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Abstract

Plants defend themselves against herbivorous insects with the induced production of volatiles that attract the enemies of the herbivores. In this thesis I used an ecogenomic approach to study the role of signal-transduction pathways that regulate the induction of herbivore-induced plant volatiles (HIPV) and the effects of HIPVs on parasitoid host-finding behavior. To this end, I have combined transcriptomics, metabolite analyses and insect behavioral analyses.

Nine *Arabidopsis thaliana* accessions were screened for the emission of HIPVs. The accessions varied in the emission rate of *Pieris rapae*–induced volatiles after folivory or treatment with the herbivory-mimicking plant hormone jasmonic acid (JA). The relevance of this observed variation in the emission of JA-induced volatiles for host location was tested with *Diadegma semiclausum* parasitoids. Furthermore, the accessions also varied in transcript levels of genes that are (putatively) involved in the production of some of the recorded HIPV-compounds.
The oxylipin JA is the key plant hormone involved in the induction of the HIPV-blend emitted in response to caterpillar folivory. Mutant plants affected in the oxylipin signal-transduction pathway were studied to assess the effects of JA and its oxylipin intermediates 12-oxo-phytodienoate (OPDA) and dinor-OPDA (dnOPDA) on HIPV emission and attraction of the parasitoid *D. semiclausum*. In contrast to the effect of JA on the induced production of HIPVs, dnOPDA and OPDA were found to have no and little effect, respectively. The HIPV-compound methyl salicylate was shown to be JA-regulated and its abundance in the headspace varied among accessions. The contribution of methyl salicylate to parasitoid attraction was investigated. Bioassays with *P. rapae*-infested transgenic plants, lacking MeSA production, showed that MeSA negatively influenced *D. semiclausum* host-finding behavior.
Mutant plants were also studied to assess whether JA and its intermediates affected the induction of genes potentially involved in defense. The different oxylipins were shown to have distinct roles in induced defense signaling. Jasmonic acid had the strongest effect on transcript levels of defense-related genes from the oxylipin- and shikimate signal-transduction pathway. Minor roles were observed for OPDA and dnOPDA in the induction of one of these genes.

Utilizing an ecogenomic approach has provided new insight into the mechanisms underlying insect-plant interactions and holds promising opportunities.

Chapter 1

Picture: Tibor Bukovinszky

General introduction

Tjeerd A.L. Snoeren

General introduction

To fend off attacks by herbivorous insects, plants have evolved various defense mechanisms that can be divided into two distinct strategies. The first strategy is constitutively present. This involves physical bariers such as thorns, wax layers or trichomes. After breaking through this barrier, herbivores face the next constitutive barrier, consisting of secondary metabolites such as digestibility reducers, repellents, and toxins. In addition, plants can enhance the presence of natural enemies of herbivorous insects by providing them shelter (Karban and Baldwin, 1997; Schoonhoven et al., 2005). The second defense strategy that plants can utilize is defense that is activated after actual herbivore feeding-damage. Plants may increase their barriers, for instance, by an increased trichome density (Agrawal et al., 2002; Traw and Dawson, 2002), or an increased concentration of secondary metabolites that are already present at lower concentrations (Karban and Baldwin, 1997; Walling, 2000). Herbivory can also induce the *de novo* production of secondary metabolites, such as volatile infochemicals (Dicke and Sabelis, 1988). Volatile infochemicals can be indirectly beneficial for the plant, since they can provide natural enemies of the herbivores, i.e. predators and parasitoids, with detectable and reliable information for localization of their prey or host (Vet and Dicke, 1992).

In this thesis, I focus on induced indirect plant defense mechanisms involving herbivore-induced plant volatile (HIPV) production. I studied the effects of altering underlying mechanisms that affect HIPV production and the role of HIPVs in attracting natural enemies to infested plants.

My PhD project was embedded in an NWO-VICI-project that used an ecogenomic approach to address the role of infochemicals in insect-plant interactions. To study induced indirect plant defense, a plant-insect system composed of crucifer plants and their related insect fauna, i.e. biting-chewing herbivores and parasitoids, was adopted as the experimental study system. Extensive knowledge is already available on characteristics and interactions of the involved species, see e.g. Steinberg et al., 1993; Geervliet et al., 2000; Bukovinszky et al., 2005; Bruinsma et al., 2007; Smid et al., 2007; Van Leur et al., 2008. In particular, the roles of HIPVs in host-finding behavior of parasitoid wasps that attack the larval stages of the herbivores are well-studied for crucifers (Gols and Harvey, 2009). Additionally, the cruciferous plant *Arabidopsis thaliana,* well established as tool in molecular genetics, shows similar responses to herbivory as other members of the brassicaceous family (Mitchell-Olds, 2001; Van Poecke and Dicke, 2004). Furthermore, many Arabidopsis genotypes (wild-types and mutants) that are altered in various defense traits

are readily available. Combined with the relatively easy generation of transgenics, this makes Arabidopsis a very interesting species to study induced indirect defense traits. The use of Arabidopsis has already resulted in the partial elucidation of signal-transduction pathways as well as biosynthetic pathways underlying the production of HIPVs. Also, Arabidopsis can be used as a stepping stone towards other crucifer species. The knowledge obtained from using Arabidopsis as a model-system for plant-insect interactions (Fig. 1) can be utilized in studies of related crops, e.g. when cloning genes of *Brassica* spp. (Zheng et al., 2007). Furthermore, microarrays developed for Arabidopsis can be exploited to investigate global gene expression in *Brassica* (Lee et al., 2004; Broekgaarden et al., 2007, 2008).

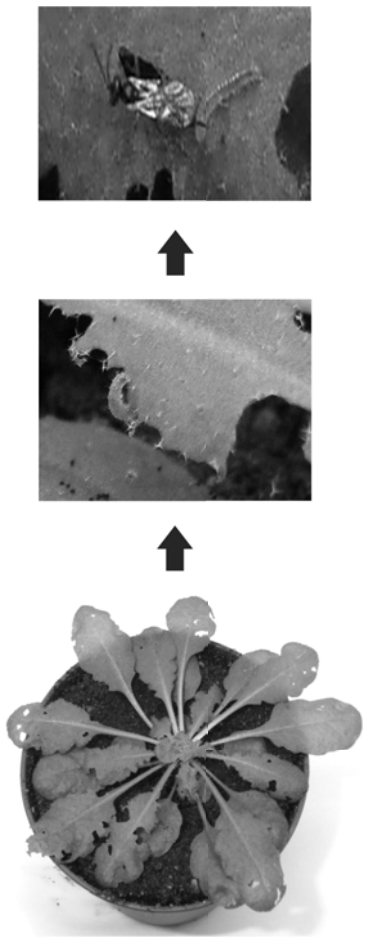

Figure 1. Tritrophic model system used in this thesis. A: *Arabidopsis thaliana* with folivory damage, B: *Pieris rapae* (L1/L2) caterpillar feeding, C: Parasitoid wasp *Diadegma semiclausum* that attempts to parasitize a *P. rapae*-larva

Within the NWO-VICI-project I took a molecular genetic approach to study the role of HIPVs in parasitoid attraction using Arabidopsis. Although the production of HIPVs is orchestrated by at least three signal-transduction pathways, the jasmonic acid (JA), the salicylic acid (SA), and the ethylene (ET) pathways (Dicke and Van Poecke, 2002), it is mainly the jasmonic acid pathway that is induced after herbivory by biting-chewing herbivores (Kessler and Baldwin, 2002; Liechti and Farmer, 2002; De Vos et al., 2005). Therefore, the role of the jasmonate pathway and effects of its modifications on parasitoid host-finding behavior is the main objective of investigation in this thesis. Using well-defined Arabidopsis genotypes (i.e. wild-types, mutants and transgenics) I gained more insight in the mechanisms that underlie HIPV production and the ecological effects of HIPVs on parasitoids.

Thesis outline

Chapter 2 reviews the current approaches to studying the role of HIPVs in insect-plant interactions. Furthermore, it discusses the future prospects of link-

ing new developments in functional genomics with ecology, which may provide novel tools to study infochemicals and plant-insect interactions in a food web context.

Chapter 3 presents a study of the genetic variation in *Pieris rapae*-induced indirect defense mechanisms among nine Arabidopsis accessions that originate from different geographic origins. We analyzed whether there is phenotypic variation among accessions in terms of herbivore-induced emission of volatiles. Furthermore, variation was screened for transcript levels of genes that are (putatively) assigned to the biosynthesis of volatile compounds. Finally, bioassays were performed to assess whether the observed variation among accessions is reflected in host-finding behavior of the parasitoid *Diadegma semiclausum*.

Chapter 4 describes the multidisciplinary approach I took to unravel the jasmonic acid pathway that underlies the induction of HIPVs in response to *P. rapae* folivory. The approach taken combines analyses of oxylipin titers, headspace analyses and investigations of parasitoid behavior. Plants mutated in different genes involved in the jasmonate pathway were studied to assess the effect of JA and its intermediates on HIPV-production and attraction of the parasitoid *D. semiclausum.*

Chapter 5 addresses the question whether JA and intermediates of the jasmonate signal-transduction pathway affect the induction of genes involved in induced defense. The mutants studied in chapter 4 were now analyzed for variation in *P. rapae*-induced transcript levels of genes in both the JA and SA signal-transduction pathways.

In **Chapter 6**, I took a slightly different molecular genetic approach. In this chapter, I used a transgenic Arabidopsis line altered in the biosynthesis of an HIPV compound, i.e. methyl salicylate. The emission rate of the selected volatile compound varied among Arabidopsis accessions (chapter 3) and was also found to be influenced by alterations of the jasmonate pathway (chapter 4). Therefore, variation in the emission of this compound might influence parasitoid behavior. In this chapter, I investigated the contribution of methyl salicylate to the attraction of the parasitoid *D. semiclausum* towards the caterpillar-induced plant volatile blend.

Chapter 7 summarizes and discusses the most important results from this thesis. In this chapter I also consider the future perspectives for this molecular genetic approach to the ecology of HIPV.

Chapter 2

Picture: Hans Smid

Ecogenomic approach to the role of herbivore-induced plant volatiles in community ecology

Tjeerd A.L. Snoeren, Peter W. de Jong, and Marcel Dicke

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Abstract

Linking novel developments in genomics with ecology provides interesting novel tools to address ecological questions in ways that have not been possible up to now. In this paper we address this issue for the ecology of infochemicals and plant-insect interactions in a food web context. Plants are at the basis of most terrestrial food webs and insects are a dominant animal group interacting with plants. Insect-plant communities are characterized by direct and indirect interactions, many of which are mediated by infochemicals. Plants respond to insect herbivory with the production of volatiles that attract the enemies of the herbivores, such as insect predators and parasitoids. Moreover, the plant volatiles may be exploited by any organism in the environment and this results in many more infochemically-mediated interactions. Thus, a food web is overlaid with an infochemical web. In the past, several manipulative tools have been developed to investigate the role of infochemicals. The rapid advancement of molecular genetics and '–omics' technologies results in interesting new tools. A recent development is the use of well-characterized genotypes that are modified in the mechanisms underlying the induced plant volatiles. These genotypes produce precisely manipulated phenotypes, that often differ in only a single gene, and can be used to investigate the effect of particular genes on specific interactions. Moreover, these genotypes can be introduced into a natural community to assess the effects of the genetic change and its resulting phenotypic change on interactions with the entire natural community. Furthermore, with the progress in microarray technology it becomes possible to assess the expressed genotype of plants in the field, which can be exploited to investigate expressed genetic variation under field conditions. These developments are expected to be only the beginning of a successful integration of –omics technologies, such as transcriptomics and metabolomics, with community ecology into the new research field of ecogenomics. In this review we present the current status and discuss the prospects for the future of an ecogenomic approach to the role of herbivore-induced plant volatiles in insect-plant community ecology.

Key words:
Phenotypic plasticity, insect-plant interactions, induced defense, infochemicals, manipulative approach, community genetics

Introduction

A principal aim of ecologists is to understand the mechanisms shaping population and community processes. The evolution of species interactions, and hence their impact on the community, can only be fully understood by the study of the genetics and dynamic processes underlying such interactions. During the last decade, developments in the study of the organization and functioning of genomes have resulted in the acquisition of novel insights in the effects of individual genetic variation and plasticity on community processes. Among the plethora of –omics, the relatively recent branch of ecogenomics holds promise for major breakthroughs in linking these two fields (Van Straalen and Roelofs, 2006).

Chemical information

Chemical cues are a major source of information for very different organisms ranging from micro-organisms to mammals (e.g. Roitberg and Isman, 1992; Kats and Dill, 1998; Tollrian and Harvell, 1999; Dicke and Grostal, 2001) that affect various behaviors underlying population dynamics and food web interactions (e.g. Roitberg and Isman, 1992; Kats and Dill, 1998; Turlings and Benrey, 1998; Dicke and Vet, 1999; Sabelis et al., 1999; Hilker and Meiners, 2002). However, the study of chemical information conveyance has been mostly restricted to studies at the level of individual organisms and the identification of the chemicals that convey the information. The influence of chemical information on food web processes has received little attention (Vet, 1998; Van der Meijden and Klinkhamer Peter, 2000; Hunter, 2002), in contrast to the influence of direct trophic interactions (Morin, 1999). Yet, circumstantial evidence indicates that chemical information from phenotypically plastic plants can have important influences on food web dynamics through indirect effects that combine bottom-up and top-down effects (Dicke and Vet, 1999; Sabelis et al., 1999; Kessler and Baldwin, 2001).

