Cell-type Specificity and Herbivore-induced Responses of Primary and Terpene Secondary Metabolism in Arabidopsis Roots

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Abstract

Plants employ diverse defense mechanisms to combat attack by harmful organisms. For instance, plants produce constitutive physical barriers or use chemical compounds such as specialized secondary metabolites to resist herbivore or pathogen invasion. Considering the cost-efficiency and energy balance between defense, growth and reproduction, defense reactions in plants have to be regulated temporally and spatially. As more cost-efficient strategies, plants may induce their defense response only in the presence of the attacker or restrict constitutive defenses to specific tissues or cells.

In this study, we investigated aspects of the spatial regulation and induced changes of primary and secondary metabolism in roots of *Arabidopsis thaliana.* Roots represent important organs for anchoring plants in the soil and taking up water and nutrients. Hence, it is assumed that roots are as well protected as aerial tissues by different defense mechanisms. The first part of this work is focused on the cell-type-specific biosynthesis of volatile terpenes in *Arabidopsis* roots. Terpenes are the most abundant specialized metabolites in plants and play an important role in plant defense against pathogens or herbivores. Terpene biosynthetic enzyme activities are often coordinated in specific tissues and cellular compartments. Fine-scale transcriptome maps of *Arabidopsis* roots

have shown that terpene biosynthesis is restricted to particular cell types. However, the reasons and significance of this cell-type specificity are not well understood. We hypothesized that the formation of terpene metabolites is not restricted to specific cells but can be supported by different cell types. We, therefore, probed the plasticity of the cell-specific formation of terpenes by swapping the expression of the terpene synthase (*TPS*) genes, *TPS08*, *TPS13* and *TPS25*, between different root cell types in the respective mutant background. To investigate the ectopic expression of *TPS*s at different levels, quantitative real-time PCR (qRT-PCR), confocal microscopy, and gas chromatography-mass spectrometry (GC-MS) were performed. We found that terpene synthase TPS08, which produces the diterpene rhizathalene and is normally expressed in the root vascular tissue, is functionally active when expressed in the epidermis or cortex, although at substantially lower levels compared to the wild type. We did not find an obvious correlation between the volatile emission level and gene transcript level of TPS08, which may be attributed to a reduced activity of the expressed TPS08-yellow fluorescent protein (YFP) fusion protein. When expression of TPS13 (producing the sesquiterpene (*Z*)-γ-bisabolene) was directed from the cortex to the epidermis or stele, *TPS13* gene expression and (*Z*)-γ-bisabolene formation was supported by these cell types although to varying levels in comparison to wild type. TPS13-YFP fluorescent signal driven by the epidermal *WER* and *GL3* promoters was primarily detected at the root tip. Terpene production was also observed for the (*E*)-β-farnesene sesquiterpene synthase TPS25 when its expression was switched from the endodermis and non-hair producing

epidermal cells to hair producing epidermal cells although only a weak fluorescent signal was detected from the expressed TPS25-mGFP protein. Together, the results provide preliminary evidence for a relaxed cell specificity of terpene biosynthesis in *Arabidopsis* roots and suggest that tissue-specific terpene metabolite patterns could change depending on different selective pressures in rhizosphere.

In the second part of this study, we performed global gene transcript profiling and primary metabolite analysis of *Arabidopsis* roots upon feeding by the generalist root herbivore, *Bradysia* (fungus gnat). In a microarray analysis, we identified 451 of 22,810 genes that were up-regulated more than 2-fold. Gene ontology (GO) analysis showed that 26% of those genes with predicted or known functions play a role in primary or secondary metabolism, while 24% are involved in cell signaling or in responses to stimulating factors, such as jasmonic acid (JA), ethylene, wounding, and oxidative stress. At the metabolite level, we observed only marginal changes of amino acid, sugar and carboxylic acid relative levels over a time course of 4 days of *Bradysia* feeding. There was a trend for increased levels of amino acids and the relative levels of sucrose were increased significantly (α=0.05) at the fourth day of feeding.

In conclusion, the study provided evidence for the induction of genes related to primary and secondary metabolism and stress responses in *Arabidopsis* roots, but showed only marginal changes at the primary metabolite level. In addition, the work indicated that the formation of terpene-specialized metabolites in *Arabidopsis* roots is not restricted

to specific cells, but can be supported by different cell types.

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General Introduction and Overview of Research Background

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1.1 Relationship between plants and arthropods and plant defense mechanisms

Plants and arthropods compose half of the known species of multicellular organisms on earth. During four-hundred million years of evolution, vascular plants and arthropods have adapted to each other at various levels in mutualistic and antagonistic interactions. For instance, angiosperms have developed numerous shapes and colors of flowers to attract different species of pollen-feeding insects, while many insects adapted morphologically to the special shapes of flowers and to recognize specific flower colors and scents (Weiss, 1990; McCall and Primack, 1992; Briscoe and Chittka, 2001; Galen and Cuba, 2001). To successfully colonize plants while escaping from their own enemies, arthropod herbivores have developed strategies such as to produce a large number of offspring and to complete a fast life cycle (Jolivet, 1998; Schoonhoven *et al.*, 2005). Besides reducing plant biomass, phytophagous arthropods provide additional challenges to plants by introducing chemicals and inducing wounding responses that alter plant defense signaling or plant development, which can make plants more susceptible for a secondary attack by microorganisms or other herbivores (Morris *et al.*, 2007). Plants also developed thick cutin or wax on the epidermis to inhibit herbivore invasion, while herbivores manage to detour the physical barriers or grow a sharp mandible (Jolivet, 1998). From the metabolic aspect, which has received a significant attention recently as a result of the technological advancements, plants may produce metabolites to attract enemies and pathogens of herbivores as indirect defense strategies, or expel herbivores directly by producing toxic metabolites. In return, herbivores try to avoid exposing themselves to enemies and develop detoxification metabolism pathway.

To cope with herbivory, plants have evolved various defensive mechanisms. Plant defenses are classified as pre-existent *versus* inducible, or direct *versus* indirect. Pre-existent, direct defenses can be physical barriers such as thick cutin or wax layers on the epidermis to reduce herbivore invasion (Heredia, 2003; Santamaria, 2013). Plants may also affect oviposition by herbivores *via* morphological changes (Horner and Abrahamson, 1992; Solarz and Newman, 1996; Agrawal and Sherriffs, 2001). In addition to physical barriers, plants deter herbivores by producing toxic metabolites or compounds that attract natural enemies of herbivores as part of an indirect defense (Van Poecke and Dicke, 2004; Rasmann et al., 2011). The metabolites used in these defenses are called secondary or specialized metabolites and include terpenes, glucosinolates, flavonoids, and alkaloids (Mithöfer and Boland, 2012; Bennett, 1994). Since such defenses are costly because they consume resources and energy from growth and reproduction, many metabolic changes are induced by herbivore feeding activity as a more cost-effective way to increase fitness (Frost, 2008).

Induced defenses are regulated by different signaling pathways. Early responses to feeding damage and wounding include the initiation of protein kinase cascades (Nakagami et al., 2005; Liu et al., 2007), production of superoxide (O_2^-), H_2O_2, NO, or other reactive oxygen species (ROS) (Apel and Hirt, 2004; Lamotte et al., 2005). ROS can function as defense compounds that directly repel herbivores or as signal components to induce defense signal cascades and initiate the production of other defense compounds. For instance, H_2O_2 is involved in the lignification process by inducing cell wall solidification. NO regulates the status of Ca^{2+} channels and activates Ca^{2+}-depended

signaling cascades (Willmott et al., 1996; Durner et al., 1998), or connects with MAPK cascades and promotes the programmed cell death (Clarke et al., 2000).

After the early response, which is normally limited to the attacked tissues or nearby tissues, plants also prepare themselves to resist further herbivory by systemic responses. Typical endogenous signals that are involved in these responses are the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (O'Donnell et al., 1996; Birkenmeier and Ryan, 1998; Stratmann, 2003). Other molecules, such as RNA and small peptides can also play a role in long-distance signal transduction between above- and below-ground tissues *via* vascular tissues. For examples, Banerjee et al. (2006) have characterized the mobile RNA dynamics as a long-distance defense signal in tomato. Each of these signaling pathways contains different components, and, they are also involved in negative or positive cross talks (Kunkel and Brooks, 2002).

One of the best studied examples for induced indirect defense is the emission of volatile organic compounds (e.g. terpenes) that attract herbivore parasites or predators for a top-down control of herbivore populations (Bruinsma & Dicke, 2008). Other indirect defense strategies include the constitutive attraction of natural enemies by offering extra nutrition, such as extrafloral nectar (Bentley, 1977), or shelters, such as leaf domatia and hollow thorns (Agrawal et al., 2000). In addition to the production of specialized metabolites, plants can slow down herbivory by the depletion of nutrients (Samach et al., 1991; Joshi et al., 2006) (Figure 1.1).

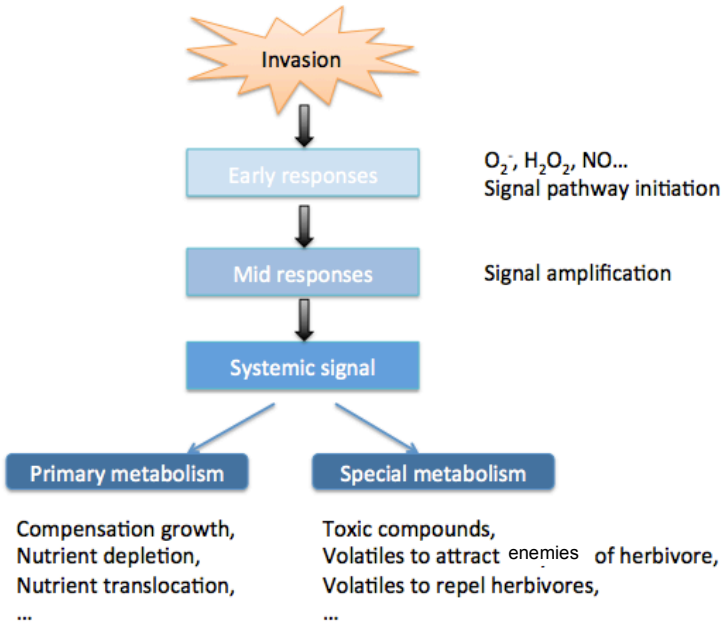

Figure 1.1 Response against herbivore invasion in plants.

Early responses can be established in wound tissues within seconds to less than one hour after attack by herbivores. Defense signaling pathways are activated and amplified in hours after feeding damage. Finally, systemic signals deliver the information to the entire plant and changes that are in response to herbivory take place in both primary and specialized metabolism.

1.2 Changes in primary metabolism in response to herbivory

Primary metabolism provides the resources and energy for plant growth and development. Since herbivores consume carbon- and nitrogen-containing primary metabolites, one defense strategy by plants is to deplete essential nutrients from the attacked tissues so that herbivores need more time to obtain enough nutrients for growth and reproduction. For example, the induced expression of proteinase inhibitors (Green and Ryan, 1973) affects the herbivore digestive system and uptake of N-sources (Kunse et al., 2010). In tomato, the expression of the gene encoding threonine deaminase is up-regulated. This enzyme degrades threonine in both the plant and the herbivore digestive system. Thus, the herbivore may face a shortage of the essential amino acid threonine, which negatively affects its growth and propagation (Samach et al., 1991; Joshi et al., 2006). Second, besides the depletion of nutrients, plants may increase their primary metabolism for compensatory growth and to finish their life cycle earlier than

normal. A third strategy is the reallocation of nutrients, which involves root-to-shoot long-distance or short-distance transport between nearby tissues. For example, plants may transfer carbon or nitrogen resources from the exposed tissues to other tissues. By this way, plants can: (i) avoid significant nutrient loss and (ii) restore their damaged tissues very quickly (Bezemer and Van Dam, 2005; Gomez et al., 2010).

1.3 Spatial and temporal regulation of defense metabolism.

As part of an optimal defense against herbivores, the formation of specialized metabolites, is not only regulated temporally, but is often restricted spatially to distinct tissues or cells that may be preferably attacked by herbivores. For instance, cannabinoid biosynthetic genes are highly expressed only in flowers of cannabinoid-producing cultivars of *Papaver somniferum* (Higashi and Saito, 2013). Many plant species in the Lamiaceae family such as peppermint and basil store volatile terpenes in glandular trichomes located on the surface of leaves. The terpenes are released when trichomes are damaged and repel potential pathogens or herbivores (Pichersky and Gershenzon, 2002). In *Arabidopsis*, volatile terpenes are emitted from tissues without accumulation in specialized cells. However, different terpenes are exclusively produced in specific organs without a major overlap between tissues (see section below).

While some terpenes are produced constitutively, quite a few terpenes are made following induction by herbivory, pathogens, wounding, or phytohormones (Turlings et al., 1990; Dicke et al., 1999; Agrawal and Sherriffs, 2001; De Vos et al., 2005).

1.4 Biosynthesis of volatile terpenes in *Arabidopsis*

Terpenes are the most abundant specialized metabolites in plants and play an important role in plant defense against pathogens or herbivores. All terpenes are biosynthesized from a common C_5-isoprenoid unit precursor, isopentenyl diphosphate (IPP). There are two pathways of IPP biosynthesis in plants, the mevalonate pathway (MVA) and the methylerythritol phosphate (MEP) pathway, which take place in the cytosol and plastid, respectively (Fig. 1.2). In the MVA pathway, the initial substrates are three molecules of acetyl coenzyme A (acetyl-CoA) and they are condensed to a C_6 molecule, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), by acetoacetyl-CoA thiolase (AACT). HMG-CoA is then converted to mevalonate as a precursor of IPP and its allylic isomer, dimethylallyl diphosphate (DMAPP). In the MEP pathway, the initial substrates are pyruvate and glyceraldehyde-3-phosphate. They are condensed to the C_5 unit, 1-deoxy-D-xylulose-5-phosphate (DXP) by DXP synthase 1 (DXS1). DXP is then converted to IPP and DMAPP in six enzymatic steps (Tholl and Lee, 2011).

In the next step of the core terpene biosynthetic pathways, IPP and DMAPP, are condensed to different prenyl diphosphates (Gershenzon and Kreis, 1999; Tholl, 2006; Tholl and Lee, 2011). Formation of geranyl diphosphate (GPP, C_{10}) or geranylgeranyl diphosphate (GGPP, C_{20}), which are the precursors of monoterpenes (C_{10}) and diterpenes (C_{20}), respectively, occurs in plastids (Bohlmann et al., 1998; Davis and Croteau, 2000; Tholl, 2006; Vaughan, 2010; Lee, 2011), while farnesyl diphosphate (FPP, C_{15}), the precursors for sesquiterpenes or triterpenes are made in the cytosol or possibly in peroxisomes (Figure 1.2) (Tholl and Lee, 2011).

Prenyl diphosphates are then converted into the final terpene products by enzymes called terpene synthases (TPSs). The reaction of most class I type TPSs starts with the cleavage of the diphosphate moiety resulting in a carbocation intermediate, which undergoes a series of structural rearrangements to produce the final terpene product. By contrast, class II TPSs start their reaction with the protonation of the double bond at the opposite end of the molecule from the diphosphate moiety (Figure 1.3 A&B). Generally, a single TPS enzyme may produce a blend of terpene compounds. For example, the *Arabidopsis* 1,8-cineole synthase TPS23 (At3g25830) produces a mixture of monoterpenes (Figure 1.3 C) (Chen et al., 2004b).

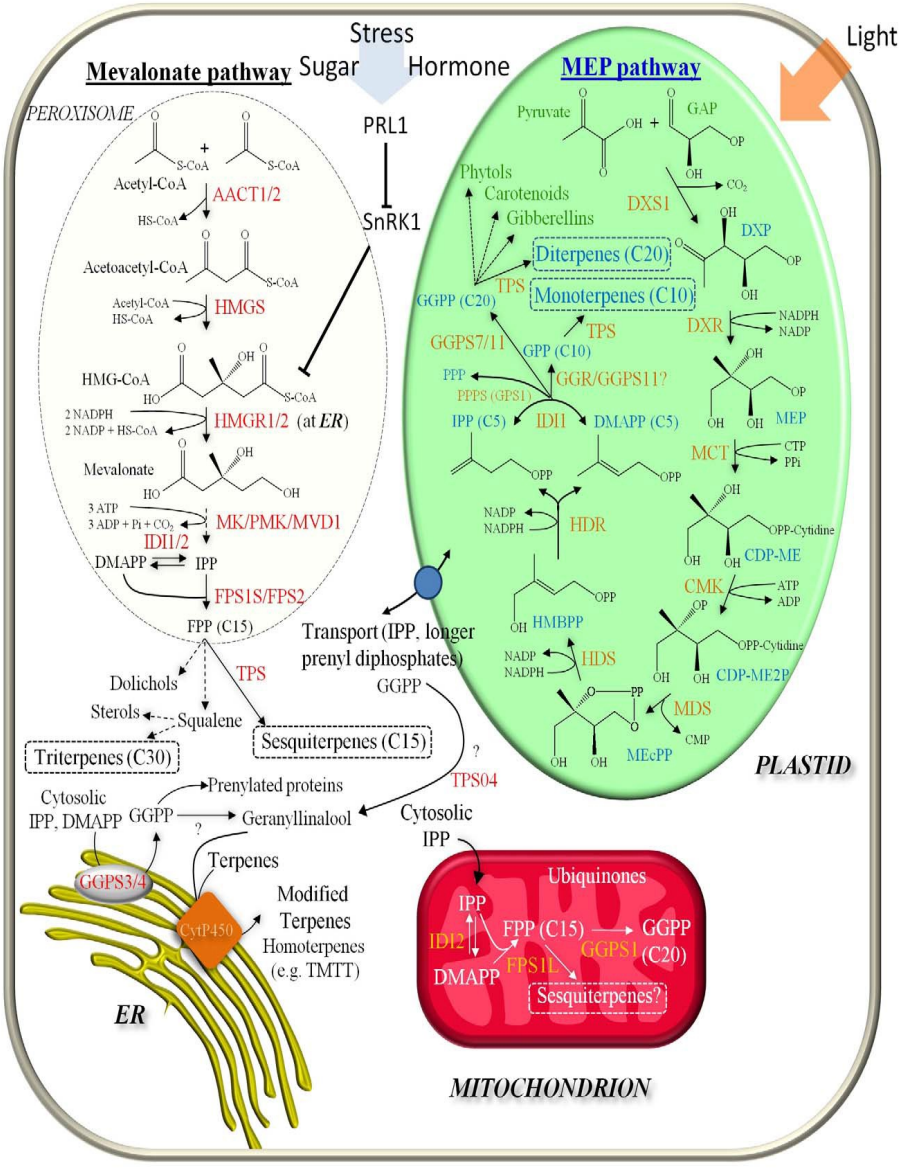

Figure 1.2 Terpene biosynthesis pathway in the plant cell (Tholl and Lee, 2011).

