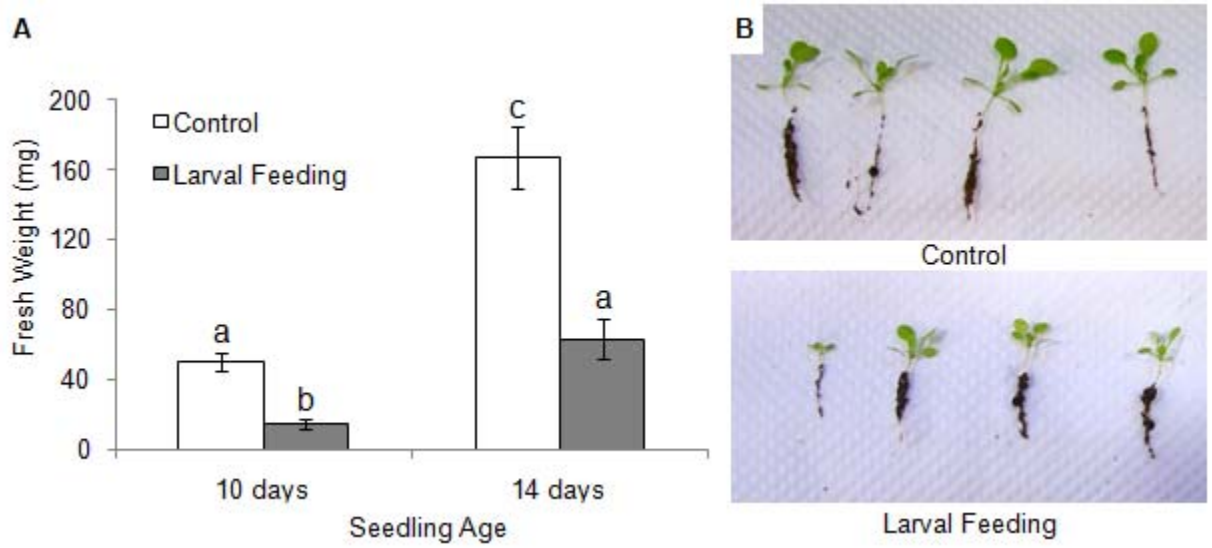


**Figure 2.8. Effect of *Bradysia* feeding on *Arabidopsis* seedlings grown in potting mix.** (A) Ten and 14-day-old *Arabidopsis* seedlings treated with 10 *Bradysia* larvae were significantly reduced in plant biomass. Values represent averages ± standard error. Letters indicate significant differences between seedling ages and treatments (one-way ANOVA and Tukey-Kramer HSD on log(x) transformed data, n= 8-10, $P < 0.0001$). (B) Developing *Arabidopsis* seedlings challenged by root herbivore feeding were noticeably stunted in growth.

## Discussion

### Arabidopsis *aeroponic root culture provides conditions suitable for root herbivory*

Investigating root herbivory has remained a challenge in the study of belowground plant-organism interactions because of limited access to root tissue for subsequent analysis. Several culture methods have been developed previously to make *Arabidopsis* roots more accessible for molecular and biochemical studies. These methods are primarily based on growing plants in liquid culture for optimal root biomass production but not suitable for investigating root herbivory (Gibeaut*, et al.* 1997, Hetu *et al.* 2005). We tested whether hydroponically grown roots removed from liquid culture could be used for *Bradysia* feeding experiments; however, larvae did not actively feed on these roots. Similarly, larvae did not feed on roots of plants grown in solid sterile media, but instead burrowed into the agar and suffocated.

The technique presented here of growing *Arabidopsis* in aeroponic clay granulate represents a simple culture method, which can be established under ordinary growth conditions without the use of sophisticated bubble stones, air pumps or sprayers. The culture requires 8 weeks to progress from seed to mature plants, but beyond transferring the plants from soil to aeroponic substrate, only watering by dipping tubes in Hoagland's solution is necessary.

A sufficient growth period of 4 weeks on potting mix is required prior to the transfer of plants to aeroponic culture. Growing seedlings directly on rockwool placed on top of the clay substrate is less suitable since plants are easily stressed by over- or under-watering. A similar problem has been encountered in establishing hydroponic cultures and is considered a major limitation in the success of germination and seedling survival in this system (Gibeaut*, et al.* 1997, Hetu*, et al.* 2005). Within 2 to 3 days after transplanting, plants adapt to the aeroponic

conditions and grow as phenotypically healthy plants with no obvious signs of impaired roots growth or damage after 4 weeks (Figure 2.3A-D).

Aeroponically-grown plants produce a root mass comparable to that obtained in other *Arabidopsis* culture systems (Table 2.1). For example, plants grown for 24 days in axenic liquid culture supplemented with (1 to 3 %) sucrose yield approximately 1 g of root fresh weight (Hetu et al., 2005), which is similar to that of aeroponically grown roots (Table 2.1). When compared to *Arabidopsis* grown under hydroponic conditions, the average root-to-shoot ratio in aeroponic culture (0.45) is approximately twice the ratio observed for hydroponically-grown plants (Gibeaut*, et al.* 1997). Thirty percent of the total aeroponic plant biomass is comprised of root tissue while 15 to 25% of total hydroponic plant biomass has been attributed to root tissue (Table 2.1). The higher percentage of root mass under aeroponic conditions may be the result of increased aeration in the root environment (Padgett and Leonard 1993, Spek 1981). Moreover, enhanced root growth might be caused by the intermittent nutrient supply in aeroponic culture as opposed to continuous nutrient availability in hydroponic systems.

**Table 2.1. *Arabidopsis thaliana* growth in aeroponic culture compared to liquid cultures**

| | | Root | | Shoot | |
|---|---|---|---|---|---|
| | Age (Days) | Fresh weight | Dry Weight | Fresh Weight (mg) | Dry weight |
| Aeroponic | 52(28[a]) | 984 ± 61 | 92 ± 6 | 2,824 ± 180 | 203 ± 10 |
| Hydroponic[b] | 32 | 498 ± 57 | 32 ± 3 | 1,160 ± 69 | 121 ± 37 |
| Hydroponic[b] | 48 | 2,916 ± 164 | 211 ± 10 | 10,940 ± 499 | 1,195 ± 37 |
| Axenic[c] | 24 | 1,100 | - | - | - |

[a] Number of days in aeroponic culture. [b][18]. [c][22]

**Bradysia *larval feeding significantly reduces root biomass of mature* Arabidopsis *plants and affects survival and growth of seedlings***

Fungus gnats are common greenhouse pests and frequently infest *Arabidopsis* growth rooms. While feeding of *Bradysia* larvae on *Arabidopsis* seedlings has been previously reported (McConn*, et al.* 1997), the extent of feeding damage on mature wild type plants has not been investigated. When placed in aeroponic clay substrate deprived of other food sources, larvae quickly began to feed on root tissue. Within 2 to 4 days of feeding, larvae significantly reduced root mass by 37% (Figure 2.3E-H). Despite the considerable damage of tissue belowground, plants appeared to be less affected aboveground. However, *Bradysia*-damaged plants had a reduced shoot mass, which was due to the loss of water suggesting that water absorption and most likely nutrient absorption were compromised by root herbivore damage.

In the presented experiments, approximately 200 to 300 larvae in the second and third instar were applied per plant. Considering that only 20 to 25 gnats released into an area with 15 pots containing *Arabidopsis* plants can yield 300 to 500 larvae per pot within 14 days (McConn*, et al.* 1997), it was within reason that this density can occur under greenhouse conditions. The observed root damage in aeroponic culture is most likely magnified due to the absence of other food sources such as organic matter. *Bradysia* larvae primarily consumed root tips, root hairs, and young lateral roots (Figure 2.5). Larval feeding on older roots preferably removed the epidermis and cortex but none of the vascular tissue and only parts of the endodermis. This feeding preference indicates that *Bradysia* larvae tend to avoid consuming roots with secondary growth and increased cell wall lignification. Moreover, differences in formation and concentration of constitutive and induced secondary defense metabolites such as glucosinolates

and terpenoids can contribute to the observed differential feeding behavior and overall root consumption (Vaughan and Tholl, unpublished results). The role of direct defense metabolites was also demonstrated in spinach roots, where *Bradysia* larvae did not cause any significant reduction in root mass but led to a dramatic up-regulation of defense compounds (Schmelz*, et al.* 2002).

*Arabidopsis* seedlings grown in potting mix were severely affected by *Bradysia* larval feeding. A moderate infestation (Harris*, et al.* 1996) of 10 larvae per plant reduced seedling survival by 50% or less depending on the plant age at the time the biotic stress was introduced. Surviving seedlings were much smaller and appeared to have reduced growth. This reduction in plant growth can be attributed to larval root consumption and limited uptake of nutrients but may also reflect trade-offs between resource allocation to defense and growth (Agrawal *et al.* 1999, Zavala *et al.* 2004).

To our knowledge, a bioassay for studying belowground herbivory on *Arabidopsis* roots has not previously been reported. We propose that *Arabidopsis* grown in aeroponic culture and the generalist herbivore *Bradysia* (*B. coprophila* and *B. impatiens*) can be used as a novel system to investigate belowground plant defense responses to herbivore attack. The chemistry of plant defense against root herbivores is one of the most neglected aspects of root biology (Rasmann and Agrawal 2008). Using this system, *Arabidopsis* mutants with altered secondary metabolite profiles can be applied to study the biological effect of individual defense compounds belowground (Gigolashvili*, et al.* 2007).

## Conclusions

We have established a method of growing *Arabidopsis* in an aeroponic clay granulate culture system, which can be used to investigate belowground herbivory and allows easy preparation of roots for chemical and molecular analysis upon insect feeding. We have shown that *Bradysia* larvae actively feed on mature *Arabidopsis* in aeroponic culture and cause severe root damage that affects both root biomass and water absorption. Furthermore, *Arabidopsis* seedlings challenged by *Bradysia* larvae show decreased survival and growth. This root-herbivore system can be used to study the biochemistry, molecular regulation, and function of root defense compounds in response to belowground herbivory.

## References


**Agrawal, A.A., Strauss, S.Y. and Stout, M.J.** (1999) Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. *Evolution*, **53**, 1093-1104.

**Birch, A.N.E., Griffiths, D.W., Hopkins, R.J., Smith, W.H.M. and McKinlay, R.G.** (1992) Glucosinolate responses of swede, kale, forage and oilseed rape to root damage by turnip root fly (*Delia-floralis*) larvae. *Journal of the Science of Food and Agriculture*, **60**, 1-9.

**Blossey, B. and Hunt-Joshi, T.R.** (2003) Belowground herbivory by insects: Influence on plants and aboveground herbivores. *Annual Review of Entomology*, **48**, 521-547.

**Chen, H., Wilkerson, C.G., Kuchar, J.A., Phinney, B.S. and Howe, G.A.** (2005) Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 19237-19242.

**Cloyd, R.A. and Zaborski, E.R.** (2004) Fungus gnats, *Bradysia* spp. (Diptera : sciaridae), and other arthropods in commercial bagged soilless growing media and rooted plant plugs. *Journal of Economic Entomotogy*, **97**, 503-510.

**De Deyn, G.B. and Van der Putten, W.H.** (2005) Linking aboveground and belowground diversity. *Trends in Ecology and Evolution*, **20**, 625-633.

**Gibeaut, D.M., Hulett, J., Cramer, G.R. and Seemann, J.R.** (1997) Maximal biomass of *Arabidopsis thaliana* using a simple, low-maintenance hydroponic method and favorable environmental conditions. *Plant Physiology*, **115**, 317-319.

**Gigolashvili, T., Berger, B., Mock, H.P., Muller, C., Weisshaar, B. and Fluegge, U.I.** (2007) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. *Plant Journal*, **50**, 886-901.

**Harris, M.A., Gardner, W.A. and Oetting, R.D.** (1996) A review of the scientific literature on fungus gnats (Diptera: Sciaridae) in the genus *Bradysia*. *Journal of Entomological Science*, **31**, 252-276.

**Harris, M.A., Oetting, R.D. and Gardner, W.A.** (1995) Use of entomopathogenic nematodes and a new monitoring technique for control of fungus gnats, *Bradysia coprophila* (Diptera, Sciaridae), in floriculture. *Biological Control*, **5**, 412-418.

**Hetu, M.F., Tremblay, L.J. and Lefebvre, D.D.** (2005) High root biomass production in anchored *Arabidopsis* plants grown in axenic sucrose supplemented liquid culture. *Biotechniques*, **39**, 345-+.

**Hunter, M.D.** (2001) Out of sight, out of mind: the impacts of root-feeding insects in natural and managed systems. *Agricultural and Forest Entomology*, **3**, 3-9.

**Jagdale, G.B., Casey, M.L., Canas, L. and Grewal, P.S.** (2007) Effect of entomopathogenic nematode species, split application and potting medium on the control of the fungus gnat, *Bradysia difformis* (Diptera : Sciaridae), in the greenhouse at alternating cold and warm temperatures. *Biological Control*, **43**, 23-30.

**McConn, M., Creelman, R.A., Bell, E., Mullet, J.E. and Browse, J.** (1997) Jasmonate is essential for insect defense *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 5473-5477.

**Meers, T.L. and Cloyd, R.A.** (2005) Egg-laying preference of female fungus gnat *Bradysia sp nr. coprophila* (Diptera : Sciaridae) on three different soilless substrates. *Journal of Economic Entomology*, **98**, 1937-1942.

**Padgett, P.E. and Leonard, R.T.** (1993) Contamination of ammonium-based nutrient solutions by nitrifying organisms and the conversion of ammonium to nitrate. *Plant Physiology*, **101**, 141-146.

**Pechan, T., Cohen, A., Williams, W.P. and Luthe, D.S.** (2002) Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 13319-13323.

**Rasmann, S. and Agrawal, A.A.** (2008) In defense of roots: A research agenda for studying plant resistance to belowground herbivory. *Plant Physiology*, **146**, 875-880.

**Rasmann, S., Kollner, T.G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J. and Turlings, T.C.J.** (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature*, **434**, 732-737.

**Schmelz, E.A., Grebenok, R.J., Ohnmeiss, T.E. and Bowers, W.S.** (2002) Interactions between *Spinacia oleracea* and *Bradysia impatiens*: A role for phytoecdysteroids. *Archives of Insect Biochemistry and Physiology*, **51**, 204-221.

**Spek, L.Y.** (1981) Influence of nitrate and aeration on growth and chemical-composition of *Zea-mays-L*. *Plant and Soil*, **63**, 115-118.

**van Dam, N.M.** (2009) Belowground herbivory and plant defenses. *Annual Review of Ecology Evolution and Systematics*, **40**, 373-391.

**van Dam, N.M. and Raaijmakers, C.E.** (2006) Local and systemic induced responses to cabbage root fly larvae (*Delia radicum*) in *Brassica nigra* and *B. oleracea*. *Chemoecology*, **16**, 17-24.

**Van Der Putten, W.H.** (2003) Plant defense belowground and spatiotemporal processes in natural vegetation. *Ecology*, **84**, 2269-2280.

**Zaborski, E.R. and Cloyd, R.A.** (2004) Method for quantitative sampling of fungus gnat larvae in soilless growing media. *Journal of Economic Entomology*, **97**, 678-686.

**Zavala, J.A., Patankar, A.G., Gase, K. and Baldwin, I.T.** (2004) Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata*. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 1607-1612.

**Zhu-Salzman, K., Luthe, D.S. and Felton, G.W.** (2008) Arthropod-inducible proteins: Broad spectrum defenses against multiple herbivores. *Plant Physiology*, **146**, 852-858.

# Chapter III

## The root-specific *Arabidopsis* terpene synthase AtTPS08 catalyzes the formation of the volatile diterpene rhizathalene involved in defense against belowground herbivory

Contributors:

Austin Wray, undergraduate research assistant, aided in the cloning of *AtTPS*08 into pET102/D-TOPO vector

Whitnee Askew, laboratory technician, assisted with the initial protein expression assays

Christopher O'Donnell, undergraduate research assistant, aided in the screening of T-DNA insertion lines for homozygous plants

Ashley Spencer, undergraduate research assistant, helped with aeroponic plant preparations

(Targeted for publication in **The Plant Journal**)

# Chapter III

# The root-specific *Arabidopsis* terpene synthase AtTPS08 catalyzes the formation of the volatile diterpene rhizathalene involved in defense against belowground herbivory


## SUMMARY

Despite the substantial impact of root herbivores on plant health and survival, research investigating the interactions between plants and belowground herbivores has largely been neglected. Specialized metabolites, such as terpenes, play an important role in direct plant defense by deterring or intoxicating herbivores or in indirect defense by attracting natural parasitoids and predators. However, little is known of the biological function(s) of terpene metabolites with regard to root herbivore defense. Here we describe a novel volatile diterpene, which we have named rhizathalene that is constitutively produced in roots of *Arabidopsis thaliana*. Using a reversed genetics approach, we have identified and characterized the root-specific terpene synthase AtTPS08 responsible for the specific formation of rhizathalene. AtTPS08 is a class I enzyme that shows high affinity for geranylgeranyl diphosphate (GGPP) and is targeted to the root leucoplast. *AtTPS*08 promoter activity is specifically localized to the stele and root tips. Using aeroponically grown *Arabidopsis* plants and larvae of the opportunistic root-herbivore *Bradysia* spp. (fungus gnats), we demonstrate that roots of *AtTPS*08 gene knock-out plants, which are deficient in rhizathalene formation, are more susceptible to insect herbivory. Our work provides the first evidence of a role for volatile diterpenes in belowground direct defense against root-feeding insects.

## INTRODUCTION

Nearly all terrestrial plants depend on roots as life-supporting organs. Consequently, more than 50% of the net primary production of plants is commonly allocated to roots (Fogel 1985). Root herbivores represent a constant belowground threat to plants by disrupting the uptake of water and mineral nutrients or limiting the storage of carbohydrates (Blossey and Hunt-Joshi 2003, van Dam 2009, Van Der Putten 2003). Moreover, root damage by herbivores can influence organismal communities both below- and aboveground (Kaplan*, et al.* 2008a, Van Der Putten 2003). Despite the importance of roots for plant growth and fitness, knowledge of root defense mechanisms and the interaction between plants and belowground herbivores is limited.