Herbivore-induced plant volatiles are infochemicals that mediate many interactions in a plant-insect community, both above- and below-ground (Dicke and Vet, 1999; D'Alessandro and Turlings, 2005; Rasmann et al., 2005). These volatiles that plants produce in response to damage inflicted by herbivores affect various interactions of the plant with community members (Dicke and Vet, 1999; Dicke, 2000; Strauss et al., 2001). For instance, herbivore-induced plant volatiles may deter or attract herbivores, but they also indirectly affect carnivore-herbivore interactions through attraction of carnivores. Differential responses by different carnivore species may mediate the degree to which they compete for the same resource or interact through intraguild predation on each other. Carnivorous arthropods largely rely on herbivore-induced plant volatiles in locating herbivores or their microhabitat from a distance. Moreover, herbivore-induced plant volatiles can also affect herbivore-plant and carnivore-

herbivore interactions on neighboring plants through their effect on the neighbor's phenotype (Dicke and Vet, 1999; Sabelis et al., 1999; Dicke et al., 2003a). These herbivore-induced plant volatiles (HIPV) represent phenotypically plastic responses of plants to herbivory that result in changes in interactions among individuals in the insect-plant community.

Insect-plant communities

Approximately 50% of all insect species feed on plants and the other half consists of carnivores and detritivores (Schoonhoven et al., 2005). Insect-plant communities are complex entities that consist of hundreds of species (Rott and Godfray, 2000). In addition to the large number of species, genetic variation among species members as well as phenotypic plasticity of individuals add to the complexity of insect-plant interactions in communities (Agrawal, 2001).
Insect-plant communities are highly dynamic. Population sizes of insects may quickly change and food webs can show drastic quantitative changes within a season (Rott and Godfray, 2000). Moreover, interactions between two community members may influence interactions between various others. Many species show phenotypic plasticity that is induced by the interaction with community members and phenotypic changes may be specific for the interaction that induces them (Agrawal, 2001). Thus, dynamic food web changes can be amplified through their effects on interactions between members of the food web.
When studying a plant-insect community, ecologists may focus on the food web that connects different species in a direct way according to trophic relationships exclusively. Communities also contain indirect interactions between species from different trophic levels, mediated by a third species. For instance, plants may attract the enemies of herbivorous insects with plant volatiles that are induced by feeding damage inflicted by the herbivores.
Both direct and indirect interactions in a community are mediated by chemical information (infochemicals, (Dicke and Sabelis, 1988)). Every member of a community produces infochemicals that can influence direct interactions between the producer of the cues and the organisms that have a trophic relationship with the producer. Moreover, an infochemical that is released into the environment can be exploited by any organism of the community to meet its own needs. As a result, infochemicals mediate ample indirect interactions as well (Turlings and Benrey, 1998; Dicke and Vet, 1999; Sabelis et al., 1999). Thus, a food web is overlaid with an infochemical web. This infochemical web is more reticulate than the food web as the food web only comprises direct interactions whereas the infochemical web also comprises indirect interactions (Dicke and Vet, 1999; Shiojiri et al., 2001).

A major challenge for biology in the 21st century is to integrate research approaches that address different levels of biological organization: i.e. from subcellular processes all the way to community processes (Fig. 1). A pressing issue in ecology is to understand how interactions among individual organisms

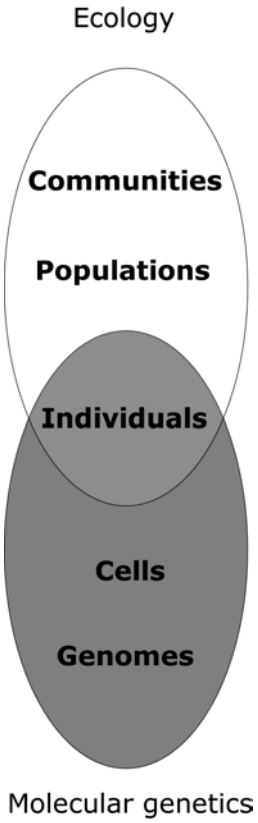

Figure 1. Molecular genetics and community ecology are involved at different levels of biological integration and overlap at the level of the individual.

influence food webs and community dynamics. Evolutionary ecologists aim at understanding how genetic differences affect the fitness of individuals in complex communities. This can now be addressed through a novel integration of approaches: from ecogenomics, through behavioral ecology to community ecology. The major question of evolutionary ecology, i.e. how individual genes affect an individual's performance, can now for the first time be investigated as such without the confounding influence of other genetic differences between individuals. This is the topic of this review in which we focus on infochemicals and the community ecology of plant-insect interactions.

Manipulative tools for investigating the infochemical web

To develop manipulative tools, knowledge of underlying mechanisms is of great importance. Investigating community dynamics and the role of the infochemical web requires precise manipulative tools. Ideally one should be able to modify individual components of the infochemical web and assess the resulting effects on community processes. Various manipulative tools have been developed over the past decades. Each of these tools is based on mechanistic information on the production of, and response to, the infochemicals (Dicke et al., 2003b). Here we will briefly review the different tools developed.

Ecologists initially started to investigate the effects of individual chemicals and mixtures of specific cues (Dicke et al., 1990c; Whitman and Eller, 1990; Turlings et al., 1991; Birkett et al., 2000). The chemicals used were derived from analytical studies that deciphered the composition of HIPV blends. Surprisingly, individual compounds from HIPV blends were found to attract carnivorous arthropods although the compounds were not specific indicators of their herbivorous prey or hosts (Dicke et al., 1990c; Whitman and Eller, 1990). This was followed by experiments where more compounds from the HIPV blend were offered simultaneously (Turlings et al., 1991). More recently, HIPV blends were fractionated and the fractions were investigated for their effects on arthropods (Turlings and Fritzsche, 1999; Van den Boom, 2003; D'Alessandro and Turlings, 2005).

For investigating the effects of infochemicals on individual interactions or overall community effects, the use of (blends of) pure chemicals can be a first step, if the compounds of interest are available as pure compounds. This allows well-defined application of stimuli (Dicke et al., 1990c; Whitman and Eller,

1990; Turlings et al., 1991; Kessler and Baldwin, 2001; D'Alessandro and Turlings, 2005; D'Alessandro et al., 2006). Yet, although this can provide valuable information, the application of synthetic chemicals cannot mimic the natural dynamics of volatile emission. For compounds that are not synthetically available or for complex blends the use of elicitors may be a valuable tool to investigate the effect on individual interactions (Dicke et al., 1999). Elicitors can induce a subset of the blend of herbivore-induced plant volatiles (Koch et al., 1999). This allows to assess the role of individual compounds against a complex odor background by supplementing the partially induced blend with individual compounds (De Boer and Dicke, 2004; De Boer et al., 2004). Yet, also in this case the natural dynamics of volatile emission will not be mimicked. The use of inhibitors that block specific steps in the signaling- or biosynthetic pathways (Zeidler et al., 1998; Koch et al., 1999) is more likely to leave the non-inhibited signaling and biosynthetic pathways intact. Therefore, the non-affected pathways are likely to retain their natural dynamics. However, a disadvantage may be that chemicals that accumulate in the last step before the inhibited step have physiological side-effects or are redirected into another biosynthetic pathway. A metabolomic analysis may provide more insight into this (Bezemer and van Dam, 2005).

Plant physiological- and biochemical knowledge revealed that induced plant defenses are orchestrated by three main signaling pathways, the octadecanoid pathway, the shikimate pathway and the ethylene pathway (Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002). These three signaling pathways are also involved in the induction of infochemicals (Horiuchi et al., 2001; Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002; Van Poecke and Dicke, 2004). Mechanistic knowledge of the biosynthetic and signaling pathways provides interesting options for manipulating the emission of plant volatiles through specific elicitors and inhibitors (Koch et al., 1999). During the last decade molecular genetic information on the induction of plant volatiles has rapidly accumulated. This allows the use of mutants or plants that have been genetically modified in the biosynthetic (Kappers et al., 2005; Schnee et al., 2006) or signaling pathways (Thaler et al., 2002; Van Poecke and Dicke, 2002; Ament et al., 2004; Kessler et al., 2004) with consequences for the emission of volatiles.

The most interesting genotypes are those that differ in a limited number of well-defined genes. Such genotypes have often been unavailable, but recent molecular genetic developments are changing this drastically. With the rapidly expanding knowledge of genomics and molecular genetics specific genotypes are being developed in which the expression of a single gene is modified. These genotypes provide exciting new tools to ecologists. To a large extent this is already true for model plant species of molecular biologists such as *Arabidopsis thaliana*. However, these tools are rapidly being developed for ecological model species as well (Kessler et al., 2004; Schmidt et al., 2004).

Molecular ecological approach to community ecology

All manipulative tools mentioned in the previous paragraph have been derived from information on mechanisms. In this paragraph we will specifically address the potential of a molecular genetic approach. It has yielded interesting results already (Baldwin et al., 2001; Degenhardt et al., 2003; Dicke et al., 2004) and holds many promises for the future.

Novel transcriptomic developments have allowed the assessment of global responses of plants to environmental changes at the level of gene expression. This has shown that the attack by biotic agents can lead to drastic rearrangements of gene expression (Reymond et al., 2000; Schenk et al., 2000; Hermsmeier et al., 2001; Reymond et al., 2004; De Vos et al., 2005). Attack results in up-regulation of defence-related genes and down-regulation of genes involved in photosynthesis (Hermsmeier et al., 2001), which provides a mechanistic explanation for the growth-defense trade-off that is well-known to ecologists (Herms and Mattson, 1992). Furthermore, it has become clear that responses of the same plant species to different attacker species can be very different and that induced signal-transduction pathways influence each other (Voelckel and Baldwin, 2004a; De Vos et al., 2005). As a consequence, the effects of combinations of species that attack the same plant can have quite surprising effects on the expressed plant phenotype (De Vos, 2006).

The analysis of plant volatiles is undergoing developments that extensively improve our knowledge of the metabolomic changes in plants in response to attack. With the application of more sensitive analytical equipment the composition of the induced blends appears to be more complex than was known before and with novel real-time techniques for data sampling and analysis also the dynamics of plant volatile emission can be registered in much more detail (for review see, Tholl et al., 2006). Moreover, the technological developments in metabolomics result in the recording of large sets of metabolites (Fiehn, 2002). Combining transcriptomic and metabolomic analyses in plants that are not a genomic model species can lead to the identification of genes involved in the biosynthetic pathways that lead to the production of herbivore-induced plant volatiles (Mercke et al., 2004). Two subtractive cDNA libraries, enriched in cDNA fragments up- or down-regulated by herbivore infestation can be made from the leaves of infested and uninfested conspecific plants. Randomly selected clones from these libraries can be used to make a cDNA microarray. Subsequently, cDNAs prepared from mRNA from plants of several different treatments at different time points can be hybridized to the clones on the microarray. When induced volatile compounds were collected from the same leaves from which the mRNA was collected gene expression profiles can be analyzed in combination with volatile production data in order to gain insight in the possible involvement of the studied genes in the synthesis of those volatiles. By grouping clones on the microarray and the herbivore-induced volatiles into a number of clusters, biosynthetic genes clustered with the product of that pathway can be found. The cDNA fragments on the microarray can then be

used to screen the cDNA library to clone the gene (Mercke et al., 2004).
A logical next step in the -omics development is to link transcriptomics and metabolomics to the assessment of the ecological function of the identified genes and resulting infochemicals (Fig. 2). Transgenics and natural mutants in the genes of ecological interest can be important tools to study the effect of characteristics on the plant-insect community. This can be done by investigating the expression of phenotypes of an organism under different conditions, also termed 'phenomics' (Edwards and Batley, 2004; Kahraman et al., 2005). First steps in this research field have been made, e.g. related to the behavioral responses by arthropod predators and parasitoids towards altered herbivore-induced volatile production (Van Poecke and Dicke, 2002; Bouwmeester et al., 2003; Ament et al., 2004; Kessler et al., 2004; Kappers et al., 2005; Schnee et al., 2006). Approaches in this research can roughly be divided into two groups. The first consists of alterations in the biosynthesis of induced plant volatiles (Bouwmeester et al., 2003; Fäldt et al., 2003; Kappers et al., 2005; Schnee et al., 2006). The second group consists of alterations in the signal-transduction pathways that regulate volatile biosynthesis (Van Poecke and Dicke, 2002; Ament et al., 2004; Kessler et al., 2004).

Biosynthetic manipulations

Molecular genetic information on biosynthetic pathways has accumulated rapidly in recent years (Dudareva et al., 2004; Aharoni et al., 2005; Fridman and Pichersky, 2005). This allows for the manipulation of the plant phenotype in terms of infochemical production (Bouwmeester et al., 2003; Degenhardt et al., 2003; Arimura et al., 2004a; Kappers et al., 2005; Tholl et al., 2005; Schnee et al., 2006).
For instance, identification of genes encoding key enzymes in the biosynthesis of herbivore-induced terpenoids has been exploited to transfer these genes to *Arabidopsis thaliana* (Kappers et al., 2005; Schnee et al., 2006). As a result the transgenic plants emitted one or two (Kappers et al., 2005), or six or more terpenoids (Schnee et al., 2006). The transgenic plants attracted significantly more carnivorous arthropods such as the predator *Phytoseiulus persimilis* (Kappers et al., 2005) and the parasitoid *Cotesia marginiventris* (Schnee et al., 2006) than the wild-type. These studies clearly demonstrated the ecological effects of particular terpenoids that are components of a complex blend of HIPV and thus revealed an ecological function for the genes involved. These results are likely to be a starting point for future studies on the role of these genes in a community context rather than in an isolated ecological interaction. For such studies individuals of the original plant species, varying in the degree of gene expression dynamics will be of great value.