Two major pathways of IPP biosynthesis are shown in this figure. The MEP pathway takes place in plastids and produces precursors (GPP, GGPP) of monoterpene and diterpene, or other terpenes, while MVA pathway is mainly in the cytosol and produces the precursor (FPP) of sesquiterpenes. Possible exchange of precursors between those two pathways happens on the membrane of plastids. Precursors are then modified by terpene synthases and become the final terpene product. Biotic or abiotic stress may induce biosynthesis of some terpenes, while the others may be produced constitutively.

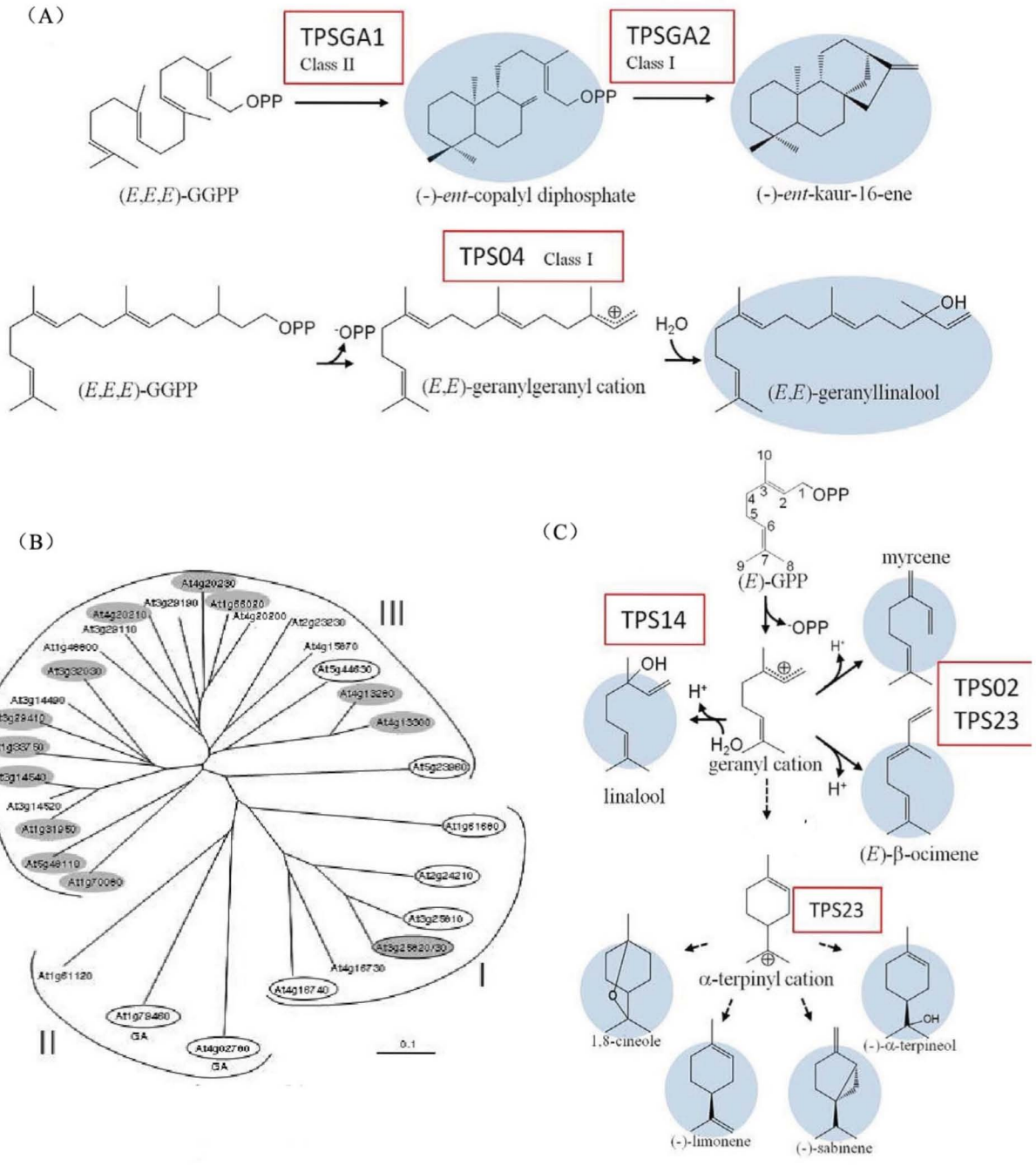

Figure 1.3 Position of several TPSs in terpene biosynthesis pathway and TPS classification (modified from Tholl, 2006).

(A) The TPSs that produce (-)-*ent*-kaur-16-ene and (*E,E*)-geranyllinalool and their classes. **(B)** TPSs in Arabidopsis have been classified to three clades; **(C)** The position of TPS14, TPS02 and TPS23 on the monoterpene biosynthesis pathway and their product.

The *TPS* gene family can be divided into seven subfamilies, a, b, c, d, e/f, g and h according to phylogenic analysis (Chen et al., 2011). Three of the subfamilies, TPS-a, TPS-b and TPS-g are angiosperm specific and produce either monoterpenes (TPS-b and TPS-g) or sesquiterpenes/diterpenes (TPS-a). TPS-c subfamily contains the most conserved TPSs among land plants and the corresponding enzymes function as diterpene synthases. TPS-d has been found only in gymnosperms. Enzymes in the TPS-e and TPS-f subfamilies are conserved among vascular plants and produce diterpenes. The youngest member of the TPS subfamily is TPS-h containing enzymes found in *Selaginella moellendorffii* that encode bifunctional diterpene synthases (Chen, 2011; Tholl and Lee, 2011).

Several studies have shown that terpene formation is primarily regulated by the expression of TPSs. For instance, *TPS* gene expression directs terpene biosynthesis to different types of cells. In *Arabidopsis*, there are 14 TPSs that are primarily expressed in roots (Tholl and Lee, 2011). Rhizathalene synthase (TPS08) converts GGPP to a blend of diterpenes, with rhizathalene as the main product. This enzyme is almost exclusively present in the pericycle of root vascular tissue. TPS25 uses FPP as a substrate and produces a blend of sesquiterpenes with β-farnesene as the main product (Huh, 2011). (*Z*)-γ-Bisabolene is the main volatile molecule in a blend of sesquiterpenes that is synthesized by FPP-utilizing TPS13. TPS25 is constitutively expressed to some extent in the cortex, endodermal cells and epidermal non-hair cells, while TPS13 is expressed in the cortex or endodermis, with some expression taking place in the stele in young root tissues closer to the actively growing root tip. Figure 1.4 shows the cell type specificity of several TPSs. Stronger expression of TPS25 and TPS13 occurs under herbivory or

treatment with JA (Huh, unpublished).

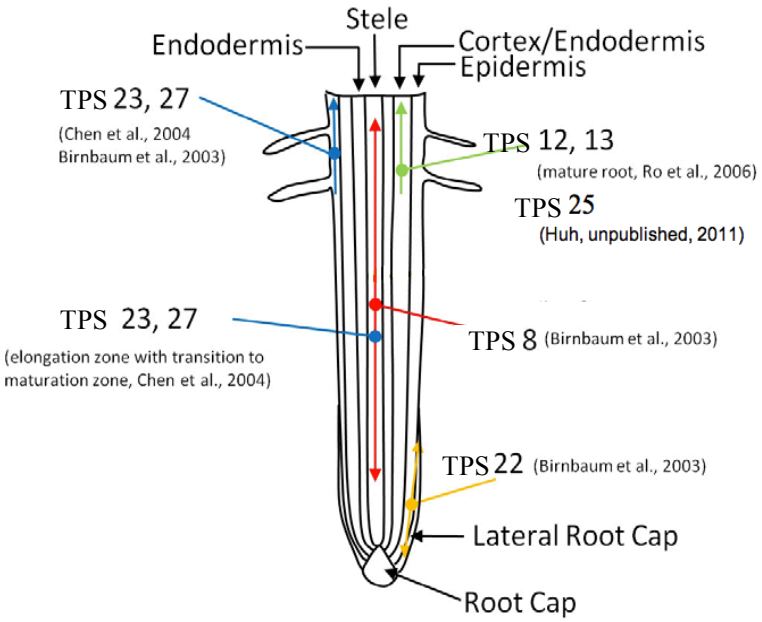

Figure 1.4 Tissue specificity of several terpene synthase genes (TPSs) in the *Arabidopsis* root (Tholl and Lee, 2011 modified).

TPS23 and *TPS27* express in both epidermal cells and stele. *TPS12* and *TPS13* can be found in cortex/endodermis. TPS25 also expresses in cortex/endodermis. TPS08 expresses only in the stele. TPS22 expresses only in the lateral root cap.

1.5 Plant defense in the rhizosphere

A recent survey of root chemical defenses suggests that roots are well protected against belowground attackers as aerial tissues by producing a diversity of different secondary metabolites (Rasmann and Agrawal, 2008a). Depending on the plant species as well as developmental and environmental factors, profiles of preformed or induced root chemicals can be similar or different to those in shoots (Rasmann and Agrawal, 2008a). Secondary metabolites induced by root herbivores include alkaloids, glucosinolates, and terpenes (Bezemer et al., 2003; Hol et al., 2004; Van Dam et al., 2004; Van Dam and E., 2006; Rasmann and Agrawal, 2008a). A detailed study in

spinach has shown that root herbivore feeding increases phytoecdysteroid levels in roots, which negatively affects *Bradysia* larval feeding performance (Schröder et al., 2002). Advances have been achieved in the understanding of indirect belowground chemical defenses by investigating the emission of volatiles such as terpenes that attract insect-parasitizing nematodes (Tol et al., 2001; Rasmann et al., 2005a). In general, antimicrobial, antifeedant, or insecticidal activities of phytochemicals from roots have been almost exclusively studied with *in vitro* bioassays (e.g. Puckhaber *et. al.* (2002) applying purified compound standards of varying concentrations), but these investigations have not been taken to the molecular level using mutant lines to test the function of these compounds *in vivo*. The role of JA and ethylene in host resistance against root pathogens and insects is well accepted (Okubara and Paulitz, 2005; Browse and Howe, 2008), but more information is required for understanding the fine-tuning of chemical defense responses according to the different signaling signatures of root-attacking organisms. Finally, the relationship between primary metabolite pools and root secondary metabolism requires additional attention. Schwachtje *et al.* (2006) demonstrated enhanced sugar translocation from shoots to roots in response to foliar feeding, which leads to questions of the possible movement of primary metabolites to shoots upon root herbivory.

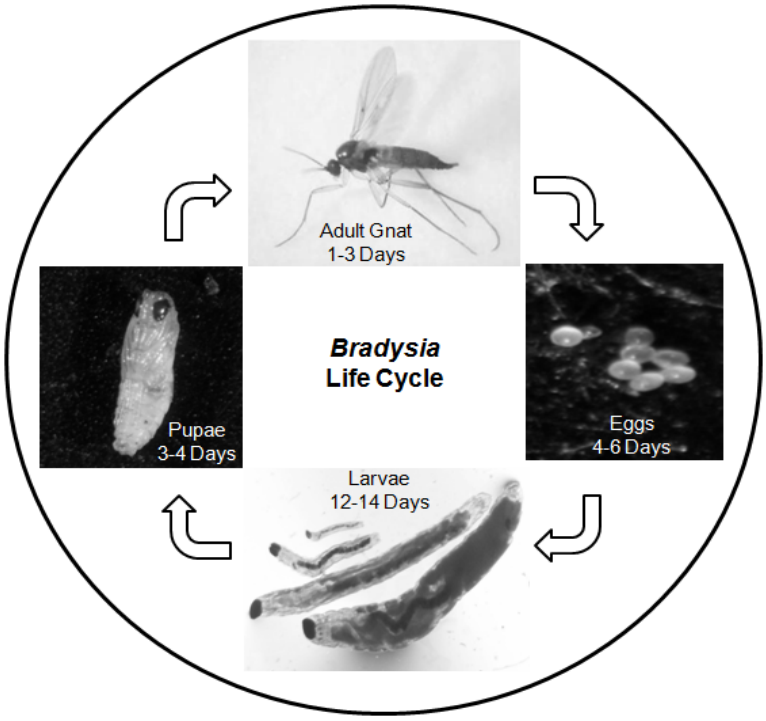

Figure 1.5 Bradysia life cycle. Males of *Bradysia* species (spp.) emerge one day before females. There is a 24 hour pre-oviposition period following female emergence, in which the mating occurs. Males usually live longer than females, which die soon after oviposition. Eggs hatch in 4 to 6 days and larvae feed in the soil for 12 to 14 days while developing 4 instars. Larvae then pupate for 3 to 4 days before subsequently emerging as adults. The life cycle is largely dependent on the temperature. At 20 to 25°C the cycle is usually completed in approximately 20 to 25 days (Harris et al., 1996; Cloyd and Zaborski, 2004).

1.6 *Bradysia* is a below ground herbivore of *Arabidopsis*

Larvae of darkwinged fungus gnats are root-feeding herbivores and common greenhouse pests. *Bradysia coprophila* (Lintner) and *Bradysia impatiens* (Johannsen) are two major species of dark-wing fungus gnats (Jagdale et al., 2007). Fungus gnat larvae can cause major damage on plant roots especially fine roots and roots of seedlings (Springer and Carlton, 1993). Larvae often chew and strip roots, thereby negatively impacting water and nutrient absorption. Roots may lose almost half of their biomass when extreme infestations happened. High reproduction rates contribute to the harmful effects of *Bradysia* spp. on plants. Adult female gnats lay between 250 and 1000 eggs

before death after 1 to 3 days. Larvae emerge within 4 to 6 days and feed in the soil for 12 to 14 days before pupating, during which they live on roots. The life cycle is completed within 20 to 25 days at 20 to 25°C (Figure 1.5) (Cloyd and Zaborski, 2004).

1.7 Overview of research

Plants have developed intricate mechanisms to protect themselves from herbivory. For example, they may synthesize and emit small molecular weight volatiles to directly or indirectly repel herbivores; changes may also happen at the level of primary metabolism and metabolite transport. This study was guided by the hypothesis that root defense responses and mechanism at the levels of both primary and specialized metabolism are highly regulated and a result of plant co-evolution or adaptation with herbivores. The overall goal of this work was to understand the regulation and molecular changes in *Arabidopsis* plants in response to herbivory with a focus on: (i) probing the cell type specificity of volatile terpene formation in *Arabidopsis* for gaining a better understanding of the plasticity of terpene biosynthesis and (ii) the change of gene expression and primary metabolism in *Arabidopsis* roots in response to *Bradysia* larvae feeding.

The specific objectives of this study were to:

A. Probe the cell type specificity of three terpene synthase genes in *Arabidopsis* roots

B. Detect the response of *Arabidopsis* roots at the transcriptional and metabolic levels to feeding by a generalist herbivore, *Bradysia* spp.

In Chapter II, the cell type specificity of three terpene synthase genes, *TPS08*, *TPS13* and *TPS25*, which have putative defense function in *Arabidopsis* roots, has been observed

by swapping the genes among different cell types. The analysis of gene expression was conducted at the levels of gene expression and abundance of TPS metabolites.

In Chapter III, *Arabidopsis* plants grown in aeroponic culture were challenged with the root-feeding insect *Bradysia*. Microarray and quantitative real-time PCR analyses were performed to determine differences in gene expression. Root metabolite extractions coupled with gas chromatography-mass spectrometry (GC-MS) analysis of relative metabolite levels were conducted to examine changes in central carbon and nitrogen metabolism.

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Chapter II

Probing the Cell-type Specificity of Terpene Formation

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Abstract

Terpenes are the most abundant specialized metabolites in plants and play an important role in plant defense against pathogens and herbivores. Terpene biosynthetic enzyme activities are often coordinated in specific tissues and cellular compartments. Fine-scale transcriptome maps of *Arabidopsis* roots have shown that terpene biosynthesis is restricted to specific cell types, which may be a consequence of different selective pressures by beneficial or harmful organisms. We address the question to what extent terpene metabolism is restricted to specific cell types by substrate availability, transcriptional, and post-transcriptional regulation. To this end, we probed the cell type specificity of terpene biosynthesis by changing the expression of terpene synthase genes from their endogenous site of expression to different root cell types using cell type specific promoters. We show that the terpene synthase TPS08, which produces the diterpene rhizathalene and is normally expressed in the root vasculature, is functionally active when expressed as a YFP-fusion protein in the epidermis or cortex, although at substantially lower levels than in wild type. We did not find any relationship among the emission of volatiles, protein levels, and gene expression levels of TPS08. When expression of TPS13 (producing the sesquiterpene γ-bisabolene) was directed from the cortex to the epidermis or stele in the form of a YFP fusion protein, terpene emission rates and *TPS13* transcript levels were different among the transgenic plants although we observed a high fluorescent signal from the epidermal cells of root tips under control of both the *WER* and *GL3* promoters. Terpene production was also observed for the (*E*)-β-farnesene sesquiterpene synthase TPS25 when its expression was switched from

the endodermis and non-hair producing epidermal cells to hair producing epidermal cells. The spatial shift was observed for transcript accumulation. However, the TPS25-mGFP showed only very weak fluorescent signal. Overall, the results indicate relaxed cell specificity of terpene biosynthesis in roots and suggest that tissue-specific terpene metabolite patterns could change depending on different selective pressures during the interaction of roots with the soil environment.

2.1 Introduction

Plants have evolved different mechanisms to interact with organisms in their environment. Defense strategies against harmful organisms include physical barriers (Jander and Howe, 2008; Trumble et al., 1993), such as morphological changes in response to herbivory (Bazzaz et al., 1987), and chemical defenses that operate directly or indirectly (Bennett and Wallsgrove, 1994; Schoonhoven et al., 2005; Walling, 2008). To maintain an energy balance between defense and growth, plants need to regulate their defenses temporally and spatially (Kempema et al., 2007; Erb et al., 2009). For an optimal and most efficient defense and to avoid potential cytotoxic effects of defense compounds, chemical defenses may occur as induced responses or be restricted as constitutive defenses to specific tissues and cells (Dudareva et al., 2004). For instance, many plant species in the *Lamiaceae* family, such as peppermint and basil, store volatile terpenes in glandular trichomes located on the surface of the leaves. Their volatiles are only released when these trichomes are broken (Pichersky and Gershenzon, 2002).