Plants deploy chemical defense or specialized metabolites to directly affect herbivore performance or indirectly attract natural enemies of herbivores (Birch*, et al.* 1992, De Moraes*, et al.* 2001, Kessler and Baldwin 2001, Mercke*, et al.* 2004, Rasmann*, et al.* 2005, Turlings *et al.* 1990, Vancanneyt*, et al.* 2001). Terpenes constitute the largest class of plant defense chemicals, whose functions have been investigated primarily in aboveground plant organs. For example, low-molecular weight, volatile, monoterpenes ($C_{10}$) and sesquiterpenes ($C_{15}$) that are released from plants upon feeding damage can deter or intoxicate insects (De Moraes*, et al.* 2001, Kessler and Baldwin 2001, Vancanneyt*, et al.* 2001) or function as signals to attract parasitoids and predators of arthropod herbivores (Mercke*, et al.* 2004, Rasmann*, et al.* 2005, Turlings*, et al.* 1990). Defensive roles of terpenes produced in roots have so far been primarily been associated with antimicrobial and allelopathic activities. In rice, several diterpenes ($C_{20}$) such as momilactones with assumed functions as phytoalexins or allelochemicals are found in root exudates (Kato-Noguchi *et al.* 2008b, Kong *et al.* 2006, Toyomasu *et al.* 2008). Similarly, cotton

roots exude gossypol terpene aldehydes, and oat roots release triterpene exudates with antimicrobial activity in response to pathogen infection (Hunter*, et al.* 1978, Osbourn 2003, Osbourn*, et al.* 2003). Recent studies have provided evidence for the defensive role of terpene metabolites against root herbivores. In response to herbivore damage, maize roots release the volatile sesquiterpene (*E*)-β-caryophyllene as a belowground signal to attract insect-parasitizing nematodes (Rasmann*, et al.* 2005). Despite these advances, possible anti-herbivore activities of many other root-specific terpene or specialized metabolites have not been described *in vivo*. In addition, a better understanding of the cell-type specificity and molecular regulation of these metabolic defenses is necessary to assess their role in defense against different types of root-attacking herbivores.

*Arabidopsis thaliana* represents a suitable model system to conduct such studies since its genome contains 32 terpene synthase (*TPS*) genes (Aubourg*, et al.* 2002), 15 of which are expressed primarily or exclusively in the roots (Birnbaum*, et al.* 2003, Chen *et al.* 2003). Terpene synthases catalyze the formation of monoterpenes, sesquiterpenes or diterpenes from the prenyl diphosphate precursors geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphsophate (GGPP), respectively (Bohlmann*, et al.* 1998, Davis and Croteau 2000, Tholl 2006). Prenyl diphosphates are synthesized by sequential condensation of isopentenyl diphosphate (IPP) five-carbon units via the mevalonate pathway in the cytosol or the methylerythritol phosphate (MEP) pathway in plastids (Chen*, et al.* 2003, Dudareva*, et al.* 2004). Sesquiterpene formation occurs primarily in the cytosol, while monoterpenes and diterpenes are synthesized predominantly in plastids. Two identical *Arabidopsis* TPSs (AtTPSs), AtTPS23 (At3g25820) and AtTPS27 (At3g25830), which produce the monoterpene 1,8-cineole, were shown to be constitutively expressed in epidermal cells of *Arabidopsis* roots (Chen*, et al.* 2004).

Two other duplicated genes *AtTPS*12 (At4g13280) and *AtTPS*13 (At4g13300) encoding (*Z*)-γ-bisabolene sesquiterpene synthases are constitutively expressed in the root cortex and sub-epidermal layers (Ro*, et al.* 2006). None of the other eleven root expressed *AtTPSs,* which exhibit cell-type specific expression patterns according to fine-scale transcript analyses (Birnbaum*, et al.* 2003, Brady *et al.* 2007), have been characterized for their biochemical and biological activities.

Here we show that the terpene synthase AtTPS08 is responsible for the formation of the diterpene hydrocarbon rhizathalene in *Arabidopsis* roots. Rhizathalene is constitutively emitted from roots of plants grown in potting substrate and under non-soil culture conditions. Rhizathalene synthase represents a class I diterpene synthase, which is specifically expressed in the vascular tissue and root tips and is targeted to root leucoplasts. Using bioassays with larvae of the common opportunistic root herbivore *Bradysia* (fungus gnat), we further demonstrate that roots of *AtTPS*08 gene knock-out plants, which are deficient in rhizathalene formation, are more susceptible to insect herbivory. Our studies show that volatile diterpenes are involved in direct belowground plant defense.

## RESULTS

### Arabidopsis *roots emit volatile diterpenes*

From the 15 *AtTPS* genes that are expressed constitutively in *Arabidopsis* roots (Chen et al., 2003; Birnbaum et al., 2003; Ro et al., 2006), five (*AtTPS*08, At4g20210; *AtTPS*17, At3g14490; *AtTPS*20, At5g48110; *AtTPS*26, At1g66020; *AtTPS*29, At1g31950) were previously predicted to function as diterpene synthases (Aubourg et al., 2002). Therefore, we investigated

the possible formation of diterpenes in *Arabidopsis* roots grown under different culture conditions. Plants were grown in potting mix (2 weeks), in hydroponic culture (4 weeks), and in axenic culture supplemented with 1% sucrose (3 weeks) (Figure 3.1a). In addition, we established a novel aeroponic system, in which plants were cultivated for 4 weeks in plastic tubes filled with clay granulate and moistened with nutrient solution (Figure 3.1a). Under the assumption that some of the diterpenes produced in *Arabidopsis* roots were volatile, we applied automated solid phase microextraction (SPME) to collect volatiles from roots that were detached from plants grown under the different culture conditions. Four diterpene olefins were detected by gas chromatography-mass spectrometry (GC-MS) analysis of volatiles emitted from hydroponically grown roots (Figure 3.1b). The mass spectrum of the predominant diterpene compound resembled that of a tricyclic hydrocarbon diterpene (Figure 3.1c) but did not match the spectra of other known diterpene compounds listed in the NIST/Wiley libraries or in other databases and references. Therefore, we named these diterpene compounds rhizathalene A to D (rhiza- originating from the Greek word for "roots", and thale for the *Arabidopsis* common name, thale cress).

Quantitative analysis of the detected diterpenes by solvent extraction of root tissue and subsequent GC-flame ionization detection (FID) showed highest concentrations of rhizathalene A in hydroponically grown roots of approximately 900 ng per g fresh weight (Figure 3.1d). By comparison, only half the amount of rhizathalene was found in roots grown in sterile liquid culture and approximately 150 to 200 ng of rhizathalene A per g fresh weight were detected in roots from plants grown under aeroponic culture conditions and in potting substrate, respectively (Figure 3.1d). Compounds B, C, and D were only found in roots of hydroponically grown plants. Other volatile terpenes that were detected in small amounts from *Arabidopsis* roots were the

monoterpene 1,8-cineole and the sesquiterpene (*Z*)-γ-bisabolene (data not shown), which have been described as products of the root-expressed terpene synthases AtTPS23/27 and AtTPS12/13, respectively.

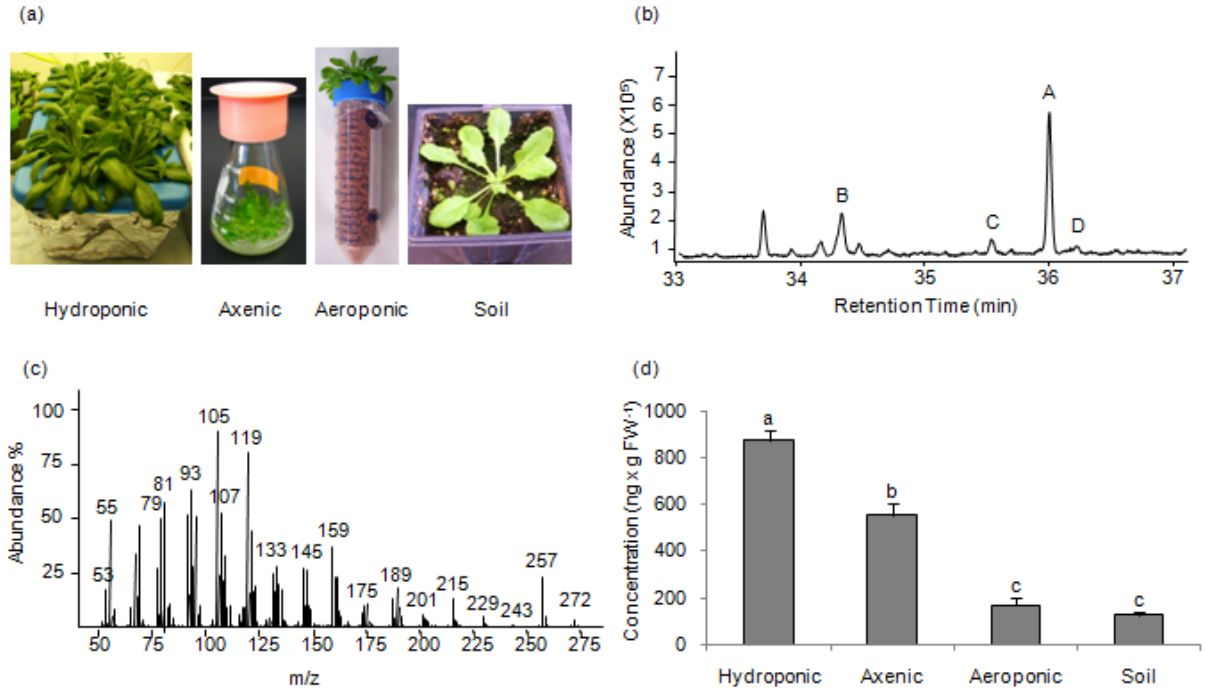


**Figure 3.1. Diterpene volatile emission from *Arabidopsis* roots.** (a) *Arabidopsis* culture systems used for root volatile analysis. (b) Total ion GC-MS chromatogram of volatiles collected from 1 g of hydroponically grown roots using SPME. Four diterpene compounds were detected designated rhizathalene A to D (peaks A to D). (c) Mass spectrum of rhizathalene A. (d) Concentrations of rhizathalene A in roots grown under the different culture conditions. Diterpenes were extracted with organic solvent and quantified by GC-FID. Letters above bars indicate significant differences (one-way ANOVA and Tukey-Kramer HSD, n= 3, $P < 0.01$). Values represent averages ± standard error.

### *A* AtTPS*08 T-DNA insertion line lacks the formation of rhizathalene*

To identify the *AtTPS* gene responsible for rhizathalene formation, roots from gene knockout lines of all the putative root-specific diterpene synthases were analyzed for the absence of rhizathalene A. Only a single line (salk_125194) with a T-DNA insertion in exon 6 of the gene *AtTPS*08 (At4g20210) (Figure 3.2a) did not produce rhizathalene A (Figure 3.2b). No *AtTPS*08 transcript was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in the roots of this mutant in comparison to wild type plants (Figure 3.2c). By contrast, a T-DNA insertion in the 3'-untranslated region (UTR) of *AtTPS*08 in line salk_112521 did not affect *AtTPS*08 transcript levels or the formation of rhizathalene A (Figure 3.2b, c). To confirm that the observed phenotype was due to the T-DNA insertion in AtTPS08 and not an additional insertion the genome of salk_125194, a southern blot analysis was performed. Results from DNA-hybridization were consistent with those expected for a single insertion in the *AtTPS*08 gene (Figure 3.S1) which supported the hypothesis that *AtTPS*08 was involved in rhizathalene synthesis.

### *Complementation analysis of* AtTPS*08 loss-of-function plants*

To complement the *AtTPS*08 loss-of function phenotype, the mutant line salk_125194 was transformed with the *AtTPS*08 gene under control of the constitutive cauliflower mosaic virus (*CaMV*) *35S* promoter (*Pro35S*). Transgenic plants from 3 independent transformations showed constitutive emission of rhizathalene A from flowers and leaves consistent with accumulation of *AtTPS*08 mRNA in these organs (Figure 3.3a, b). Interestingly, rhizathalene A

could not be detected in roots despite the presence of a full-length transcript for the *AtTPS*08 transgene (Figure 3.3a, b).

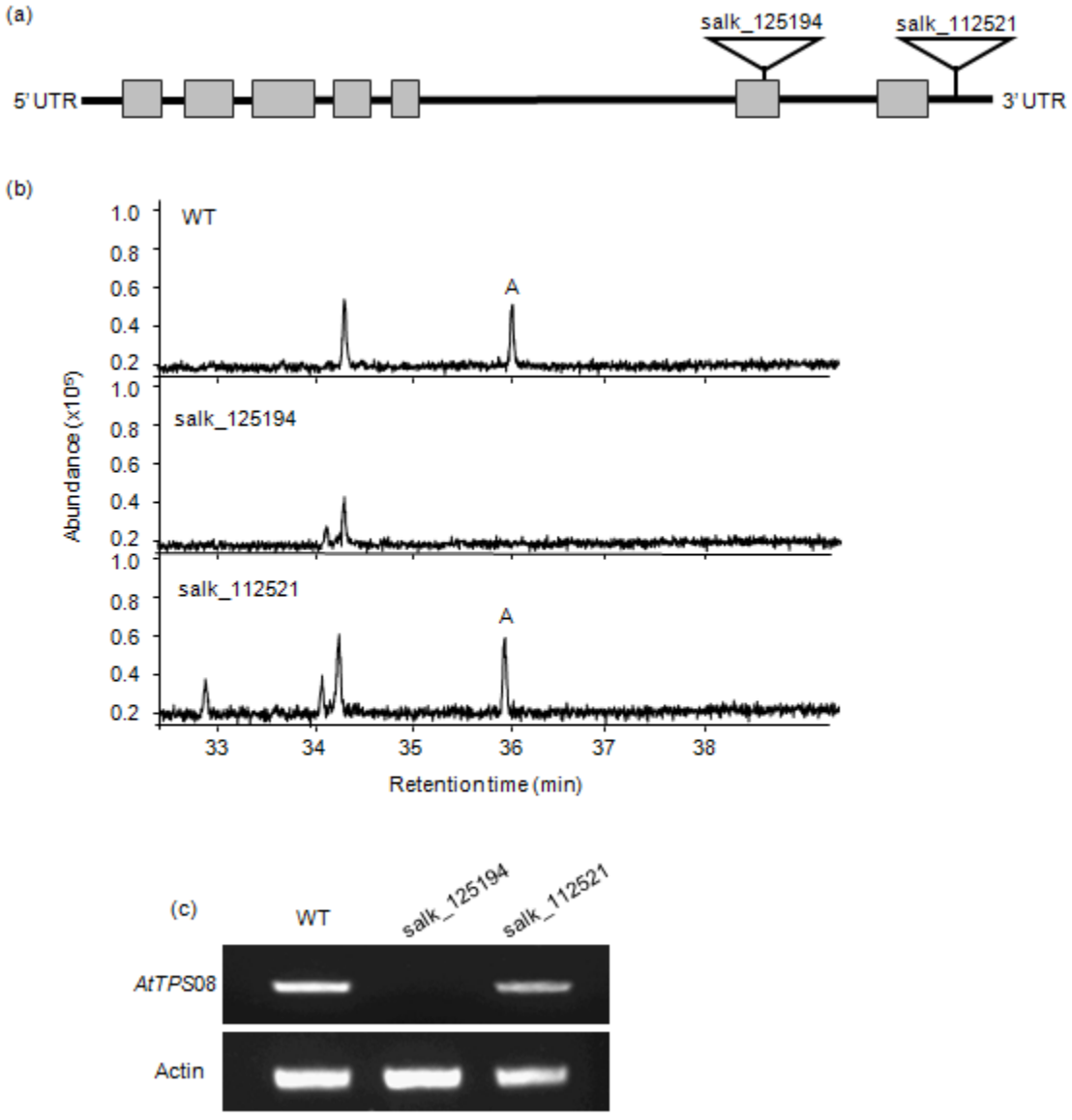


**Figure 3.2. A *AtTPS*08 T-DNA insertion line lacks the formation of rhizathalene A.**
(a) Positions of T-DNA insertions in the *AtTPS*08 *(At4g20210)* gene. Exons are represented by the gray boxes. Introns and untranslated regions (UTR) are represented by the black line. The two independent T-DNA insertions are indicated as triangles. (b) Total ion GC-MS chromatograms of rhizathalene A emitted from roots of wild type (WT) and T-DNA insertion lines grown in axenic culture. (c) RT-PCR analysis of *AtTPS*08 transcripts in roots of wild type and T-DNA insertion lines.

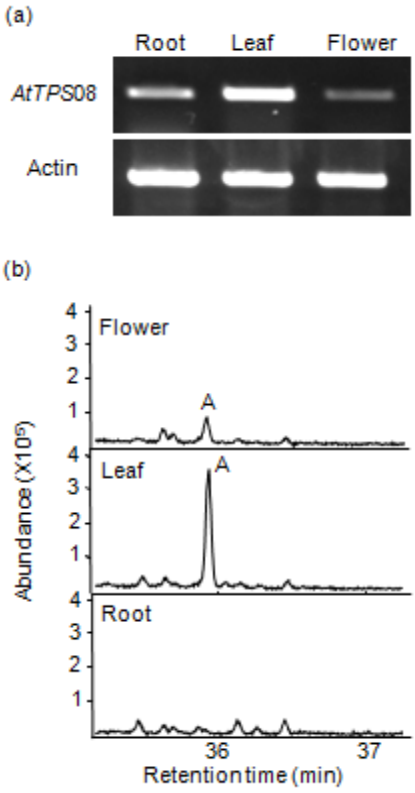


**Figure 3.3. Complementation analysis of the *AtTPS*08 T-DNA insertion line salk_125194.** *AtTPS*08 was expressed in salk_12594 under the control of the *CaMV 35S* promoter. (a) RT-PCR *AtTPS*08 transcript analysis in flowers, leaves and roots of a *Pro35S:AtTPS*08 transformant. Similar results were obtained from 3 independent lines. (b) GC-MS analysis of volatiles emitted from flowers, leaves and roots of the same *Pro35S:AtTPS*08 transgenic line grown in hydroponic culture. Complementation of *AtTPS*08 resulted in the emission of rhizathalene from flowers and leaves but did not restore diterpene volatile emission in roots.