Signal-transduction manipulations

The signal-transduction pathways underlying induced plant responses, including herbivore-induced volatile production, have been intensively studied in several plant species, especially Arabidopsis, tomato and tobacco (Pieterse and

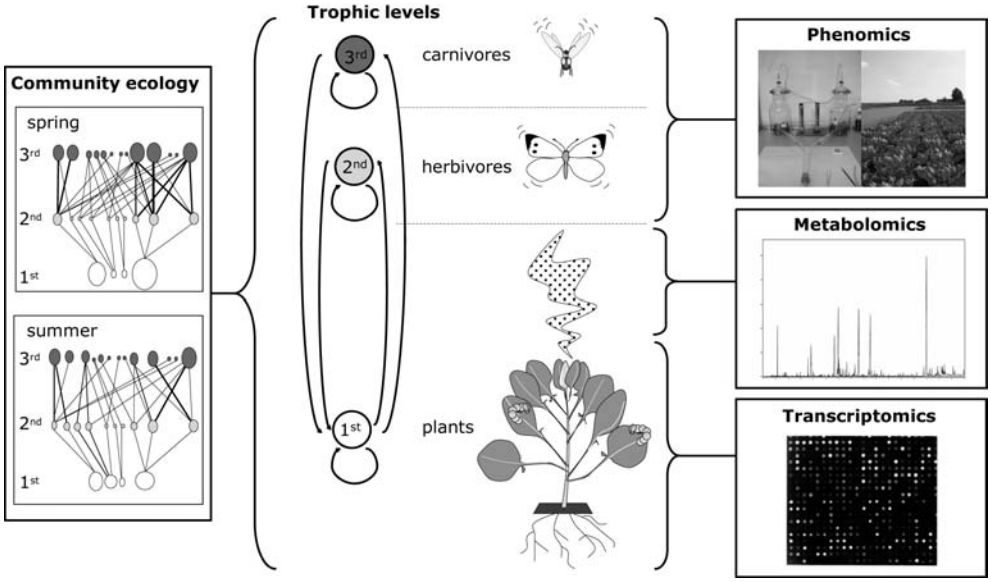

Figure 2. The application of –omics technologies, such as transcriptomics and metabolomics, and analyses of the range of phenotype expression in individual interactions (phenomics) to the study of community ecology of insect-plant communities. Herbivore damage to plants results in the emission of volatiles that can influence interactions of the plant with organisms at different trophic levels.
Interactions (arrows) between members of the same or different trophic levels may be affected by the induced volatiles and this may have consequences for the quantitative food web, in which each species is represented by a circle (circle size indicates population size and the width of the lines connecting species indicates the relative degree of trophic interaction between the two connected species).

Van Loon, 1999; Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002). The natural tomato mutant *def-1* is deficient in jasmonic acid as a result of a mutation early in the octadecanoid pathway. This mutant does not produce induced metabolites such as proteinase inhibitors involved in direct defense (Howe et al., 1996) or volatile terpenoids involved in indirect defense (Thaler et al., 2002; Ament et al., 2004). The *def-1* mutant is compromised in the attraction of carnivorous arthropods and this can be restored by the application of exogenous jasmonic acid (Thaler et al., 2002; Ament et al., 2004). Knowledge of the molecular genetics of signal-transduction pathways can be used to generate transgenic genotypes that are altered in the underlying mechanism leading to induced plant volatiles. For instance, genotypes changed in certain steps in the octadecanoid (Van Poecke and Dicke, 2002; Halitschke and Baldwin, 2003) or shikimate pathway (Van Poecke and Dicke, 2002) have been used in studies on the attraction of arthropods to HIPV. Arabidopsis plants compromised in the octadecanoid or shikimate pathway that were infested with caterpillars of the small cabbage white (*Pieris rapae*) were less attractive to the parasitoid *Cotesia rubecula* than caterpillar-infested wild-type plants (Van Poecke and Dicke, 2002).

Molecular ecology of HIPVs: two examples

Herbivore-induced plant volatiles have been well-studied in crucifers. This includes behavioral ecology of crucifer-caterpillar-parasitoid interactions (Wiskerke and Vet, 1994; Geervliet et al., 2000; Vos et al., 2001; Shiojiri et al., 2002), as well as molecular genetics and analytical chemistry of induced crucifer volatiles (Mattiacci et al., 1995; Geervliet et al., 1997; Van Poecke et al., 2001). Over the last decade *A. thaliana*, the model plant of molecular geneticists, has been increasingly used by ecologists (for reviews see Mitchell-Olds, 2001; van Poecke and Dicke, 2004). This has yielded novel insight into the role of specific genes in ecological interactions of the crucifer Arabidopsis with insects (e.g., Stotz et al., 2000; Van Poecke and Dicke, 2002; Aharoni et al., 2003). With respect to induced plant volatiles interesting genotypes are being developed, which can no longer produce certain induced plant volatiles (e.g. Chen et al., 2003).

However, from an ecological point of view Arabidopsis may not be the most interesting plant because of its short life cycle very early in the season. Yet, interesting ecological field work on Arabidopsis-attacker interactions has been carried out (Yano and Ohsaki, 1993; Mauricio, 1998; Arany, 2006). Moreover, this plant is a valuable species as a stepping stone towards other brassicaceous plants. For instance, certain types of microarrays developed for Arabidopsis can be used to investigate global gene expression in *Brassica* (Lee et al., 2004). Moreover, there are many parallels between Arabidopsis and other brassicaceous species in the context of HIPVs (van Poecke and Dicke, 2004). Therefore, Arabidopsis is an interesting species for investigating the role of induced plant volatiles on community ecology both from a methodological and a conceptual point of view. In our laboratory we have intimately integrated projects on Arabidopsis-attacker interactions with projects on the community ecology of brassicaceous plants and their associated insects. For *Brassica* we compare the effects of genotypes that differ in the degree of parasitoid attraction on community composition (Poelman et al., in press.) and investigate the differences between the genotypes in the underlying mechanisms at the molecular and metabolite level (Broekgaarden et al., 2007; Poelman et al., 2008).

An excellent example demonstrating the value of a molecular approach to community ecology is the work by Kessler, Baldwin and colleagues (Baldwin et al., 2001; Kessler et al., 2004; Kessler, 2006). They have taken the solanaceous species *Nicotiana attenuata* as their ecological model plant and have developed extensive molecular tools to investigate and manipulate mechanisms underlying induced responses to attackers. Their methods and tools include cloned genes, dedicated microarrays, anti-sense (as) knock-out genotypes, in addition to methods to assess changes in secondary metabolites such as nicotine, volatiles and others (Paschold et al., 2006). They have made three plant lines that are knocked out for one of three different genes of the octadecanoid signal-transduction pathway and placed these in a common garden in their native habitat (Kessler et al., 2004). This showed that eliminating the functional ex-

pression of the *LOX-3* gene results in drastic changes in interactions with the natural insect community: the amount of herbivory increased substantially and the invasion of a new herbivore species, *Empoasca* sp., was the main cause for this. This herbivore did not feed on wild-type plants under natural conditions. Another new herbivore that had never before been recorded from *N. attenuata,* is the western cucumber beetle *Diabrotica undecimpunctata tenella.* In laboratory tests *Empoasca* sp. and *D. undecimpunctata* preferred to feed on *as-lox* plants over wild-type *N. attenuata* plants. The *as-lox* plants had a lower emission rate of the herbivore-inducible terpenoid *cis*-α-bergamotene. However, it remains to be investigated whether the change in herbivore-induced *cis*-α-bergamotene is the cause of the change in acceptance by herbivorous insects. Yet, previous research employing the application of synthetic volatiles or JA as elicitor has shown that plant volatiles are important in the interactions of *N. attenuata* with herbivorous and carnivorous insects under field conditions and result in direct and indirect protection against herbivores (Kessler and Baldwin, 2001).

Future prospects

Although the ecogenomics approach clearly holds promise to ecologists, there are some important developments to be made. The most important of these questions plus the ecological questions to which they relate are given below:

1. The most important hurdle to be taken is that, to date, the best genomic model systems do not include the best ecological model systems, either because the ecology of the genomics models is little studied or because important ecological questions cannot be addressed by using the genomics models. Moreover, the ecogenomic approach has so far been applied to a limited number of systems. Two main solutions to this problem exist. One is to investigate an ecological model species that is closely related to a molecular genetic model species and use the latter as a stepping stone. This has been explained for using Arabidopsis to develop an ecogenomic approach to *Brassica* species. Information on Arabidopsis gene sequences can be used to help in cloning of genes for *Brassica* (Zheng et al., 2007) and 70-mer oligonucleotide microarrays developed for Arabidopsis can be exploited to investigate global gene expression in *Brassica* (Lee et al., 2004). The other solution is to use Suppression Subtractive Hybridization (SSH) to build a library of differentially expressed clones that can be used to develop a dedicated microarray and to clone genes involved in the expression of an induced plant phenotype (Mercke et al., 2004).

2. The technological developments of molecular biology have provided ecologists with a 'digital' tool to compare two genotypes in which a certain gene is either functionally expressed or not. Using the variation between independently transformed lines allows studying variation of phenotypic expression for candidate genes (Halitschke and Baldwin, 2003). Yet, ecologists are ultimately inte-

rested in quantitative natural variation. A major challenge for molecular ecologists is to identify genotypes that differ quantitatively in the expression of specific genes. This may be done by developing molecular expression markers based on sequence information for the gene(s) of interest. When genotypes have been selected that express quantitative variation in gene expression, novel tools will become available to be used in manipulative experiments under field conditions. The relative effects of community members on these genotypes can be used to assess the effects of the gene on an individual's fitness. This will yield significant information beyond that gained from current qualitative manipulations resulting from knocking out genes.

3. New methodology in quantitative food web analysis has been developed in recent years (Rott and Godfray, 2000; Lewis et al., 2002; Forup and Memmott, 2005). This quantitative food web analysis provides valuable information beyond connectance food webs. This methodology captures community dynamics in space and time (Rott and Godfray, 2000) and has proven to be a useful tool for comparative analysis where the effects of particular phenotype changes are addressed (Omacini et al., 2001). This methodology can be used to address issues such as the effect of a single gene on community composition and dynamics. Genotypes that have been characterized in terms of transcriptomic and metabolomic profiles can be quantitatively compared in terms of food web structure. This can be used to evaluate the effects of single genes on food web structure similarly to the evaluation of the effects of an endophytic fungus on the parasitoid-host food web (Omacini et al., 2001).

4. In this review, we have illustrated the potential of ecogenomics to understand the consequences of (plasticity in) expression of genes for community processes, assuming that the shape of a particular interspecific interaction is ubiquitous. Whereas this approach enables the linking of sub-cellular processes to particular community processes or structures, a challenge for the future lies in the implementation of these results in a spatial framework. Populations are generally highly structured, giving rise to complex mosaic-patterns of species interactions (Thompson, 2005). This will have consequences for variation across local communities. To address this issue, ecogenomics may again provide important tools. Association- and correlation studies (Epperson, 1993; Sinervo and Svensson, 2002; Purugganan and Gibson, 2003), 'natural selection mapping' (Kohn et al., 2000), and population genomics (Black et al., 2001) enable the estimation of variable selection at (sets of) loci, distinguishing this from processes that act on the whole genome, such as migration and genetic drift. Such an ecogenomics approach provides insights that complement those of the community- and ecosystem genetics approach as proposed by (Whitham et al., 2003). Whereas the latter focuses on the study of patterns of inheritance of traits involved in 'extended phenotypes' (i.e. traits that are likely to have community and ecosystem consequences), ecogenomics provides an experimental approach, enabling the testing of the involvement of candidate genes in

processes affecting the community. It is the integration of these types of studies with the previously described careful dissection of species interactions and their effect on communities that is likely to form both a highly fruitful approach and a major challenge in the search for a unified explanation of community dynamics.

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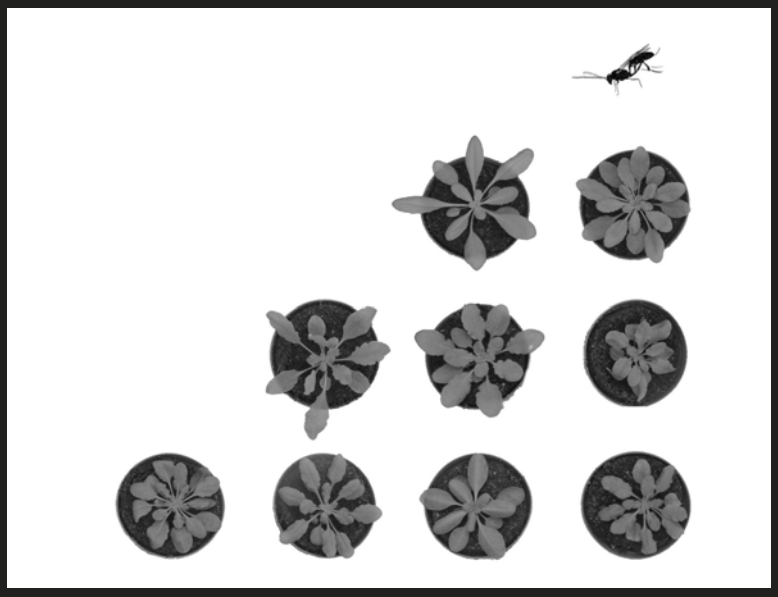
Chapter 3



Picture: Tjeerd Snoeren

Natural variation in herbivore-induced volatiles in *Arabidopsis thaliana*

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Abstract

Naturally-occurring genetic variation in the emission of plant volatiles that are released upon herbivory by the biting-chewing caterpillar *Pieris rapae* or in response to jasmonic acid (JA) treatment in leaves of *Arabidopsis thaliana* was studied by investigating nine accessions (ecotypes). A total of 73 compounds in the headspace were compared, which resulted in quantitative differences in the emission rates of individual compounds among the accessions. Moreover, variation in the emission of volatile compounds after JA treatment was reflected in the behavior of the parasitoid *Diadegma semiclausum* when they were offered the headspace volatiles of several combinations of accessions in two-choice experiments. Yet, we could not correlate wasp behavior to the emission rates of individual compounds. The complex variation in odor blends is likely to interfere with identifying the contribution of individual compounds.
Accessions also differ in transcript levels of genes that are (putatively) associated with the emission of plant volatiles. Genes *BSMT1* and *Cyp72A13* could be connected to the emission of methyl salicylate and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), respectively.