This work examined the stringency of cell-type specificity of secondary metabolism by using the terpene biosynthesis in *Arabidopsis* roots as the model system. Terpenes are the most abundant specialized metabolites in plants. All terpenes are biosynthesized from the common C_5-isoprenoid precursor, isopentenyl diphosphate (IPP). The enzymatic reaction starts with the binding of the reactive C_5-precusor dimethylallyl pyrophosphate (DMAPP), which is derived from IPP. DMAPP is then condensed with multiple IPPs to form prenyl diphosphates of different chain length. Geranyl diphophate (GPP, C_{10}) and geranylgeranyl diphosphate (GGPP, C_{20}) are produced in plastids, while farnesyl

diphosphate (FPP, C_{15}) is made in the cytosol (Bohlmann et al., 1998; Gershenzon and Kreis, 1999; Davis and Croteau, 2000). Prenyl diphosphates are then converted to terpenes by enzymes called terpene synthases (TPSs) (Gershenzon and Kreis, 1999; Lee, 2011). Several *TPS* genes are expressed exclusively in roots. For example, the 1,8-cineole synthase TPS23 is expressed in epidermal cells of *Arabidopsis* roots (Chen et al., 2004). Ro *et al.* (2006) showed that the γ-bisabolene synthases TPS12 and TPS13 are specifically expressed in the cortex and endodermis of mature *Arabidopsis* roots and in the root elongation and differentiation zones of the stele. Further characterization of mutant lines in the Tholl lab has shown that TPS13 is largely responsible for the formation of γ-bisabolene *in vivo*. Recent studies in the Tholl lab have also identified two β-farnesene sesquiterpene synthases, TPS22 and TPS25, with expression patterns in the cortex, endodermal cells and also in non-hair epidermal cells (N-cells) (Huh, 2011). In addition to their constitutive expression, *TPS12*, *TPS13* and *TPS25* are also regulated temporally by wounding or the defensive phytohormone, such as JA (Ro et al., 2006; Huh, 2011). Moreover, the terpene synthase TPS08, which produces the unusual semi-volatile diterpenes rhizathalenes, was found to be constitutively expressed in the stele of the *Arabidopsis* root. Rhizathalenes have antifeedant activities against root herbivores such as fungus gnat (*Bradysia spp.*) larvae (Vaughan et al., 2013). The described expression patterns have been derived from *TPS* promoter-GUS experiments and are generally in agreement with fine-scale cell-specific microarray analyses of *Arabidopsis* roots (Birnbaum et al., 2003; Brady et al., 2007).

Although it is well known that the biosynthesis of terpene metabolites often occurs in specific cells (Tholl and Lee, 2011; Vaughan et al., 2013), the stringency for maintaining

this cell-type specificity is not well understood. We hypothesize that the biosynthesis of different terpenes can be supported by different cell types. This hypothesis has been investigated by swapping different root cell-specific promoters to alter cell-type specific expression of *TPS08*, *TPS13,* and *TPS25* (Table 2.1 and Figure 2.1). Functional gene expression in the different cell types was analyzed at transcriptional, translational, and metabolic levels by using quantitative real-time PCR, confocal microscopy, and SPME/GC-MS.

Table 2.1 Background information of the chosen promoters.

Abbreviation	Promoters	Localization	Function
TPS08	TPS08	Stele	Rhizathalene synthase (Vaughan *et.al*., 2013)
TPS23	TPS23	Epidermis	1,8-cineole synthase (Chen *et.al*., 2004)
CO2	CO2	Cortex	Inhibitor, lipid-transfer protein (Heidstra *et.al*., 2002)
GL3	GLABRA3	Epidermis, hair (H)-cell	bHLH protein regulating H-cell development (Bernhardt *et.al*, 2005)
WER	WEREWOLF	Epidermis, non-hair (N)-cell	MYB-related protein regulating epidermal cell patterning (Lee *et.al.*, 1999)

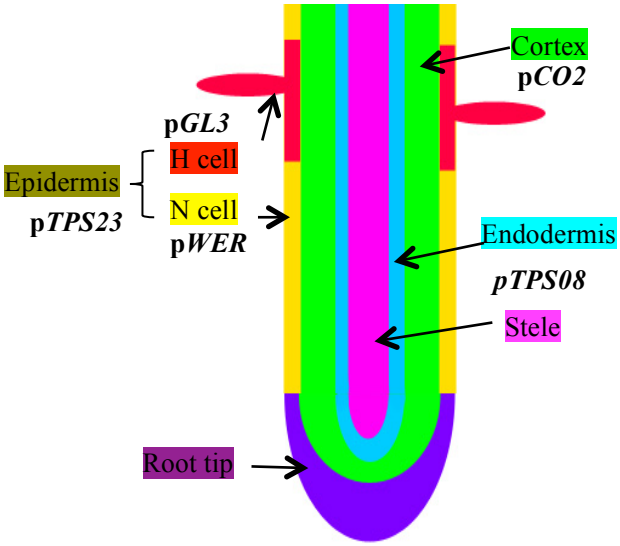

Figure 2.1 Expression patterns of the chosen promoters in roots of *Arabidopsis thaliana*.

Promoter *TPS23* drives gene expression in epidermal cells; promoters *GL3* and *WER* express in H-cells and N-cells of the epidermis, respectively. *CO2* is a cortex specific gene and *TPS08* expresses specifically in the stele.

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2.2 Materials and methods

Plant materials and culture conditions

Transgenic plant lines. cDNAs of *TPS08*, *TPS13* and *TPS25* were cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen, Carlasbad CA, USA). Promoters were cloned from the genomic DNA of *Arabidopsis* WT Columbia into pDONR/P4-P1R (Invitrogen). The generation of the *pGL3*::*TPS25-mGFP* construct was done by recombination cloning of the promoter, the *TPS25* coding region, and *mGFP* (in pENTR/P1-P2R vector) into the pB7m34GW destination vector (Karimi et al., 2007). Other constructs were generated by recombining the promoter and the targeted gene into the destination vector pB7Y24, which contains the *YFP* coding sequence (Appendix II).

The recombination reactions were performed with the RL-reaction kit (Invitrogen). Transformation used *Agrobacterium tumefaciens* strain GV3101 and followed Valvekens' protocol (Karimi et al., 2002; Valvekens et al., 1988). T2 plants were cultivated and used for all the assays in this paper.

Plant materials The knock-out mutant alleles for *TPS08* and *TPS13* used in this study were SALK_125194 and SALK_011441, respectively, obtained from the ABRC stock center (Alonso et al., 2003). The *tps22/tps25* double-knockout mutant was established by Dr. Jung-Hyun Huh. T2 transgenic plants, including *pTPS23*::*TPS08-YFP*, *pCO2*::*TPS08-YFP*, *pGL3*::*TPS08-YFP*, *pWER*::*TPS08-YFP*, *pTPS08*::*TPS13-YFP*, *pGL3*::*TPS13-YFP, pWER*::*TPS13-YFP, pGL3*::*TPS25-mGFP* and two complementary lines, *pTPS08*::*TPS08-YFP* and *pTPS25*::*TPS25-YFP* were grown for the assays. Wild type *Arabidopsis* ecotype Columbia was also used in this study as the control.

Growth conditions An axenic culture system was used in this study. All seeds were surface sterilized prior culturing (Schiefelbein and Somerville, 1990). Plants that were used for confocal microscopy assay were grown in 12-well plates with 2 ml of half-strength Murashige and Skoog, 2% (w/v) sucrose for 5 days. For quantitative real-time PCR and volatile assays, approximately 20 to 25 seeds of plants were germinated on a 2 cm^2 plastic mesh square (Small Parts Inc., Logansport, IN) on the same plate for one week and then transferred to 0.5× MS liquid medium with 2% sucrose in axenic conditions for an additional 3 weeks (Hetu *et al.* 2005). All plants were grown under 10 h/14 h day/night light cycle, 150 μmol m^{-2} sec^{-1} at 20 °C - 21°C.

Solid-phase microextraction (SPME)/GC-MS

One gram of root tissues was harvested for analysis of volatile compounds. SPME/GC-MS was performed according to Vaughan (2010).

Confocal Microscopy

Optical sections of fresh roots were recorded with an inverted fluorescence microscope (Axiovision; Zeiss, Jena, Germany). Fresh roots were observed on a confocal laser scanning microscope (LSM510, Zeiss, Jena, Germany) with the HeNe laser and LP560 filter and with a 40× water immersion objective. mGFP/YFP fluorescence was excited at 488 nm and autofluorescence was excited at 543 nm. Confocal images were captured with the built-in software, Zen2009, and processed further by Photoshop version CS5.0.

qRT-PCR

Total RNA was isolated by the Trizol method (Piotr Chomczynski, 1987) and treated with RQ1 DNase (Promega). Reverse transcription was performed by using GoScript reverse transcriptase (Promega). Gene-specific primers were designed for quantitative RT-PCR (Appendix Table III), which produce approximately 100 bp fragments. The SYBR green method has been used to perform the qRT-PCR. PCR conditions were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for targeted fragment amplification. The fluorescence detection point was set at 60 °C for 10 s. The qRT-PCR was performed on an ABI 7300 Real-Time PCR instrument.

2.3 Results

Ectopic expression of *TPS08* in different root cell types

As described above, the rhizathalene synthase TPS08 is primarily expressed in the root stele and the root tip (Vaughan et al., 2013). We changed the cell type specific expression of *TPS08* by expressing the gene in the epidermis and the cortex (Table 2.2). To this end, we used the epidermal promoters *GLABRA3* (*pGL3*), which is active in the epidermis and regulates root-hair cell (H-cell) formation (Bernhardt et al., 2005); the *WEREWOLF* promoter (*pWER*) that functions in epidermal non-hair cells (N-cells) (Lee and Schiefelbein, 1999); and the epidermal promoter of the 1,8-cineole synthase, *TPS23* (Chen et al., 2004a). Expression of *TPS08* in the cortex was achieved under the *CO2* promoter (*pCO2*) (Oh et al., 1996). In addition to these promoters, we used the native *TPS08* promoter to complement the T-DNA insertion *tps08-1* mutant (Vaughan et al., 2013), which was used as a background line for all the *TPS08* transgenic plants.

Table 2.2 Combinations of chosen promoters and target genes.

Targeted Genes	*TPS 08*					*TPS 25*		*TPS 13*		
Chosen Promoters	*TPS08*	*TPS23*	*CO2*	*GL3*	*WER*	*TPS25*	*GL3*	*TPS08*	*GL3*	*WER*

Gene expression analysis of TPS08 in the roots of different transgenic lines by quantitative real-time PCR

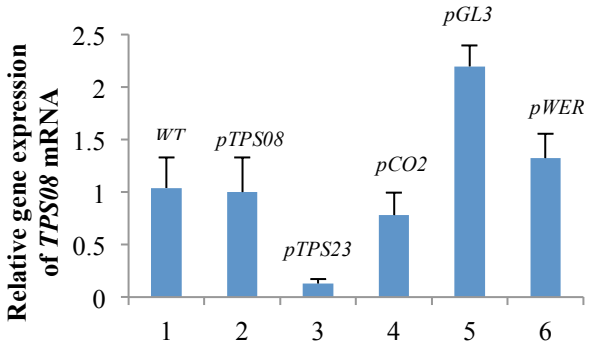
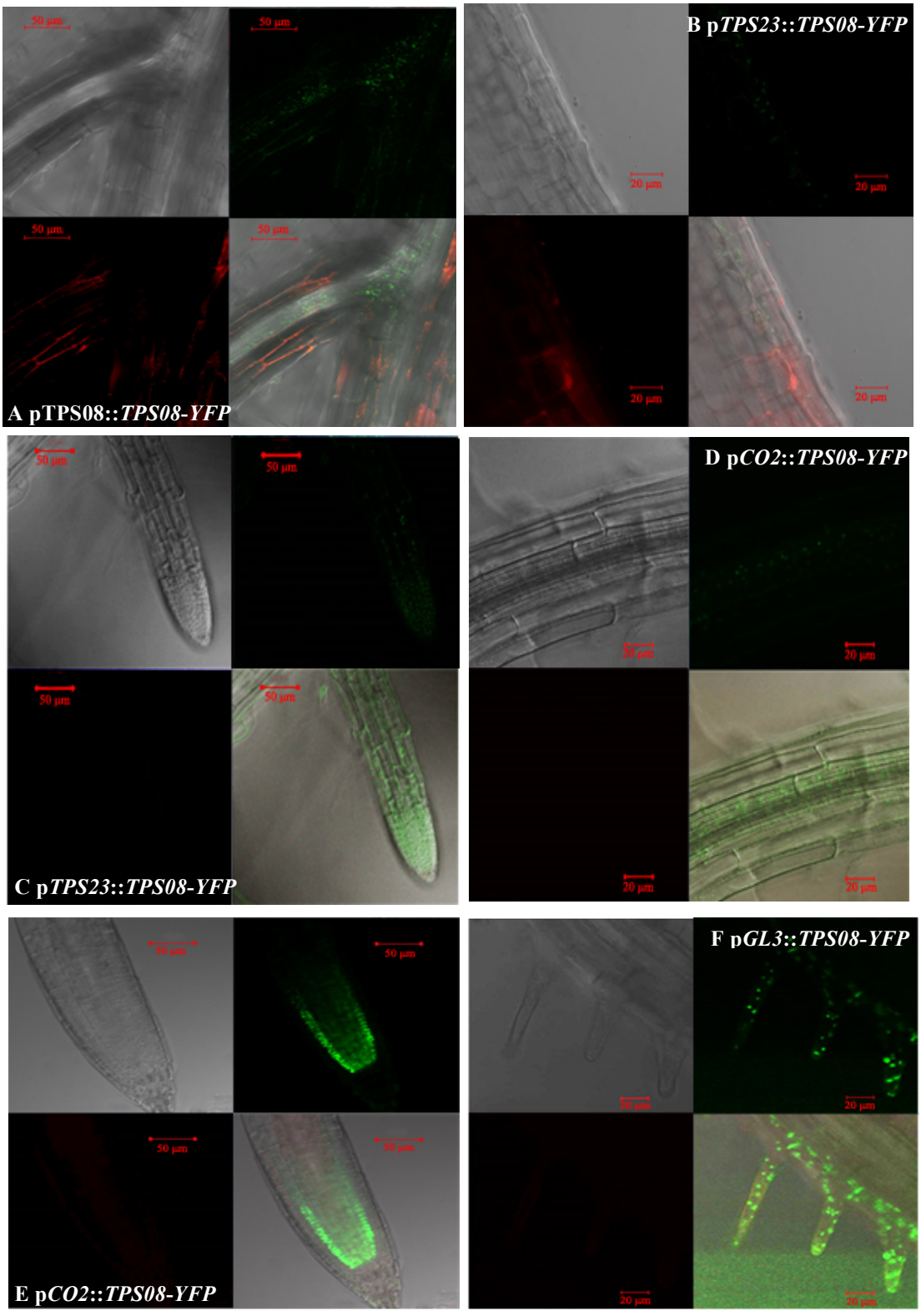


Figure 2.2 *TPS08* gene expression in *Arabidopsis* roots of transgenic lines and WT.
Expression of *TPS08* mRNA in *Arabidopsis* root tissues was examined by quantitative RT-PCR analysis and is presented as relative mRNA expression (means of ΔΔCt±SEM, *n=3*). The expression level of *TPS08* in wild type plants was set to 1. **1**, WT (Col); **2**, *pTPS08*::*TPS08-YFP*; **3**, *pTPS23*::*TPS08-YFP*; **4**, *pCO2*::*TPS08-YFP*; **5**, *pGL3*::*TPS08-YFP*; **6**, *pWER*::*TPS08-YFP*.

Basta-resistant plants (T2 generation) were generated carrying the full length *TPS08* transgene in C-terminal fusion with YFP under the control of the promoters listed in Table 2.2. Approximately 20-25 seedlings representing a mix of transgenic homozygous and heterozygous offspring of a single T1 plant were grown on a mesh on Basta-containing medium and then transferred to sterile liquid medium to promote root growth. Roots were harvested after 4 weeks and analyzed for expression of the *TPS08* transgene. Transcript levels represent the average transcript abundances from all plants per replicate. Previous studies showed the absence of the *TPS08* transcript in the *tps08-1* knockout mutant (Vaughan et al., 2013). Complementation of the *tps08* mutant by using a 2.3 kb native *TPS08* promoter fragment restored wild type transcript levels. Similar expression values were obtained under the control of the cortex-specific *CO2* promoter and the non-hair epidermal cell specific *WER* promoter, while two-fold higher transcript levels were found for the hair-cell specific *GL3* promoter and substantially lower transcript abundance was observed under control of the 1,8-cineole synthase (*TPS23*) epidermal promoter (Figure 2.2).

TPS08-YFP protein expression in transgenic Arabidopsis *roots*

We then performed confocal microscopy to analyze whether a TPS08-YFP fusion protein was detectable in the different cell types according to the cell type specific promoters (Figure 2.2). In transgenic plants that expressed the TPS08-YFP protein under the control of the native *TPS08* promoter (Figure 2.3 A), the fluorescent signal was found in plastids of the root stele. Fluorescence in *pTPS23*::*TPS08-YFP* was observed in epidermal cells in roots and root tips (Figure 2.3B, C). Strong fluorescence was also observed in the cortex of root tips in *pCO2*::*TPS08-YFP* plants (Figure 2.3 D), while only a very weak signal was detected in the cortex of the other root zones (Figure 2.3 E) Bright fluorescence was detected in H-cells of *pGL3*::*TPS08-YFP* plants (Figure 2.3 F). Protein expression was also detected in the epidermal cells of root tips in *pGL3*::*TPS08-YFP* plants (Figure 2.3 G). In *pWER*::*TPS08-YFP*, the fusion protein was found in the epidermal layer in the root tip and in older developmental zones (Figures 2.3 H, I).