### ***Functional characterization of* AtTPS*08***

To confirm the catalytic activity of the AtTPS08 enzyme, a 1678 bp cDNA of *AtTPS*08 lacking 123 bp, which encode a putative 41 amino acid plastidial transit peptide, was cloned into the *Escherichia coli* (*E. coli*) expression vector pET102/D-TOPO. The partially purified recombinant AtTPS08 enzyme converted GGPP into several diterpene compounds (Figure 3.4a). The major enzymatic product was identified as rhizathalene A. Mass spectra from three of the less abundant compounds were identical to those of rhizathalene B, C and D, which had been detected in roots of hydroponic plants (Figure 3.1a). Incubation of the AtTPS08 recombinant enzyme with the substrates GPP and FPP led to the formation of small amounts of the

monoterpenes linalool and geraniol and the sesquiterpenes α-farnesene and γ-bisabolene, respectively. However, these compounds were also detected in extracts of *E. coli* cells carrying the empty pET102/D-TOPO vector and thus were considered non-specific products.

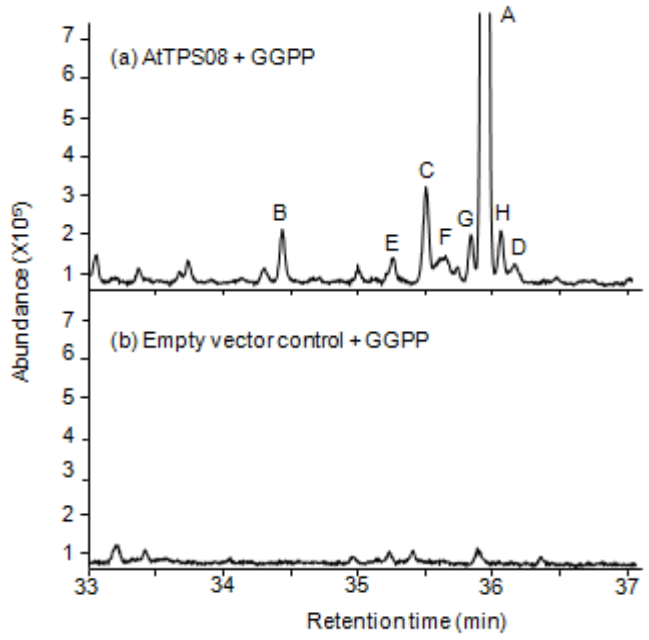


**Figure 3.4. Enzyme assay of AtTPS08.** (a) GC-MS chromatogram of diterpene compounds produced from GGPP with partially purified recombinant AtTPS08 enzyme. AtTPS08 catalyzes the formation of rhizathalene A as the major product and seven minor diterpene products. Peaks A-D correspond to the same compounds as shown in Figure 3.1b. (b) GC-MS chromatogram obtained with an extract from *E. coli* carrying the empty expression vector. The extract was subjected to the same purification and assay procedures.

Kinetic characterization of the partially purified recombinant AtTPS08 protein showed a low $K_m$ value for GGPP of 0.8 ±0.2 μM. $V_{max}$ was 4.6 ± 0.6 pKat/mg and $k_{cat}$ was $3x10^{-4}$±$4x10^{-5}$ $sec^{-1}$ resulting in a $k_{cat}/K_m$ value of 0.5 ± 0.1 $sec^{-1}$/μM. The activity of AtTPS08 was dependent on the presence of $Mg^{2+}$ as a divalent metal ion.

To elucidate the molecular structure of rhizathalene A, larger amounts of the diterpene compounds were produced by transformation of the *E. coli* C41 (DE3) strain with the pET102/D-TOPO-*AtTPS08* vector construct. Strain C41(DE3) contained an additional pACYCDuet-rAgGGPS plasmid carrying the pseudomature GGPP synthase from grand fir (*Abies grandis*), rAgGGPS, obtained from Dr. Reuben Peters at Iowa State University (Cyr *et al.*

2007). Rhizathalene compounds were collected in the headspace of 2 L bacterial cultures co-expressing the GGPP synthase and AtTPS08 by applying a closed-loop stripping procedure as described previously (Tholl *et al.* 2006). After elution of the volatile compounds from the collection traps, rhizathalene A was purified by liquid chromatography-mass spectrometry (LC-MS) fraction collection to reach a purity of more than 95%. LC/UV analysis of the purified diterpene compound showed absorbance at 205 nm. The compound is currently being analyzed by proton nuclear magnetic resonance (NMR) and 2D-NMR: COSY, HSQC and HMBC by Dr. Francis Webster at Syracuse University.

***Subcellular localization of AtTPS08***

Since diterpenes are primarily synthesized in plastids, we questioned whether AtTPS08 is targeted to leucoplasts of *Arabidopsis* root cells. TargetP and ChloroP algorithms predicted a plastidial transit peptide of AtTPS08 of 41 amino acids. To determine the subcellular localization of the AtTPS08 protein, transgenic plants were generated in the AtTPS08 knockout background expressing AtTPS08 in C-terminal fusion with the green fluorescent protein (GFP) under the control of the *CaMV 35S* promoter. Analysis of root tissue from four independent *Pro35S:AtTPS08-GFP* lines showed green fluorescence specifically in leucoplasts (Figure 3.5a,b). No fluorescence was detected from wild type roots (Figure 3.5c). GFP protein expressed without any fusion was located in the cytosol (data not shown).

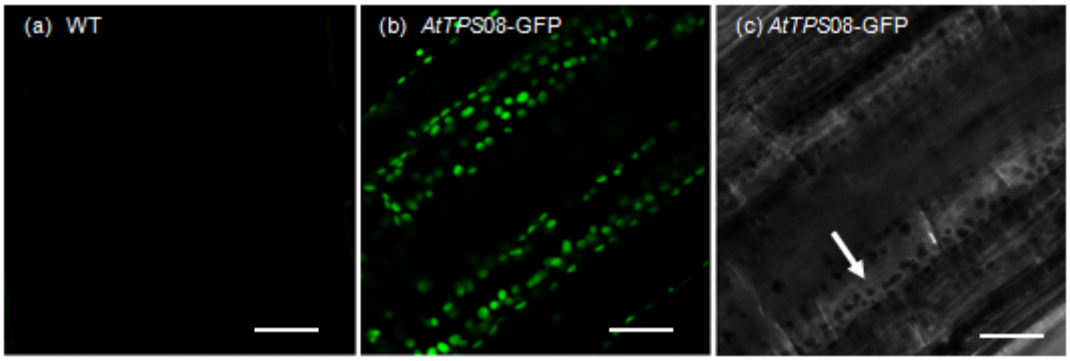


**Figure 3.5. Subcellular localization of an AtTPS08-GFP fusion protein.** Fluorescent image of wild type (WT) roots (a) and roots of plants transformed with the *AtTPS*08-*GFP* construct (b). GFP is specifically located in leucoplasts. (c) Corresponding bright-field image of (b) with leucoplast indicated by an arrow. Images were obtained by confocal laser scanning microscopy from 20 day old soil grown roots. Scale bar: 25 μm.

### AtTPS*08 promoter tissue-specific activity in the root stele*

Tissue specific activity of the *AtTPS*08 promoter was examined *in planta* by the staining of transformed *Arabidopsis* plants carrying a *ProAtTPS*08:β-glucuronidase (*GUS*) fusion. GUS staining was observed exclusively in the roots of at least three independent lines in the $T_2$ generation. In seedlings grown on sterile MS medium, weak GUS staining was detected in the stele and at the tips of both primary and secondary roots (Figure 3.6a-c). In comparison, hydroponically grown *ProAtTPS*08:*GUS* plants showed considerably stronger GUS activity in the same tissues and cell types consistent with the higher transcript levels of *AtTPS*08 observed under this culture condition (Figure 3.6d-f). No GUS staining was detected in wild type roots grown in hydroponic culture (Figure 3.6g, h).

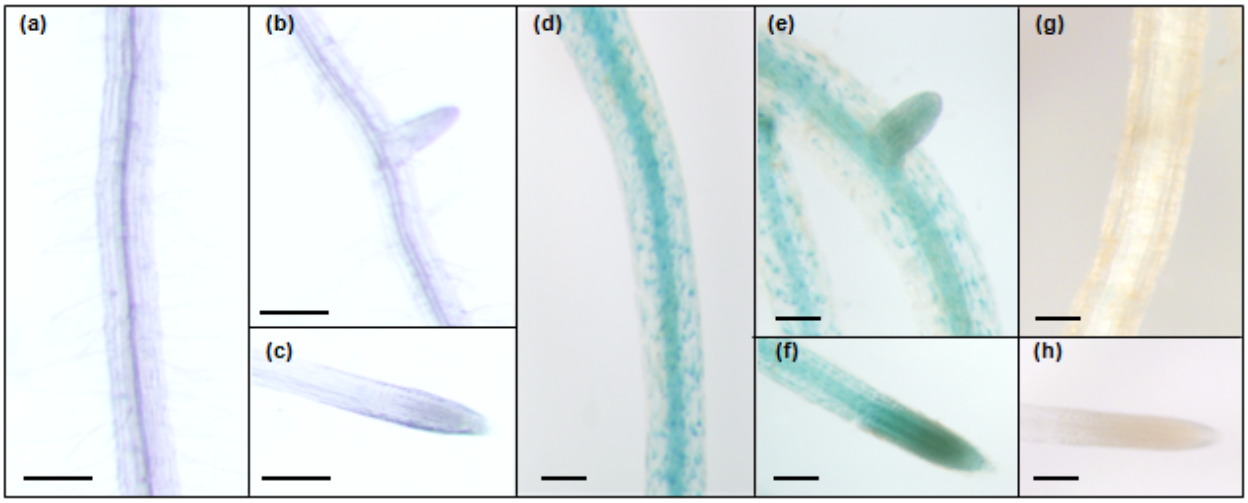


**Figure 3.6. Tissue-specific expression of GUS activity in roots of *ProAtTPS*08*:GUS* plants.** Histochemical GUS staining was observed in the stele, in emerging secondary roots and root tips. Images of histochemically stained *ProAtTPS*08:*GUS* roots from seedlings grown on half strength MS plates for 15 days (a-c), from hydroponically grown *ProAtTPS*08*:GUS* plants (d-f) or from hydroponically grown wild type plants (g-h). Scale bar: 200 µm. Results were representative for at least 4 independent lines.

### AtTPS*08 knockout mutants are more susceptible to* Bradysia *larval feeding*

To investigate the possible defense activity of rhizathalene against belowground herbivores, we established an aeroponic clay pellet culture system that maintains a realistic belowground environment for the herbivore while providing easy access to the root tissue (Figure 3.7). Wild type and *AtTPS*08 mutant plants were grown in aeroponic culture for 4 weeks and subsequently treated with 200 to 300 *Bradysia* larvae of the second and third instar, which were allowed to feed on the roots for 5 days. The number of larvae is equivalent to the offspring of a single fungus gnat female (Harris*, et al.* 1996). After removal of the clay granules, feeding damage on roots was examined under a dissecting microscope (Figure 3.8). Roots of wild type plants were visibly damaged. Portions of the root epidermis and cortex were removed and several root tips were severed. Generally, larvae avoided feeding on the vascular tissue and parts

of the endodermis (Figure 3.8e-h). By contrast, feeding damage on roots of the *AtTPS*08 knockout line was much more severe. Large areas of the root tissue were completely stripped of the epidermis and cortex. The endodermis and phloem were consumed leaving only a string of lignified xylem cells (Figure 3.8i-l).

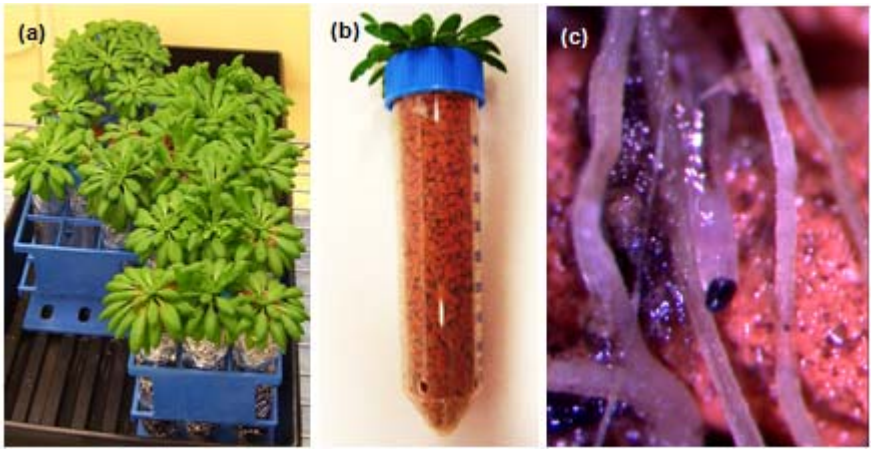


**Figure 3.7. Aeroponic culture system for investigating belowground herbivory on *Arabidopsis.*** (a,b) Clay substrate culture tubes with approximately 8-week-old *Arabidopsis* plants. Plants were initially grown in soil for 4 weeks prior to their transfer to aeroponic culture. (c) Image of *Bradysia* larva feeding on *Arabidopsis* roots in aeroponic culture.

The amount of root mass remaining after *Bradysia* feeding was compared to that of non-infested control plants (Figure 3.9a). Wild type and mutant plants showed a reduction of root mass but significantly less root tissue was recovered from *AtTPS*08 knockout plants in comparison to wild type plants and the *AtTPS*08 T-DNA insertion line salk_112521, which produced rhizathalene at wild type levels ($P < 0.0001$ and $P < 0.01$, respectively) (Figure 3.9a). The percentage of root mass consumed from control plants ranged between 32% and 48%, while 56 to 63% of root tissue was consumed from *AtTPS*08 knockout plants, amounting to 10% more than that of controls (Figure 3.9b).

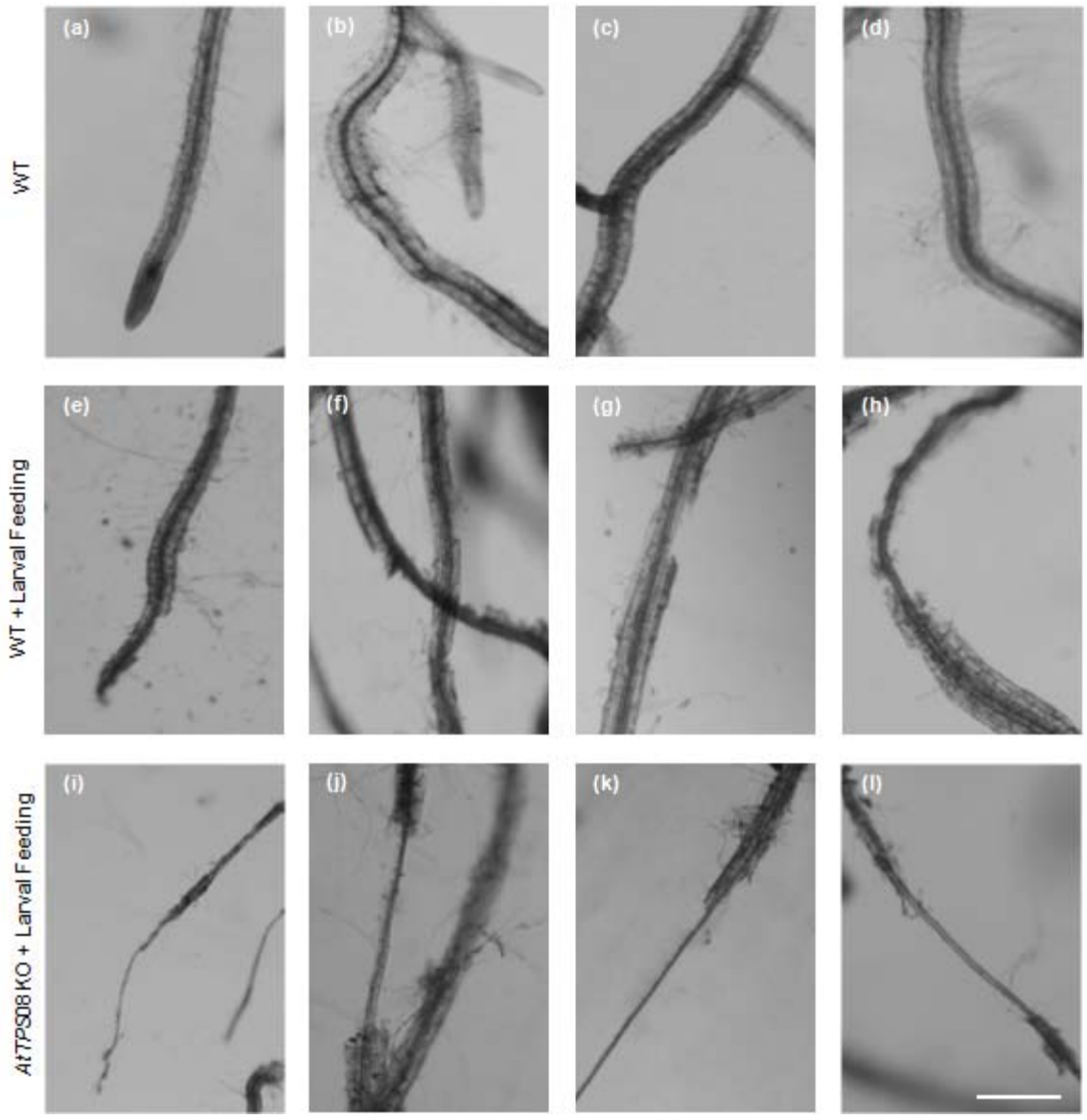


**Figure 3.8. *Bradysia* larval feeding damage on roots of wild type and *AtTPS*08 gene knockout plants in aeroponic culture.** (a-d) Undamaged roots of wild type (WT) plants. (e-h) *Bradysia* feeding damage on WT roots. Parts of the root epidermis and cortex were removed and tips of several secondary roots were severed. (i-l) Herbivore damage on roots of the *AtTPS*08 knockout (KO) mutant salk_125194. Large areas of the root were severely damaged. In addition to the epidermis and cortex, the endodermis and phloem were consumed, leaving only a string of lignified xylem. Scale bar: 250 µm.