Key words
Herbivory, herbivore-induced volatile, qRT-PCR, gene transcript level

Introduction

In nature, plants are challenged by a wide range of herbivorous insects. Herbivory can severely reduce survivorship and reproduction of native plants, and in crops insect herbivore infestation can result in severe yield losses. Plants have developed a multitude of defense strategies, which include pre-existing physical and chemical barriers, induced defenses that are activated upon attack, and tolerance mechanisms. Plants exhibit variation in these traits, which comprises both intra-individual phenotypic plasticity, and genetic polymorphisms among individuals and populations. The plasticity of inducible defenses allows individual plants to adapt to changing environments (Agrawal, 2001). Polymorphism in traits for resistance against herbivory may result from varying selection pressures among populations (Meyers et al., 2005). Induced responses allow plants to be cost-effective and also to diminish the risk that herbivores adapt to plant defenses (Agrawal and Karban, 1999; Heil, 2008; Steppuhn and Baldwin, 2008).

Herbivore-induced defense responses can be subdivided into direct defense and indirect defense. Induced direct defense encompasses the production of anti-digestive proteins or toxic secondary metabolites such as glucosinolate derivatives that influence the performance and survival of the herbivore (Karban and Baldwin, 1997; Walling, 2000). Induced indirect defense comprises the production of, for instance, herbivore-induced plant volatiles (HIPV) that attract the natural enemies of the herbivore (Dicke and Hilker, 2003).
A central issue in ecology is to understand how interactions among individual organisms influence food webs and community dynamics. Therefore, evolutionary ecologists aim to understand how genetic variation affects the fitness of individuals in plant-insect communities. Its major underlying question, i.e. how the expression of individual genes affects an individual's phenotype and its performance, can nowadays be investigated without the confounding influence of other genetic variation between individuals by using specific mutants. This approach has also been addressed for defense genes, e.g. genes involved in HIPV and glucosinolate production (Kessler and Baldwin, 2004; Kappers et al., 2005; Schnee et al., 2006; Mumm et al., 2008a).

Herbivore-induced production of plant volatiles has been studied in a wide variety of plant species. These studies include the behavioral ecology of plant-herbivore-carnivore interactions, as well as molecular genetics and analytical chemistry of HIPVs (D'Alessandro and Turlings, 2006; Snoeren et al., 2007). Plant physiological and biochemical explorations have revealed that herbivore-induced plant defenses are orchestrated by at least three interconnecting signal-transduction pathways, the jasmonic acid (JA), the salicylic acid (SA), and the ethylene pathways (Pieterse and Dicke, 2007; Kazan and Manners, 2008). Induction of infochemicals (Dicke and Sabelis, 1988) also depends upon these three signaling pathways (Horiuchi et al., 2001; Kessler and Baldwin, 2002;

Van Poecke and Dicke, 2002). The signal-transduction pathways underlying HIPV production have been studied for several plant species, e.g. *Arabidopsis thaliana*, tomato, Lima bean and tobacco (Dicke et al., 1999; Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002; Ament et al., 2004). The available knowledge of the molecular genetics of signal-transduction pathways has been used to generate transgenic genotypes that are altered in the underlying mechanisms leading to induced plant volatiles, which are studied in the context of the attraction of carnivorous arthropods (Van Poecke and Dicke, 2002; Halitschke and Baldwin, 2003; Kessler et al., 2004; Snoeren et al., 2009 chap. 4). In addition, molecular insight into the herbivore-induced biosynthesis of plant volatiles, allowed for the modification of the emission of volatile compounds. These modified plants were used to study arthropod behavior in response to plants differing in just a single gene (Bouwmeester et al., 2003; Kappers et al., 2005; Tholl et al., 2005; Schnee et al., 2006; Loivamäki et al., 2008).

Arabidopsis is an important model plant to study herbivore-induced plant defense responses (Mitchell-Olds, 2001; Van Poecke and Dicke, 2004; Snoeren et al., 2007). However, to date, functional analysis of genes and the dissection of traits are mostly limited to three Arabidopsis accessions, i.e. the laboratory accessions Columbia (Col), Landsberg *erecta* (L*er*), and Wassilewskija (WS). Although presently a range of screening techniques have resulted in the functional analysis of many genes, defining new gene functions is limited by the range of variation that is explored, including variation in HIPV-blend. So far, exploring genetic variation among accessions was limited because most attention had been given to mutants that express qualitative variation and not to quantitative variation.

Phenotypic variation between Arabidopsis accessions is abundant for various traits and enables almost every Arabidopsis accession to be distinguished from others when they are grown together and compared under similar environmental conditions (Alonso Blanco and Koornneef, 2000; Kliebenstein et al., 2001; Kover and Schaal, 2002; Koornneef et al., 2004). Genetic variation has increasingly been associated with gene transcription in early defense signaling and secondary metabolism (Gao et al., 2008; Steppuhn et al., 2008; Wu et al., 2008), and has been reported for resistance to herbivores (Kusnierczyk et al., 2007; Broekgaarden et al., 2008; Steppuhn et al., 2008). Also, variation in gene transcription was demonstrated for Arabidopsis accessions treated with the phytohormone methyl jasmonate (Matthes et al., 2008).

To our knowledge, exploring genetic variation in HIPV emission with respect to their role in indirect defense has so far primarily been explored for crop cultivars (see e.g. (Loughrin et al., 1995; Krips et al., 2001; Scutareanu et al., 2003; Hoballah et al., 2004; Bukovinszky et al., 2005; Nissinen et al., 2005; Lou et al., 2006). Studying this kind of traits is particularly complex, as the

defense trait involves many chemical compounds (Dicke et al., 1990c; Turlings et al., 1991; Mattiacci et al., 1995). In several studies, mutants and transgenics have been used to unravel signal-transduction and biosynthetic pathways in indirect defense (Van Poecke and Dicke, 2002; Aharoni et al., 2003; Ament et al., 2004; Shiojiri et al., 2006a; Snoeren et al., 2009 chap. 4). However, in order to be able to use newly available marker technologies that allow characterization and positioning of loci that control these types of traits (Lambrix et al., 2001; Kliebenstein et al., 2002), exploring the range of genetic variation for HIPVs is a prerequisite.

Here, we address the genetic variation in caterpillar-induced indirect defense, i.e. HIPV emission, among nine Arabidopsis accessions originating from different geographic origins. The objectives of this study were: (1) to screen for variation in the herbivore-induced emission of volatiles, (2) to study the variation in transcription levels of genes putatively involved in volatile production, and (3) to assess the effects of induced volatile emission on the attraction of parasitoid wasps.

Materials and Methods

Plant and insect material

Nine *Arabidopsis thaliana* (L.) Heynh. accessions, either obtained from NASC (http://nasc.nott.ac.uk/) (An-1= N944, C-24= N906, Cvi= N8580, Kond= CS6175, L*er*= NW20, the Sendai stock centre in Japan (Kyo-1= JW137) or collected in Sweden by members of the Wageningen Genetics Laboratory (Eri-1= CS22548) were used. Seeds from Col-0 and WS were provided by P. Reymond (Lausanne, Switserland). Seeds were germinated in sandy Arabidopsis soil (Lentse potgrond BV, Lent, Netherlands), and cultivated in a growth chamber at 21 ± 2 °C , 50-60% relative humidity (RH), and a L8:D16 photoperiod with 80-110 µmol m^{-2} s^{-1} PPF. The soil was heated to 90 °C for at least two hours prior to sowing of the plants. Two-week-old seedlings were transferred from seed trays to plastic cups (5 cm in diameter) filled with similar soil. Plants were watered twice a week. When plants were full-grown vegetative plants, i.e. after 6 to 8 weeks since sowing, they were used for experiments. To prevent infestation by sciarid larvae, the soil was treated weekly with *Steinernema feltiae* entomopatogenic nematodes (Koppert B.V. Berkel en Rodenrijs, the Netherlands).

The herbivore *Pieris rapae*, the small cabbage white, was reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera*, cv Cyrus) in a growth chamber (L16:D8; 20 ± 2 °C and 70% RH).
Parasitoid wasps, *Diadegma semiclausum,* were reared as described by Bukovinszky et al. (2005). Emerging wasps were provided *ad libitum* with water and honey, and are referred to as 'naïve' wasps as they had neither received exposure to plant material, nor obtained oviposition experience. The parasitoid is known to be attracted to the volatiles that are emitted by *P. rapae* -infested Arabidopsis Col-0 plants (Loivamäki et al., 2008).

Plant treatment

Defense responses were induced by 24 h of herbivore-feeding or by spraying the plant with the phytohormone jasmonic acid (JA) 24 h before the experiment. Plants were infested by equally distributing 20 first-instar *P. rapae* larvae per plant over the fully expanded leaves. To check for variation in induced volatiles due to differences in leaf tissue consumption by caterpillars, we included a treatment that mimicked the effect of herbivory by spraying with a JA solution. Plants were completely sprayed with a total volume of 5 ml of 1.0 mM (±)-JA (Sigma-Aldrich) aqueous solution.

Caterpillar-feeding

To assess the areas of leaf tissue consumed by the caterpillars, on three experimental days five plants of each accession were infested by equally distributing 20 first-instar *P. rapae* larvae over the fully expanded leaves of each plant. Twenty-four hours after infestation individual leaves were cut, taped on paper and scanned with a Hewlett-Packard scan jet 3570c. Original leaf shapes were reconstructed using drawing software Paint.NET v3.30, Microsoft Corporation. Quantification of consumed leaf tissue area was performed using Winfolia pro 2006a, Regent Instruments (Québec, Canada). A one-way ANOVA with an LSD post-hoc test was used to test whether the consumed leaf areas differed between the accessions (SPSS 15.0, Chicago, USA).

Headspace collection and volatile analysis

Dynamic headspace sampling was carried out in a climate room (20 ± 2 °C, 70% RH; L8:D16 photoperiod and 90-110 µmol photons m^{-2} s^{-1} PPFD at canopy height). Twenty-four hours before sampling, pots were removed, roots and soil were carefully wrapped in aluminum foil, and four plants were placed together in a 2.5 L glass jar. The glass jars were then covered with insect-proof gauze. Just before headspace collection, the gauze was removed and jars were closed with a Viton-lined glass lid having an inlet and outlet. Inlet air was filtered by passing through stainless steel cartridges (Markes, Llantrisant, UK) filled with 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, USA). Volatiles were trapped by sucking air out of the jar at a rate of 100 ml min^{-1} through a similar cartridge filled with 200 mg Tenax TA. Headspace volatiles for all treatments were collected during 3.5 h. Fresh weights of all rosettes were determined immediately after the experiments. On each experimental day, headspace samples of three or four accessions of each treatment were collected simultaneously. For each accession, 5 (An-1, C-24, Cvi, Eri-1, Kond, Kyo-1, L*er*) or 6 (Col-0, WS) replicates for each experimental condition (control, caterpillar-infested, or JA-treated) were collected and analyzed.

Headspace samples were analyzed with a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, USA) quadrupole mass spectrometer. Before desorption of the volatiles, the Tenax cartridges were dry-purged with nitrogen at

30 ml min^{-1} for 20 min at ambient temperature to remove water. Volatiles were desorbed from the cartridges using a thermal desorption system at 250 °C for 3 min (Model Ultra Markes Llantrisant, UK) with a helium flow of 30 ml min^{-1}. Analytes were focused at 0 °C on an electronically-cooled sorbent trap (Unity, Markes, Llantrisant, UK). Volatiles were transferred in splitless mode to the analytical column (Rtx-5ms, 30 m, 0.25 mm i.d., 1.0 µm film thickness, Restek, Bellefonte, USA) by rapid heating of the cold trap to 250 °C. The GC was held at an initial temperature of 40 ^{o}C for 3.5 min followed by a linear thermal gradient of 10 ^{o}C min^{-1} to 280 ^{o}C and held for 2.5 min with a column flow of 1 ml min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 45-400 m/z with a scan rate of 3 scans s^{-1}. Compounds were identified using the deconvolution software AMDIS (version 2.64, NIST, USA) in combination with NIST 98 and Wiley 7th edition spectral libraries and by comparing their retention indices with those from the literature (Adams, 1995). For quantification, characteristic quantifier ions were selected for 84 compounds (SOM Table 1). Metalign software (PRI-Rikilt, Wageningen, the Netherlands) was used to align peaks of chromatograms of all samples and integrate peak areas for the quantifier ions. Peak areas of all compounds were corrected for the fresh weight of the leaf rosettes. Overall volatile profiles were analyzed using Principal Component Analysis (GeneMath XT 2.0) after log_{10} transformation of the data and subtracting the average value of all treatments. PCA involves the calculation of the Eigenvalue decomposition of a data covariance matrix and we used it to visualize differences between accessions and treatments based on the total volatile profile.