A pTPS08::*TPS08-YFP*
B p*TPS23*::*TPS08-YFP*
C p*TPS23*::*TPS08-YFP*
D p*CO2*::*TPS08-YFP*
E p*CO2*::*TPS08-YFP*
F p*GL3*::*TPS08-YFP*

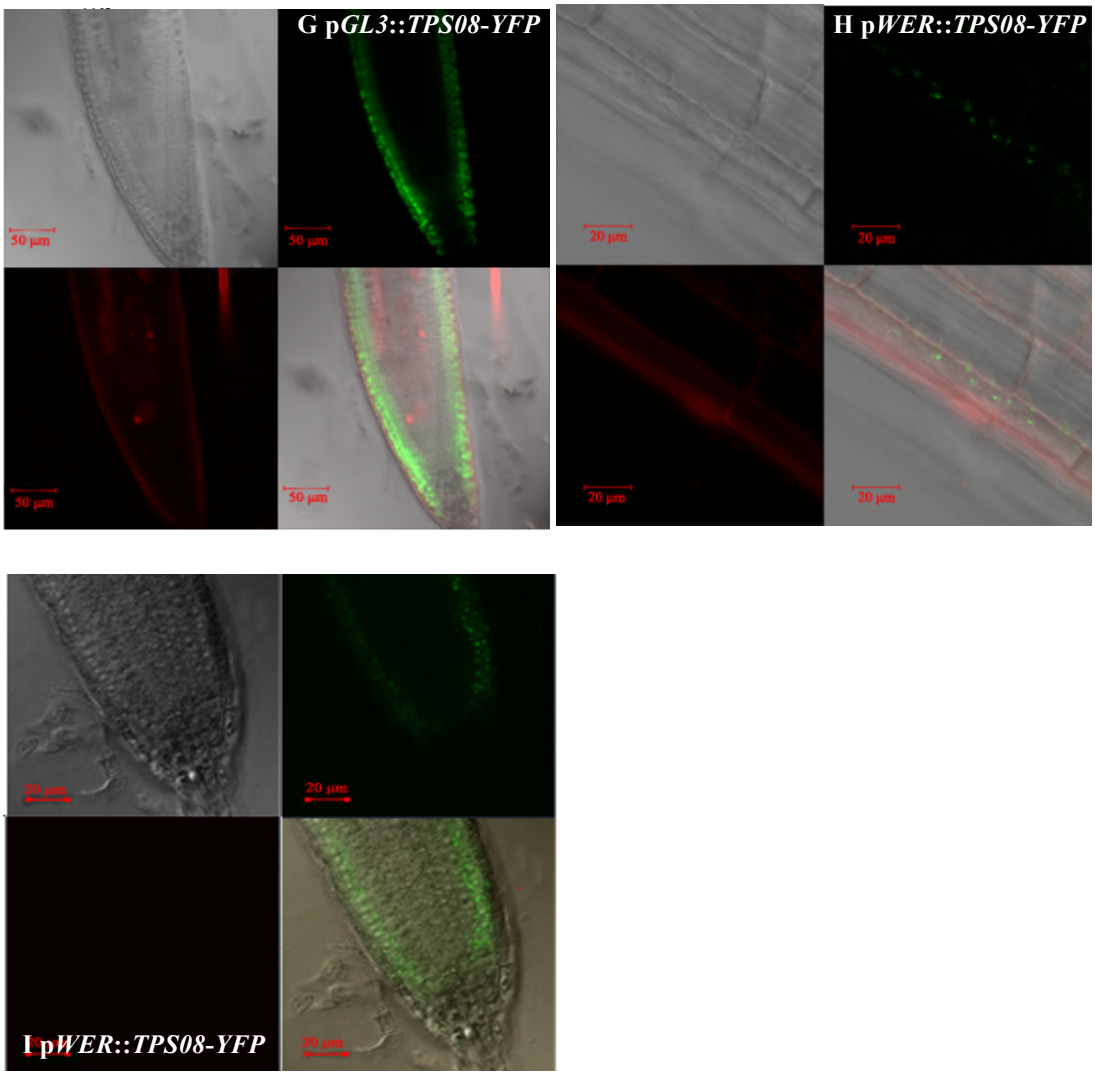


Figure 2.3 Cell type specificity of TPS08-YFP protein expressed under the control of different promoters.

YFP was detected by confocal microscopy. Green color indicates YFP fluorescence. Red indicates auto-fluorescence from plants. Grey images represent images under transmission Images were overlaid to indicate the localization of fluorescence signal in roots.
(A) Images of roots from plants expressing enhanced YFP fused to TPS08 under the control of the *TPS08* promoter; **(B-I)** Images of TPS08-YFP in roots of transgenic plants expressing TPS08 under the different root-specific promoters.

Diterpene analysis of the different transgenic lines

To determine whether the gene transcript and the observed fluorescence protein levels would correlate with the emission of rhizathalene, we performed headspace analysis of volatiles on 1 g of root tissue using SPME/GC-MS. *pWER*::*TPS08-YFP* plants had the most emission of rhizathalene among all transgenics. Overall, we found very limited emission of rhizathalene including the TPS08 complementation line (Figure 2.4). This finding indicates that the fusion of the TPS08 protein to YFP might have affected its enzymatic activity.

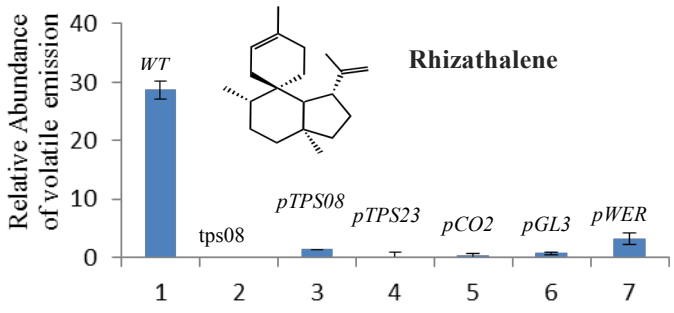


Figure 2.4 Rhizathalene emission from Arabidopsis roots.

Rhizathalene volatile emission from 1 g of axenic roots using solid phase microextraction (SPME). **1**, WT; **2**, *tps08*; **3**, *pTPS08*::*TPS08-YFP*; **4**, *pTPS23*::*TPS08-YFP*; **5**, *pCO2*::*TPS08-YFP*; **6**, *pGL3*::*TPS08-YFP*; **7,** *pWER*::*TPS08-YFP*.

Ectopic expression of the (*Z*)-γ-bisabolene synthase TPS13 in different cell types

(*Z*)-γ-bisabolene synthase TPS13 is primarily expressed in the root cortex, endodermal cells in mature roots, and vascular tissues of younger roots closer to the actively growing root tip (Ro et al., 2006). TPS13 expression is induced by JA treatment and the primary enzyme for (*Z*)-γ-bisabolene formation in *Arabidopsis* roots. We changed the cell type-specific expression of *TPS13* by using the epidermal promoters *GLABRA3* (*pGL3*) (Bernhardt et al., 2005) and *WEREWOLF* (*pWER*) (Lee and Schiefelbein, 1999).

We also used the stele-specific promoter *TPS08* (Vaughan et al., 2013), although the overlap with endogenous expression in younger root zones was expected. A *tps13* knockout mutant was confirmed to have a knock-down phenotype and used for the transformation of all promoter constructs (Table 2.2).

Gene expression analysis of TPS13 in the roots of different transgenic lines by quantitative real-time PCR

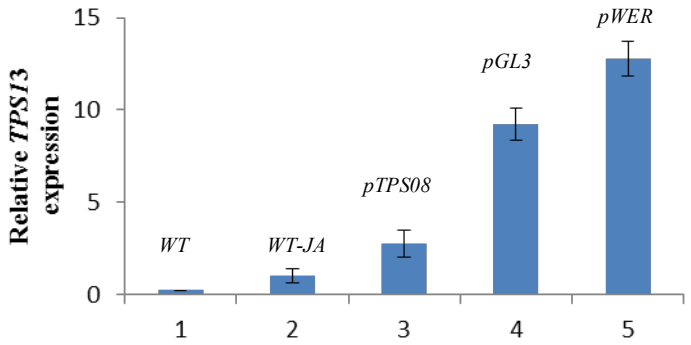
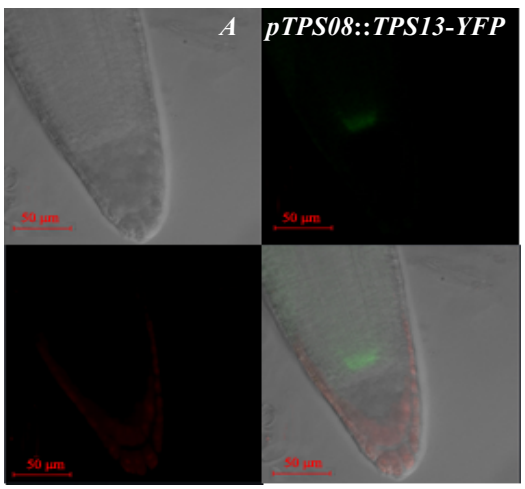
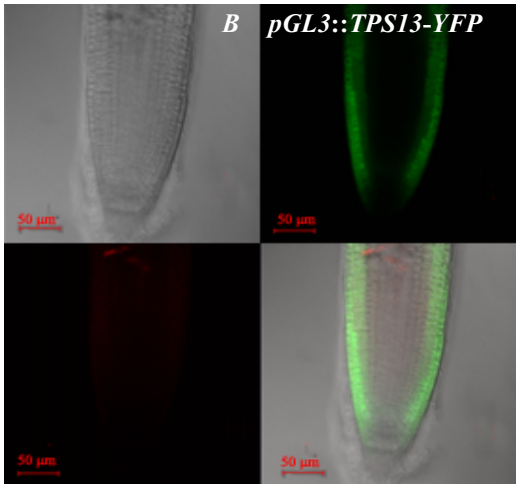


Figure 2.5 TPS13 gene expression in Arabidopsis roots of transgenics and WT.

Expression of *TPS13* mRNA in *Arabidopsis* root tissues, that were harvested after 4-week cultivation, was examined by quantitative RT-PCR analysis and is presented as relative mRNA expression (means of ΔΔCt±SEM, *n=3*). The expression level of *TPS13* in wild type plants that have been induced by JA was set to 1. **1**. WT mock; **2**. WT induced by JA; **3**. p*TPS08*::*TPS13-YFP;* **4**, p*GL3*::*TPS13-YFP*; **5**. p*WER*::*TPS13-YFP*.

T2 plants were selected and grown as described for the TPS08 plant lines. Roots were harvested after 4 weeks and analyzed for expression of the *TPS13* transgene. Transcript levels represent the average of transcript abundances from all plants per replicate (Figure 2.5). Wild type mock and JA-treated plants were used as the positive control. Three-fold higher transcript levels were found for the stele-specific *TPS08* promoter than those in WT induced by JA, while approximate 9-fold and 13-fold higher expression values were obtained under the control of the epidermal promoters *GL3* and *WER*, respectively. A low level expression of *TPS13* was observed also in wild type plants without any induction.





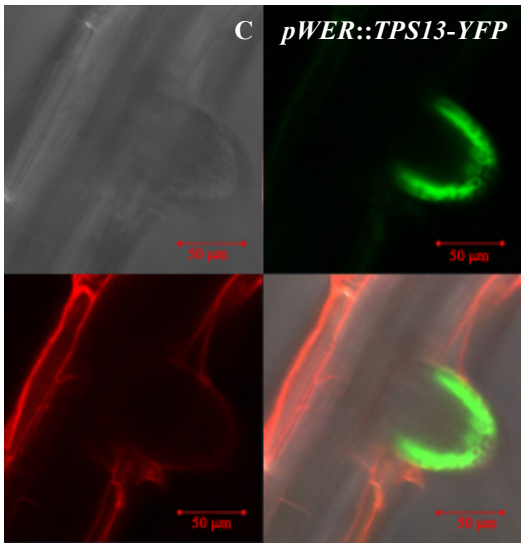


Figure 2.6 Cell type specificity of TPS13-YFP protein expressed under the control of different promoters.

YFP was detected by confocal microscopy. Green color indicates YFP fluorescence. Red color indicates auto-fluorescence. Grey images represent images under transmission light. Images were overlaid to indicate the localization of fluorescence signal in roots.
(A-C) Images of TPS13-YFP in roots of transgenic plants expressing TPS13 under the different root-specific promoters.

TPS13-YFP protein expression in transgenic Arabidopsis *roots*

Confocal microscopy was performed to observe whether a TPS13-YFP fusion protein was detectable in the different cell types in accordance with the cell type specificity of the used promoters (Fig 2.6) Fluorescence was detected in the stele of *pTPS08*::*TPS13-YFP* root tips (Figure 2.5A). A very weak or no signal was detected in the other root zones (data not shown). The TPS13-YFP protein was detected in epidermal cells of *pGL3*::*TPS13-YFP* root tips (Figure 2.6. B), but not in the other root tissues. A

bright fluorescent signal was observed at the initiation site of lateral roots in the *pWER*::*TPS13-YFP* line (Figure 2.6. C). However, only a weak signal was detected in root tips or other root zones in these lines (data not shown).

Sesquiterpene analysis of the different transgenic lines

To determine whether gene transcript and the observed fluorescence protein levels would correlate with the emission of (*Z*)-γ-bisabolene, we performed headspace analysis of volatiles from root tissue by SPME/GC-MS (Figure 2.7). *pWER*::*TPS13-YFP* plants had the highest emission of (*Z*)-γ-bisabolene among all transgenics, even compared to the wild type plants treated JA. *pGL3*::*TPS13-YFP* plants had relatively high levels of bisabolene emission, however, less than WT induced by JA. Background (*Z*)-γ-bisabolene has been detected in wild type plants without JA induction. However, it is less than the induced plants. Compared to WT, low levels of (*Z*)-γ-bisabolene emission were observed in transgenic *pTPS08*::*TPS13-YFP* lines. However, it is still higher than *tps13* non-induced plants.. Because the promoters of *GL3*, *TPS08* and *WER* are constitutively active, plants that

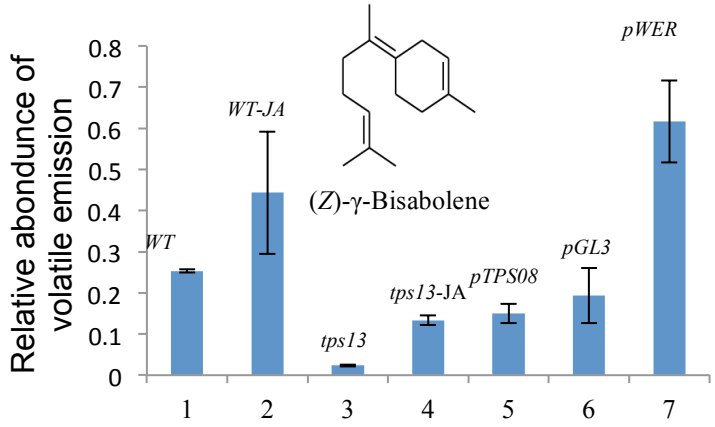


Figure 2.7 (Z)-γ-Bisabolene emission from Arabidopsis roots.

(*Z*)-γ-Bisabolene emissions from 1 g of axenic roots using SPME. **1**. WT mock; **2**. WT induced by JA; **3**. *tps13*; **4**. *tps13* JA induced; **5.** p*TPS08*::*TPS13;* **6.** p*GL3*::*TPS13*; **7**. p*WER*::*TPS13*.

were transformed with the construct containing these three promoters were not treated with JA.

Ectopic expression of the (*E*)-β-farnesene synthase *TPS25* in epidermal hair cells

Gene expression analysis of TPS25 in the roots of different transgenic lines by quantitative real-time PCR

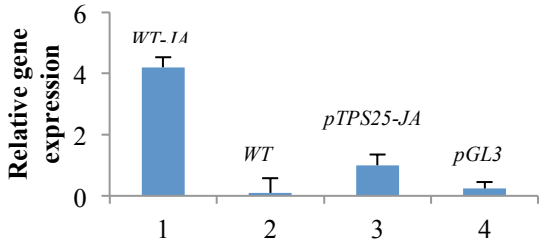


Figure 2.8 Transcript levels of TPS25.

Expression of *TPS25* mRNA in *Arabidopsis* root tissues were examined by quantitative RT-PCR analysis and is presented as relative mRNA expression (means of ΔΔCt±SEM, *n=3*). The transcript levels of the TPS25 complementation line was set to 1. **1**, WT, JA induced; **2**, WT, mock; **3**, p*TPS25*::*TPS25-YFP*, JA induced; **4**, p*GL3*::*TPS25-mGFP* non-induced.

Basta-resistant plants were generated carrying the full length *TPS25* transgene in C-terminal fusion with mGFP/YFP under the control of the promoters listed in Table 2.2. tps 22/tps25 double knockout mutants were used as the background. Transcript analysis of TPS25 in *Arabidopsis* roots treated with JA showed no functional full-length transcripts of *TPS25* in background plants (Huh, 2011). The plants were grown and cultured as described before. Transcript levels represent the average of transcript abundances from all plants per replicate (Figure 2.8). A 2.0 kb native *TPS25* promoter fragment was combined with TPS25-mGFP to generate a complementation line. JA was used to treated the wild type plants and the *pTPS25::TPS25-YFP* line. Four-fold higher expression values were obtained in wild type plants that have been induced by JA, while

two-fold less transcript levels were found for the hair-cell specific *GL3* promoter and very low transcript abundance was observed in WT without JA induction.

TPS25-mGFP protein expression in transgenic Arabidopsis roots

To analyze whether a TPS25-mGFP or TPS25-YFP fusion protein was detectable in the different cell types according to the cell type specific promoters, confocal microscopy was employed (Figure 2.9) In transgenic plants that expressed the TPS25-YFP protein under the control of the native *TPS25* promoter, the fluorescent signal was found close to the stele, possibly in the endodermis. However, we did not detect a fluorescent signal in

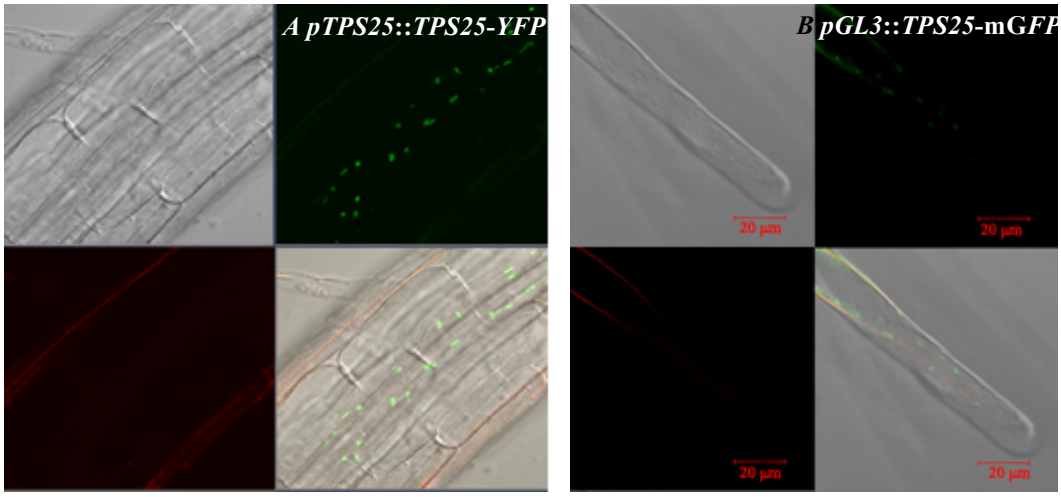


Figure 2.9 Cell type specificity of TPS25-YFP and TPS25-mGFP protein expressed under the control of different promoters.

YFP was detected by confocal microscopy. Green color indicates YFP fluorescence. Red color indicates auto-fluorescence of plants. Grey images transmission light images. Images were overlaid to indicate the localization of fluorescence signal in roots.
(A) Images of roots from plants expressing enhanced YFP fused to TPS08 under the control of the TPS25 promoter; (B) Images of TPS25-mGFP in roots of transgenic plants expressing TPS25 from the *GL3* promoter.

N-cells in this experiment (data not shown). Protein expression was also detected in the outside layer of root tips in *pGL3*::*TPS25-mGFP* plants (data not shown), however, the signal was very weak.

*Emission of (*E*)-β-farnesene from transgenic plants*

To determine whether gene transcript and the observed fluorescence protein levels would correlate with the emission of (*E*)-β-farnesene, we measured farnesene emission from roots of the different transgenic lines using SPME/GC-MS (Figure 2.10). Wild type plants that were induced with JA had the highest emission of (*E*)-β-farnesene among all plants. JA-treated *pTPS25*::*TPS25-YFP* plants had also comparatively high levels of emission, however, it was less than WT induced by JA. Background levels of (*E*)-β-farnesene were detected in wild type plants prior to JA induction, but they were much less than those from the induced roots. Interestingly, substantial emission of (*E*)-β-farnesene was observed in roots of the transgenic p*GL3::TPS25-mGFP* line without JA treatment.