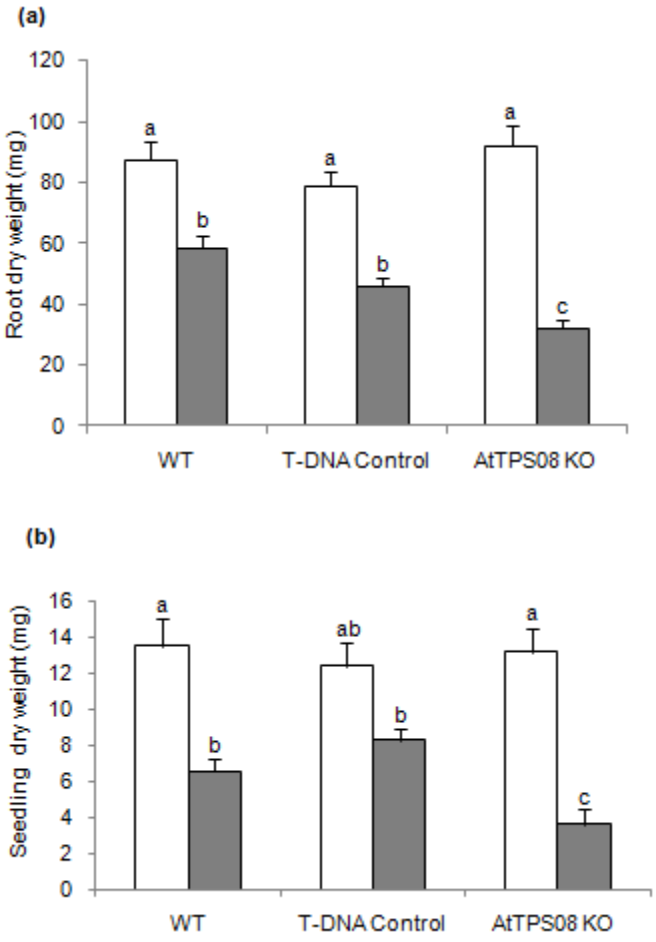


**Figure 3.9. *Arabidopsis* plants lacking rhizathalene production are significantly more susceptible to *Bradysia* root herbivory.** (a) Mature wild type (WT) and *AtTPS*08 mutant plants grown in aeroponic culture were infested with 200 to 300 *Bradysia* larvae for 5 days. Average dry root mass remaining after *Bradysia* feeding (gray bars) compared to untreated control plants (white bars). Significantly less root mass was recovered from the *AtTPS*08 knockout (KO) mutant (salk_125194) in comparison to WT plants and the T-DNA insertion control line salk_112521 (Figure 3.2a), which produces rhizathalene at wild type levels. Cumulative log transformed data from two independent experiments were analyzed with one-way ANOVA and Tukey-Kramer HSD; n = 24 for WT and *AtTPS*08 KO, n =12 for the T-DNA control line, $P < 0.01$. (b) *Arabidopsis* seedlings grown in soil were infested with 10 larvae per plant for 7 days. Average seedling mass of plants infested with *Bradysia* larvae (gray bars) compared to non-infested control plants (white bars). Seedlings treated with *Bradysia* larvae showed reduced growth. Seedlings of *AtTPS*08 plants were significantly smaller than WT and T-DNA control (salk_112521) plants subjected to the same stress. Cumulative log transformed data from two independent experiments were analyzed with one-way ANOVA and Tukey-Kramer HSD, n = 20, $P < 0.01$.

We also tested the effect of *Bradysia* feeding on wild type and mutant plants at the seedling stage. Fourteen-day-old seedlings planted in potting mix were subjected to a more moderate infestation of ten larvae per plant. The seedling biomass of wild type and *AtTPS*08 mutants challenged by larval feeding were compared to non-damaged controls (Figure 3.9c). All seedlings treated with *Bradysia* larvae showed reduced growth. However, seedlings of the *AtTPS*08 knockout line consistently showed significantly less biomass after larval feeding than wild type seedlings and those of the salk_112521 control line ($P < 0.01$ and $P < 0.001$) (Figure 3.9c). The percent reduction in seedling biomass was between 31% and 50% for wild type plants and the salk_112521 line. By comparison, reduction of biomass was significantly higher for seedlings of the *AtTPS*08 mutant, ranging between 62% and 72% (Figure 3.9d).

### *Expression of* AtTPS08 *in response to larval feeding and jasmonate treatment*

Many terpene synthases that are involved in the defense against herbivores show induced expression in response to mechanical wounding or feeding damage (Gomez *et al.* 2005, Ro*, et al.* 2006, Schnee *et al.* 2002). Using semi-quantitative RT-PCR, *AtTPS*08 expression levels from aeroponically grown wild type *Arabidopsis* roots exposed to 5 days of *Bradysia* larval feeding were compared to the expression levels of un-infested roots. No increased *AtTPS*08 mRNA levels were found in response to larval feeding for this particular time point (Figure 3.10a). We also tested whether *AtTPS*08 expression could be induced by treatment with the defense hormone jasmonic acid (JA). *AtTPS*08 transcript levels were slightly induced upon treatment with 100 μM JA for 24 hours in axenic culture (Figure 3.10b), but this increase did not correspond to a significant change in the amount of rhizathalene produced (data not shown). However, a more

stringent correlation between diterpene production and the extent of *AtTPS*08 transcription was observed in roots of plants grown under different culture conditions. The highest *AtTPS*08 transcript levels were found in roots of hydroponically grown plants corresponding to the highest concentrations of rhizathalene A in these roots, whereas the lowest levels of *AtTPS*08 mRNA and rhizathalene A were present in roots of plants grown in potting mix (Figures 3.1a, 10c).

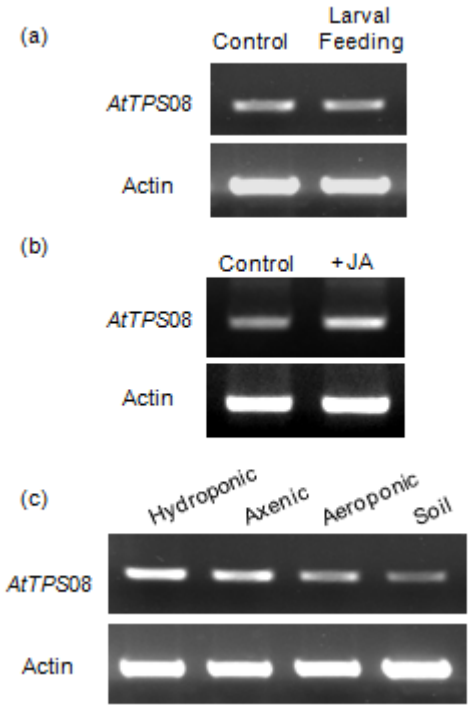


**Figure 3.10. Transcript analysis of *AtTPS*08 by semi-quantitative RT-PCR.** (a) *AtTPS*08 transcript levels in aeroponically grown roots upon 5 days of *Bradysia* feeding. (b) Transcript levels of *AtTPS*08 in roots of axenic culture treated with 100 µM jasmonic acid. (c) *AtTPS*08 transcript levels in roots from plants grown under different culture conditions as shown in Figure 3.1a.

## DISCUSSION

### Arabidopsis *roots produce volatile diterpenes*

We have found that roots of the *Arabidopsis* ecotype Columbia produce at least four related volatile diterpenes that have, to the best of our knowledge, not been reported so far in this

or any other plant species. Because the compounds are the first diterpene hydrocarbons detected in *Arabidopsis*, we named them rhizathalene A to D. Root-specific formation of terpenes has been reported among gymnosperms (Huber *et al.* 2005) and angiosperms such as carrot, oat, rice and cotton (Hunter*, et al.* 1978, Osbourn*, et al.* 2003, Senalik and Simon 1987). Rhizathalene A was found at a concentration of maximal 0.9 µg per g *Arabidopsis* root fresh weight. Nanogram concentrations of momilactone diterpenes have also been detected in the roots of rice seedlings (Kato-Noguchi *et al.* 2008a, Toyomasu*, et al.* 2008). Approximately 1.5 to 35-fold higher concentrations were reported for hemigossypol and gossypol, respectively, in roots of cotton seedlings (Howell *et al.* 2000). The content of sesquiterpenes in carrot roots (Hampel *et al.* 2005) or diterpene acids in Douglas fir (Huber*, et al.* 2005) was 100-200 fold higher than that of rhizathalene when calculated per gram fresh weight. The comparatively lower concentrations of diterpenes in *Arabidopsis* and rice roots may in part be due to the absence of secretory cells or oil ducts, which allow an accumulation of potentially autotoxic terpene metabolites as observed in carrot and conifer roots. Moreover, the detected *Arabidopsis* diterpene olefins are not glycosylated and therefore cannot be stored in the vacuole at higher concentrations.

The amount of rhizathalene produced in *Arabidopsis* roots was dependent on the culture conditions. Roots grown in hydroponic or sterile liquid cultures contained 3 to 4.5 fold more diterpene compound than those cultivated in potting mix or in clay substrate (Figure 3.1d). The increased formation of diterpenes in liquid culture may be caused by the continuous nutrient availability under these conditions. Fertilizer effects on terpene production have previously been reported. For example, hydroponically grown aromatic plants were found to contain a higher content of essential oils than soil-grown plants (Davtyan 1976, Ormeno *et al.* 2008). Differences in aeration and oxygen availability to roots may also contribute to the differential formation of

rhizathalene although only a slight increase in gene expression of the rhizathalene synthase AtTPS08 has been reported under hypoxic conditions in microarray datasets.

***AtTPS08 catalyzes the formation of rhizathalenes***

We demonstrated that the *Arabidopsis* gene *AtTPS*08 encodes a TPS, which produces rhizathalenes *in vitro* and is responsible for their root-specific formation *in vivo*. A phylogenetic comparison of *Arabidopsis* diterpene synthases and other plant diterpene synthases shows that AtTPS08 clusters with the predicted *Arabidopsis* diterpene synthases AtTPS17, 20, 26 and 29 (38% to 60% sequence identities) and casbene synthase from *Ricinus communis* (32% sequence identity), all belong to the TPS-a subfamily (Aubourg et al., 2002) (Figure 3.S2). Lower homology was found between AtTPS08 and diterpene synthases of the TPS-c to f subfamilies and labdane-related diterpene synthases from rice (Figure 3.S2). The closer similarity of AtTPS08 to casbene synthase and the presence of the highly conserved aspartate-rich DDXXD sequence motif at the C-terminal domain of the AtTPS08 protein indicate its function as a class I diterpene synthase. Class I terpene synthases initiate their enzymatic reaction by $Mg^{2+}$-ion assisted ionization of the prenyl diphosphate substrate, facilitated by the DDXXD mitif (Davis and Croteau 2000, Starks *et al.* 1997). We predict that the AtTPS08 enzyme, similar to *Ricinus* casbene synthase or taxadiene synthase from *Taxus brevifolia*, converts GGPP into rhizathalene via a class I type reaction (Mau and West 1994, Wildung and Croteau 1996). This assumption is further supported by the absence of a DXDD motif in the central domain of AtTPS08, which is characteristic for class II diterpene synthases or bifunctional class I/class II enzymes (Peters and Croteau 2002, Peters *et al.* 2001, Sun and Kamiya 1994). These enzymes initially protonate the

prenyl diphosphate substrate such as in the conversion of GGPP to copalyl diphosphates (CPP) catalyzed by CPP synthases of the TPS-c subfamily. In the case of bifunctional enzymes such as abietadiene synthase from grand fir in the conifer TPS-d family, a CPP-intermediate formed from GGPP is further converted to the final terpene end product by a type I reaction (Martin *et al.* 2004). AtTPS08 also differs from class I kaurene synthases involved in gibberellin biosynthesis (TPS-e subfamily) and the related labdane-related diterpene synthases from rice in the way that these enzymes use *ent*- or *syn*-CPP as substrates but do not accept GGPP (Wilderman *et al.* 2004, Xu *et al.* 2007).

The molecular weight of the AtTPS08 protein of 69 kDa is similar to that of other terpene synthases in the modern class I family, which do not contain the 200 amino acid N-terminal domain of the phylogenetically older CPP synthases and conifer diterpene synthases (Mau and West 1994, Peters and Croteau 2002, Wildung and Croteau 1996). As many other terpene synthases, recombinant AtTPS08 has a low apparent $K_M$ value for its prenyl diphosphate substrate GGPP indicating a high affinity for this substrate. Apparent $K_M$ values lower than 1 μM were reported for *Arabidopsis* kaurene synthase (AtKS) (Xu*, et al.* 2007) and abietadiene synthase (Ravn et al., 2002), although these reactions were performed with the substrate CPP. $K_M$ values of recombinant class-I type casbene and taxadiene synthases for GGPP are somewhat higher in the range of 2 to 3 μM (Hill et al., 1996; Williams et al., 2000). However, the apparent $k_{cat}$ value for AtTPS08 is 30 to 100 fold lower than those observed for taxadiene and casbene synthase, respectively, resulting in an overall 10 to 50 fold lower corresponding catalytic efficiency.

***Rhizathalene synthase is located in root leucoplasts and its promoter exhibits specific activity in the vascular tissue and root tips***

With the exception of *Arabidopsis* geranyllinalool synthase, which is located in the cytosol (Herde et al., 2008), all of the plant diterpene synthases characterized so far were shown to carry an N-terminal transit peptide for a presumed protein transport into plastids (Bohlmann*, et al.* 1998). Similarly, rhizathalene synthase contains a 41 amino acid predicted plastidial transit peptide. Subcellular localization of a constitutively expressed AtTPS08-GFP fusion protein confirmed targeting of the AtTPS08 enzyme to plastids. The result strongly indicates that AtTPS08 produces rhizathalenes from GGPP in root leucoplasts. Further analysis is required to determine the role of different GGPP synthases expressed in *Arabidopsis* roots and the contribution of the MEP and mevalonate pathways in root leucoplast-specific formation of diterpenes.

*AtTPS*08 promoter:GUS reporter activity was observed specifically in the root vasculature and in the tips of primary and secondary roots (Figure 3.6). GUS staining is largely in agreement with results obtained from cell-type specific gene transcript profiles in *Arabidopsis* roots, although only low *AtTPS*08 transcript levels or none were reported in the elongation zone and the meristem, respectively (Birnbaum*, et al.* 2003, Brady*, et al.* 2007). High-resolution transcript maps further indicated *AtTPS*08 expression exclusively in the xylem-pole pericycle and the procambium (Brady*, et al.* 2007). Cell-type specificity is apparent for several other *TPSs* expressed in *Arabidopsis* roots. The 1,8-cineole synthases AtTPS23 and 27 are primarily expressed in epidermal cells, while expression of the (*Z*)-γ-bisabolene synthases AtTPS12 and 13 occurs predominantly in the cortex. A highly coordinated expression of the genes of the thalianol

triterpene biosynthesis gene cluster was found in the root epidermis (Field and Osbourn, 2008). At*TPS*08 appears to be co-expressed with four genes encoding cytochrome P450 (CytP450) enzymes (At4g15380, At3g20110, At5g4258, At5g3611). We cannot completely exclude that these enzymes catalyze a conversion of rhizathalenes into alcohol or acid derivatives. However, no accumulation of rhizathalenes was observed when *Arabidopsis* hairy roots were treated with different inhibitors of CytP450 enzymes (data not shown). Interestingly, when the *AtTPS*08 gene was expressed under the control of the constitutive *CaMV 35S* promoter, no diterpene product was detected in roots despite the presence of a full length *AtTPS*08 mRNA, which indicates possible feedback and posttranslational regulatory effects.

***Rhizathalene biosynthesis contributes to a direct defense against* Bradysia *root herbivores***

To investigate the possible defense activities of rhizathalenes against herbivores, we conducted feeding experiments with larvae of the opportunistic root herbivore *Bradysia* spp. (fungus gnat). *Bradysia coprophila* and *Bradysia impatiens* feed on organic matter and soil fungi but will also consume plant roots (Harris*, et al.* 1996). *Bradysia* larvae can be particularly destructive to *Arabidopsis* seedlings and jasmonate-insensitive mutants with impaired root defense (McConn*, et al.* 1997). The consequences of feeding damage are most apparent when larvae reach high densities which can be quite frequent considering their short life cycle of 25 days with adult female gnats laying as many as 1000 eggs (Jagdale*, et al.* 2007).

Feeding assays were performed in an aeroponic culture system, which allows easy separation of roots from clay granulate without mechanical damage upon completion of the assay (Figure 3.7). An *Arabidopsis* transgenic line carrying a single T-DNA inserted in the *AtTPS*08

gene and lacking rhizathalene formation was significantly more susceptible to larval feeding than wild type plants and those of a T-DNA insertion line that was unaffected in rhizathalene production (Figure 3.8a, b). A comparison of root tissue damage on wild type plants and the *AtTPS*08 gene knockout line revealed clear differences in the extent and type of feeding damage. In wild type roots, larvae removed primarily the epidermis and cortex at the site of feeding, while larvae appeared to persist feeding at damaged sites of roots of the *AtTPS*08 mutant by removing additional cell layers leaving behind only the xylem (Figure 3.9e-h, i-l). The xylem was not consumed most likely because of the higher degree of cell lignification. The observed difference in feeding behavior suggests that rhizathalene functions as a deterrent against *Bradysia* larvae.

Differences in biomass reduction by *Bradysia* feeding similar to those found in aeroponic culture were observed between seedlings of the *AtTPS*08 mutant and control plants grown in soil (Figure 3.8c, d) indicating a defensive role of rhizathalene at an early stage of development. Defense by rhizathalene diterpenes is primarily constitutive since transcription of *AtTPS*08 was unaffected by larval feeding and only slightly induced upon jasmonic acid treatment (Figure 3.10a, b). Diterpene formation in *Arabidopsis* roots most likely contributes to other more prominent chemical defenses such as glucosinolates. However, our results clearly show that the formation of relatively low levels of specialized metabolites, when confined to specific cell types in plant roots, can considerably affect feeding patterns of root herbivores.