Emitted quantities of individual volatile components were analyzed for significant changes between plant treatments using a t-test. Individual volatiles were analyzed for significant differences in emission rates between accessions within each of the treatments, using one-way ANOVA followed by a Dunnett T3 post-hoc analysis (SPSS 15.0). Differences in emission of volatile compounds between treatments for each accession were analyzed for significance using a one-way ANOVA.

Quantitative RT-PCR analysis

A qRT-PCR analysis was used to screen for differences in the expression of JA and *P. rapae*-induced genes putatively involved in volatile production in Arabidopsis. Leaf material was collected by selecting two almost fully expanded leaves with feeding damage, from which caterpillars and their products were removed. For JA- and non-treated plants, similar leaves were collected. Five plants, i.e. ten leaves, were used per replicate. Collected leaf material was immediately flash frozen in liquid nitrogen and stored at -80 °C. As control plants we used uninfested plants that were sprayed with water and that were otherwise treated similar to the infested plants.

Pooled leaf samples were ground in liquid nitrogen and total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). One µg of total

RNA was treated with DNaseI (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA-free total RNA was subsequently converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Gene-specific primers were designed using Beacon Designer 7.0 (Premier Biosoft Int) for seven Arabidopsis genes based on sequences obtained from the TIGR Arabidopsis database. Primer sequences are shown in Table 1. Primers were tested for gene specificity by performing melt curve analysis on a MyIQ Single-Color Real-Time PCR Detection System (BioRad). PCR products were sequenced to confirm the amplification of the gene of interest. Sequence results were checked by a BLAST search in the Arabidopsis TIGR database (data not shown).

Table 1. Sequences of *Arabidopsis thaliana*-derived primers used in quantitative Real Time-PCR analyses.

Gene	***AGI-codes***	***Forward primer (5′ à 3′)***	***Reverse primer (5′ à 3′)***
β-Actin	At3g46520	GGAGAAGATTTGGCATCACAC	TGGCAACATACATAGCAGGAG
HPL1	At4g15440	ACATCGCTGAGAACGGTTG	CAAGAGGCTGAGGAACTACG
BSMT1	At3g11480	TGGTCACTACTACGAAGAAGATG	GAGCATTGGTTCACTAACAGC
TPS03	At4g16740	GCCACCATCCTCCGTCTC	CCAAGCCACACCGATAATTCC
TPS04	At1g61120	TCGCAGCACACACCATTG	GAGCAGCACGGAGTTCATC
CYP72A13	At3g14660	GATGGCAATGACACTGATTCTAC	GATAAGAGGAGCACCGAACTG
CYP82G1	At3g25180	ATCAGACAGCACATCCATCAC	GCCGACACTATTATCAATCTCTTC

Quantitative RT-PCR analysis was carried out in optical 96-well plates with a MyIQ Single-Color Real-Time PCR Detection System (BioRad), using SYBR Green to monitor dsDNA presence. Each reaction contained 10 µl 2x SYBR Green Supermix Reagent (BioRad), 10 ng cDNA and 300 nM of the gene-specific primers in a final volume of 20 µl. All qRT-PCR experiments were performed in duplicate. The following PCR programme was used for all PCR analyses: 95 °C for 3 min; 40 cycles of 95 °C for 30 s and 60 °C for 45 s. Threshold cycle (Ct) values were calculated using the MyIQ Optical System software (version 2.0, BioRad). Subsequently, Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of the constitutively expressed gene β-actin from the Ct value of the gene of interest. *β-Actin* is widely used as a reference gene in expression studies and its absolute expression level was not influenced by *P. rapae*-infestation nor by JA-application in our study (data not shown). Experiments were repeated four times and the differences in normalized gene expression ($2^{-\delta Ct}$) between treatments were statistically analyzed using a one-way ANOVA with an LSD post-hoc test (SPSS 15.0).

Behavioral assays using parasitoid wasps

The effect of JA-induced volatile production on parasitoid behavior was compared for different accessions in a closed-system Y-tube olfactometer as described by Bukovinszky et al. (2005). In short, filtered air was led through activated charcoal and split into two air streams (4 L min^{-1}) that were led through five-liter glass cuvettes containing the odor source each consisting of four plants. The olfactometer was illuminated with artificial light from above at an intensity of 60 ± 5 µmol m^{-2} sec^{-1} PPFD. All experiments were conducted in a climatized room (20 ± 2 °C).

Naive, 3-7 days-old female *D. semiclausum* were individually transferred from the cage into the Y-tube olfactometer using a glass tube. Upon release in the olfactometer, parasitoid behavior and parasitoid choice for one of the two odor sources was observed. Parasitoids that did not make a choice within ten minutes after release or did not choose for one of the two arms of the olfactometer within five minutes were considered as non-responding individuals, and were excluded from preference analysis. After every three parasitoids tested, the odor sources were interchanged to compensate for any unforeseen asymmetry in the set-up.

Parasitoid preference for accessions treated with JA was statistically analyzed using a Chi-square test, with the null-hypothesis that parasitoids did not have a preference for any of the two odor sources.

Results

Leaf damage by Pieris rapae

P. rapae caterpillars consumed 3.5 ± 0.2% (WS) to 7.4 ± 0.5% (C-24) of the total leaf area in 24 hours (Table 2). As the accessions differed in total leaf area per plant (varying from 1935 ± 98 mm^2 for C-24 to 4427 ± 179 mm^2 for WS; mean ± SE) we also determined the absolute amount of leaf tissue area consumed per plant, which varied from 88 ± 7.5 mm^2 (An-1) to 195 ± 10.2

Accession	*Mean area remaining per plant (%) ± SE*	*Mean leaf area consumed per plant (mm^2) ± SE*	
An-1	96.4 ± 0.5	88 ± 7.5	a
C-24	92.6 ± 0.5	139 ± 5.2	ce
Col-0	94.2 ± 0.4	165 ± 7.9	d
Cvi	95.2 ± 0.5	135 ± 8.5	c
Eri-1	94.8 ± 0.3	195 ± 10.2	b
Kond	93.6 ± 0.5	190 ± 6.9	b
Kyo-1	94.8 ± 0.4	158 ± 8.6	de
L*er*	94.0 ± 0.4	157 ± 6.7	de
WS	96.5 ± 0.2	152 ± 5.3	cde

Table 2. Leaf damage by *Pieris rapae*-feeding on plants from nine accessions of *Arabidopsis thaliana*. Data show the percentage of leaf that remained after 24 h of infestation, and the leaf area that was consumed by the caterpillars. Mean leaf areas consumed per plant were statistically analyzed using a one-way ANOVA followed by LSD post-hoc. Accessions followed by different letters indicate a significant difference (P<0.05).

mm^2 (Eri-1) (mean ± SE)(Table 2). Accession An-1 had the lowest area of leaf tissue consumed per plant compared to all other accessions (ANOVA LSD: $P<0.001$). From accessions Eri-1 and Kond significantly more leaf area was consumed when compared to accessions An-1, C-24, Col-0, Cvi, Kyo-1, L*er*, and WS (Table 2). To exclude variation in induced volatiles as a result of differences in the amount of damage, we included the JA-treatment to expose plants to a similar level of induction.

Volatile profiling in different accessions

A principal component analysis (PCA) for the total volatile profile showed that the first three principal components explain 60% of the observed variation.
Figure 1 shows the principal component analysis for the total volatile profile of all accessions and treatments based on a loading plot of 73 different compounds. The data show that the overall volatile profile alters when plants are induced by *P. rapae* herbivory or JA treatment compared to non-treated plants. No clear difference is observed for volatile emission patterns induced by JA treatment or *Pieris* infestation when all 73 compounds are taken into account for all nine accessions (Fig. 1).

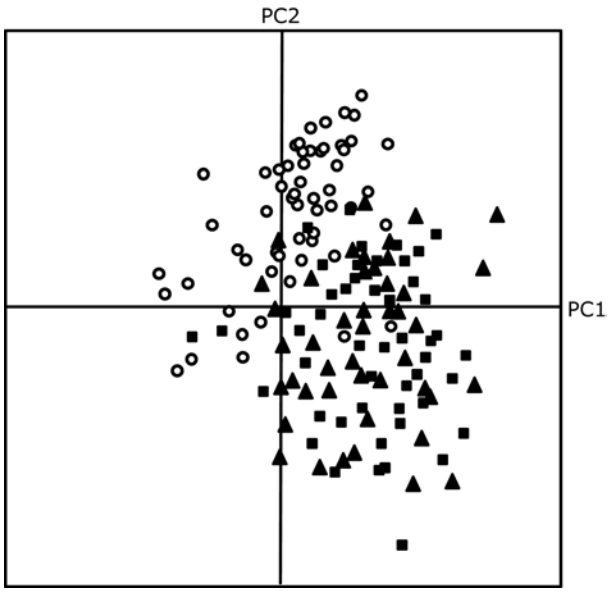

Figure 1. Principal Component Analysis (PCA) of volatiles emitted by nine *Arabidopsis thaliana* accessions infested with *Pieris rapae* (■), treated with JA (▲),or left untreated (control, ❍). The PCA shows the first and second PC.

To get an impression of the natural variation among accessions in herbivore-induced volatiles, accessions were analyzed separately for *P. rapae* infestation or JA treatment (Fig. 2). The first PC is not included, since it mainly separated samples according to the day of headspace collection (batch variation) more than to biological variation. We therefore present the graph of the second and third principal component for both treatments. For *P. rapae* infestation (Fig. 2A), principal components 2 to 6 explain 52.5% of the variation in the total dataset, whereas for JA-treated plants (Fig. 2B), 59.3% of the variation can be explained by the principal components 2-7. The remaining variation could not be assigned to biological variation as individual data points did not cluster per accession anymore. The PCA analysis in both treatments shows that individual samples of each accession

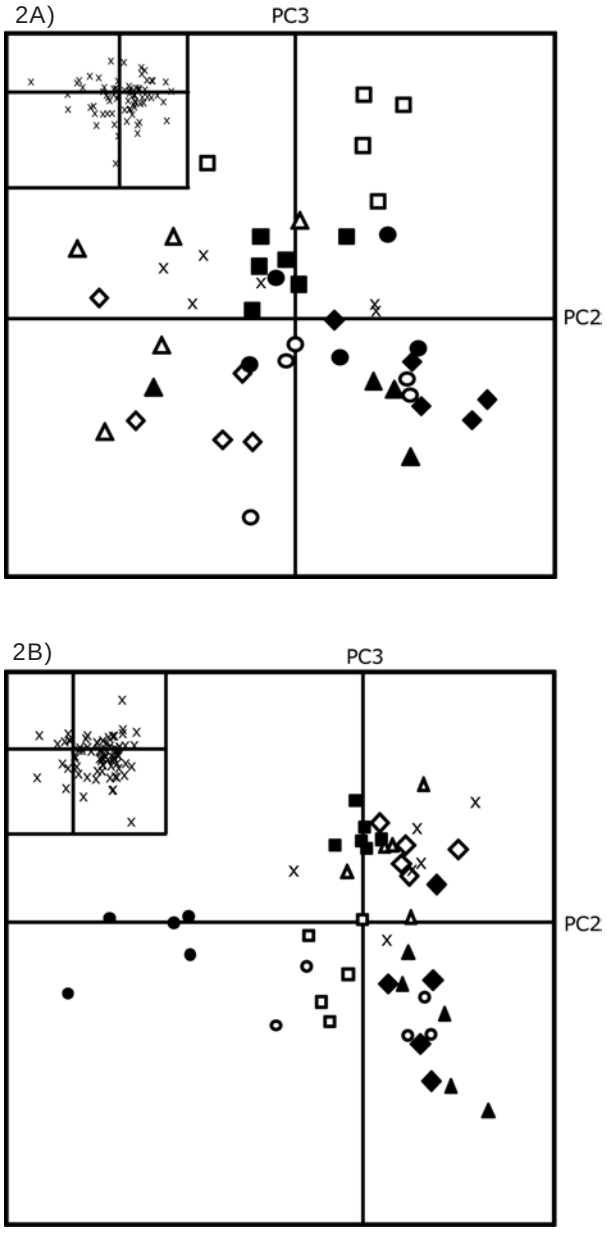

Figure 2. Principal Component Analysis (PCA) of volatiles emitted by nine *Arabidopsis thaliana* accessions infested with *Pieris rapae* (A) or treated with JA (B). The PCA shows the second and third PC. m, An-1; l, C-24; o, Cvi; n, Col-0; ◇, Eri-1; u, Kond; Δ, L*er*; p, Kyo-1; x, WS. The PCA shows the second and third PC.

cluster together, although the relative position of the accessions is different for plants in the *P. rapae* infestation and JA treatment groups. It implies that *P. rapae* herbivory and JA treatment have different effects on individual volatile compounds. This is also supported by ANOVA analysis (α=0.05) for differences in induction of individual compounds between both treatments (SOM Table 4).