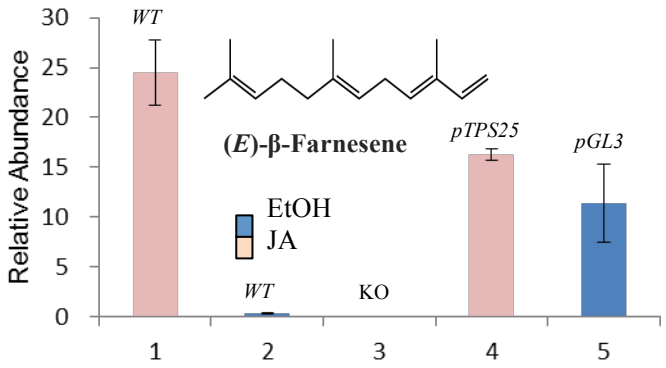


Figure 2.10 (*E*)-β-Farnesene emission from *Arabidopsis* roots.

(*E*)-β-Farnesene emissions from 1 g of axenic grown roots using SPME in the presence and absence of JA (100 µM, 24 h). **1**&**2**. WT; **3**, *tps22*/*tps25*; **4**, *pTPS25*::*TPS25-YFP*; **5**, *pGL3*::*TPS25-mGFP*.

2.4 Discussion

Cell type specificity of gene expression

Spatial regulation of chemical defenses in plants is assumed to be a result of contact and co-evolution with herbivores and pathogens (Reymond et al., 2004; Jander and Howe, 2008; Prasad et al., 2012). The diversity of terpene metabolites that, like many other

secondary metabolites, mediate plant-environment interactions arises through the evolution of large gene families of TPS proteins (Chen et al., 2011). The divergence of species-specific TPS families is a consequence of gene duplication and neo-functionalization. As part of this process, cell type specificity may emerge by changes in promoter sequence and activity. For example, both *TPS22* and *TPS25* produce a blend of sesquiterpenes with (*E*)-β-farnesene as the major product. For both genes, GUS staining was detected mainly in primary and lateral roots and to some extent in leaves and flowers (Huh, 2011). However, *TPS22* is mainly expressed in the root tip, while the *TPS25* promoter activity appeared in the cortex and possibly vascular tissues in the root elongation zone, but not in the root tip (Huh, 2011).

Our experiments to express *TPS* genes under different cell type-specific promoters led to the observation of different transcript levels of the target genes. These differences can simply be the result of different strengths in promoter activities or could be a consequence of differences in regulatory elements, post-transcriptional regulation, including RNA stability in the different cell types. To further investigate these aspects, transcript abundance of the endogenous genes driven by the individual promoters need to be determined and compared to the respective *TPS* transcript levels. Overall, expression of all three *TPS* genes was possible in all cell types investigated and no cell type was found that entirely suppressed the transcription of these *TPS* genes.

Analysis of the YFP signal provided some confirmation for expression of the TPS proteins in the different cell types. For example, the *GL3* promoter has been proven to drive gene expression in H-cells. From the fluorescent image of *pGL3::TPS08-YFP*, we could detect a bright signal in root hairs of the plant. Fluorescent signals were also detected in TPS13 and TPS25-YFP plants under control of the *GL3* promoter. However, they were much weaker with the possible reason of incorrect protein folding. Overall, by using confocal microscopy observation only, the YFP fusion lines did not allow for an exact quantitative analysis of protein abundance. Additional experiments are required to determine protein levels by Western blot or ELISA analysis of myc-tagged TPS proteins. Transgenic plant lines expressing these proteins have been established.

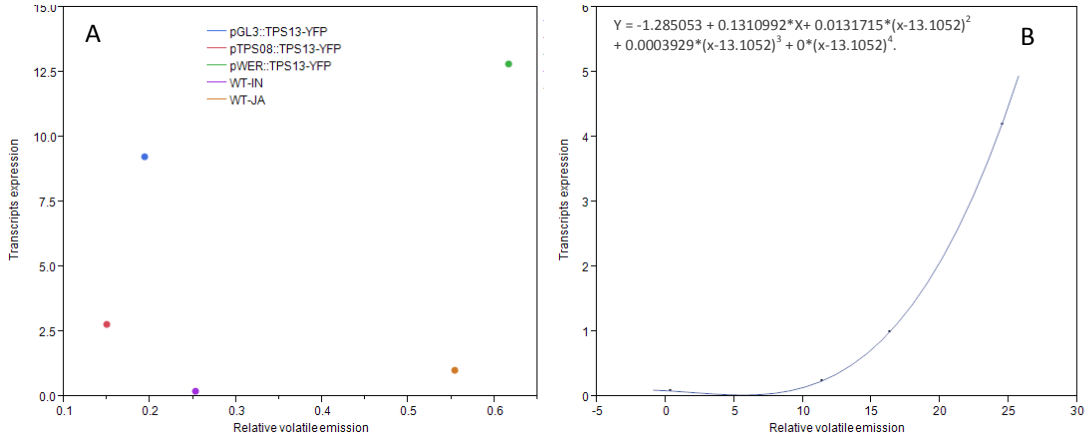


Figure 2.11 Correlation between TPS13 transcript and volatile compound emission levels.

(A) Points were plotted with *TPS13* transcripts level of each line as Y axis and relative (*Z*)-γ-bisabolene volatile emission as X axis. No correlation between gene transcript levels and volatile emission was observed. **(B)** Points were plotted with the *TPS25* transcripts level of each line as Y axis, X axis is (*E*)-β-farnesene emission; volatile emission increased with gene transcript levels in a sigmoidal fashion.

We could not find any correlations between gene transcript and metabolite levels for all TPS08-YFP fusion lines. Since this was also the case for the complementation line,

we assume that the protein fusion affected the enzymatic function of TPS08. Thus, similar analyses need to be performed with lines expressing TPS08 fused to a myc tag or without any fusion tags. In *pTPS08*::*TPS13-YFP* or *pGL3*::*TPS13-YFP* lines, the TPS13-YFP transcript levels were higher than in wild type plants that were induced with JA. However, these lines had less bisabolene emission than the JA-treated WT roots (Figure 2.11 A). One possible reason may be substrate limitation although TPS13 shows some endogenous expression in the stele of the differentiation and elongation zones. Another possibility is a limited stability of the TPS13-YFP fusion protein in these cell types. Relatively good correlations between transcripts and metabolite levels were found for TPS25 in complementation lines with the native promoter and when expressed in epidermal N-cells (Figure 2.11 B). The latter result indicates that sufficient substrate (FPP) for the formation of β-farnesene is available in leucoplasts of epidermal cells.

In conclusion, the presented work provides preliminary evidence for the plasticity of *TPS* gene expression and terpene formation in different root cell types. Additional experimentation is required using several independent plant lines and expressions of the *TPS* genes with a smaller protein tag to allow an accurate quantification of proteins and prevent the loss of protein function due to potential aberrant protein folding.

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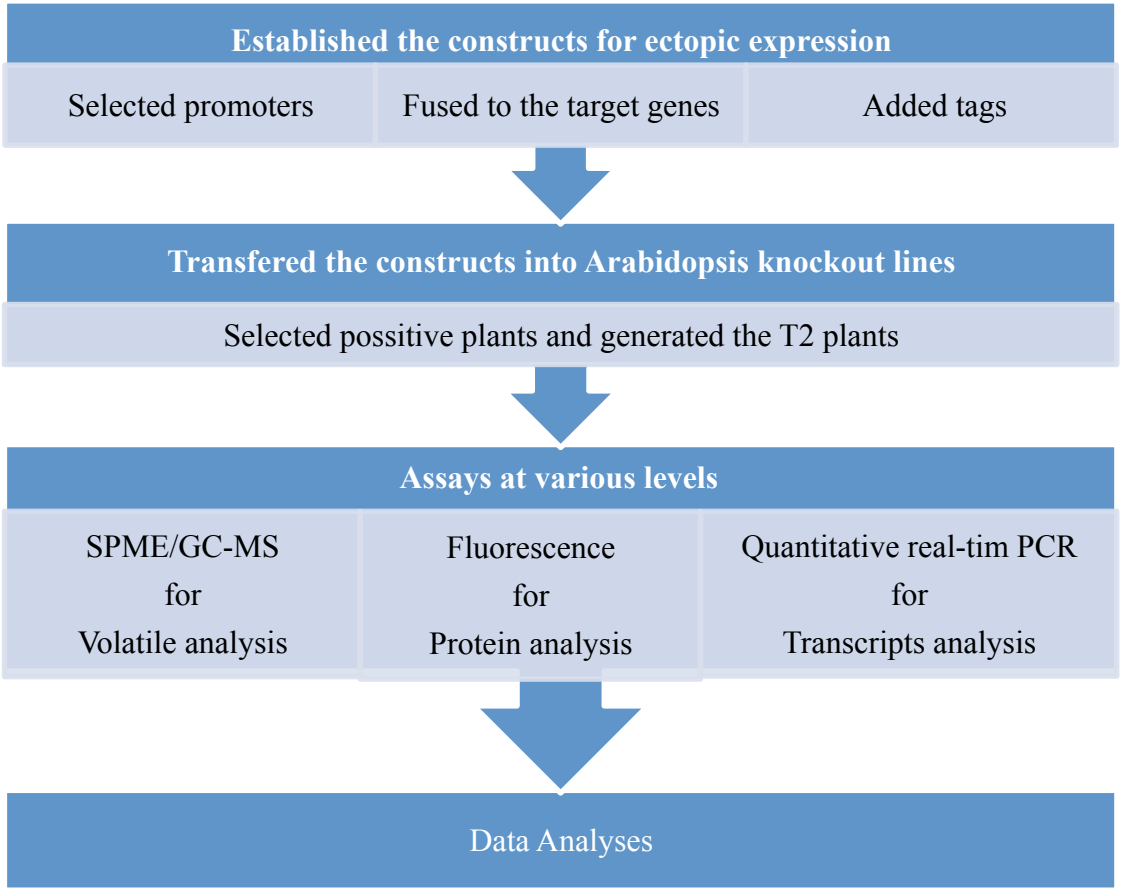
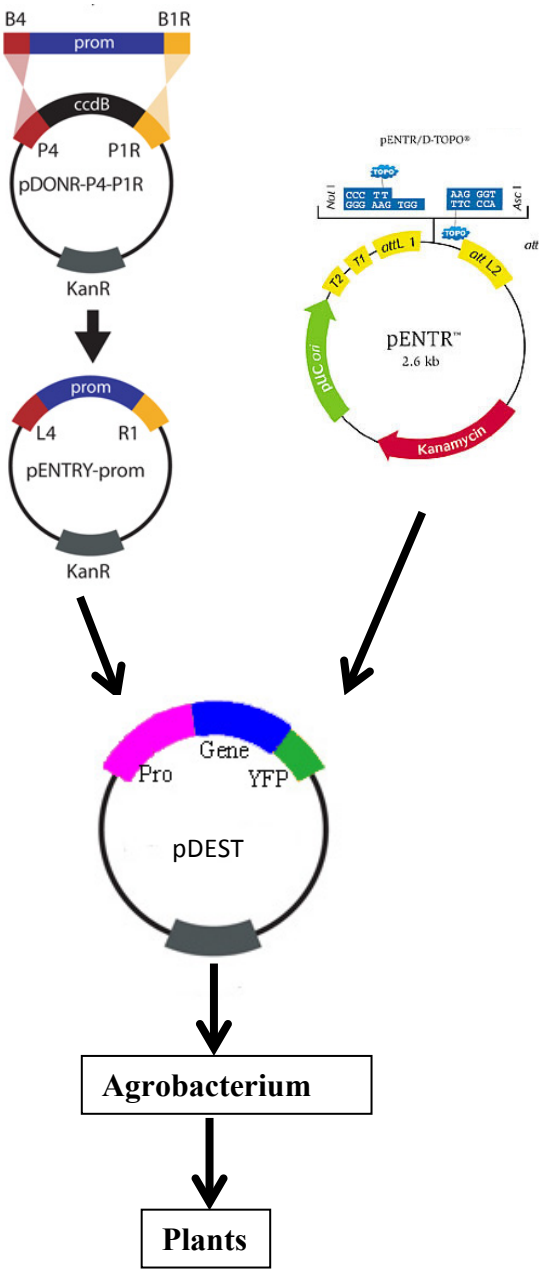
Appendix A

Flow chart of experiment



Appendix B

Vector structures



Appendix C

TableA1 Primers for quantitative Real-Time PCR

Name	ID	Primer Name	Primer Sequences
TPS08	At4g20210	TPS08 Q-RT F	AGAGAATTGGCGTCAGAAGG
		TPS08 Q-RT R	ACCTCACCAACTTCCATGTAC
TPS13	At4g13300	TPS13 Q-RT F	CGATGATTCGGAGTTAGACGC
		TPS13 Q-RT R	CCTCTTTGATGTCTCATCATCACC
TPS25	At3g29410	TPS25 Q-RT F	AAATGACATTGTCACGTTCGAG
		TPS25 Q-RT R	CGCTGCTTCTTTGGTGACACCA
UBC	At5g25760	UBC Q-RT F	AGTCCTGCTTGGACGCTTCA
		UBC Q-RT R	GAAGATTCCCTGAGTCGCAGTT

Chapter III

Analysis of Transcriptional and Metabolic Changes in Arabidopsis Roots in Response to Herbivory by Fungus gnat (Bradysia) Larvae

Chapter III Analysis of Transcriptional and Metabolic Changes in *Arabidopsis* Roots in Response to Herbivory by Fungus gnat (*Bradysia*) Larvae

Abstract

Plant defense against belowground herbivory is essential to maintain efficient uptake of water and nutrients. Despite growing evidence of root-specific chemical defenses against herbivores and the interactions between herbivore-induced above and belowground defense systems, a comprehensive understanding of the metabolic, biochemical, and molecular responses of roots to herbivory is missing. We developed an *Arabidopsis thaliana* aeroponic culture system that allows the generalist root herbivore *Bradysia* (fungus gnat) to feed on plant roots in a soil-like environment. Microarray analysis was used for profiling transcript changes in response to feeding for 4 days. Genes involved in signaling pathways, wounding, or other stimuli were identified as potential defensive genes. Moreover, several genes involved in primary metabolism, such as dark inducible 11 (*DIN11)*, dark inducible 2 (*DIN2)*, pyruvate orthophosphate dikinase (*PPDK)*, were up-regulated more than 2 fold after a 4-day feeding period. Microarray results were in part confirmed by quantitative real-time PCR. Responses in central carbon and nitrogen metabolism in roots were also characterized by performing GC-MS analysis. However, only marginal changes in the representative primary metabolite levels were observed due to a large within group variance. Sucrose was the only metabolite showing statistically significant changes in its relative steady-state levels in response to *Bradysia* larvae feeding for 4 days.

3.1 Introduction

Roots are the main plant organs for absorbing water and nutrients. Therefore, it is crucial for plants to employ efficient mechanisms to protect roots against agents causing various biotic stresses such as soil-borne pests. Several strategies have been described about how plants cope with belowground herbivory (Rasmann and Agrawal, 2008b). One such strategy involves the constitutive or induced formation of secondary metabolites. For example, (*E*)-β-caryophyllene, a sesquiterpene that is emitted from maize roots, strongly attracts entomopathogenic nematodes that attack larvae of the Western corn rootworm, *Diabrotica virgifera* (Rasmann et al., 2005b). The diterpene, rhizathalene, which is produced by *Arabidopsis* roots, plays a role in directly repelling larvae of the opportunistic root herbivore *Bradysia* (fungus gnat) (Vaughan et al., 2013). Glucosinolates present another classical example of plant direct defense mainly in the family *Brassicaceae*. These compounds are converted into toxic breakdown products by the enzyme myrosinase, which is stored in specialized plant cells and released when plant tissue is damaged (Hopkins et al., 2009).

Plant responses to herbivory also involve changes in primary metabolism. These changes can relate to growth compensation (Trumble et al., 1993; Agrawal, 2000). Moreover, nitrogen- and carbon-resources such as free or protein-bound essential amino acids and carbohydrates are important nutrients for herbivores; thus, their depletion or re-allocation can affect herbivory. For instance, the reallocation of stored resources from roots to shoots sustains plant regrowth and reproduction after attacked by herbivores (De Jong and Van Der Meijden, 2000). Plants also reallocate their carbon resources away from the site of foliar damage to be stored in roots and used later for seed production. For

example, C sources were shown to be exported from leaves of *Populus nigra* L. (Salicaceae) in response to feeding by *Lymantria dispar* (gypsy moth) (Babst et al., 2008).

Relatively few studies have investigated global transcriptional and metabolic changes in roots. But several examples have been presented for transcriptome changes in response to herbivory by generalist and specialist insects on aboveground tissues (Masclaux et al., 2012). We have recently developed an aeroponic culture system based on a calcine clay substrate that is suitable for *in situ* feeding assays with *Bradysia* on *Arabidopsis* roots (Vaughan et al., 2011; Vaughan et al., 2013). At the same time, this culture system allows the recovery of clean root tissue. We have used this system to examine metabolic and transcriptional changes in roots in response to herbivore attack. We hypothesize that belowground herbivory leads to root chemical defense responses mediated by different signaling pathways and possible changes in primary metabolism to re-allocate or alter C- and N-resources. The primary objectives of this study were to:

(i) Examine transcriptional changes in roots over a time course of four days of *Bradysia* feeding by a preliminary microarray experiments followed by real time PCR for selected genes and;

(ii) Analyze potential changes in the abundance of primary metabolites including amino acids, sugars, and carboxylic acids.

3.2 Materials and Methods

Plant material and culture conditions

Arabidopsis thaliana ecotype Columbia (Col-0 6000) was cultivated in an aeroponic culture system. The culture protocol was modified from the method described by Vaughan et al. (2010) as follows: *Arabidopsis* seedlings were grown on small moist Jiffy-7 peat soil pellets (Jiffy, Canada) (Figure 3.1 A). Approximately 100 seedlings were placed in a sealed Petri dish, vernalized at 4 °C for 3 days, and then transferred to a growth chamber for germination. The culture conditions were 20 °C to 21°C, with 10 h/14 h day/night light cycle and 150 μmol m^{-2} sec^{-1} photosynthetically active radiation (PAR). Seedlings with the first pair of true leaves were each transferred to a 50 ml plastic conical tube (Fisher scientific, Suwanee, GA) filled with Seramis® clay granules (Seramis, Germany). The clay granules were moistened with Hoagland's solution modified from Gibeaut (1997) (Appendix IV). Tubes were wrapped with aluminum foil to reduce growth of algae. Four 3.5 mm diameter holes were drilled around the bottom of the tube to allow medium exchange. Two 5 mm diameter holes were drilled at the middle of the tube for releasing larvae. The tubes were then placed in a rack (Figure 3.1 B), which was sealed with plastic wrap until the roots of plants had grown into the clay granulate. Clay granules were kept moist by submerging the culture tubes in Hoagland's solution for approximately 30 minutes every other day. Eight-week old plants that started to bolt were used for feeding experiments.