## EXPERIMENTAL PROCEDURES

### *Plant material*

*Arabidopsis* plants were grown under controlled conditions at 22 to 25°C, 150 μmol $m^{-2}$ $sec^{-1}$ photosynthetically active radiation (PAR) and a 10 h light – 14 h dark photoperiod. All wild type plants were ecotype Columbia (Col-0 6000). Seeds of At4g20210 T-DNA insertion lines, salk_125194 and salk_112521, were ordered from the ABRC stock center (Alonso *et al.* 2003). Plants were grown in potting mix (90% Sunshine mix #1 [Sun Gro Horticulture, Bellevue, WA], 10% sand) for 2 to 4 weeks.

Non-soil grown *Arabidopsis* culture conditions included hydroponic culture (Gibeaut*, et al.* 1997), axenic culture (Hetu*, et al.* 2005) and aeroponic clay granulate culture system. Hydroponic cultures were established by transferring 4-week-old plants grown in potting mix to plastic containers containing Hoagland's solution as previously described (Gibeaut*, et al.* 1997). Constant aeration was supplied by house air with an aquarium bubble stone. Axenic cultures were prepared by following the procedures described by Hetu et al. (2005). Approximately 20 to 25 surface sterilized seeds were germinated on a 2.5 $cm^2$ plastic screen (Small Parts Inc, Logansport, IN) placed on half-strength solid Mursashing and Skoog (MS) medium containing 1% sucrose. A week after germination the seedlings and screen were transferred to a 125 ml flask with 10 ml liquid MS medium supplemented with 2% sucrose. Flasks were placed on a rotary shaker under the same light conditions above and allowed to grow for 2 weeks. Two days before harvesting the root tissue, the MS medium was changed to reduce the sucrose content to 1%. Hormone treatments with jasmonic acid (JA) were applied to axenically grown roots by

added 100 μM JA (Sigma Aldrich, St. Louis, MO) directly to the liquid medium. Treated tissue was collected 24 h after application of the hormone.

***Reagents and Radiochemicals***

Unlabeled GPP (geranyl diphosphate), FPP (farnesyl diphosphate) and GGPP (geranylgeranyl) diphosphate were purchased from Echelon Biosciences Incorporated (Salt Lake City, UT, USA). Tritium labeled GGPP ([1-$^{3}$H]-GGPP ~ 0.74 TBq mmol$^{-1}$) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). All other reagents or solvents were obtained from Fisher Scientific, Sigma-Aldrich, Invitrogen and Fluka, unless otherwise stated. All primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA).

**Arabidopsis *aeroponic culture system***

Cultures were prepared in 50 mL plastic conical tubes (Fisher Scientific, Suwanee, GA) with five 3.5 mm diameter holes around the bottom and a 25 mm hole in the cap. The tubes were filled with Seramis® clay granules (www.seramisuk.co.uk.) and the exterior was wrapped with aluminum foil. Tops were sealed with plastic wrap, which was punctured in the center and held in place by the caps. Tubes were submerged in Hoagland's solution (Gibeaut*, et al.* 1997) to moisten the clay granules. Four-week-old *Arabidopsis* plants grown in potting mix were carefully removed with their roots from the pots, and the roots were repeatedly submerged in water to remove potting substrate. A half inch cube of rockwool (Hydroempire, Grodan, Los Angeles, CA) was placed around the base of the rosette and plants were transferred to the culture

tubes. Plants were held in place by the plastic wrap and rockwool until the roots had grown into the clay granules. Clay granules were moistened by submerging the bottom half of the tubes in Hoagland's solution for 10 to 15 min every other day. Plants were grown for 4 weeks until the initiation of primary shoot production.

**Bradysia *(fungus gnat) culture***

Fungus gnat larvae were collected from greenhouse infested soil using potato slices (Harris*, et al.* 1995) to establish a colony. The colony was maintained in 8 L plastic containers with a screened opening in the lid. The culture medium consisted of 4 L moist Sunshine mix #1 enriched with 1.5 kg of shredded potato (Cloyd and Zaborski 2004, Zaborski and Cloyd 2004). Cultures were kept under controlled greenhouse conditions at 21 to 23°C and indirect sunlight. Every 3 to 4 weeks, 0.5 L of medium (containing *Bradysia* larvae, pupae, adults and eggs) was transferred to a fresh container of soil and potato mix. Specimens were identified as a mixed colony of *Bradysia coprophila* (Lintner) and *Bradysia impatiens* (Johannsen).

**Bradysia *feeding experiments with plants grown in aeroponic culture***

Larvae were isolated from the culture medium using a modified flotation/extraction procedure previously described by Zaborski and Cloyd (2004). Approximately 1 L of *Bradysia* culture medium containing larvae was placed into a 2 L wide-mouth Erlenmeyer flask filled with tap water. The flask was inverted and placed on top of a 2 L graduated cylinder filled with cold water so that the mouth of the flask opened just below the surface of the water in the cylinder.

After larvae sank out of the flask into the cylinder, the water was poured off and replaced by a 1.5 M $MgSO_4$ solution. Larvae of all four instars floating to the surface were collected with a sieve, rinsed with water and placed in a Petri dish on moist filter paper without food for 20 to 24 h prior to feeding assays.

For application of larvae to aeroponically grown plants, tube caps were carefully opened avoiding any leaf damage. Approximately 200 to 300 selected second- and third-instar larvae, collected as described above, were transferred to a single culture tubes in 1 mL of Hoagland's solution pipetted into the clay substrate. First and fourth instar larvae were excluded from the experiments because they were too small to count and too close to pupation, respectively. During the feeding experiment, plants were watered every other day by applying 10 mL of Hoagland's solution through the top of the tube. Cultures were kept in a screened box under the same conditions described above. Five days after larval feeding, roots were removed from the tubes and the clay granules by submerging the tubes in water. The procedure allowed a simple separation of roots from the clay substrate without visible wounding damage. Roots were then dried with Kimwipes (Fisher Scientific, Suwanee, GA) and the fresh weight recorded. After drying for two days at room temperature, the dry weight was measured. For data analysis, the weights from two independent experiments were combined and log transformed before performing a one-way analysis of variance (ANOVA) followed by Tukey-Kramer HSD, n = 24 for WT and *AtTPS*08 KO, n =12 for T-DNA control. The percentage of root mass consumed was calculated for each individual plant in comparison to its untreated control. The percentages were analyzed for differences by one-way ANOVA and Tukey-Kramer HSD.

**Bradysia *feeding experiments with seedlings grown in potting mix***

Seedlings were grown in individual pots (8 cm x 8 cm x 10 cm) under the described conditions. Fourteen-day-old seedlings were challenged with 10 second- and third-instar *Bradysia* larvae isolated and transferred to the pots as described above. Larvae that did not move into the potting substrate within 5 min of the transfer were removed from the experiment and replaced. Pots with treated and control seedlings were then randomly placed in separate net enclosures and kept under growth room conditions as described above. After 7 days of larval feeding, seedlings were removed from the soil and roots were rinsed in water. Seedlings were allowed to air dry for 2 days to record dry weight. Cumulative log transformed data from two independent experiments were analyzed with one-way ANOVA and Tukey-Kramer HSD, n = 20. The percentage of biomass reduced was calculated for each individual plant in comparison to its non-infested control. The percentages were analyzed by one-way ANOVA and Tukey-Kramer HSD.

***Volatile collection and analysis***

Volatiles were collected from roots by automated solid phase microextraction (SPME) using a AOC-5000 Shimadzu autosampler. One gram of root tissue was detached and placed in a 20 mL screw cap glass vial (Supelco, Bellefonte, PA). Volatiles were adsorbed in the headspace with a 100 μM polydimethylsiloxane (PDMS) fiber (Supelco, Bellefonte, PA) for 30 min at

30°C. Volatile analysis was performed using a gas chromatograph (GC-2010, Shimadzu) coupled with a quadrupole mass spectrometer *(*GC-MS-QP2010S*,* Shimadzu). Samples were separated with a Rxi$^{TM}$-XLB GC column (Restek, Bellefonte, PA) of 30 m x 0.25 mm i.d. x 0.25 μM film thickness. Helium was the carrier gas (flow rate, 1.4 mL/min), a 2:1 split injection with an injection temperature of 240°C was used, and a temperature gradient of 5°C min$^{-1}$ from 40°C (2 min hold) to 240°C (2 min hold) was applied.

The mass spectra of the volatile diterpene compounds were compared to those of published reference terpene spectra in the terpene library of MassFinder (Konig *et al.* 1999) and in the National Institute of Standards and Technology (NIST) MS database (Powell *et al.* 2005).

Authentic diterpene standards of pimara-8(9),15(16)-diene, syn-pimara-7,15-diene, ent-pimara-8(14),15-diene, isopimara-8(14),15-diene, isopimara-7-15-diene, syn-stemod-13(17)-ene, ent-cassa-7,12-diene, abieta-8,12-diene, ent-kaur-16-ene, moanool, sclareol, and cembrene were analyzed by GC-MS, but their retention times and mass specta did not match any of the *Arabidopsis* root diterpenes.

Quantification of rhizathalene A was conducted by gas chromatography-flame ionization detection (GC-FID) analysis of organic root extracts (see below). A Shimadzu 2010 GC-FID was used with the same column and temperature program as described above for volatile analysis on the GC-MS. For quantification, the peak of rhizathalene was integrated and the amount of compound was calculated based on an external calibration curve generated with the authentic diterpene standard, cembrene (Acros Organics, Geel, Belgium).

### *Organic extraction and quantitative analysis of* Arabidopsis *root terpenes*

Volatile terpenes were extracted from 2 g of roots grown under the different culture conditions. Root tissue was rinsed with water, dried with tissue paper and immediately submerged in 10 mL ethyl acetate. Roots were then ground with a mortar and pestle in the presence of the organic solvent and 10 mL de-ionized water. Then the ground material was mixed with 90 mL of each ethyl acetate and de-ionized water in a separatory funnel for 5 min. After phase separation, the organic phase was collected and concentrated to approximately 100 µL under a gentle air flow. Following the addition of 5 mL hexane, the extract was passed through a small silica column overlaid with magnesium sulfate and preconditioned with hexane. The flow through and a second 4 mL fraction were combined and concentrated again to approximately 250 µL. One microliter of the extract was analyzed by GC-MS and GC-FID analysis.

### *Isolation and cloning of* AtTPS*08 cDNA*

Total RNA was isolated from 150 mg of axenically-grown *Arabidopsis* roots using the TRIzol method (Chomczynski and Mackey 1995). Two micrograms of total RNA were reverse transcribed into cDNA using oligo($dT_{20}$) primers (IDT, Coralville, IA) and Superscript II reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Two primers, 5-CACCATGGAAGCCATAAAAACTTTTT-3 (P1) and 5-ATTCAAATACGAATCGGGGTAATAAAC-3 (P2) corresponding to the start and the end of the coding region of *AtTPS*08 and AccuPrime *Pfx* polymerase (Invitrogen, Carlsbad, CA) were

used for PCR amplification of the *AtTPS*08 cDNA. The resulting PCR product was cloned into the pET102/D-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturer's protocol.

***Genotyping of plant material***

Mutants carrying a T-DNA insertion in the *AtTPS*08 gene were identified in the insertional mutant population obtained from the ABRC stock center (Sessions et al., 2002). The presence of the T-DNA insertion in line salk_12594 was confirmed by PCR and sequencing of the right and left border PCR products. To determine the copy number of T-DNA insertions in the salk_12594 line, genomic DNA was isolated from leaves according to Rogers and Bendich (1985). Fifty micrograms of DNA were digested with *Bam*HI and *Hin*dIII and separated by 0.7% agarose gel electrophoresis. After capillary transfer to a nitrocellulose membrane, hybridization was carried out using a digoxigenin (DIG) labeled T-DNA specific probe designed with the following primers: 5'-GTTTTCTATCGCGTATTAAATG-3 (P3) and 5-GTAATAATTAACATGTAATGCATGAC-3 (P4). DNA hybridization was performed following the protocol previously published by Smith and Summers (1980). Five µl of DIG labeled DNA molecular weight marker II (Roche, Indianapolis, IN) was used to estimate the fragment sizes detected by chemiluminescence.

***Complementation Analysis***

The *AtTPS*08 open reading frame was amplified from *AtTPS*08 cDNA using primers P1 and P2. The amplicon was cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen,

Carlsbad, CA) and then recombined into the pK7WG2 binary vector containing the constitutive *CaMV 35S* promoter (Karimi *et al.* 2002). Cloning methods were as described by manufacturer's protocol. The *Pro35S*:*AtTPS*08 construct was introduced into *Agrobacterium tumefaciens* strain GV3101, and transformed into salk_125194 plants by floral vacuum infiltration (Bechtold *et al.* 1993). Transformants were screened on half strength Murashige and Skoog (MS) plates with 1% (w/v) sucrose and 0.1 mg/ml kanamycin.

***Heterologous expression in* E.coli *and purification of AtTPS08 protein***

A truncated version of *AtTPS*08 without 123 bp encoding a predicted 41 amino acid N-terminal plastidial transit peptide was amplified from the *AtTPS*08-pET102/D-TOPO construct by PCR using the forward primer 5-CACCCGTTTGAAGGCAACTAGAGC-3 (P5) and the reverse primer 5-TCCAATACGAATCGGGGTAATAAAC-3 (P6). The PCR product was cloned into the pET102/D-TOPO expression vector according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) to generate a translational N-terminal fusion to thioredoxin and a C-terminal fusion to a $His_6$-tag. The resulting plasmid was transformed into *E. coli* BL21 Codon Plus cells (Stratagene, La Jolla, CA). A 5-ml overnight culture from a single transformant was used to inoculate 500 ml of Luria–Bertani (LB) medium containing ampicillin (100 μg/ml). The culture was grown at 37°C to an $OD_{600}$ of 0.5 to 0.6 and then induced overnight at 18°C with 1 mM isopropyl *β-D*-1-thiogalactopyranoside (IPTG). Cells were extracted according to (Chen*, et al.* 2003) and the AtTPS08 protein was partially purified from crude bacterial extract on a 0.5 mL Ni-NTA agarose column (Invitrogen, Carlsbad, CA) as described by Tholl et al. (2004). Elution fractions were desalted into assay buffer (10 mM 3-(*N*-morpholino)-2-

hydroxypropanesulfonic acid (MOPSO), pH 7.0, 10% [v/v] glycerol, and 1 mM DTT) for enzyme characterization.

***Terpenes synthase assays and enzyme characterization***

Enzyme assays were conducted in a total volume of 1 mL, containing 960 µl partially purified enzyme extract, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.2 mM and 0.1 mM of the phosphatase inhibitors $NaWO_4$ and NaF, respectively, and 60 µM GGPP. The assay was performed for 30 min at 30°C in a 10 ml screw cap vial sealed with PFTE/Silicone seta (Supelco, Bellefonte, PA). Enzyme products were collected during assay incubation by automated SPME and subsequently analyzed by GC-MS as described above.

For enzyme characterization, assays were carried in a final volume of 50 µl with 1 µg partially purified AtTPS08 enzyme, and 10 µM of [1-$^3$H]GGPP (0.74 TBq mmol$^{-1}$). Buffer and salt concentrations were as described above. The reaction was incubated for 30 min at 30°C and the products were extracted with 300 µl hexane. Total radioactivity of the reaction products was determined by scintillation counting. To determine the divalent metal ion dependency of AtTPS08, assays were performed without the addition of $MgCl_2$ and $MnCl_2$. For kinetic assays, appropriate enzyme concentrations and incubation times were determined so that the reaction velocity was linear during the reaction time period. To determine the $K_M$ value for GGPP, six different concentrations of [1-$^3$H]-GGPP were applied. Assays were conducted in three replicates. Calculation of $K_M$ and $V_{max}$ values was performed by Hanes plot analysis using the Hyperbolic Regression Analysis (HYPER 1.01) software (J.S. Easterby, University of Liverpool).

***Structural elucidation of rhizathalene A***

To conduct structural analysis of rhizathalene A, larger amounts of the diterpene were produced *in vivo*. The truncated *AtTPS*08- pET102/D-TOPO construct was transformed into *E. coli* strain C41(DE3) (Invitrogen, Carlsbad, CA), which carried a recombinant pseudomature GGPP synthase (GGPS) from grand fir (*Abies grandis*) rAgGGPS in the pACYCDuet vector (Cyr*, et al.* 2007). The GGPP synthase vector construct was obtained from Dr. Reuben Peters (Iowa State University). Bacteria were grown at 37°C to an $OD_{600}$ of 0.7 to 0.9 and induced with 0.5 mM IPTG overnight at 24°C. The induced bacterial culture (250 ml) was transferred to a desiccator and the diterpene volatile was collected in the culture headspace using 25 mg Super-Q traps and a closed-loop stripping procedure described previously (Donath and Boland 1995, Tholl*, et al.* 2006). Compounds were eluted from the volatile traps twice with 100 µL $CH_2Cl_2$, the eluate was concentrated under nitrogen to a few microliters and then resuspended in 1 mL of 100% methanol. The procedure was repeated 10 times to obtain sufficient quantities of diterpene compound for NMR analysis.

Next, rhizathalene A was purified by liquid chromatography-mass spectrometry (LC-MS) (Walters) using a X-Bridge column (4.6 x 250 mm, 5 µm particle size). A solvent gradient of 80 to 90% methanol in 1% (v/v) formic acid was applied over 5 min and the final concentration was held for 20 min; the flow rate was 0.8 ml $min^{-1}$. The injection volume varied from 20 to 100 µl. The fraction containing rhizathalene A at 95 to 99% purity was collected between 16 and 17.5 min as determined by MS analysis.

### *Construction and analysis of the* **ProAtTPS*08*:GUS** *reporter gene fusion*

A 2.2 kb *AtTPS*08 promoter fragment was amplified by PCR from genomic DNA using the forward primer 5-CACCAGATACTTACGTAAATCATGTG-3 (P7), and the reverse primer 5-CAACATTTGATAACCTACTTAAC-3 (P8). The amplicon was cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA) and recombined into the binary vector pKGWFS7 (Karimi*, et al.* 2002) with Gateway LR Clonase II (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. The construct was introduced into the *Agrobacterium tumefaciens* GV3101 strain, which was used to transform *Arabidopsis* (ecotype Columbia) plants by floral vacuum infiltration (Bechtold*, et al.* 1993). Histochemical GUS assays were performed as previously described (Jefferson *et al.* 1987).