To identify those volatile compounds that are emitted in different rates between accessions within a treatment, one-way ANOVA analysis (α=0.05) was carried out for each compound, followed by a Dunnett T3 post-hoc test. Table 3 shows those compounds that are significantly different between accessions within a treatment. The differences are also presented in SOM Table 2, but then from the perspective of a comparison among accessions. The 28 (out of 73) compounds in Table 3 belong to various chemical classes, including green leaf volatiles (GLV) [(*E*)-2-hexenal, (*E*)-3-hexenal, (*Z*)-3-hexenyl acetate, (*Z*)-2-penten-1-ol, (*Z*)-3-hexen-1-ol, pentan-2-ol], terpenoids [(*E*)-ß-ocimene, (*Z*)-ß-ocimene, 3-carene, ß-myrcene, α-pinene, α-phellandrene (monoterpenes); (*E*)-nerolidol, ß-sesquiphellandrene, ß-acoradiene, ß-bisabolene, (*E*)-ß-farnesene, (*E,E*)-α-farnesene, α-humulene (sesquiterpenes), (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT)

Table 3. Volatile compounds that are emitted in significantly different rates among nine accessions of *Arabidopsis thaliana* infested with 20 first instar larvae of *Pieris rapae*, treated with JA or left untreated (control). Individual volatiles were analyzed for significant differences between accessions within one treatment, by using one-way ANOVA followed by a Dunnett T3 post-hoc analysis. Significance: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Compound	Control	Sign.	*P. rapae*	Sign.	JA	Sign.
(*E*)-2-hexenal			An-1 > L*er*	*		
			C-24 > L*er*	*		
(*E*)-3-hexenal	WS > Col-0	**				
	WS > C-24	*				
(*Z*)-3-hexenyl acetate			Cvi > An-1	***		
			Cvi > C-24	***		
(*Z*)-2-penten-1-ol	WS > Col-0	*				
(*Z*)-3-hexen-1-ol			An-1 > Col-0	***		
			An-1 > Cvi	*		
			Cvi > Col-0	*		
			Eri-1 > Col-0	**		
			Kond > C-24	*		
			Kond > Col-0	***		
			Kond > Cvi	**		
			WS > Col-0	***		
			WS > Cvi	*		
hexanoic acid	C-24 > L*er*	***				
pentan-2-ol	C-24 > Kyo-1	*				
	Cvi > Kyo-1	*				
(*E*)-nerolidol	C-24 > Col-0	*	Col-0 > WS	*		
			Kond > WS	*		
(*E*)-ß-ocimene			Kond > C-24	***	An-1 > Col-0	*
			Kond > Col-0	**	An-1 > Kyo-1	**
			WS > Col-0	*	C-24 > Kyo-1	*
			WS > C-24	**	Cvi > Kyo-1	*
					Eri-1 > Kyo-1	*
					Kond > Col-0	***
					Kond > Cvi	*
					Kond > Eri-1	***
					Kond > Kyo-1	***
					WS > Col-0	***
					WS > Eri-1	**
					WS > Kyo-1	***
(*Z*)-ß-ocimene	Kond > An-1	**	An-1 > Col-0	*	An-1 > Kyo-1	*
	Kond > Cvi	*	Kond > Col-0	*	C-24 > Col-0	*
			L*er* > *C-24*	*	C-24 > Kyo-1	*
			L*er* > *Col-0*	**	Cvi > Col-0	*
			WS > Col-0	*	Cvi > Kyo-1	**
			WS > C-24	*	Kond > Col-0	**
					Kond > Eri-1	*
					Kond >Kyo-1	***
					Kond > L*er*	*
					WS > Col-0	*
					WS > Kyo-1	**
decanal			Col-0 > An-1	*		
			Col-0 > Cvi	*		
			Eri-1 > An-1	*		
			Eri-1 > Cvi	*		
			WS > An-1	***		
			WS > Cvi	**		
3-carene	An-1 > Col-0	***				
ß -acoradiene			Cvi > Kyo-1	*		
DMNT	C-24 > Col-0	***			C-24 > Col-0	**
	Kond > Col-0	***			Kond > Col-0	*
	WS > Col-0	**			WS > Col-0	***
ß-bisabolene			An-1 > Kond	***	C-24 > WS	*
			C-24 > Kond	***	Eri-1 > WS	*
			Kyo-1 >Kond	***		

Compound	Control	Sign.	*P. rapae*	Sign.	JA	Sign.
(E)-ß-farnesene	Col-0 > L*er*	**	Col-0 > C-24	***		
	Kond > L*er*	*	Cvi > C-24	*		
			Cvi > C-24	*		
			Kond > C-24	**		
			Kyo-1 > C-24	***		
			WS > C-24	*		
ß-myrcene			Cvi > An-1	*	Cvi > An-1	*
			Eri-1 > An-1	*	Cvi > Col-0	*
			Kond > An-1	*	Cvi > L*er*	*
			L*er* > *An-1*	*	Eri-1 > Col-0	*
ß-myrcene					Eri-1 > L*er*	*
					Kond > An-1	***
					Kond > C-24	**
					Kond > Col-0	***
					Kond > L*er*	**
					Kond > WS	*
ß-sesquiphellandrene			An-1 > Eri-1	*	An-1 > Cvi	*
			An-1 > Col-0	*	An-1 > Eri-1	*
			An-1 > C-24	*	Kond > Cvi	*
					Kond > Eri-1	*
TMTT			Col-0 > C-24	**	An-1 > Cvi	*
			Col-0 > Cvi	**	Kond > Cvi	**
			Kond > C-24	*	Kyo-1 > Cvi	***
			Kond > Cvi	*	L*er* > *Cvi*	**
			WS > Cvi	**		
			WS > C-24	*		
(*E,E*)-α-farnesene			An-1 > Cvi	**	Eri-1 > C-24	**
			An-1 > Col-0	*	Eri-1 > WS	**
			An-1 > C-24	*	Kond > C-24	*
			Eri-1 > Cvi	**		
			Kyo-1 > C-24	*		
			Kyo-1 > Col-0	*		
			Kyo-1 > Cvi	***		
			Kyo-1 > L*er*	*		
α-humulene			C-24 > Kyo-1	**		
α-phellandrene	Cvi > Col-0	*				
	Kond > Col-0	*				
α-pinene					Kond > An-1	*
					L*er* > *An-1*	*
benzaldehyde			An-1 > C-24	**		
			Col-0> C-24	*		
			Kond > C-24	*		
			L*er* > *C-24*	**		
ethyl salicylate					Cvi > An-1	***
					Cvi > Eri-1	***
					L*er* > *An-1*	***
					L*er* > *Eri-1*	***
geranyl acetone			Kond > Col-0	*	WS > Eri-1	*
indole			Cvi > C-24	***		
MeSA	An-1 > WS	*	An-1 > Eri-1	*	Col-0 > Cvi	***
	Eri-1 > WS	*	An-1 > Kond	**	Col-0 > Eri-1	*
	Kyo-1 > WS	**	An-1 > Kyo-1	*	Kond > Cvi	**
			An-1 > Cvi	***	Kyo-1 > Cvi	**
			C-24 > Cvi	***	L*er* > *Cvi*	***
			Col-0 > Cvi	***	L*er* > *Eri-1*	**
			Col-0 > Eri-1	*	L*er* > *Kond*	*
			Col-0 > Kond	**	L*er* > *Kyo-1*	*
			Col-0 > Kyo-1	*	WS > Cvi	***
			Eri-1 > C-24	***	Ws > Eri-1	**
			Kyo-1 > Cvi	**		
			L*er* > *Cvi*	***		
			L*er* > *Kond*	*		
			WS > Cvi	***		

(homoterpenes)], phenolic compounds (MeSA, ethyl salicylate) and indole, benzaldehyde, hexanoic acid, decanal and geranyl acetone.

The total amount of volatiles emitted after herbivory increased for all accessions from 1.1 fold (*P. rapae*-infested C-24) to 4.6 fold (JA-treated Kond) the amount emitted by the non-treated controls. The total amount of volatiles emitted after *P. rapae* infestation or JA treatment was highest in accessions L*er*, Kond and WS. The composition of the volatiles blends, i.e. the relative contribution of compounds that belong to different chemical classes, differed substantially between different accessions. *P. rapae*-infestation increased the proportion of GLVs in the total blend in all accessions, except for Kond and WS, although the absolute amount of GLVs did increase in these accessions. JA treatment increased GLV emission in all accessions, except An-1, C-24 and Kond, resulting in a relatively lower amount of GLVs emitted by JA-treated plants of these accessions. The highest proportion of GLVs was found in the blend of JA-treated Kyo-1 plants, amounting to more than 25% of the total volatiles. All accessions showed a higher contribution of GLVs in the blend of *P. rapae*-infested plants compared to JA-treated plants.
Both Kond and WS showed a stronger increase in the emission of monoterpenes than the other accessions. After JA-treatment more than half of the total blend consisted of monoterpenes in WS and even more (68%) in Kond. After *P. rapae* infestation 39% and 54% of the volatile blend consisted of monoterpenes in WS and Kond, respectively. In contrast, although *P. rapae* infestation and JA treatment increased the absolute emission of monoterpenes, the contribution of this class of compounds to the total blend was lowest in Kyo-1, 10% and 7% for *P. rapae*-infested and JA-treated plants, respectively.
Herbivory increased the absolute emission of sesquiterpenes in most accessions, except for *P. rapae*-infested C-24 and Col-0 and JA-treated Cvi plants. About 20% of the volatile blend of JA-treated plants of accessions Eri-1 and Kyo-1 consisted of sesquiterpenes, which is the highest proportion of sesquiterpenes found in the accessions included in this study. In contrast, *P. rapae* infestation resulted in only 3% (Eri-1) and 9% (Kyo-1) of sesquiterpenes in the total volatile blend. Remarkably, JA treatment failed to increase sesquiterpene emission in the blend of Cvi, whereas *P. rapae* feeding did increase sesquiterpene emission in Cvi (10%).

Six of the volatile compounds that may be related to the genes for which we analyzed expression levels in this study are discussed in more detail below. Fig. 3 shows the induced emission rates of these compounds for both treatments (*P. rapae* and JA) in the nine accessions.
Several GLVs are emitted in larger amounts from *P. rapae*-infested plants than from non-treated plants (Fig. 3A; SOM Table 3). JA treatment resulted in only small increases in the emission of the GLV compounds compared with non-treated controls. The GLV (*Z*)-3-hexen-1-ol contributed most to the observed variation among the accessions (Fig. 3). In particular accessions An-1, that had

the highest emission rate of (*Z*)-3-hexen-1-ol, and Col-0, that showed lowest emission, contributed to the observed variation among accessions after herbivory.

All accessions, except Cvi, showed significantly higher MeSA emission after *P. rapae*-infestation than from uninfested plants (Fig. 3B; SOM Table 3), and all plants treated with JA emitted MeSA in higher amounts than non-treated plants except for accessions An-1, Cvi and Eri-1 (Fig. 3B; SOM Table 3). Accessions varied significantly in the emission rates of MeSA after herbivore damage, particularly accession An-1 emitted more and accession Cvi less than the others (Fig. 3B). After JA treatment, the largest amount of MeSA was emitted by accession L*er* while the lowest amount was emitted by accession Cvi (Fig. 3B).

Emission of various monoterpenes varied between accessions after *P. rapae* infestation and JA treatment. Figure 3 shows data for two of them, i.e. ß-myrcene and (*Z*)-ß-ocimene. *P. rapae* infestation induced ß-myrcene in accessions C-24, Cvi and Kond and JA treatment did so in Cvi, Kond and WS. (*Z*)-ß-ocimene was induced in all accessions by *P. rapae* infestation and JA treatment, except in Kyo-1 and in Col-0 after JA treatment (Fig. 3D; SOM Table 3). The highest emission rates of ß-myrcene, (*Z*)-ß-ocimene, and several other monoterpenes occurred in induced Kond plants (Fig. 3C, D). The lowest emission of (*Z*)-ß-ocimene occurred in *P. rapae*-infested and JA-treated Kyo-1 plants. *P. rapae*-infested Kyo-1 plants were also lowest in emission of ß-myrcene, whereas JA-treated Col-0 plants showed lowest ß-myrcene emission. Other monoterpenes that were emitted quantitatively differently between the two treatments were α-terpineol (An-1, C-24,), nerol (C-24, Eri-1), 3-carene, limonene, α-phellandrene (all in Cvi) and linalool (Eri-1, WS) (SOM Table 4).

The emission rates of the homoterpene TMTT were significantly higher in *P. rapae*-infested Col-0, Eri-1, and WS plants and JA-treated An-1, Col-0, and Eri-1 plants than in non-treated plants (Fig. 3E; SOM Table 3). Within each treatment group accessions did differ in the emission of TMTT. After herbivory Col-0 plants emitted the largest amount of TMTT and Cvi the lowest. Treatment of plants with JA resulted in highest TMTT emission rates in L*er* and lowest in Cvi (Fig. 3E).

The emission of (*E,E*)-α-farnesene was significantly induced after herbivory in accessions An-1, Eri-1, and Kyo-1, whereas after JA-treatment accessions An-1, Col-0, Eri-1, and Kyo-1 emitted more (*E,E*)-α-farnesene than non-treated control plants (Fig. 3F; SOM Table 3). Herbivory induced the highest emission rate of (*E,E*)-α-farnesene in An-1 plants while WS plants had the lowest emission rate of (*E,E*)-α-farnesene after herbivory. JA-treatment resulted in Eri-1 plants emitting most (*E,E*)-α-farnesene and Cvi plants the lowest amount (Fig. 3F).