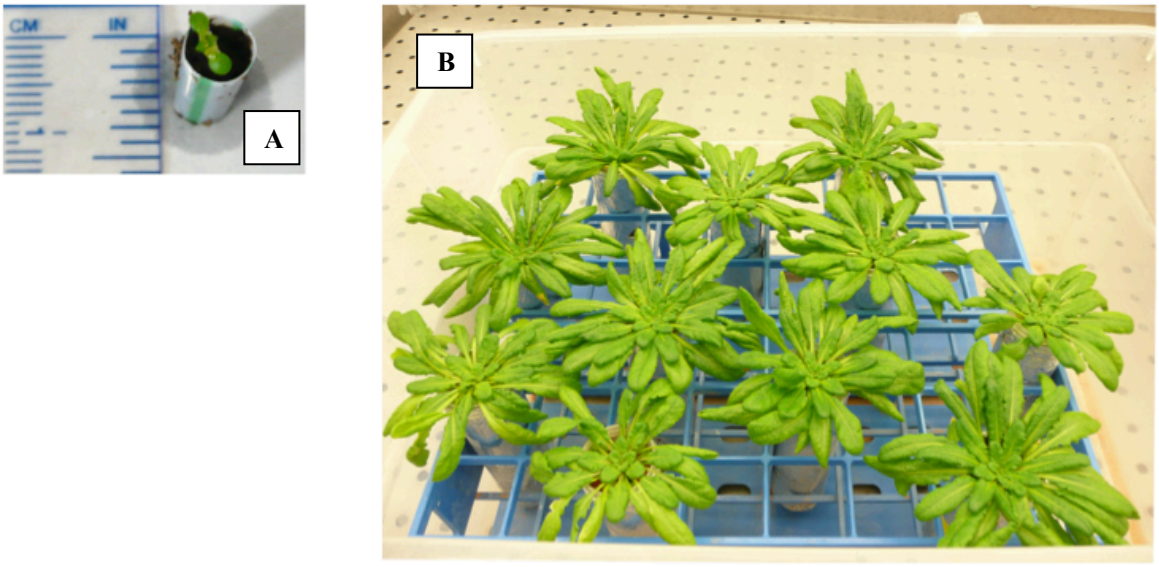


Figure 3.1 Aeroponic culture system for investigating belowground herbivory on *Arabidopsis.*

(A) *Arabidopsis* seedling grown on Jiffy substrate; (B) seven-week old plants grown in aeroponic culture.

Bradysia *(fungus gnat) culture and isolation*

Approximately one hundred fungus gnat larvae were picked from the soil of contaminated pots by using toothpicks and subsequently used to establish a laboratory colony. The colony was maintained in 8 L plastic containers that were covered by mesh cloth. The culture medium was composed of 2 L of moist Sunshine mix #1 medium and enriched with approximate 1 kg of shredded potato (Cloyd, 2004). Cultures were kept under controlled conditions at 21 to 23 °C with 10 h/14h day/light photoperiod. Half of the medium mixture, which included *Bradysia* larvae, pupae, adults and eggs, was transferred to fresh potato medium every 3-4 weeks.

Bradysia larvae were isolated from the soil-potato medium following the procedure previously described by Vaughan (Vaughan, 2010). Briefly, 1 L of *Bradysia* culture was placed into a 2 L wide-mouth Erlenmeyer flask and mixed well with warm tap water.

After the larvae were released in the water, the flask was inverted and placed in a 2 L graduated cylinder filled with cold water. Denser material including fungus gnat larvae sank from the flask into the cylinder while most of the medium remained in the flask. After approximately 5 minutes, the water, larvae and remaining organic matter in the cylinder were poured into an 8 L 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 into the sediment. Due to the low density of water compared to the $MgSO_4$ solution, fungus gnat larvae were floating on the surface, while most of the remaining organic matter sank to the bottom. Larvae were then collected and transferred to another container. After rinsing in water, larvae were placed on moist filter paper in a small container and starved overnight prior to the feeding assay.

Feeding experiment and biomass measurement

Bradysia feeding assays followed the procedure developed by Vaughan (2010). Eight-week old *Arabidopsis* plants were challenged with fungus gnat larvae. After overnight starving, larvae were submerged in the dish with 1 ml of Hoagland's solution and then released with a Pasteur pipette into the clay granules. Approximately 200 to 300 second- and third-instar larvae were collected and transferred to a single aeroponic culture tube as the experimental (feeding) group. Around 3/4 of larvae were released from the top of the tubes and ¼ were released from side holes. Feeding assays were performed for 12 hours, 1 day, 2 days and 4 days. For each time point, 6 plants were challenged as the feeding group and 6 plants were set as the control group. The experiment was repeated independently three times.

To harvest roots at different times of feeding, the whole culture tube was submerged into Hoagland liquid medium and clay granules were removed completely from plant roots. Roots and rosettes were separated, dried briefly on paper towels, and the fresh weight of roots and rosettes was recorded. One hundred milligrams of fresh tissue were then removed from each root and stored in a 1.5 ml microcentrifuge tube for gene expression analysis. The remaining sample was lyophilized for three days and the dry weight was recorded. All samples were stored at -80 °C for the subsequent experiments. For data analysis, a one-way analysis of variance (ANOVA) was performed on the ratio of root to rosette dry weight. Because 100 mg of root tissue was removed before lyophilization, the total root dry weight was calculated as:

$$Total\ root\ dry\ weight = Measured\ root\ dry\ weight \times \frac{Root\ fresh\ weight}{(Root\ fresh\ weight - 100\ mg)}.$$

RNA isolation

Total RNA was extracted by using the Trizol method (Piotr Chomczynski, 1987) following the manufacturer's (Invitrogen, Carlsbad CA, USA) protocol. Approximately 50 mg of root tissue was ground under liquid nitrogen using a plastic pestle in a 1.5 ml centrifuge tube and then immediately extracted with Trizol.

To avoid genomic DNA contamination, RQ1 RNase-Free DNase (Promega, Medison WI, USA) was added to the extracted RNA sample. RNA quality and quantity were measured with a Nanodrop spectrophotometer (BioRad).

Microarray analysis

Five hundred nanograms of total RNA from each plant in the same group were pooled. To fit the RNA quality requirements for microarray analysis, the pooled RNA samples were cleaned using an RNeasy kit (Qiagen, Venlo, Netherlands). Conversion into labeled cDNA and hybridization were performed at the Virginia Bioinformatics Institution (VBI) following standard protocols. The raw data analysis, including R^2 statistic and slope calculation, normalization, and significant analysis were performed in collaboration with Dr. Rick Jensen (Department of Biological Sciences). After obtaining the raw data, gene expression was scored by GCOS software (Affymetrix). GeneSpring (SiliconGenetics) and JMP were used for further statistical analysis. Candidate genes that showed significant changes in root-specific expression were selected for further qRT-PCR assays.

qRT-PCR

The mRNA from each sample was treated with M-MLV reverse transcriptase (Promega, Medison WI, USA). Five hundred nanograms of total RNA were used as template in a 10 µl reaction.

Gene specific primers of *DIN11*, *SCPL30* (serine carboxypeptidase-like 30), *THA1* (*threonine aldolase 1*), *PPDK* and *JAZ10* (jasmonate-ZIM-domain protein 10) were designed for qRT-PCR (Table 3.1). The housekeeping ubiquitin-conjugating gene (*UBC*) was used as the internal standard. Approximately 8 ng of cDNA and 60 nmol of each gene-specific primer was used in a 20 µl volume. Ten µl 2×SYBR Green PCR Master Mix (Applied Biosystems, UK) was added to every reaction. Reactions were performed in a 7300 Real-time PCR System (AB Applied Biosystems®). The amplification curve

and absolute *Ct* values were obtained from the build-in software. Mean value of $2^{(-\Delta\Delta Ct)}$ were used to present the fold changes of gene expression.

Table 3.1. Gene-specific primers for quantitative real-time PCR.

Name	ID	Primer Name	Primer Sequences	Tm (°C)
DIN11	At3g49620	DIN11 Q-RT F	CTACTACCAGTTATCGACATCAGTCC	59
		DIN11 Q-RT R	CCCATGACCAATCACGTAGAAG	60
SCPL30	At4g15100	SCPL30 Q-RT F	AGTTACGCAGGAATATACGTACCC	58
		SCPL30 Q-RT R	CATAATCCACCCAGCCTCTC	58
THA1	At1g08630	THA1 Q-RT F	CTTCCATTGCACTTGGAGTTC	58
		THA1 Q-RT R	TTGCGAACCAACGATTACAGAT	60
PPDK	At4g15530	PPDK Q-RT F	GCCGTGAAGATAGCAGTTGATA	58
		PPDK Q-RT R	GGATCATGAAACTGTGGGTGA	59
JAZ10	At5g13220	JAZ10 Q-RT F	GGTGAAATTATGAAGGTCGCTAATG	60
		JAZ10 Q-RT R	CTTGCGATGGGAAGATCTCC	60
UBC	At5g25760	PEX4 Q-RT F	AGTCCTGCTTGGACGCTTCA	60
		PEX4 Q-RT R	GAAGATTCCCTGAGTCGCAGTT	60

Gas chromatography-mass spectrometry analysis

Polar metabolites were extracted following a procedure adapted from (Collakova et al., 2008) Internal standards, 10 nmol ribitol in H_2O for carbohydrates and 10 nmol norvaline in 5 mM HCl for amino acids, were added to each 2 ml microcentrifuge tube and dried completely under vacuum (speed-vac) prior to extraction and derivatization. Two milligrams of dry root samples were transferred to each tube and accurate weights were recorded for normalization. The tissue was then extracted in 200 µl of 10 mM HCl for 5 minutes using three ø 4 mm glass beads per sample and a paint shaker. Following centrifugation at the max speed for 5 min to separate the aqueous phase from the tissue debris, two aliquots of 80 µl of the supernatant were each transferred to a glass insert (250 µl pulled point glass inserts, Agilent, Santa Clare CA, USA) in 1.5 ml glass vials (Agilent, Santa Clare CA, USA) and dried thoroughly with N_2 at 50 °C.

Amino acids and carbohydrates are not volatile and require derivatization prior to GC analysis. Two different methods were used to obtain complementary and unique information about changes in relative steady-state metabolite levels in response to herbivore feeding. Amino acids and carboxylic acids were derivatized with 25 µl MTBSTFA (N-tert- butyldimethylsilyl-N-methyltrifluoroacetamide) + 1% TBDMS (tert–butyldimethylsilyl) (Pierce, Rockford IL, USA) in pyridine (v/v=1:1) for 30 minutes at 50 °C. Polar metabolites in general (sugars, carboxylic acids, and amino acids) were derivatized first with 15 µl MOX·HCl (Acid methoxylamine) (Pierce, Rockford IL, USA) for 2 h at 50 °C and then with 15 µl of MSTFA (N-methyl-N-trifluoroacetamide)+1% TMCS (trimethyl silyl) (Pierce, Rockford IL, USA) for 30 minutes at 50 °C.

Gas chromatography (GC) was performed with an Agilent 7890A GC equipped with a 30 m DB-5 MS column (0.25 mm × 0.25 µm). The flow of the carrier gas was 1 ml/min Analysis-scan and positive EI mode was performed by using an Agilent 5975C series quadrupole mass spectrometer (MS). One microliter of derivatization product was injected for GC-MS. Conditions for GC/MS followed the procedure outlined in Appendix V Table A1.1.

Fragments having identified mass-to-charge (*m/z*) ratios were used to quantify the levels of metabolites. Peak integration was first processed automatically in Enhanced MSD ChemStation software (Agilent Technologies). Integrated areas were then verified manually with Quantitative Edit in the same software. First normalization was performed by comparison to the internal standard, and the relative amount of each metabolite was then normalized again by the samples' weight. JMP Pro 10 software (SAS Institute) was used for ANOVA analysis on the changes of metabolites in response to feeding damage.

Relative metabolite levels of individual samples were used in PCA on correlations using the same software.

3.3 Results

Analysis of biomass changes upon Bradysia feeding

Arabidopsis was cultivated in an aeroponic culture system for 8 weeks and then challenged with *Bradysia* for up to 4 days. Roots were harvested at 12 h, 1 d, 2 d and 4 d time points. We then examined the extent of the feeding damage by determining the ratio of root to shoot dry weight of *Bradysia*-exposed plants and controls over the time course of four days. Average root to shoot mass ratios decreased by approximately 15 to 25% in comparison to controls; however, these changes were not statistically significant ($a<0.05$, Figure 3.2).

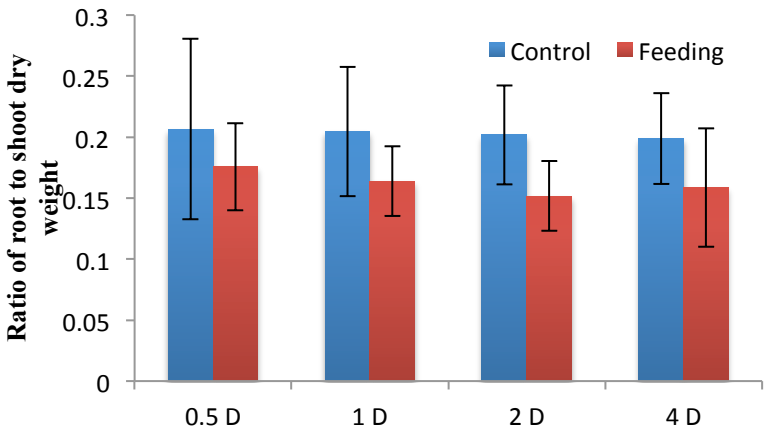


Figure 3.2 Effect of Bradysia feeding on root to shoot mass ratios.

Plants were harvested at four time points. Roots and rosettes were detached and lyophilized. To avoid the effect from differences in plant size, the ratio of root to shoot dry weight was determined. Data are means ± SD of three parallel experiments.

Gene expression differences in Arabidopsis roots in response to herbivory

Microarray analysis

Three independent feeding experiments were performed for microarray analysis. In

each experiment, *Arabidopsis* roots were harvested 4 days after *Bradysia* feeding. Total RNA was isolated and reversely transcribed for the assay. After raw data processing, the expression of a large percentage of genes was found to be unchanged or only marginally changed. Nevertheless, we identified 451 genes from 22810 microarray gene probes, whose expression was up-regulated more than 2 fold (>1 in log2 ratio) in comparison to the controls. Forty gene probes were down-regulated more than 2 fold.

We further used GO online software to categorize and classify the differentially expressed genes based on their function (Table 3.2, Figure 3.3). Several genes were identified that are known to respond to stimuli, such as wounding, phytohormones, sugar shortage, and so on, or are involved in cellular and metabolic processes (Figure 3.3). Many of the up-regulated genes were related to wound-induced and defense signaling responses mediated by the hormones JA or ethylene, such as lipoxygenases involved in jasmonate biosynthesis (*LOX1*) and genes that encode JAZ repressor proteins or ethylene response factors. Overlap in the hormone-regulated responses of these genes lead to their categorization in more than one group such as *LOX1* being classified as wound-, JA-, and oxidative stress- responsive gene (Table 3.2). The induced expression of JA- and ethylene-dependent genes suggests that root responses to herbivory similarly to those in aboveground tissues are regulated in a large part by the JA and ethylene signaling pathways.

We also found evidence for the up-regulation of genes involved in glucosinolate biosynthesis and triterpene biosynthesis. For example, sulfotransferase 2A (*ST2A*) and the arabidiol triterpene synthase (*AtPen1*) were up-regulated by factors of 5.85 and 2.4, respectively. Interestingly, several genes predicted to respond to herbivory, such as genes

involved in flavonoid biosynthesis, showed no or only marginally up-regulated expression. On the other hand, several unexpected genes were strongly induced. For instance, transcript levels of the genes *DIN 2* and *DIN 11* that are normally controlled by changes in sugar metabolism and show dark-induced response in leaves were up-regulated 17-60 fold (4-6 in log2 ratio) after feeding. This pointed to possible changes in carbohydrate metabolism upon *Bradysia* feeding. We also found other genes related to carbohydrate, N- and amino acid metabolism and N-mobilization that were up-regulated in response to *Bradysia* larvae feeding, showing in Table 3.3.

Table 3.3 Up-regulated genes in response to herbivory related to C- or N-metabolism

Gene	Name	Metabolic pathway involved in
GAPB	Glyceraldehyde bisphosphate dehydrogenase subunit B	Carbon-resource metabolism (Kwon et al., 1994)
BGLU18	Beta-glucosidase 18	Carbon-resource metabolism (Yamada et al., 2009)
SCPL30	Serine dependent carboxypeptidase	Protein degradation (Fraser et al., 2005)
THA1	Threonine aldolase	Threonine degradation (Joshi et al., 2006)
ATBCAT-2	Branched chain amino acid aminotransferase	Branched-chain amino acid metabolism (Binder, 2010)
PPDK	Pyruvate orthophosphate dikinase	Nitrogen mobilization (Parsley and Hibberd, 2006)

Groups of transcription factors and other signaling components also revealed different expression after feeding (Table 3.2).

Table 3.2 Representative genes and changes of their gene expression levels based on microarray and GO analysis.

Wounding response		
Public ID	Gene	Ratio
At4G35770	SEN1	4.29
At5G13220	JAZ10	3.91
AT1G43160	RAP2.6	3.23
At5G20230	ATBCB	2.34
At1G55020	LOX1	2.08
At1G80840	WRKY40	1.90
At1G76650	SML38	1.86
At1G06160	ORA59	1.80
At2G46510	ATAIB	1.38
At3G61190	BAP1	1.34
At1G72450	JAZ6	1.27
At2G24850	TAT3	1.23
At5G10300	MES5	1.10
At4G34710	ADC2	1.08
At1G17420	LOX3	1.04
At1G74950	TIFY10B	1.02

JA signaling		
Public ID	Gene	Ratio
At4g35770	SEN1	4.29
At5g24780	VSP1	4.27
At5g13220	JAZ10	3.91
At5g07010	ST2A	2.55
At1g70700	TIFY7	2.45
At1g17380	JAZ5	2.42
At3g15500	ANAC055	2.17
At4g37150	MES9	2.13
At1g55020	LOX1	2.08
At3g25760	AOC1	1.84
At2g34600	JAZ7	1.82
At1g06160	ORA59	1.80
At3g23240	ERF1	1.74
At2g31180	MYB14	1.59
At3g09940	MDHAR	1.59
At3g06490	MYB108	1.53
At1g72450	JAZ6	1.27
At2g24850	TAT3	1.23
At1g28480	GRX480	1.14
At4g34710	ADC2	1.08
At1g17420	LOX3	1.04
At1g74950	TIFY10B	1.02

Ethylene signaling		
Public ID	Gene	Ratio
At2g47190	MYB2	2.04
At2g44840	ERF13	1.92
At1g06160	ORA59	1.80
At3g23240	ERF1	1.74
At2g31180	MYB14	1.59
At3g06490	MYB108	1.53
At2g31230	ATERF15	1.33
At2g38210	PDX1	1.20
At4g17490	ATERF6	1.01

Oxidative stress		
Public ID	Gene	Ratio
At4g35770	SEN1	4.29
At2g29450	ATGSTUS	2.42
At1g55020	LOX1	2.08
At4g30775	ERD5	1.82
At3g09940	MDHAR	1.59
At5g16970	AT-AER	1.54
At1g20630	CAT1	1.28
At3g49120	PRXCB	1.08
At3g03190	ATGSTF11	1.02

>3 fold up-regulated		
Public ID	Gene	Ratio
At3g49620	DIN11	5.96
At4g15100	SCPL30	4.95
At1g08630	THA1	4.91
At4g35770	SEN1	4.29
At5g24780	VSP1	4.27
At3g60140	DIN2	4.08
At4g15530	PPDK	4.08
At5g22300	GAPB	3.96
At5g13220	JAZ10	3.91
At1g30135	JAZ8	3.49
At1g52400	BGLU18	3.42
At1g10070	ATBCAT-2	3.25

Glucosinolate and triterpene biosynthesis		
Public ID	Gene	Ratio
At5g07010	ST2A	2.55
At5g48180	NSP5	1.45
At1g62560	FMO GS-OX3	1.28
At4g15340	AtPen1	1.26
At1g65860	FMO GS-OX1	1.06
At1g62540	FMO GS-OX2	1.04

*Data are the mean of gene expression levels of three independent feeding experiments. The ratio represents signal of gene expression level changes in

Log2 ratio.