### *Fusion of* **AtTPS*08*** *with GFP*

The full length *AtTPS*08 cDNA was transferred from the pENTR/D-TOPO vector to the binary vector pK7FWG2 using Gateway LR Clonase II as described above. This construct established a C-terminal translational fusion of the AtTPS08 protein with enhanced green florescent protein (eGFP) (Karimi*, et al.* 2002). Generation of transgenic plants was completed as described above. Two-week-old transformants grown on half strength MS, 1% (w/v) sucrose and 0.1 mg/ml kanamycin were used for fluorescence imaging by confocal microscopy.

***Transcript analysis by reverse transcriptase–PCR***

Crude RNA was extracted from 150 mg of root tissue and reverse transcribed into cDNA as previously described above. One µl of cDNA was used for PCR amplification of *AtTPS*08 with the internal forward primer 5-GAAATGGATGCGCTTAGGAAA-3 (P9) and reverse primer 5-CTCTCCAGACTATTGGTGAGG-3 (P10). Primers P1 and P2 were used to verify *AtTPS*08 expression in *Pro35S*::*AtTPS*08 plants. PCR was performed in 30 cycles with 0.2 µM of each primer, 0.5 mM of each dNTP, and 0.5 U *Taq* polymerase (New England Biolabs, Ipswich, MA). Reactions with primers 5-ATGAAGATTAAGGTCGTGGCAC-3 (P11) and 5-GTTTTTATCCGAGTTTGAAGAGGC-3 (P12) for *Actin 8* were performed to judge the equality of cDNA template concentrations.

***Statistical Analysis***

Specific data analysis performed has been described with the individual experiments. Analysis of variance was accomplished using the JMP (SAS Institute Inc.) statistical software.

***Phylogenetic Analysis***

The amino acid sequence alignment of *Arabidopsis* and other plant diterpene synthase proteins was produced with ClustalW (Lasergene 8) and exported as a Nexus file. Phylogenetic analysis of the data set was conducted using maximum parsimony in PAUP* (D.L. Swofford, 2002,

Florida State University). Maximum Parsimony analyses were conducted using heuristic tree searches with tree bisection-reconnection (TBR) branch-swapping and 1000 random addition sequence replicates. Support for the clades was obtained by performing bootstrap (BS) (Felsenstein 1985) searches with 1000 replicates and 10 random sequence replicates. Tree was compiled using TreeGraph2 (Stover and Muller 2010).

## ACKNOWLEDGMENTS

We wish to thank Dr. Raymond J. Gragné at the USDA Systematic Entomology Laboratory in Washington, DC for the identification of *Bradysia* species. We thank Dr. Mehdi Ashraf-Khorassani at the Virginia Tech Department of Chemistry for aiding in the purification of the diterpene compound. We are greatful to Dr. Reuben Peters at Iowa State University for providing the many diterpene standards and the rAgGGPS construct. We are grateful to Dr. Francis Webster, Dr. David Kiemle and Dr. Robert Coates for the NMR analysis and interpretation.

**SUPPLEMENTAL MATERIALS**

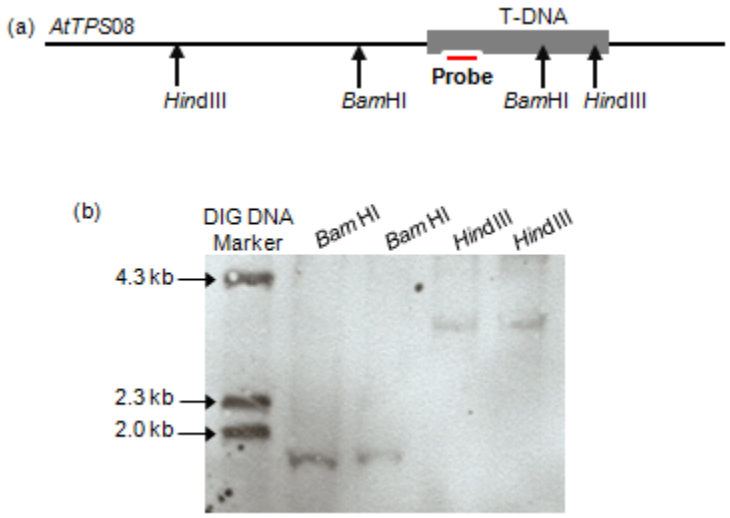


**Figure 3.S1. Southern blot analysis of T-DNA copy numbers in genomic DNA of salk_125194.** (a) Positions of the hybridization probe and restriction sites of *Hin*dIII and *Bam*HI relative to the position of the T-DNA insertion in *AtTPS*08. The black line represents the *AtTPS*08 gene and the T-DNA insertion is depicted as a gray box. The expected fragment sizes were estimated to be 1.8 kb and 3.8 kb for digestion with *Bam*HI and *Hin*dIII, respectively. (b) Southern blot analysis of salk_125194 genomic DNA digested with *Bam*HI and *Hin*dIII. The probe hybridization pattern is consistent with a single T-DNA copy specifically located in the *AtTPS*08 gene. Blotting conditions and probe labeling are described under Experimental Procedures.

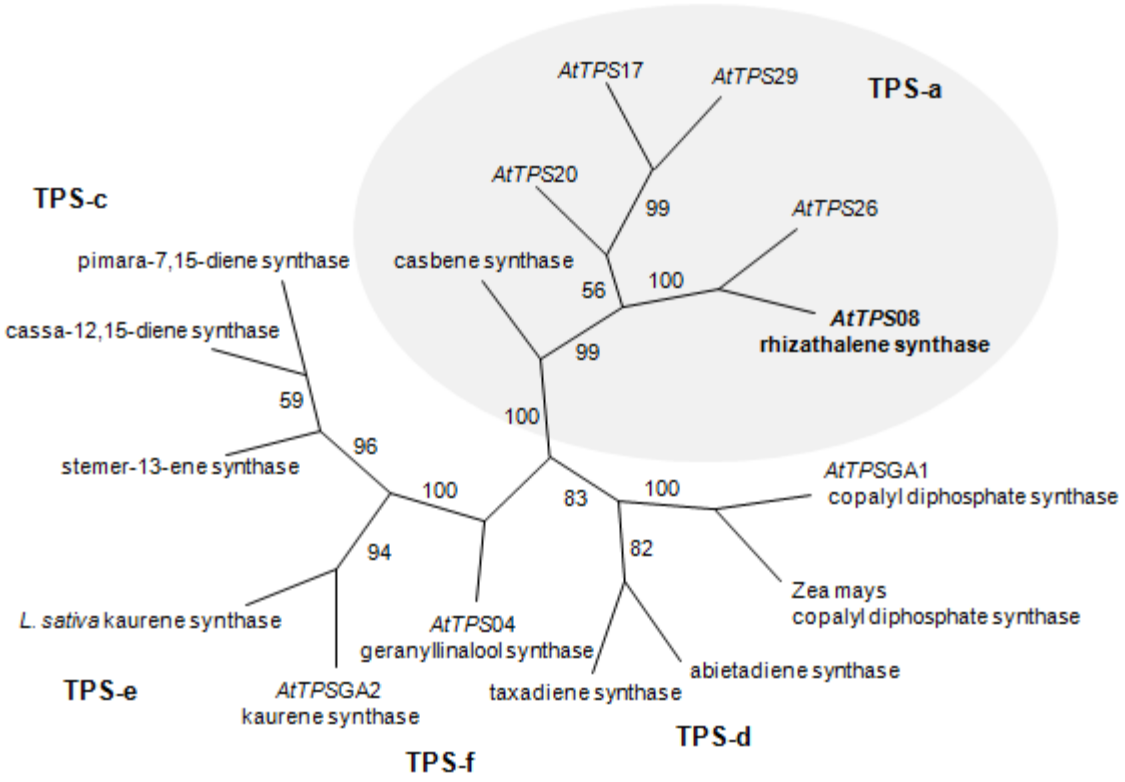


**Figure 3.S2. Phylogenetic tree of putative *Arabidopsis* diterpene synthases and representative diterpene synthases of known function.** A neighbor joining tree based on the degree of amino acid sequence similarity between characterized and putative *Arabidopsis* diterpene synthases and selected diterpene synthases from other plants. TPSs clustering together with AtTPS08 are highlighted in gray. *AtTPS*08 rhizathalene synthase is shown in bold. The TPS subfamilies are also indicated is bold, TPS-a,c-f (TPS-b contains monoterpenes and is not in this tree).The phylogenetic tree was generated using PAUP 4.0 software. Accession numbers of non-*Arabidopsis* diterpene synthases represented in the phylogenetic tree are: *Abies grandis* abietadiene synthase (U50768); *Latuca sativa* copalyl diphosphate synthase (AB031204); L. sativa ent-kaurene synthase (AB031205); *Oryza sativa* cassa-12,15-diene synthase (AB089272); *Oryza sativa* pimara-7,15-diene (AY616862); *Oryza sativa* stemer-13-ene synthase (AB118056); *Ricinus communis* casbene synthase (L32134); *Taxus brevifolia* taxadiene synthase (U48796); *Zea mays* copalyl diphosphate synthase (L37750).

## REFERENCES


**Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C. and Ecker, J.R.** (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653-657.

**Aubourg, S., Lecharny, A. and Bohlmann, J.** (2002) Genomic analysis of the terpenoid synthase (AtTPS) gene family of *Arabidopsis thaliana*. *Molecular Genetics and Genomics*, **267**, 730-745.

**Bechtold, N., Ellis, J. and Pelletier, G.** (1993) In-planta Agrocacterium-mediated gene-transfer by infiltration of adult *Arabidopsis thaliana* plants. *Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie,* **316**, 1194-1199.

**Birch, A.N.E., Griffiths, D.W., Hopkins, R.J., Smith, W.H.M. and McKinlay, R.G.** (1992) Glucosinolate responses of swede, kale, forage and oilseed rape to root damage by turnip root fly (*Delia-floralis*) larvae. *Journal of the Science of Food and Agriculture*, **60**, 1-9.

**Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W. and Benfey, P.N.** (2003) A gene expression map of the *Arabidopsis* root. *Science*, **302**, 1956-1960.

**Blossey, B. and Hunt-Joshi, T.R.** (2003) Belowground herbivory by insects: Influence on plants and aboveground herbivores. *Annual Review of Entomology*, **48**, 521-547.

**Bohlmann, J., Meyer-Gauen, G. and Croteau, R.** (1998) Plant terpenoid synthases: Molecular biology and phylogenetic analysis. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 4126-4133.

**Brady, S.M., Orlando, D.A., Lee, J.Y., Wang, J.Y., Koch, J., Dinneny, J.R., Mace, D., Ohler, U. and Benfey, P.N.** (2007) A high-resolution root spatiotemporal map reveals dominant expression patterns. *Science*, **318**, 801-806.

**Chen, F., Ro, D.K., Petri, J., Gershenzon, J., Bohlmann, J., Pichersky, E. and Tholl, D.** (2004) Characterization of a root-specific *Arabidopsis* terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole. *Plant Physiology*, **135**, 1956-1966.

**Chen, F., Tholl, D., D'Auria, J.C., Farooq, A., Pichersky, E. and Gershenzon, J.** (2003) Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. *Plant Cell*, **15**, 481-494.

**Chomczynski, P. and Mackey, K.** (1995) Modification of the TRI- reagent procedure for isolation of RNA from polysaccharide-and proteoglycan-rich sources. *Biotechniques*, **19**, 942-945.

**Cloyd, R.A. and Zaborski, E.R.** (2004) Fungus gnats, *Bradysia* spp. (Diptera : sciaridae), and other arthropods in commercial bagged soilless growing media and rooted plant plugs. *Journal of Economic Entomology.*, **97**, 503-510.

**Cyr, A., Wilderman, P.R., Determan, M. and Peters, R.J.** (2007) A modular approach for facile biosynthesis of labdane-related diterpenes. *Journal of American Chemical Society*, **129**, 6684-+.

**Davis, E.M. and Croteau, R.** (2000) Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. *Biosynthesis: Aromatic Polyketides, Isoprenoids, Alkaloids*, pp. 53-95.

**Davtyan, G.S.** (1976) The productivity of medicinal, essential oil and condiment plants grown under open-air hydroponics. *Horticultural abstracts*, **46**, 619-629.

**De Moraes, C.M., Mescher, M.C. and Tumlinson, J.H.** (2001) Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature*, **410**, 577-580.

**Donath, J. and Boland, W.** (1995) Biosynthesis of acyclic homoterpenes - enzyme selectivity and absolute-configuration of the nerolidol precursor. *Phytochemistry*, **39**, 785-790.

**Dudareva, N., Pichersky, E. and Gershenzon, J.** (2004) Biochemistry of plant volatiles. *Plant Physiology*, **135**, 1893-1902.

**Gibeaut, D.M., Hulett, J., Cramer, G.R. and Seemann, J.R.** (1997) Maximal biomass of *Arabidopsis thaliana* using a simple, low-maintenance hydroponic method and favorable environmental conditions. *Plant Physiology*, **115**, 317-319.

**Gomez, S.K., Cox, M.M., Bede, J.C., Inoue, K., Alborn, H.T., Tumlinson, J.H. and Korth, K.L.** (2005) Lepidopteran herbivory and oral factors induce transcripts encoding novel terpene synthases in *Medicago truncatula*. *Archives of Insect Biochemistry and Physiology*, **58**, 114-127.

**Hampel, D., Mosandl, A. and Wust, M.** (2005) Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. *Phytochemistry*, **66**, 305-311.

**Harris, M.A., Gardner, W.A. and Oetting, R.D.** (1996) A review of the scientific literature on fungus gnats (Diptera: Sciaridae) in the genus *Bradysia*. *Journal of Entomological Science*, **31**, 252-276.

**Harris, M.A., Oetting, R.D. and Gardner, W.A.** (1995) Use of entomopathogenic nematodes and a new monitoring technique for control of fungus gnats, *Bradysia coprophila* (Diptera, Sciaridae), in floriculture. *Biological Control*, **5**, 412-418.

**Hetu, M.F., Tremblay, L.J. and Lefebvre, D.D.** (2005) High root biomass production in anchored *Arabidopsis* plants grown in axenic sucrose supplemented liquid culture. *Biotechniques*, **39**, 345-+.

**Howell, C.R., Hanson, L.E., Stipanovic, R.D. and Puckhaber, L.S.** (2000) Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. *Phytopathology*, **90**, 248-252.

**Huber, D.P.W., Philippe, R.N., Godard, K.A., Sturrock, R.N. and Bohlmann, J.** (2005) Characterization of four terpene synthase cDNAs from methyl jasmonate-induced Douglas-fir, *Pseudotsuga menziesii*. *Phytochemistry*, **66**, 1427-1439.

**Hunter, R.E., Halloin, J.M., Veech, J.A. and Carter, W.W.** (1978) Exudation of terpenoids by cotton roots. *Plant and Soil*, **50**, 237-240.

**Jagdale, G.B., Casey, M.L., Canas, L. and Grewal, P.S.** (2007) Effect of entomopathogenic nematode species, split application and potting medium on the control of the fungus gnat, *Bradysia difformis* (Diptera : Sciaridae), in the greenhouse at alternating cold and warm temperatures. *Biological Control*, **43**, 23-30.

**Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W.** (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal*, **6**, 3901-3907.

**Kaplan, I., Halitschke, R., Kessler, A., Rehill, B.J., Sardanelli, S. and Denno, R.F.** (2008) Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecology Letters*, **11**, 841-851.

**Karimi, M., Inze, D. and Depicker, A.** (2002) GATEWAY((TM)) vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci.*, **7**, 193-195.

**Kato-Noguchi, H., Ino, T. and Ota, K.** (2008a) Secretion of momilactone A from rice roots to the rhizosphere. *Journal of Plant Physiology*, **165**, 691-696.

**Kato-Noguchi, H., Ota, K. and Ino, T.** (2008b) Release of momilactone A and B from rice plants into the rhizosphere and its bioactivities. *Allelopathy Journal*, **22**, 321-328.

**Kessler, A. and Baldwin, I.T.** (2001) Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, **291**, 2141-2144.

**Kong, C.H., Li, H.B., Hu, F., Xu, X.H. and Wang, P.** (2006) Allelochemicals released by rice roots and residues in soil. *Plant and Soil*, **288**, 47-56.

**Konig, W.A., Bulow, N. and Saritas, Y.** (1999) Identification of sesquiterpene hydrocarbons by gas phase analytical methods. *Flavour and Fragrance Journal*, **14**, 367-378.

**Martin, D.M., Faldt, J. and Bohlmann, J.** (2004) Functional characterization of nine Norway spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily. *Plant Physiology*, **135**, 1908-1927.

**Mau, C.J.D. and West, C.A.** (1994) Cloning of casbene synthase cDNA - evidence for conserved structural features among terpenoid cyclases in plants *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 8497-8501.

**McConn, M., Creelman, R.A., Bell, E., Mullet, J.E. and Browse, J.** (1997) Jasmonate is essential for insect defense Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 5473-5477.

**Mercke, P., Kappers, I.F., Verstappen, F.W.A., Vorst, O., Dicke, M. and Bouwmeester, H.J.** (2004) Combined transcript and metabolite analysis reveals genes involved in spider mite induced volatile formation in cucumber plants. *Plant Physiology*, **135**, 2012-2024.

**Ormeno, E., Baldy, V., Ballini, C. and Fernandez, C.** (2008) Production and diversity of volatile terpenes from plants on calcareous and siliceous soils: Effect of soil nutrients. *Journal of Chemical Ecology*, **34**, 1219-1229.

**Osbourn, A.E.** (2003) Saponins in cereals. *Phytochemistry*, **62**, 1-4.

**Osbourn, A.E., Qi, X.Q., Townsend, B. and Qin, B.** (2003) Dissecting plant secondary metabolism - constitutive chemical defences in cereals. *New Phytologist*, **159**, 101-108.

**Peters, R.J. and Croteau, R.B.** (2002) Abietadiene synthase catalysis: Conserved residues involved in protonation-initiated cyclization of geranylgeranyl diphosphate to (+)-copalyl diphosphate. *Biochemistry*, **41**, 1836-1842.