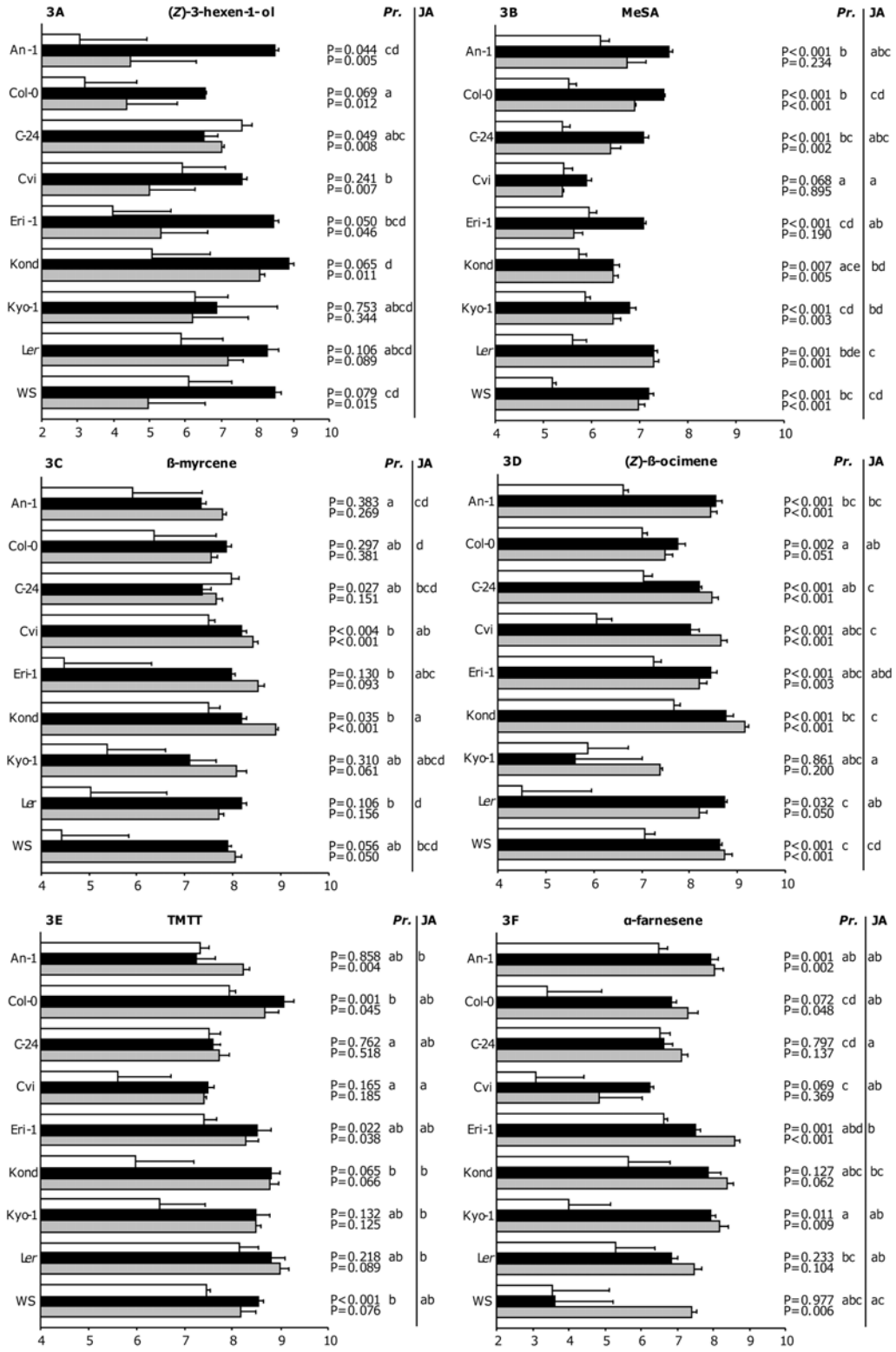
3A
(Z)-3-hexen-1-ol
Pr.
JA
An-1
Col-0
C-24
Cvi
Eri-1
Kond
Kyo-1
Ler
WS
P=0.044 cd
P=0.005
P=0.069 a
P=0.012
P=0.049 abc
P=0.008
P=0.241 b
P=0.007
P=0.050 bcd
P=0.046
P=0.065 d
P=0.011
P=0.753 abcd
P=0.344
P=0.106 abcd
P=0.089
P=0.079 cd
P=0.015
2 3 4 5 6 7 8 9 10
3B
MeSA
Pr.
JA
P<0.001 b
P=0.234
P<0.001 b
P<0.001
P<0.001 bc
P=0.002
P=0.068 a
P=0.895
P<0.001 cd
P=0.190
P=0.007 ace
P=0.005
P<0.001 cd
P=0.003
P=0.001 bde
P=0.001
P<0.001 bc
P<0.001
4 5 6 7 8 9 10
3C
ß-myrcene
Pr.
JA
P=0.383 a
P=0.269
P=0.297 ab
P=0.381
P=0.027 ab
P=0.151
P<0.004 b
P<0.001
P=0.130 b
P=0.093
P=0.035 b
P<0.001
P=0.310 ab
P=0.061
P=0.106 b
P=0.156
P=0.056 ab
P=0.050
cd
d
bcd
ab
abc
a
abcd
d
bcd
4 5 6 7 8 9 10
3D
(Z)-ß-ocimene
Pr.
JA
P<0.001 bc
P<0.001
P=0.002 a
P=0.051
P<0.001 ab
P<0.001
P<0.001 abc
P<0.001
P<0.001 abc
P=0.003
P<0.001 bc
P<0.001
P=0.861 abc
P=0.200
P=0.032 c
P=0.050
P<0.001 c
P<0.001
bc
ab
c
c
abd
c
a
ab
cd
4 5 6 7 8 9 10
3E
TMTT
Pr.
JA
P=0.858 ab
P=0.004
P=0.001 b
P=0.045
P=0.762 a
P=0.518
P=0.165 a
P=0.185
P=0.022 ab
P=0.038
P=0.065 b
P=0.066
P=0.132 ab
P=0.125
P=0.218 ab
P=0.089
P<0.001 b
P=0.076
b
ab
ab
a
ab
b
b
b
ab
4 5 6 7 8 9 10
3F
α-farnesene
Pr.
JA
P=0.001 ab
P=0.002
P=0.072 cd
P=0.048
P=0.797 cd
P=0.137
P=0.069 c
P=0.369
P=0.001 abd
P<0.001
P=0.127 abc
P=0.062
P=0.011 a
P=0.009
P=0.233 bc
P=0.104
P=0.977 abc
P=0.006
ab
ab
a
ab
b
bc
ab
ab
ac
2 3 4 5 6 7 8 9 10

Figure 3. Volatile emission (Log_{10}(peak area units.$FW^{-1}.h^{-1}$)+6 + SE)(5-6 replicates) of nine *Arabidopsis thaliana* accessions infested with *Pieris rapae* (*Pr.*) infested (black bars), treated with JA (grey bars), or left untreated (control bars). Behind bars that represent *P. rapae* and JA-induced emission of volatiles, *P* values are given for the induction (t-test). Accessions followed by a different letters - given for both treatments separately - differ significantly ($P<0.05$) (determined by post-hoc analysis).

Transcript profiling in different accessions

To investigate whether variation in emission of volatiles can be explained by differences in transcript levels of genes involved in their biosynthesis, transcript profiling of six genes that are putatively involved in the biosynthesis of plant volatiles was performed for the nine accessions after *P. rapae*-feeding or JA-treatment (Fig. 4).

For *HPL1*, which encodes a hydroperoxide lyase and is a member of the CYP74B cytochrome P450 family (Bate et al., 1998), induced transcript levels were observed after JA as well as after herbivore treatment for most accessions. The accessions An-1 and L*er* showed only just insignificantly *P. rapae*-induced transcript levels, whereas the accessions Cvi and Eri-1 only induced *HPL1* transcript levels after JA treatment (Fig. 4A). The *HPL1* transcript levels after herbivory did not differ among the accessions. In contrast, JA-treatment resulted in transcript level variation among the accessions. JA-induced *HPL1* transcript levels were highest in accession WS and lowest in accession Cvi.

Transcript levels of *BSMT1*, encoding a SABATH enzyme that methylates both salicylic acid and benzoic acid (Chen et al., 2003), were significantly induced in all accessions after 24 h of *P. rapae* feeding (Fig. 4B). JA treatment also led to higher transcript levels of *BSMT1* when compared to non-treated plants in all accessions. Quantitative differences among the accessions were found in *BSMT1* transcript levels after *P. rapae* treatment. The highest transcript level was observed in Cvi, and the lowest in WS. In contrast, no variation in JA-induced *BSMT1* transcript levels was observed among accessions.

The terpene synthase gene *TPS3* was selected for transcriptional analysis as this gene is annotated to be a (*E*)-ß-ocimene, (*Z*)-ß-ocimene, and β-myrcene synthase (Fäldt et al., 2003). Transcript levels of *TPS3* were higher in treated plants compared to control plants in most accessions (Fig. 4C). However, this was not the case for *P. rapae*-damaged Kyo-1 and L*er* as well as for JA-treated C-24, Cvi and Eri-1 plants. Irrespective of plant treatment, accessions did not differ among each other in induced *TPS3* transcript levels.

Another terpene synthase, *TPS4* encodes a geranyllinalool synthase, which is supposed to catalyze the formation of geranyllinalool, the intermediate in the biosynthesis of TMTT (Herde et al., 2008). In addition, two Cytochrome P450 genes were included in this analysis that have been postulated to be involved in the conversion of geranyllinalool to TMTT, i.e. *CYP72A13* (Bruce et al., 2008)

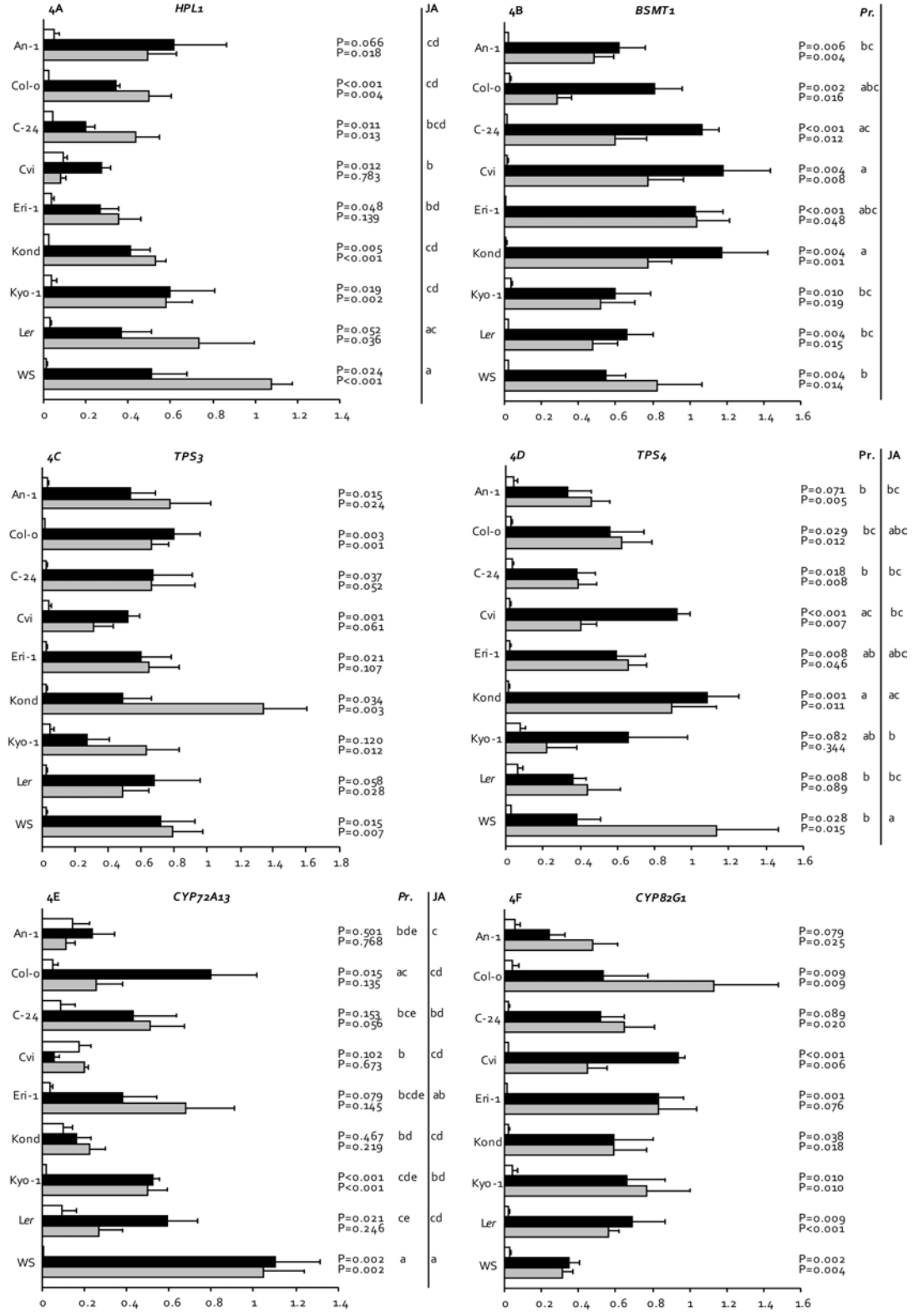
4A
HPL1
JA
An-1
Col-0
C-24
Cvi
Eri-1
Kond
Kyo-1
Ler
WS
P=0.066 P=0.018 cd
P<0.001 P=0.004 cd
P=0.011 P=0.013 bcd
P=0.012 P=0.783 b
P=0.048 P=0.139 bd
P=0.005 P<0.001 cd
P=0.019 P=0.002 cd
P=0.052 P=0.036 ac
P=0.024 P<0.001 a
0 0.2 0.4 0.6 0.8 1 1.2 1.4
4B
BSMT1
Pr.
P=0.006 P=0.004 bc
P=0.002 P=0.016 abc
P<0.001 P=0.012 ac
P=0.004 P=0.008 a
P<0.001 P=0.048 abc
P=0.004 P=0.001 a
P=0.010 P=0.019 bc
P=0.004 P=0.015 bc
P=0.004 P=0.014 b
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6
4C
TPS3
P=0.015 P=0.024
P=0.003 P=0.001
P=0.037 P=0.052
P=0.001 P=0.061
P=0.021 P=0.107
P=0.034 P=0.003
P=0.120 P=0.012
P=0.058 P=0.028
P=0.015 P=0.007
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8
4D
TPS4
Pr.
JA
P=0.071 P=0.005 b bc
P=0.029 P=0.012 bc abc
P=0.018 P=0.008 b bc
P<0.001 P=0.007 ac bc
P=0.008 P=0.046 ab abc
P=0.001 P=0.011 a ac
P=0.082 P=0.344 ab b
P=0.008 P=0.089 b bc
P=0.028 P=0.015 b a
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6
4E
CYP72A13
Pr.
JA
P=0.501 P=0.768 bde c
P=0.015 P=0.135 ac cd
P=0.153 P=0.056 bce bd
P=0.102 P=0.673 b cd
P=0.079 P=0.145 bcde ab
P=0.467 P=0.219 bd cd
P<0.001 P<0.001 cde bd
P=0.021 P=0.246 ce cd
P=0.002 P=0.002 a a
0 0.2 0.4 0.6 0.8 1 1.2 1.4
4F
CYP82G1
P=0.079 P=0.025
P=0.009 P=0.009
P=0.089 P=0.020
P<0.001 P=0.006
P=0.001 P=0.076
P=0.038 P=0.018
P=0.010 P=0.010
P=0.009 P<0.001
P=0.002 P=0.004
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6