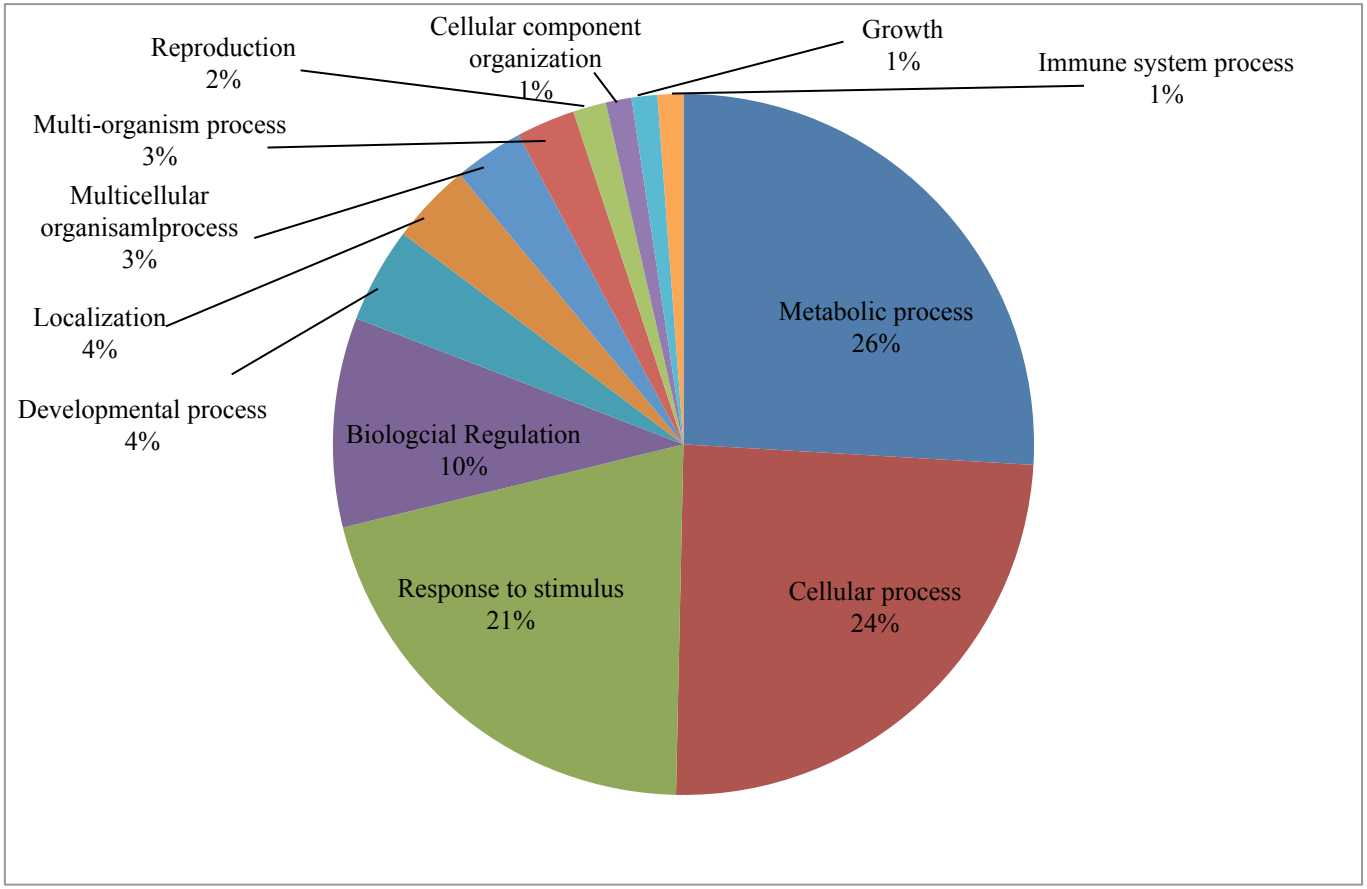


Figure 3.3 Pie-chart representation of GO annotation results for molecular function with a total number of gene counts of 451, which had potential responses to herbivory in this experiment.

Quantitative real-time PCR results

Three independent feeding experiments were conducted for QRT-PCR and metabolite analysis. Root samples were harvested at 12 hours, 1 day, 2 days and 4 days after larval feeding. For each time point, 6 plants were challenged as the feeding group and 6 plants were set as the control group. Although numerous changes of average gene transcript levels were observed from the three experiments, these changes were not considered to be significant (α=0.05) due to variation between experiments. In addition, the analysis of only a single time point (4 days) was not sufficient to predict genes transcriptional changes at earlier time points. To verify and further examine transcriptional differences over the full time course, we chose five of the most strongly induced genes involved in defense and primary metabolism to conduct additional quantitative RT-PCR analysis.

The two dark inducible genes, *DIN11* and *DIN2* were strongly up-regulated in the first 12 h of feeding activity. By the second day of feeding, expression of the *DIN* genes dropped back to a similar level as controls or even less and remained low throughout the experiment. *JAZ10* transcript levels showed a similar response although with an overall less pronounced induction. Despite previous micro-array results indicating an up-regulation of *THA1* and the *PPDK* genes, no such changes were observed by quantitative PCR; instead, these genes were found to be largely down-regulated (Figure 3.4). Because of the large variation of transcript levels between the three experiments, no significant differences were observed for most genes and time points. All genes showed

an unexpected drop in transcript levels in comparison to controls on day 2, which will be discussed below.

Changes of amino acid levels in Arabidopsis roots in response to herbivory

We were interested in changes in the relative steady-state levels of amino acids, as such changes may indicate differences in N-resource allocation or nutrient depletion in roots upon herbivory. Essential amino acids, such as threonine, leucine, phenylalanine, were of a special interest since they cannot be made by the herbivore and are needed for its normal growth and development. Concentrations of the six selected amino acids (Figure 3.5) were on average slightly increased on days one to four; however, these changes were not statistically significant on day 4 ($\alpha>0.05$).

Changes of carbohydrate and carboxylic acid levels in Arabidopsis roots in response to herbivory

In addition to potential changes in the levels of free amino acids, we determined possible changes in the level of sugars and carboxylic acids in response to *Bradysia* feeding by using GC-MS. Differences in the level of these metabolites could indicate changes in C-resource availability related to changes in central carbon metabolism. Figure 3.6 shows relative level of monosaccharides including mannose, arabinose and xylose; the disaccharides, sucrose and fructose, and the carboxylic acids citrate, malate, and glycerate. Overall, we did not find significant differences in the level of these metabolites in the first two days. A significant increase in level was only observed for sucrose on day 4 of the feeding experiment ($\alpha>0.05$).

Moreover, to investigate the internal relationship among metabolites, we performed principle component analysis (PCA) on all the metabolites that have been profiled. However, the results showed that there was no strong correspondance among metabolites in response to herbivores (date not shown).

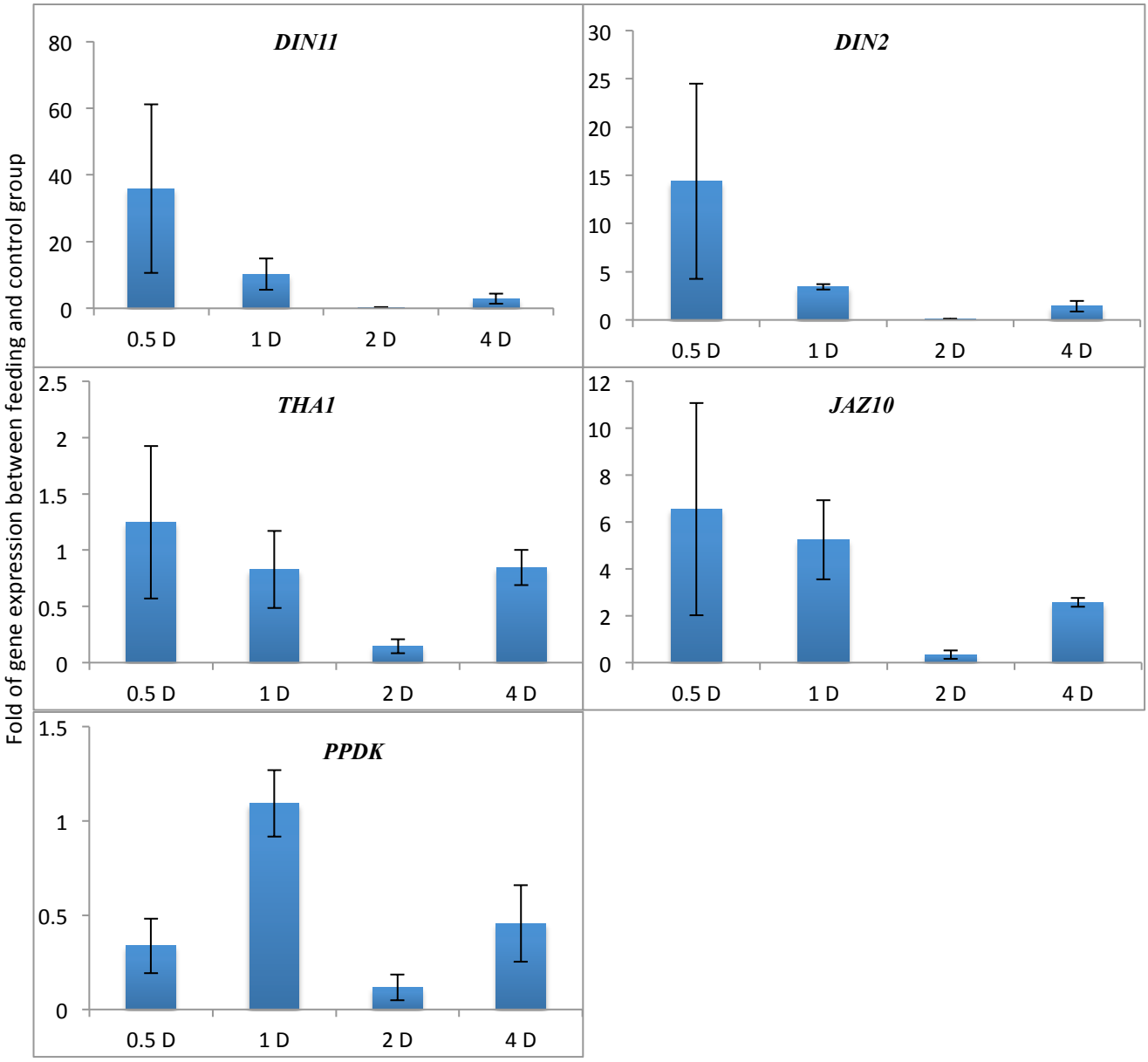


Figure 3.4 Relative differences of transcript levels of target genes between feeding and control groups.

Five-hundred ng of mRNA were isolated from six plants each and pooled. Expression of selected genes mRNA in *Arabidopsis* root tissues was examined by quantitative RT-PCR analysis and is presented as relative mRNA expression (means of ΔΔCt±SEM, *n=3*).

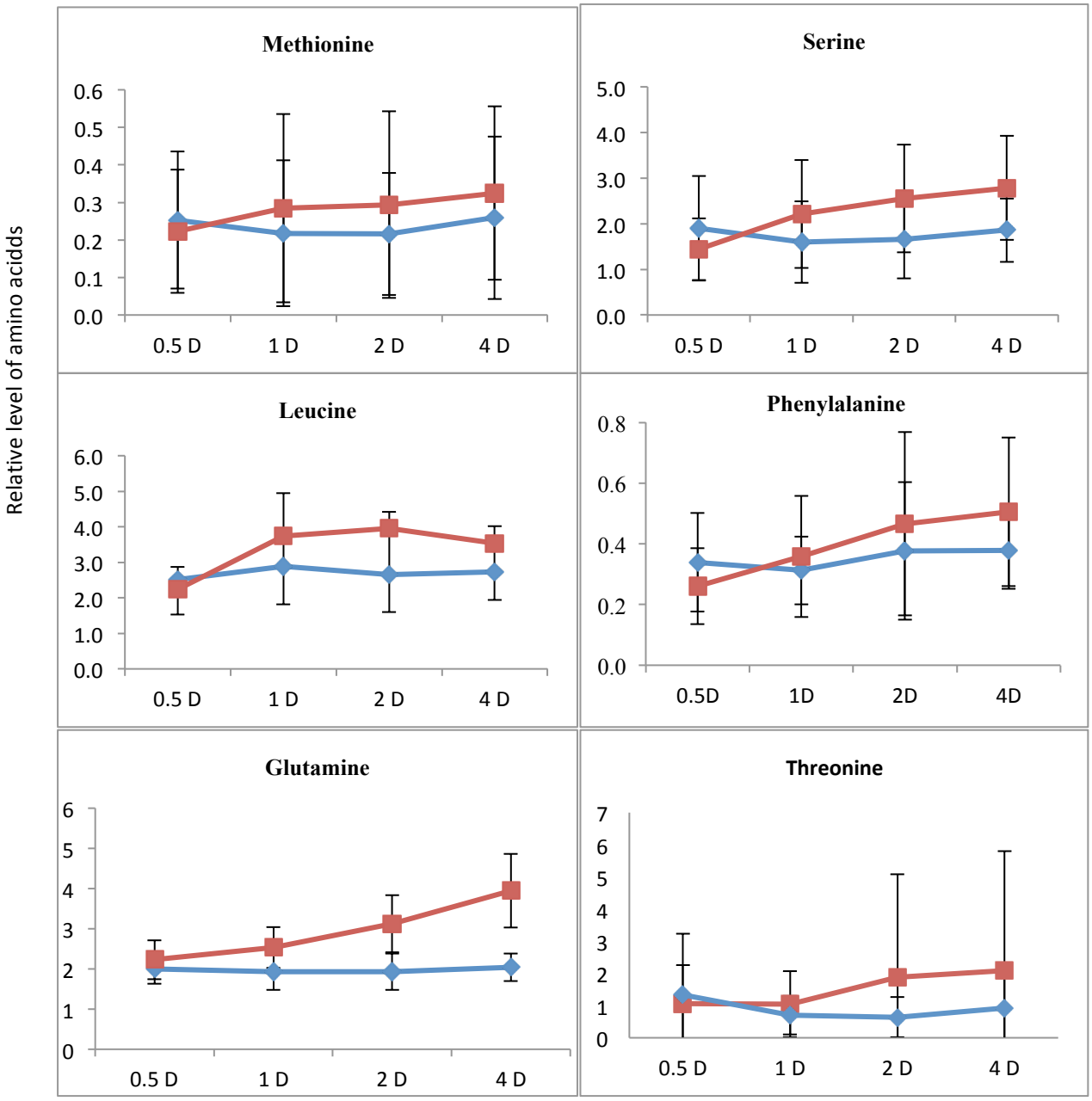


Figure 3.5 Relative changes in the level of six amino acids in Arabidopsis roots upon *Bradysia* feeding.

Time course of the changes in the levels of serine and five essential amino acids are shown. Amino acid abundance signals were normalized by using the internal standard norvaline and root dry weight. Red lines and squares are the feeding group; blues lines and diamonds are the control group. Values are means ± standard deriation (n=15). D is the number of day after feeding.

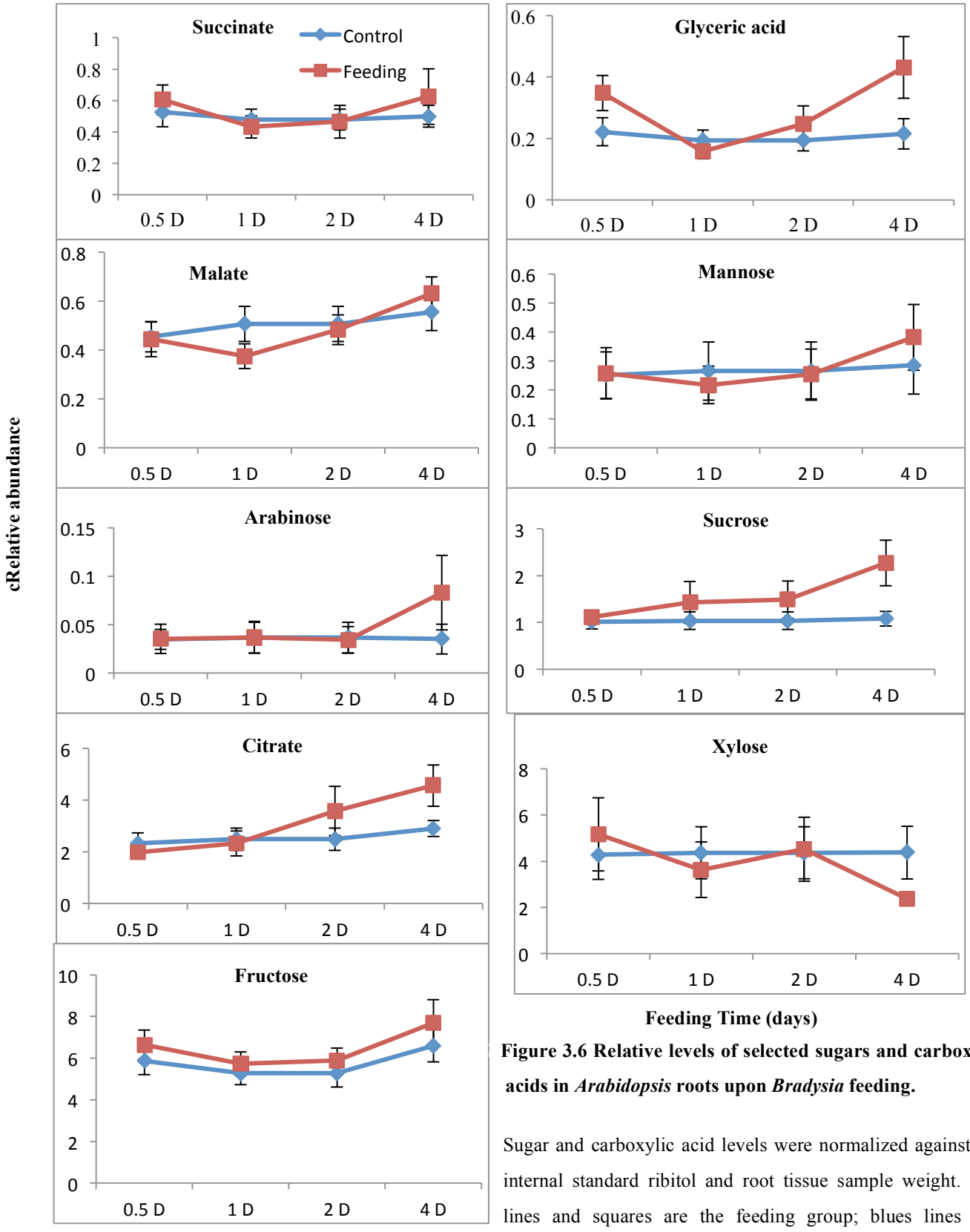


Figure 3.6 Relative levels of selected sugars and carboxylic acids in *Arabidopsis* roots upon *Bradysia* feeding.