**Peters, R.J., Ravn, M.M., Coates, R.M. and Croteau, R.B.** (2001) Bifunctional abietadiene synthase: Free diffusive transfer of the (+)-copalyl diphosphate intermediate between two distinct active sites. *Journal of American Chemical Society*, **123**, 8974-8978.

**Powell, C.J., Jablonski, A. and Salvat, F.** (2005) NIST databases with electron elastic-scattering cross sections, inelastic mean free paths, and effective attenuation lengths. *Surface and Interface Analysis*, **37**, 1068-1071.

**Rasmann, S., Kollner, T.G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J. and Turlings, T.C.J.** (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature*, **434**, 732-737.

**Ro, D.K., Ehlting, J., Keeling, C.I., Lin, R., Mattheus, N. and Bohlmann, J.** (2006) Microarray expression profiling and functional characterization of AtTPS genes: Duplicated *Arabidopsis thaliana* sesquiterpene synthase genes At4g13280 and At4g13300 encode root-specific and wound-inducible (Z)-gamma-bisabolene synthases. *Archives of Biochemistry and Biophysics*, **448**, 104-116.

**Schnee, C., Kollner, T.G., Gershenzon, J. and Degenhardt, J.** (2002) The maize gene terpene synthase 1 encodes a sesquiterpene synthase catalyzing the formation of (*E*)-beta-farnesene, (*E*)-nerolidol, and (*E,E*)-farnesol after herbivore damage. *Plant Physiology*, **130**, 2049-2060.

**Senalik, D. and Simon, P.W.** (1987) Quantifying intraplant variation of volatile terpenoids in carrot. *Phytochemistry*, **26**, 1975-1979.

**Starks, C.M., Back, K.W., Chappell, J. and Noel, J.P.** (1997) Structural basis for cyclic terpene biosynthesis by tobacco 5-epi-aristolochene synthase. *Science*, **277**, 1815-1820.

**Sun, T.P. and Kamiya, Y.** (1994) The *Arabidopsis* GA1 locus encodes the cyclase ent-kaurene synthetase-a of gibberellin biosynthesis. *Plant Cell*, **6**, 1509-1518.

**Tholl, D.** (2006) Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Current Opinion in Plant Biology*, **9**, 297-304.

**Tholl, D., Boland, W., Hansel, A., Loreto, F., Rose, U.S.R. and Schnitzler, J.P.** (2006) Practical approaches to plant volatile analysis. *Plant Journal*, **45**, 540-560.

**Toyomasu, T., Kagahara, T., Okada, K., Koga, J., Hasegawa, M., Mitsuhashi, W., Sassa, T. and Yamane, H.** (2008) Diterpene phytoalexins are biosynthesized in and exuded from the roots of rice seedlings. *Bioscience Biotechnology and Biochemistry*, **72**, 562-567.

**Turlings, T.C.J., Tumlinson, J.H. and Lewis, W.J.** (1990) Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science*, **250**, 1251-1253.

**van Dam, N.M.** (2009) Belowground herbivory and plant defenses. *Annual Review of Ecology Evolution and Systematics*, **40**, 373-391.

**Van Der Putten, W.H.** (2003) Plant defense belowground and spatiotemporal processes in natural vegetation. *Ecology*, **84**, 2269-2280.

**Vancanneyt, G., Sanz, C., Farmaki, T., Paneque, M., Ortego, F., Castanera, P. and Sanchez-Serrano, J.J.** (2001) Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 8139-8144.

**Wilderman, P.R., Xu, M.M., Jin, Y.H., Coates, R.M. and Peters, R.J.** (2004) Identification of syn-pimara-7,15-diene synthase reveals functional clustering of terpene synthases involved in rice phytoalexin/allelochemical biosynthesis. *Plant Physiology*, **135**, 2098-2105.

**Wildung, M.R. and Croteau, R.** (1996) A cDNA clone for taxadiene synthase, the diterpene cyclase that catalyzes the committed step of taxol biosynthesis. *Journal of Biological Chemistry*, **271**, 9201-9204.

**Xu, M.M., Wilderman, P.R., Morrone, D., Xu, J.J., Roy, A., Margis-Pinheiro, M., Upadhyaya, N.M., Coates, R.M. and Peters, R.J.** (2007) Functional characterization of the rice kaurene synthase-like gene family. *Phytochemistry*, **68**, 312-326.

**Zaborski, E.R. and Cloyd, R.A.** (2004) Method for quantitative sampling of fungus gnat larvae in soilless growing media. *Journal of Economic Entomology*, **97**, 678-686.

# Chapter IV

# Final Discussion and Conclusions

# Chapter IV

# Final Discussion and Conclusions

## Introduction

Plants have developed two distinct but integrated structural elements: shoots to access the atmosphere for fixing carbon and roots to penetrate the soil for water and nutrient absorption (Erb*, et al.* 2009). Herbivores feeding on roots affect plant performance with consequences to both natural and agricultural ecosystems (Van Der Putten 2003). Root herbivory is often underestimated due to difficulties associated with quantifying root damage (Hunter 2001). Unless the root herbivore causes substantial crop mortality or directly attacks the harvested tissue, their presence and related damage is often overlooked. Currently, the most common mode of below ground pest control is through the application of chemical pesticides, but there is increasing interest in exploiting plant natural defenses to develop alternative strategies in pest control (Koellner*, et al.* 2008). However, for almost two decades now, review articles have emphasized the extreme paucity of research regarding the effects of root-feeding insects and the degree to which roots gain protection from herbivores by secondary metabolites. (Blossey and Hunt-Joshi 2003, Brown 1990, Hunter 2001, MoronRios *et al.* 1997, Rasmann and Agrawal 2008). The continued discussion and demand regarding such research without major advancements reflects methodological difficulties rather than lack of consideration and interest.

The primary goal of this work was to gain a better understanding of chemical defense mechanisms in plant roots and how they affect root herbivores. We have focused our study on the root-specific formation of terpene metabolites in the model plant *Arabidopsis thaliana*. A

combined biochemical and molecular analysis of wild type and mutant plants impaired in terpene production was used to elucidate correlations between the biosynthesis of terpenes in roots and their *in vivo* defensive activities. To investigate these correlations, a bioassay was developed, which allows determining feeding damage by the opportunistic root herbivore *Bradysia* on aeroponically and soil-grown plants at different developmental stages. Using this experimental approach, we have identified and characterized a terpene synthase (AtTPS08), which is responsible for the formation of the volatile diterpene compound rhizathalene in *Arabidopsis* roots. Rhizathalene contributes to a direct chemical defense of roots as shown in comparative *Bradysia* feeding experiments with wild type and mutant plants impaired in the formation of the diterpene compound. Interestingly, the tissue-specific expression of rhizathalene synthase in the root stele correlates with its role in mitigating insect feeding on the root vascular tissue. Our studies have created new opportunities for an in-depth analysis of root chemical defenses, particularly of aspects related to the spatial and temporal regulation of belowground defenses and their impact on aboveground plant tissues. In this chapter, we review the significance of our findings in the light of several of these topics and discuss the usefulness of the developed experimental system for future studies at a more holistic scale.

## Discovery of volatile diterpenes with constitutive formation in *Arabidopsis* roots

Focusing on the largest and most diverse class of defense metabolites in *Arabidopsis*, a detailed biochemical and functional assessment of belowground terpene defense metabolites was initiated with an emphasis on volatile compounds. We detected a group of related volatile

diterpene hydrocarbons called rhizathalene A to D, all of which are produced by the root-expressed terpene synthase AtTPS08. Preliminary results from mass spectral comparison and nuclear magnetic resonance (NMR) analysis suggest that the predominant enzyme product rhizathalene A is a tricyclic labdane-related hydrocarbon diterpene that, to our knowledge, has not been described from other plant species. Most characterized tricyclic diterpene compounds are either synthesized by class I TPSs directly from the substrate copalyl diphosphate (CPP) such as the labdane-type diterpenes found in rice, or they are catalyzed by bifunctional class I/II type enzymes from GGPP via a CPP intermediate formed in a protonation-initiated reaction (Bohlmann*, et al.* 1998, Tholl 2006). AtTPS08 rhizathalene synthase uses GGPP as a substrate. However, its protein sequence is more closely related to class I diterpene synthases such as casbene or taxadiene synthase (Aubourg*, et al.* 2002, Mau and West 1994, Wildung and Croteau 1996), which produce monocyclic diterpenes and lack the DXDD motif required for carbon-carbon double bond protonation of GGPP. While we were unable to test CPP as a substrate because of its commercial unavailability, it is possible that AtTPS08 represents a novel type of diterpene synthase that initiates labdane-type diterpene formation by protonation of GGPP independent of the distinct DXDD motif.

Rhizathalene is constitutively synthesized in *Arabidopsis* roots similar to the monoterpene 1,8-cineole and the sesquiterpene (*Z*)-γ-bisabolene, which were also detected in the root terpene volatile blend. Similar to rice diterpenes (Kato-Noguchi*, et al.* 2008b), all of these compounds are produced in low amounts to most likely avoid autotoxicity in the absence of secretory cells or ducts, especially when compounds are released from internal cell layers rather than the epidermis. No induced formation of rhizathalene upon *Bradysia* feeding was observed. We cannot exclude that the diterpene compound is further converted into an alcohol or acid end

product (Keeling and Bohlmann 2006, Munne-Bosch and Alegre 2001) although there is currently no direct experimental evidence for such as conversion. Our finding that transgenic plants that constitutively express rhizathalene synthase produce the volatile only in the leaves and flowers may support the fact of a possible downstream conversion of rhizathalene and/or indicate a generally tight regulation of rhizathalene biosynthesis by posttranscriptional/posttranslational regulation. The fact that no induced formation of rhizathalene was observed in our system could also be due to the absence of specific elicitors from insects that specialize in root xylem or phloem feeding. Cicadas nymphs, for example, are root xylem feeders that tap into the plants vascular system and have been reported to consume an average of 1.3 L of xylem $nymph^{-1}$ $year^{-1}$ (White and Strehl 1978). Lettuce root aphids (*Pemphigus bursarius*) are phloem feeders and can occur at very high densities in the lettuce rhizosphere (as many as 64,500 individuals $m^{-1}$) and severally reduce vegetative growth (Xu*, et al.* 2007). Aphids such as these secrete enzymes in their saliva that could be defense elicitors in the plant (Brown 1990).

Interestingly, the constitutive formation of terpenes in *Arabidopsis* roots stands in contrast to the exclusively stress-induced volatile terpene emissions in leaves. With regard to the optimal defense theory, this observation suggests that roots have greater value with respect to plant performance. Throughout the annual life cycle of an *Arabidopsis* plant, root tissue may experience higher continuous pressure by belowground microbial pathogens and root herbivores than leaves. Due to the lack of experimental data, there is no clear consensus as to whether damage and loss of root or shoot tissue has greater detrimental effects on the plant (Hunter 2001) with the exception of a single study showing that the removal of root tissue had more severe effects on plant performance (Reichman and Smith 1991). Using the developed aeroponic culture

system, experiments regarding whether the loss of biomass from root or shoot tissue has greater deleterious effects on plant performance could be designed. Nevertheless, conclusions on the value of shoot and root tissues cannot be accurately made on the basis of only one class of secondary metabolites; all defense compounds must be included, and the relative amount of resources devoted to each must also be taken into consideration. Furthermore, the value of shoots and roots under natural conditions may vary depending on the growing environment and its limiting factors (Kaplan*, et al.* 2008b).

## Is the subcellular regulation of terpene synthesis in roots similar to that in leaves?

Previous studies on aerial parts of plants have shown that the biosynthesis of monoterpenes and diterpenes is separated from sesquiterpene biosynthesis by subcellular compartmentalization with monoterpene and diterpene formation generally occurring in plastids and sesquiterpene formation being restricted to the cytosol (Cheng *et al.* 2007, Dudareva*, et al.* 2004, Tholl 2006). Subcellular localization of AtTPS08 confirmed that formation of the diterpene rhizathalene in roots also resides in plastids. While this finding by itself might not be surprising, it can now be used to investigate the cross-talk between the cytosolic and plastidial terpene biosynthesis pathways in roots in comparison to those in leaves. To date, evidence of cross-talk between the *cytosolic mevalonate* and the *plastidial* MEP pathways comes from only two reports in carrot (*Daucus carota*) and coluria (*Coluria geoides*) roots (Hampel*, et al.* 2005, Skorupinska-Tudek *et al.* 2003). In the latter case, an important flux from the mevalonate pathway into plastids for the assembly of dolichols was reported, which is different from a flux

of terpene precursors from chloroplasts to the cytosol as mostly observed in leaves (Bick and Lange 2003). In *Arabidopsis*, gene transcript and protein data indicate that MEP pathway enzymes in root leucoplasts, in contrast to enzymes of the same pathway in leaves, are substantially down-regulated (Estevez et al 2000; Guevara-Garcia et al 2005). Moreover, in *Arabidopsis* leaves, the cross-talk between mevalonate and MEP pathways was shown to be light-regulated with a presumed enhanced flow of mevalonate pathway metabolites into plastids in a light-insensitive mutant (Rodriguez-Concepcion et al 2004). Recent findings in the Tholl lab have shown that rhizathalene formation in *Arabidopsis* hairy roots is not significantly reduced by treatment with the MEP pathway inhibitor fosmidomycin. Further studies using stable-isotope precursors should provide more insight in the contribution and regulation of the MEP and mevalonate pathways in *Arabidopsis* roots.

## Tissue- and cell-type specificity of root chemical defenses

One of the most intriguing aspects of the formation of terpenes in *Arabidopsis* roots is their apparent tissue or cell-type specificity. While cell-type specificity of terpene biosynthesis has been described primarily in single secretory cells, laticifers and ducts (Gang 2005, Kutchan 2005, Schilmiller *et al.* 2008), its organization at the organ- or multicellular levels is less well understood. This work showed that rhizathalene formation is restricted to the vascular tissue and root tips. Previous studies revealed that the monoterpene 1,8-cineole is synthesized in the epidermis and cortex of *Arabidopsis* roots (Chen*, et al.* 2004) while the production of (*Z*)-γ-bisabolene occurs primarily in the sub-epidermal layers and the cortex (Ro*, et al.* 2006). Preliminary data in the Tholl lab suggest that (*Z*)-γ-bisabolene is further converted into non-

volatile products in these cell layers. Other examples for root cell-type specificity of terpene formation are the biosynthesis of triterpenes in the epidermis of *Arabidopsis* and oat roots (Jenner *et al.* 2005). Such cell-type specificity requires a highly coordinated expression of genes participating in the same pathway as recently shown for the thalianol triterpene biosynthesis gene cluster (Field and Osbourn 2008). To what extent the formation of rhizathalene is coordinated with the expression of particular GGPP synthase isozymes or possible downstream CytP450 enzymes expressed will require further investigation.

It can be assumed that a cell-specific organization of defense metabolism in roots is critical for warding off soil-borne, root-attacking organisms such as nematodes, microbial pathogens and insect larvae that have different invasion and feeding mechanisms (Maron 1998, Rasmann and Agrawal 2008, Wardle 2006). For rhizathalene, we found that this compound plays a role in specifically protecting the root vascular tissue from herbivory. Although the compound is volatile, its highest concentration occurs in the cells of its biosynthetic origin and most likely in one or two of the surrounding layers of cells. Xylem and phloem are essential for water and nutrient transport, and particularly the carbohydrate transporting phloem is at high risk of attack from consumers (e.g. root aphids). Thus, it is likely that constitutive defense resources are allocated for protection of the root vascular tissue. By comparing the cell-type specific expression patterns of rhizathalene, 1,8-cineole, and (*Z*)-γ-bisabolene synthases based on promoter-GUS reporter gene studies, it can be noted that all three enzymes are co-localized in the stele at an early developmental stage of the root prior to their cell-type specific divergence of expression in developmentally older root zones. Such “mixed” expression might promote a higher defense capacity by the formation of blend of terpene compounds.

The question of why terpenes are produced in specific cells of the root and not in others may also relate to a possible interference of terpene metabolites with root development and cell speciation. For example, triterpene alcohols produced in oat mutants were shown to suppress root growth and hair formation (Mylona *et al.* 2008). A group of terpene lactones, strigolactones, that function as communication chemicals with parasitic weeds and symbiotic arbuscular mycorrhizal fungi, have also been shown to function as phytohormones involved in branching (Gomez-Roldan *et al.* 2008, Umehara *et al.* 2008). Interestingly, plants transformed with *AtTPS*08 under the control of the constitutive (*CaMV*) *35S* promoter did not produce the rhizathalene in roots, which could be related to possibly detrimental effects caused by disruption of cell-type specific expression. Another, though speculative, function of rhizathalene could be that the compound serves as a vascular signal for aerial parts of the plant. A signaling effect was shown for the diterpene, (11*E*,13*E*)-labda-11,13-diene-8α,15-diol, which activates a wound-induced protein kinase in tobacco leaves (Seo *et al.* 2003). However, this response was induced and not constitutive in nature. Questions such as "What would happen if we altered the differential expression patterns of terpene synthases in roots?" require further investigation.

## Integration and variation of root chemical defenses

Plants including *Arabidopsis* generally produce several different terpene specialized metabolites. Monoterpenes, sesquiterpenes, and diterpenes have been found in *Arabidopsis* roots and more compounds may be identified by refined extraction protocols and highly sensitive GC- or LC-MS analytical procedures. The monoterpene 1,8-cineole and the sesquiterpene (*Z*)-γ-bisabolene, which are both produced in *Arabidopsis* roots most likely exhibit defensive

activities. Based on aboveground interactions, 1,8-cineole has toxic and deterrent effects on certain insect herbivores (Tripathi *et al.* 2001).

A preliminary comparison of terpene profiles from roots of different *Arabidopsis* ecotypes has shown, that rhizathalene is specifically produced in the Columbia (Col) ecotype. Rhizathalene is absent in ecotypes such as Ler and CVI indicating that these ecotypes contain a non-functional copy of the gene or the *AtTPS*08 gene is not transcribed.