Sugar and carboxylic acid levels were normalized against the internal standard ribitol and root tissue sample weight. Red lines and squares are the feeding group; blues lines and diamonds indicate the control group. Values are means ± standar derivation (n=15). D in each figure is day.

3.4 Discussion

Bradysia feeding affects the expression of genes involved in metabolism and defense signaling

Herbivory affects plants at gene transcript (Moran et al., 2002; Reymond et al., 2004) and metabolite levels (Mercke et al., 2004; Bolton, 2009; Tholl and Lee, 2011; Kerchev et al., 2012). The responses of plants to herbivory in rhizosphere, as well as the aboveground part of plants, are regulated by sophisticated and dynamic immune responses. In a preliminary microarray experiment, we found approximately 2% of the *Arabidopsis* genes to be up-regulated in roots upon larval feeding. Although several of these genes have not been functionally annotated, GO analyses showed that 26% of those genes with predicted or known functions play a role in primary or secondary metabolism, while 24% are involved in cell signaling or in responses to stimulating factors (Figure 3.3) For example, 22 and 9 genes, with confirmed function in JA and ethylene signaling, respectively, were up-regulated more than 2-fold after feeding. Sixteen of the up-regulated genes showed responses to wounding. Nine of the up-regulated genes have been reported to be involved in oxidative stress. Compared to other studies, we obtained only a small number of genes that were up-regulated more than 2-fold (>1 in Log2 ratio, $p<0.05$). For instance, Reymond et al. (2004) identified more than 100 genes among 12,135 (8%) upon feeding of the specialist insect, *Pieris rapae*, in *Arabidopsi*s. Feeding by the generalist *Spodoptera littoralis*, caused an almost identical change of transcript

profiles as *P. rapae*. 30% of probes that changed in *Arabidopsis* leaves after treatment with aphids for 72 h (Moran et al., 2002). GO analysis showed that genes, which have responses to defensive phytohormones, wounding, or oxidative stress, were identified in all the experiments. These results in comparison to our study may indicate differences in the responses of leaves and roots to different herbivores and their extent of feeding damage.

We found the dark-inducible genes, *DIN11* and *DIN2* to be up-regulated 62- and 17-fold, respectively, based on the microarray results. *DIN*s were first characterized in leaves exposed to dark conditions and are associated with senescence and sugar depletion (Oh et al., 1996; Fujiki et al., 2000; Buchanan-Wollaston et al., 2005). *DIN11*, which encodes a putative dioxygenase, and *DIN2*, which encodes a putative beta-glucosidase, are typically induced after 24 hours of dark treatment or treatment with exogenous photosynthesis inhibitors (Fujiki et al., 2001). From the quantitative real-time PCR, the two genes showed the highest up-regulation after 12 h, which suggested a possible depletion of sugars in the roots, especially at the first 12 hours after feeding. However, the expression of the *DIN* genes then decreased substantially. Up to the second day after feeding, expression of *DIN11* and *DIN2* in feeding groups was only 1/5 and 1/10 of the control groups, respectively (Appendix Table A2). Possible correlations of *DIN* gene expression and sugar levels could not be confirmed since we did not find any substantial changes in sugar concentrations in insect damaged and control roots. It can, therefore, be assumed that *DIN* genes are induced under dark conditions by defense hormone stimuli

such as JA independently of changes in sugar levels. The decline of expression on day 2 and 4 might be a consequence of overall reduced feeding activity due to pupation of a specific number of larvae. This is also reflected in a minimal continued reduction of root biomass on these days (Figure 3.2).

Another gene that was found to be up-regulated encodes threonine aldolase THA1, which converts the essential amino acid threonine to the non-essential amino acid glycine. The mechanism is similar to the enzymatic conversion of threonine by threonine deaminase, which was found to be induced by herbivory on tomato leaves (Chen et al., 2005). We hypothesize that this conversion could cause the degradation of threonine released from hydrolyzed protein in the herbivore digestive system thereby reducing the availability of this essential amino acid (Joshi et al., 2006). Surprisingly, the expression pattern of *THA1* based on the qRT-PCR analysis showed only limited changes in expression level. This inconsistency may be caused by the fact that microarray and real time PCR results were obtained from two different sets of experiments. Also, the plants for the microarray experiment were transferred from soil to the clay culture system at week four. But for real time PCR, the plants were first cultured in a very limited amount of Jiffy medium and then transferred to the clay culture system at the seedling stage. The changes in culture environment could have affected the overall feeding activity of the larvae or the gene expression of plants.

The pyruvate orthophosphatedikinase PPDK has been implicated in linking carbon and nitrogen metabolism (Parsley and Hibberd, 2006; Taylor et al., 2010; Astley et al., 2011)

and playing a role in remobilizing nitrogen during senescence (Taylor et al., 2010). The *PPDK* gene was up-regulated according to the microarray results and showed highest expression on day 1 based on quantitative PCR results. In contrast to the *DIN* genes, it is possible that *PPDK* transcript induction declines over day 2 and 4.

We also identified several genes with function in defensive signaling. For example, jasmonates are key regulators of plant responses to feeding, wounding, and other biotic or abiotic stresses. They are also involved in for root growth and in the development of plant reproductive organs (Sandorf and Hollander-Czytko, 2002; Chen et al., 2005; Delker et al., 2007). Jasmonate ZIM-domain (JAZ) proteins are important regulators in the jasmonate signaling pathway by repressing the transcript factors of JA responsive genes (Chini et al., 2007). At least two genes in the JAZ family were up-regulated in response to herbivory. The sharp changes of *JAZ10* expression level in our experiments may relate to the degradation and restorage of JAZs and the activity of JA signaling pathway as the defense response.

We noticed an unexpected drop in the expression of all genes on day 2 of the experiments. This reduction in expression could be the consequence of overlapping signaling responses with a peak of a possible JA-antagonistic signal on day 2. Salicylic acid is known to have antagonistic activity with JA (Takahashi et al., 2004; Van der Does et al., 2013). However, it is also possible, that the observed gene reduction is an artifact of the feeding experiment based on reduced watering and possible additional stress

effects during larval feeding. Modifications in the experiment and measurements of hormone levels could provide further clarification.

Metabolic responses upon Bradysia feeding

Plant defense at the primary metabolism level may follow three strategies: 1, compensatory growth, which will cause an up-regulation of primary metabolism (Reymond et al., 2004; Rizhsky et al., 2004; Frost et al., 2008); 2, nutrient depletion, which decreases nutrients essential to the attacking organism (Chen et al., 2005; Chen et al., 2010; Masclaux et al., 2012); and 3, nutrient transfer or reallocation from feeding sites to other tissues (Bazzaz et al., 1987; Bezemer and Van Dam, 2005; Kempema et al., 2007; Masclaux et al., 2012)

Although not significant in most cases, we observed a trend for an increase in the concentration of amino acids as well as some sugars (sucrose, arabinose) and organic acids (glyceric acid, citrate). This increase might be indicative of a locally enhanced N- and C-metabolism and de novo biosynthesis of sugars and amino acids to support root respiration and defense metabolism. For example, higher concentrations of phenylalanine might suggest an increased incorporation of this amino acid into phenolic defense metabolites in roots. An increase of aromatic amino acid levels has also been observed in tomato foliage in response to herbivory by *Manduca sexta* (Gomez et al., 2012) or methyl jasmonate treatment of tobacco (Hanik et al., 2010). Alternatively, the metabolic changes could result from the degradation of proteins and carbohydrates in a response to

mobilized C- and N-resources. An increase in the levels of glutamine, a major transport amino acid, would support this notion. Studies on the foliage of solanaceous plants provide clear support for primary metabolic changes in response to herbivory (Gomez et. al. 2012).

Bradysia feeding assays led to variable results

The conducted feeding assays resulted in a relatively high within-group variation, which diminished the significance of differences. This variation could in part be due to physiological and response differences of individual plants. Such discrepancies may be caused by differences in establishing plants in the clay substrate after transfer at the seedling stage. More likely, it is possible that variation in the extent of feeding damage has caused a variable response in *Arabidopsis* roots.

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Appendix D

Recipe of adopted Hoagland's liquid medium

Make up the following Stock solutions:

1. $Ca(NO_3)_2 \cdot 4H_2O$	236.1 g/L
2. KNO_3	101.1 g/L
3. KH_2PO_4	136.1 g/L
4. $MgSO_4 \cdot 7H_2O$	246.5 g/L
5. Trace elements (make up to 1 L)	
H_3BO_3	2.8 g
$MnCl_2c4H_2O$	1.8 g
$ZnSO_4 \cdot 7H_2O$	0.2 g
$CuSO_4 \cdot 5H_2O$	0.1 g
$NaMoO_4$	0.025 g
6. Fe·EDTA (1L)	
EDTA·2Na	10.4 g
$FeSO_4 \cdot 7H_2O$	7.8 g
KOH	56.1 g

Make up 1 L of KOH, adjust pH to 5.5 using H_2SO_4. Then add EDTA·2Na and $FeSO_4 \cdot 7H_2O$.

To make 1 L Hoagland's solution from these stocks, add stock of

$Ca(NO_3)_2 \cdot 4H_2O$	7 ml
KNO_3	5 ml
KH_2PO_4	2ml
$MgSO_4 \cdot 7H_2O$	2 ml
Trace elements	1 ml
Fe·EDTA	1 ml

to 1 L water

Appendix E

Table A3.1 GC/MS conditions

Condition (°C)	Amino Acid Scan	Carbohydrate Scan
Injection	300	280
Interface	280	280
Ion Source	260	260
Oven	100 1min; to 300 in 5 min; total 25 min	60 1min; to 320 in 3 min; total 40 min

Appendix F

Table A2 Changes in six gene expression in *Arabidopsis* roots in response to herbivory

Time	DIN11		DIN2		THA1		PPDK		SCPL		JAZ10	
	Fold	SE	Fold	SE	Fold	SE	Fold	SE	Fold	SE	Fold	SE
0.5 D	35.92	25.30	14.36	10.11	1.25	0.68	0.34	0.15	7.99	5.46	6.54	4.53
1 D	10.24	4.68	3.44	0.28	0.83	0.34	1.09	0.18	7.23	2.65	5.14	1.74
2 D	0.21	0.09	0.10	0.03	0.15	0.06	0.12	0.07	0.28	0.09	0.34	0.18
4 D	2.92	1.53	1.42	0.57	0.85	0.16	0.45	0.20	13.13	5.52	2.57	0.19

Chapter IV

Final Discussion and Future Perspectives

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Plants interact with multiple beneficial organisms that mediate pollination or attack plant pests (Van Poecke and Dicke, 2004; Wilson and Holway, 2010) but also cope with harmful herbivores and pathogens. Different types of herbivores (monophagous - (Bernays and Chapman, 1994), oligophagous (Hsiao, 1988), or generalists (Jolivet, 1998; Cloyd, 2004)) feed on plant tissues above and belowground. Hence, plants have developed different constitutive and induced defense mechanisms (Baldwin, 1998; Wittstock and Gershenzon, 2002) that are limited to specific cells and tissues or expressed systemically throughout the entire plant (Schilmiller et al., 2008; Driouich et al., 2013). This study has focused on plant roots and the cell type specificity of constitutive chemical defenses as well as global gene transcript and primary metabolic changes in response to feeding by the generalist herbivore *Bradysia*.

4.1 Cell type specificity of specialized metabolism

Spatial regulation of chemical defenses in plants is assumed to be a result of contact and co-evolution with herbivores or pathogens (Reymond et al., 2004; Jander and Howe, 2008; Prasad et al., 2012). For an optimal defense and to avoid potential cytotoxic effects phytochemical defenses occur as induced responses or are restricted to specific tissues and cells (Dudareva et al., 2004; Wellmer et al., 2004). There are many examples of organ and cell-specific formations of specialized metabolites such as the root-specific biosynthesis of alkaloids (Batra et al., 2004) or the production and accumulation of

terpenes in trichomes and resin ducts (Martin et al., 2002; Gonzales-Vigil et al., 2012). As described earlier, terpene formation in *Arabidopsis* roots is restricted to specific cell layers based on information gained from fine scale transcriptional maps of terpene synthases (Brady et al., 2007). Other work reported the existence of an operon-like gene cluster in *Arabidopsis thaliana* that specifically regulates the coexpression of triterpene synthases in root-epidermal cell. It appears that selection pressure may play a role in the formation of gene clusters and cell type specificity (Field and Osbourn, 2008).

We asked the question, whether the biosynthesis of different terpenes is limited to specific cells or if it would be supported by different cell types in the roots. We found relaxed cell-type specific expression of the genes *TPS08*, *TPS13*, and *TPS25*. However, the study will require verification by additional independent transgenic lines. Also, gene transcript levels need to be compared with those of native genes under the control of the selected promoters. Moreover, the expression of myc-tagged TPS proteins will be used to circumvent possible negative effects on enzyme activity by fusion of the TPS protein to YFP.

Interestingly, we found higher levels of (*E*)-β-farnesene, the sesquiterpene product of TPS25, when the *TPS25* gene was expressed under the constitutive epidermal *GL3* promoter. The TPS25 protein has an unusual activity in the way that it makes a sesquiterpene product from the substrate FPP in plastids, which are the preferred organelles for the formation of monoterpenes and diterpenes from GPP and GGPP,

respectively. Our result indicates that fluxes leading to the FPP production in plastids of epidermal cells must be sufficiently high to support (*E*)-β-farnesene production, when expressed from the *GL3* promoter. It will be interesting to determine whether the plastidial activity of TPS is supported in other cell types using strong cell type-specific promoters.

The fact that the formation of terpenes is supported by different cells of the *Arabidopsis* root suggests that cell-specific expression patterns of *TPS* genes may easily change dependent on the interaction of the root with the soil environment and with different soil-borne organisms. It would be interesting to determine whether the changes of *TPS* gene expression in our transgenic lines have any effects in the interaction with different bacteria or herbivores. For example, ectopic production of anthocyanins in potato tubers from fruits increases its resistance to bacteria (Lorenc-Kukula et al., 2005).

Future studies could also investigate if changes in the cell type-specific expression of terpene synthases affect root developmental processes. We may observe and compare the morphological changes or the differential expression of cell-type specific markers between ectopic expression lines and wild type plants. Such effects might be less pronounced for volatile terpenes that easily diffuse different cells but could be tested with non-volatile metabolites. Finally, terpene synthases could be swapped to aerial part of plants for a broader investigation in terpene cell-type specific expression. It is interesting to note that there is almost no overlap between terpene compounds produced in aerial and

belowground *Arabidopsis* tissues. Thus, the formation of root-specific terpenes might have negative effects on the development or interactions of leaves or flowers and *vice versa*.

4.2 Plant defense responses in primary metabolism

Primary metabolic changes in response to root herbivory have not been studied extensively. From this experiment, we observed that several genes involved in plant primary metabolism were up-regulated in response to herbivory. However, from the metabolite profiling, we found only minimal changes in the level of sugars, carboxylic acids or amino acids. This suggested that changes in gene expression might not necessarily reflect the dynamics in primary metabolism. The biosynthesis of primary metabolites is crucial and therefore controlled at multiple levels (Keurentjes et al., 2008). For instance, threonine concentrations in *tha1* knockout mutants did not decline much when compared to wild-type plants, while the glycine concentration increased significantly in the mutant (Joshi et al., 2006).

Herbivore invasion may cause compensatory growth (Reymond et al., 2004; Rizhsky et al., 2004; Frost et al., 2008). We observed an increase in the level of sucrose after 4 days of challenge with *Bradysia* larvae. Sucrose is an important C source in plants and also the major transport sugar in most plants (Kühn et al., 1999). The fact of sucrose accumulation in roots after feeding may suggest that energy is required in plant defense

for tolerance or compensation growth. Nutrient transfer or reallocation from feeding sites to other tissues is another defending strategy (Bazzaz et al., 1987; Bezemer and Van Dam, 2005; Kempema et al., 2007; Masclaux et al., 2012). Except sucrose, we found only minor changes in the level of other transport metabolites such as glutamine. It is possible that we did not observe major changes in these metabolite levels because of their rapid transport or possible turnover. It would be worthwhile conducting transcriptome and metabolite profiling aboveground to further support the notion of nutrient reallocation and systemic effects. In the future, experiments could be conducted to determine the dynamics of metabolic changes and the possible reallocation of compounds from roots to shoots. Application of stable isotope labeled precursors could be done at the time of insect feeding (Matsuda et al., 2003). Also, exposure to $^{11}CO_2$ would allow monitoring changes in C-assimilation and allocation of resources from shoots to roots to help sustain an active root metabolism and nutrient uptake (Gómez et al., 2012). A broader analysis of the different amino acids could provide more information about their metabolic fates in roots in response to herbivory.

Moreover, plants may have different responses to generalist or specialist herbivores in primary metabolism (Steinbrenner et al., 2011; Ali and Agrawal, 2012). A comparison between generalists and specialists and their effects on gene expression and metabolite changes should be conducted. These studies should be done with a different plant system such as *Brassica* species that allow feeding of the cabbage maggot, since a crucifer

specialist feeds on thicker *Brassica* roots but not on *Arabidopsis* roots.

Finally, a large within group variance diminished the differences among groups. It may be caused by differences in the feeding status of individual larva. Hence, experiments could be simplified by performing treatments of Arabidopsis roots with JA, which would allow a more homogenous treatment with a defined dose of the stimulating agent (even though JA only partially mimics herbivory and only JA-dependent responses would be observed). The use of insect regurgitant would be ideal but is difficult to apply in soil.

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