On the other hand, the Tholl lab has evidence for the formation of at least five other diterpene compounds in the roots of ecotypes Ler, CVI, Kas-1, and Ws. From an evolutionary perspective this is quite interesting. Ecotype-specific differences in root terpene formation may reflect adaptations in the defense against local insect and pathogen communities. As a possible scenario, Columbia plants maintain the stele-specific formation of rhizathalene because they may be under heavy belowground selection pressure by xylem- and phloem-specific feeders. The fitness levels of other *Arabidopsis* ecotypes in response to belowground biotic stress should be further evaluated. Genetic variation and the role of the environmental conditions in the expression of defensive compounds is an important aspect that still must be addressed with regard to belowground defense (Hunter 2001, Rasmann and Agrawal 2008).

To understand root chemical defenses at a global scale, a more integrative investigation of several defense metabolites will be required. This will be particularly important for drawing accurate conclusions with regard to the defensive potential and fitness value of root tissue in comparison to aboveground plant parts. Terpenes constitute the largest class of secondary metabolites in *Arabidopsis,* but there are seven other classes of compounds that are likely to be involved in belowground defense against root herbivores (D'Auria and Gershenzon 2005): glucosinolates, indole sulfur compounds, phenylpropanoids and flavonoids, benzenoids, and

fatty acid derivatives. Glucosinolates and flavonoids were shown to be produced in *Arabidopsis* roots (Brown *et al.* 2003, Saslowsky and Winkel-Shirley 2001). Glucosinolates in particular have been reported to decrease *Delia radicum* feeding on the roots of *Brassica spp.* (*B. napus, B. oleracea, B. napobrassica*) (Hopkins*, et al.* 2009, Potter *et al.* 2000) and play a major role in the defense of *Arabidopsis* roots against *Bradysia* larvae as well (Vaughan, unpublished data). The biological functions of these compounds with regard to herbivore defense can now be investigated *in vivo* by using the developed *Bradysia* feeding assays.

## Linking above- and belowground plant defense

Despite their spatial separation, roots and shoots are connected and influence each other as well as the below- and aboveground organismal communities that depend on them. Responses to above- or belowground herbivory are not restricted to the site of attack but can spread throughout the entire plant. Hence, root herbivores can influence the production of specialized defense metabolites in aboveground tissues and thereby affect the performance of aboveground herbivores (Bezemer and van Dam 2005, Bezemer *et al.* 2004, Birch*, et al.* 1992). In general, root-chewing herbivores cause an increase in levels of plant defense compounds in aboveground tissues that are similar to those observed upon induction by leaf-chewing herbivores (Bezemer and van Dam 2005). For example, damage caused by wireworm (*Agriotes lineatus*) feeding on roots of cotton (*Gossypium herbaceum*) resulted in a more than 4 fold increase in terpene levels in leaves (Bezemer*, et al.* 2004).

The effects of aboveground herbivory on belowground defense are not as well studied. Foliar herbivory on the crucifer *Brassica nigra* leads to enhanced levels of indole glucosinolates

in roots with negative effects on the performance of a root herbivore and its parasitoid (Soler *et al.* 2007). However, other results suggest that the influence of shoot herbivory on root defense is less severe (Bezemer and van Dam 2005). For example, cabbage moth (*Mamestra brassicae)* leaf herbivory caused reduced alkaloid concentrations in ragwort roots (*Senecio jacobaea*) (Hol *et al.* 2004), and *Manduca sexta* feeding on tobacco leaves had no impact on root chemical defenses (Kaplan*, et al.* 2008b). Even if root defenses are not induced by shoot herbivory, increased nutrient allocation to roots can occur to enable re-growth after herbivore attack (Bazot *et al.* 2005, Holland *et al.* 1996, Schwachtje and Baldwin 2008, Schwachtje *et al.* 2006). Roots therefore are involved with aboveground defense to promote tolerance (Erb*, et al.* 2009). Cotton plants were also investigated under simultaneous above- and belowground herbivory. In this case, resources were primarily allocated aboveground at the expense of belowground defenses (Bezemer*, et al.* 2004). All of the described findings suggest a regulatory mechanism that involves above- and belowground signaling. Erb et al., (2009) stated that "shoot-root-shoot" signaling exists within the plant defensive system, and experimental data indicated that these signals are independent of the octadecanoid pathway (Erb*, et al.* 2009, Schwachtje*, et al.* 2006). The here developed *Arabidopsis-Bradysia* system using wild type plants and mutants impaired in defense signaling can be employed to further elucidate bottom-up and top-down systemic effects mediated by different defense signals. These studies can be expanded beyond insect-herbivory to include additional biotic stresses imposed by root and leaf microbial pathogens such as the necrotrophs *Pythium* spp. and *Pseudomonas syringae*.

In conclusion, plants are continuously being challenged by above- and belowground herbivores. Since specially separated inter-specific competition can influence host defense

strategies, a better understanding of these networks is necessary in order to fully comprehend the interactions between plants and herbivores in natural and agricultural settings.

## Conclusions

There is still a large gap in our understanding of belowground resistance to herbivores and the holistic effects caused by root damage. Roots are now being recognized as playing an essential role not only in belowground resistance but also in tolerance and defense priming of aerial tissues (Erb*, et al.* 2009, Kaplan*, et al.* 2008a, Kaplan*, et al.* 2008b). For a long time, research investigating root herbivory has lagged behind studies on shoot herbivory because of methodological difficulties. The bioassay we have developed has helped to overcome these difficulties and can easily be performed to assess the effect of herbivory on the formation and regulation of chemical defense in *Arabidopsis* roots. The method has facilitated the discovery of root-specific volatile diterpenes involved in direct defense against belowground root herbivores, and it has given insight into diterpene biochemistry in roots. Moreover, *Arabidopsis* grown in an aeroponic culture system allow access to both the shoots and roots, to which herbivores can be applied separately and simultaneously. Experiments can be designed to determine herbivore-stimulated responses on primary metabolism (e.g., sugars and amino acids) and signaling molecules (van Dam 2009). A comprehensive analysis of metabolic changes together with alterations in transcript profiles (Bino *et al.* 2004, van Dam 2009) in response to shoot-, root-, and simultaneous shoot and root-herbivory and/or pathogen infection will greatly contribute to a better understanding of the complete process behind root defense and plant defense as a whole.

Ultimately, we hope that this work and future research will contribute to protecting the world's food production through enhanced crop resistance and tolerance.

## References


**Aubourg, S., Lecharny, A. and Bohlmann, J.** (2002) Genomic analysis of the terpenoid synthase (AtTPS) gene family of *Arabidopsis thaliana*. *Molecular Genetics and Genomics*, **267**, 730-745.

**Bazot, S., Mikola, J., Nguyen, C. and Robin, C.** (2005) Defoliation-induced changes in carbon allocation and root soluble carbon concentration in field-grown *Lolium perenne* plants: do they affect carbon availability, microbes and animal trophic groups in soil? *Functional Ecology*, **19**, 886-896.

**Bezemer, T.M. and van Dam, N.M.** (2005) Linking aboveground and belowground interactions via induced plant defenses. *Trends in Ecology and Evolution.*, **20**, 617-624.

**Bezemer, T.M., Wagenaar, R., Van Dam, N.M., Van Der Putten, W.H. and Wackers, F.L.** (2004) Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *Journal of Chemical Ecology*, **30**, 53-67.

**Bick, J.A. and Lange, B.M.** (2003) Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. *Archives of Biochemistry and Biophysics*, **415**, 146-154.

**Bino, R.J., Hall, R.D., Fiehn, O., Kopka, J., Saito, K., Draper, J., Nikolau, B.J., Mendes, P., Roessner-Tunali, U., Beale, M.H., Trethewey, R.N., Lange, B.M., Wurtele, E.S. and Sumner, L.W.** (2004) Potential of metabolomics as a functional genomics tool. *Trends in Plant Science*, **9**, 418-425.

**Birch, A.N.E., Griffiths, D.W., Hopkins, R.J., Smith, W.H.M. and McKinlay, R.G.** (1992) Glucosinolate responses of swede, kale, forage and oilseed rape to root damage by turnip root fly (*Delia-floralis*) larvae. *Journal of the Science of Food and Agriculture*, **60**, 1-9.

**Blossey, B. and Hunt-Joshi, T.R.** (2003) Belowground herbivory by insects: Influence on plants and aboveground herbivores. *Annual Review of Entomology*, **48**, 521-547.

**Bohlmann, J., Meyer-Gauen, G. and Croteau, R.** (1998) Plant terpenoid synthases: Molecular biology and phylogenetic analysis. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 4126-4133.

**Brown, P.D., Tokuhisa, J.G., Reichelt, M. and Gershenzon, J.** (2003) Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana. Phytochemistry*, **62**, 471-481.

**Brown, V.K., Gange, A.C.** (1990) Insect herbivory below ground. *Advances in Ecological Research*, **20**, 1-59.

**Chen, F., Ro, D.K., Petri, J., Gershenzon, J., Bohlmann, J., Pichersky, E. and Tholl, D.** (2004) Characterization of a root-specific *Arabidopsis* terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole. *Plant Physiology*, **135**, 1956-1966.

**Cheng, A.X., Lou, Y.G., Mao, Y.B., Lu, S., Wang, L.J. and Chen, X.Y.** (2007) Plant terpenoids: Biosynthesis and ecological functions. *Journal of Integrated Plant Biology*, **49**, 179-186.

**D'Auria, J.C. and Gershenzon, J.** (2005) The secondary metabolism of *Arabidopsis thaliana*: growing like a weed. *Current Opinion in Plant Biology*, **8**, 308-316.

**Dudareva, N., Pichersky, E. and Gershenzon, J.** (2004) Biochemistry of plant volatiles. *Plant Physiology*, **135**, 1893-1902.

**Erb, M., Lenk, C., Degenhardt, J. and Turlings, T.C.J.** (2009) The underestimated role of roots in defense against leaf attackers. *Trends in Plant Science*, **14**, 653-659.

**Field, B. and Osbourn, A.E.** (2008) Metabolic diversification - Independent assembly of operon-like gene clusters in different plants. *Science*, **320**, 543-547.

**Gang, D.R.** (2005) Specialized metabolism in aromatic plants. *Abstracts of Papers of the American Chemical Society*, **229**, 083-AGRO.

**Gomez-Roldan, V., Fermas, S., Brewer, P.B., Puech-Pages, V., Dun, E.A., Pillot, J.P., Letisse, F., Matusova, R., Danoun, S., Portais, J.C., Bouwmeester, H., Becard, G., Beveridge, C.A., Rameau, C. and Rochange, S.F.** (2008) Strigolactone inhibition of shoot branching. *Nature*, **455**, 189-U122.

**Hampel, D., Mosandl, A. and Wust, M.** (2005) Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. *Phytochemistry*, **66**, 305-311.

**Hol, W.H.G., Macel, M., van Veen, J.A. and van der Meijden, E.** (2004) Root damage and aboveground herbivory change concentration and composition of pyrrolizidine alkaloids of *Senecio jacobaea*. *Basic and Applied Ecology*, **5**, 253-260.

**Holland, J.N., Cheng, W.X. and Crossley, D.A.** (1996) Herbivore-induced changes in plant carbon allocation: Assessment of below-ground C fluxes using carbon-14. *Oecologia*, **107**, 87-94.

**Hopkins, R.J., van Dam, N.M. and van Loon, J.J.A.** (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Annual Review of Entomology*, **54**, 57-83.

**Hunter, M.D.** (2001) Out of sight, out of mind: the impacts of root-feeding insects in natural and managed systems. *Agricultural and Forest Entomology*, **3**, 3-9.

**Jenner, H., Townsend, B. and Osbourn, A.** (2005) Unravelling triterpene glycoside synthesis in plants: phytochemistry and functional genomics join forces. *Planta*, **220**, 503-506.

**Kaplan, I., Halitschke, R., Kessler, A., Rehill, B.J., Sardanelli, S. and Denno, R.F.** (2008a) Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecology Letters*, **11**, 841-851.

**Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S. and Denno, R.F.** (2008b) Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology*, **89**, 392-406.

**Kato-Noguchi, H., Ota, K. and Ino, T.** (2008) Release of momilactone A and B from rice plants into the rhizosphere and its bioactivities. *Allelopathy Journal*, **22**, 321-328.

**Keeling, C.I. and Bohlmann, J.** (2006) Diterpene resin acids in conifers. *Phytochemistry*, **67**, 2415-2423.

**Koellner, T.G., Held, M., Lenk, C., Hiltpold, I., Turlings, T.C.J., Gershenzon, J. and Degenhardt, J.** (2008) A maize (*E*)-beta-caryophyllene synthase implicated in indirect

defense responses against herbivores is not expressed in most American maize varieties. *Plant Cell*, **20**, 482-494.

**Kutchan, T.M.** (2005) A role for intra- and intercellular translocation in natural product biosynthesis. *Current Opinion in Plant Biology*, **8**, 292-300.

**Maron, J.L.** (1998) Insect herbivory above- and belowground: Individual and joint effects on plant fitness. *Ecology*, **79**, 1281-1293.

**Mau, C.J.D. and West, C.A.** (1994) Cloning of casbene synthase cDNA - evidence for conserved structural features among terpenoid cyclases in plants. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 8497-8501.

**MoronRios, A., Jaramillo, V.J. and Dirzo, R.** (1997) Species composition of root-feeding macroarthropods in a subalpine grassland associated with pine forest in Mexico. *Canadian Entomologist*, **129**, 71-80.

**Munne-Bosch, S. and Alegre, L.** (2001) Subcellular compartmentation of the diterpene carnosic acid and its derivatives in the leaves of rosemary. *Plant Physiology*, **125**, 1094-1102.

**Mylona, P., Owatworakit, A., Papadopoulou, K., Jenner, H., Qin, B., Findlay, K., Hill, L., Qi, X., Bakht, S., Melton, R. and Osbourn, A.** (2008) Sad3 and Sad4 are required for saponin biosynthesis and root development in oat. *Plant Cell*, **20**, 201-212.

**Potter, M.J., Vanstone, V.A., Davies, K.A. and Rathjen, A.J.** (2000) Breeding to increase the concentration of 2-phenylethyl glucosinolate in the roots of *Brassica napus*. *Journal of Chemical Ecology*, **26**, 1811-1820.

**Rasmann, S. and Agrawal, A.A.** (2008) In defense of roots: A research agenda for studying plant resistance to belowground herbivory. *Plant Physiology*, **146**, 875-880.

**Reichman, O.J. and Smith, S.C.** (1991) Responses to simulated leaf and root herbivory by a biennial, *Tragopogon-dubius*. *Ecology*, **72**, 116-124.

**Ro, D.K., Ehlting, J., Keeling, C.I., Lin, R., Mattheus, N. and Bohlmann, J.** (2006) Microarray expression profiling and functional characterization of AtTPS genes: Duplicated *Arabidopsis thaliana* sesquiterpene synthase genes At4g13280 and At4g13300 encode root-specific and wound-inducible (*Z*)-gamma-bisabolene synthases. *Archives of Biochemistry and Biophysics*, **448**, 104-116.

**Saslowsky, D. and Winkel-Shirley, B.** (2001) Localization of flavonoid enzymes in *Arabidopsis* roots. *Plant Journal*, **27**, 37-48.

**Schilmiller, A.L., Last, R.L. and Pichersky, E.** (2008) Harnessing plant trichome biochemistry for the production of useful compounds. *Plant Journal*, **54**, 702-711.

**Schwachtje, J. and Baldwin, I.T.** (2008) Why does herbivore attack reconfigure primary metabolism? *Plant Physiology*, **146**, 845-851.

**Schwachtje, J., Minchin, P.E.H., Jahnke, S., van Dongen, J.T., Schittko, U. and Baldwin, I.T.** (2006) SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 12935-12940.

**Seo, S., Seto, H., Koshino, H., Yoshida, S. and Ohashi, Y.** (2003) A diterpene as an endogenous signal for the activation of defense responses to infection with Tobacco mosaic virus and wounding in tobacco. *Plant Cell*, **15**, 863-873.

**Skorupinska-Tudek, K., Bienkowski, T., Olszowska, O., Furmanowa, M., Chojnacki, T., Danikiewicz, W. and Swiezewska, E.** (2003) Divergent pattern of polyisoprenoid alcohols in the tissues of *Coluria geoides*: A new electrospray ionization MS approach. *Lipids*, **38**, 981-990.

**Soler, R., Bezemer, T.M., Cortesero, A.M., Van der Putten, W.H., Vet, L.E.M. and Harvey, J.A.** (2007) Impact of foliar herbivory on the development of a root-feeding insect and its parasitoid. *Oecologia*, **152**, 257-264.

**Tholl, D.** (2006) Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Current Opinion in Plant Biology*, **9**, 297-304.

**Tripathi, A.K., Prajapati, V., Aggarwal, K.K. and Kumar, S.** (2001) Toxicity, feeding deterrence, and effect of activity of 1,8-cineole from *Artemisia annua* on progeny production of *Tribolium castanaeum* (Coleoptera : Tenebrionidae). *Journal of Economic Entomology*, **94**, 979-983.

**Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T., Takeda-Kamiya, N., Magome, H., Kamiya, Y., Shirasu, K., Yoneyama, K., Kyozuka, J. and Yamaguchi, S.** (2008) Inhibition of shoot branching by new terpenoid plant hormones. *Nature*, **455**, 195-U129.

**van Dam, N.M.** (2009) Belowground herbivory and plant defenses. *Annual Review of Ecology Evolution and Systematics*, **40**, 373-391.

**Van Der Putten, W.H.** (2003) Plant defense belowground and spatiotemporal processes in natural vegetation. *Ecology*, **84**, 2269-2280.

**Wardle, D.A.** (2006) The influence of biotic interactions on soil biodiversity. *Ecology Letters*, **9**, 870-886.

**White, J. and Strehl, C.E.** (1978) Xylem feeding by periodical cicada nymphs on tree roots. *Ecological Entomology*, **3**, 323-327.

**Wildung, M.R. and Croteau, R.** (1996) A cDNA clone for taxadiene synthase, the diterpene cyclase that catalyzes the committed step of taxol biosynthesis. *Journal of Biological Chemistry*, **271**, 9201-9204.

**Xu, M.M., Wilderman, P.R., Morrone, D., Xu, J.J., Roy, A., Margis-Pinheiro, M., Upadhyaya, N.M., Coates, R.M. and Peters, R.J.** (2007) Functional characterization of the rice kaurene synthase-like gene family. *Phytochemistry*, **68**, 312-326.