Figure 4. Expression changes of six *Arabidopsis thaliana* genes in untreated (white bars), *Pieris rapae* infested (black bars) or JA-treated (grey bars) plants of nine Arabidopsis accessions. Bars represent relative expression levels (2^{-dCt}) (mean + SE), calculated from four biological replicates. Behind bars that represent *P. rapae* and JA-induced transcript levels of genes, the *P* values are given for the induction. Letters represent groups of accessions that did not significantly differ after ANOVA followed by post-hoc analysis, given for each treatment separately.

and *CYP82G1*, that show high co-expression with *TPS4* using the Correlated Gene Search Tool (http://prime.psc.riken.jp/?action=coexpression_index) of the RIKEN Plant Science Centre. *TPS4* transcript levels were significantly enhanced after *P. rapae* infestation or JA treatment in most of the accessions. In An-1 and Kyo-1 plants, *TPS4* transcript levels were marginally insignificantly induced after *P. rapae* feeding. Transcript levels in JA-treated Kyo-1 and L*er* plants were not induced. Accessions differed in their transcript levels after *P. rapae* infestation or JA treatment. Accession Kond had the highest *TPS4* transcript level after herbivory and An-1 showed the lowest. Treating plants with JA resulted in highest *TPS4* transcript levels for WS and the lowest for Kyo-1 (Fig. 4D).

CYP72A13 transcript levels showed a significant increase after *P. rapae* infestation in accessions Col-0, Kyo-1, L*er* and a marginally insignificant increase in Eri-1. JA treatment resulted in a significant increase in the transcript level of *CYP72A13* in accessions Kyo-1 and WS, whereas the transcript levels in C-24 and Eri-1 were not significant different from that in non-treated plants (Fig 4E).

CYP82G1 showed induced transcript levels after *P. rapae*-infestation and JA-treatment for all of the accessions except *P. rapae*-infested An-1 and C-24, and JA-treated Eri-1. No variation was observed among the accessions for either treatment (Fig 4F).

Consequences of variation in volatile profiles for parasitoid attraction

To investigate whether differences found in induced volatile blends between accessions affect the searching behavior of natural enemies of herbivores, bioassays were conducted using the parasitoid wasp *D. semiclausum*. This parasitoid wasp is known to respond to *P. rapae*-induced Arabidopsis volatiles (Loivamäki et al., 2008). To exclude variation in herbivore-induced emission due to differences in leaf damage, we chose to use JA for volatile induction only. Due to logistic limitations, we randomly selected six out of the nine accessions used in this study for the behavioral assays.

The volatiles emitted by accessions Cvi, Eri-1, Kond, L*er* and WS plants treated with JA attracted more *D. semiclausum* wasps than volatiles from non-treated control plants of the same accession. For Col-0, parasitoids did not discriminate between JA-treated and control plants, although there was a tendency of attraction towards the JA-treated plants (P=0.087; Fig 5A).

In two-choice experiments, JA-treated plants from different accessions were tested against each other (Fig. 5B). The JA-induced volatile blend from accession Kond was more attractive than those of Col-0 and Eri-1, but it was equally

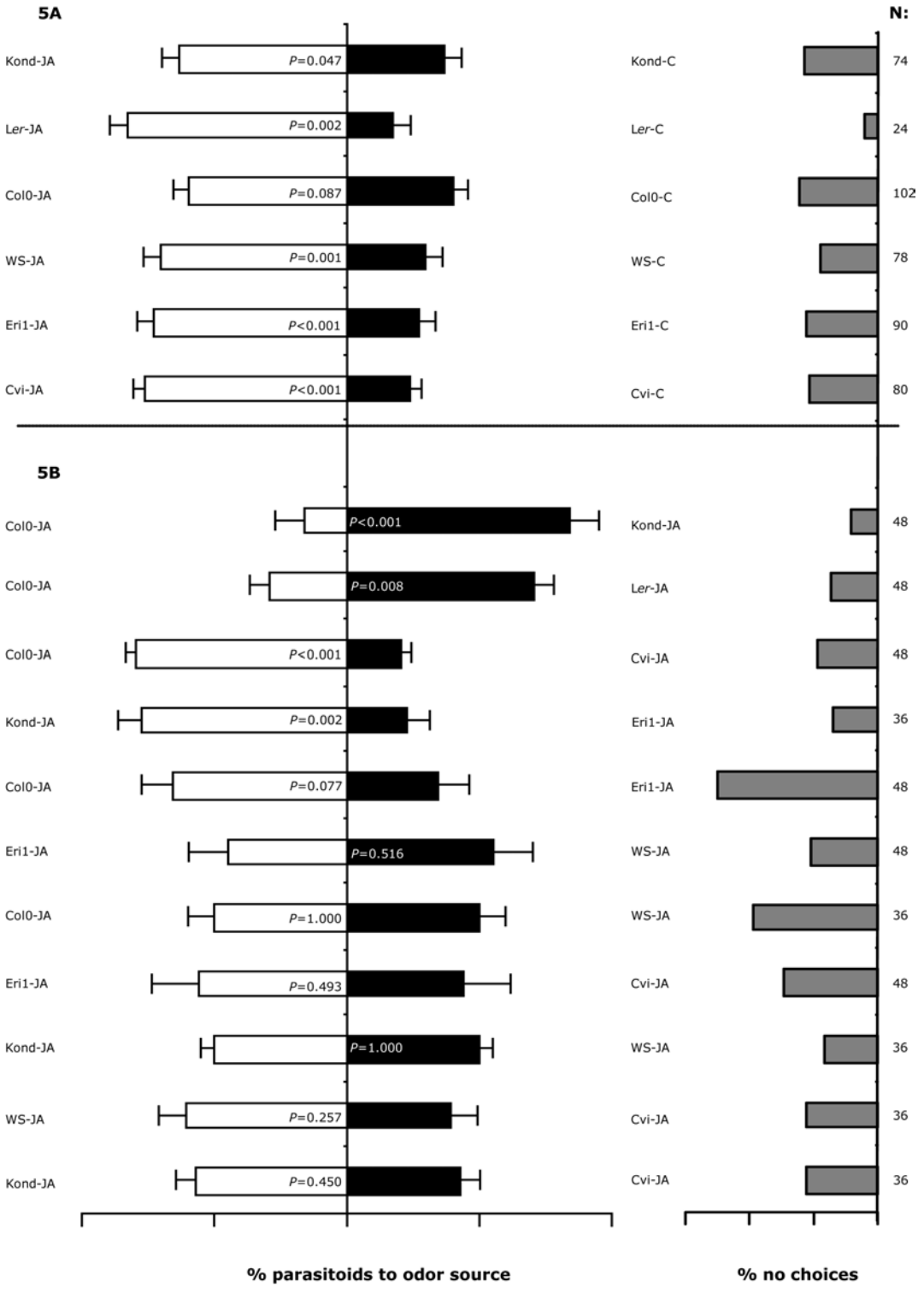
5A
N:
Kond-JA
P=0.047
Kond-C
74
Ler-JA
P=0.002
Ler-C
24
Col0-JA
P=0.087
Col0-C
102
WS-JA
P=0.001
WS-C
78
Eri1-JA
P<0.001
Eri1-C
90
Cvi-JA
P<0.001
Cvi-C
80
5B
Col0-JA
P<0.001
Kond-JA
48
Col0-JA
P=0.008
Ler-JA
48
Col0-JA
P<0.001
Cvi-JA
48
Kond-JA
P=0.002
Eri1-JA
36
Col0-JA
P=0.077
Eri1-JA
48
Eri1-JA
P=0.516
WS-JA
48
Col0-JA
P=1.000
WS-JA
36
Eri1-JA
P=0.493
Cvi-JA
48
Kond-JA
P=1.000
WS-JA
36
WS-JA
P=0.257
Cvi-JA
36
Kond-JA
P=0.450
Cvi-JA
36
% parasitoids to odor source
% no choices

Figure 5. Responses of naïve *Diadegma semiclausum* females to the volatiles of two sets of *Arabidopsis thaliana* accessions, as assessed in a Y-tube olfactometer. All plants were treated with 1mM aqueous JA-solution (JA) or left non-treated (C). Each bar represents the percentage of choices for each of the two odor sources as determined in 3-4 replicate experiments; if JA treated accessions were compared (section 5B) 12 parasitoids were tested on each replicate day, and six parasitoids when JA and non-treated treated plants were compared (section 5A): error bars indicate SE. The higher part of the figure (section 5A) depicts the percentage of choices for all tested wasps used as controls for the separate JA-treated accession comparisons. Grey bars indicate the percentage of no choice in each experiment; total number of tested parasitoids are given next to these bars (X^2 test, *P* values).

attractive as the blend of JA-treated WS and Cvi. Parasitoids preferred JA-induced volatiles from Col-0 when tested against Cvi, but Col-0 volatiles were less preferred than volatiles from JA-treated L*er* plants (Fig. 5B). A tendency was observed in parasitoid preference for Col-0 plants over Eri-1 plants (P=0.077; Fig. 5B). JA-induced WS volatiles were equally attractive as JA-induced Kond, Cvi, Col-0, and Eri-1 volatiles, and JA-induced Cvi volatiles were also as attractive as JA-induced Eri-1 and Kond volatiles.

Discussion

Currently, functional analysis of Arabidopsis genes is largely based on mutants that are selected in forward and reverse genetic studies. Alternatively, a complementary source of genetic variation is available, i.e. the naturally occurring variation among accessions. The multigenic nature underlying most of this variation has limited its analysis and applications until recently. However, the use of genetic methods developed to map quantitative trait loci (QTLs), in combination with the characteristics and resources available for molecular biology in Arabidopsis, allow this variation to be exploited up to the molecular level. Thus, the systematic exploitation of naturally occurring variation provides a complementary resource for the functional analysis of the Arabidopsis genome. Here, we present the results of an extensive study of the genetic variation in caterpillar-induced indirect defense mechanism, i.e. HIPV emission, among nine Arabidopsis accessions originating from different geographic origins.

The first line of defense that herbivores encounter upon contact with the plant, i.e. epicuticular wax loads and trichomes, was already proven to be subject to genetic variation (Larkin et al., 1996; Rashotte et al., 1997; Luo and Oppenheimer, 1999; Reymond et al., 2004). The differences in leaf area consumption by *P. rapae* larvae among accessions that were observed in the present study might have resulted from these first lines of direct plant defenses. For instance *Plutella xylostella* larvae show differences in neonate behavior on Arabidopsis wax-mutants (personal communication J.J.A. van Loon) and trichome density was reported to be directly correlated with radish resistance against *P. rapae* (Agrawal et al., 2002). Furthermore, the existing variation in glucosinolate levels among accessions (Kliebenstein et al., 2002) might explain the variation

in leaf tissue consumption we observed (Mauricio, 1998; Kliebenstein et al., 2005; Mewis et al., 2005). To exclude that the variation in volatile induction was caused by variation in leaf tissue consumption by larvae, we included an herbivory-mimicking treatment. Given that *P. rapae* caterpillars mainly induce the jasmonate pathway in Arabidopsis (De Vos et al., 2005), we chose for JA-treatment to induce plant volatile emission.

The indirect defense mechanism of plants, i.e. HIPV emission, has been studied in many plant species (Arimura et al., 2005). To date, the occurrence of variation in HIPV emission was primarily explored for crop varieties, including cotton (Loughrin et al., 1995), gerbera (Krips et al., 2001), pear (Scutareanu et al., 2003), maize (Hoballah et al., 2004), carrot (Nissinen et al., 2005), rice (Lou et al., 2006), and cruciferous crops (Bukovinszky et al., 2005). In some of these studies, the authors have demonstrated that variation in emission of HIPVs among cultivars also influences carnivorous arthropod behavior (Dicke et al., 1990b; Krips et al., 2001; Hoballah et al., 2002; Bukovinszky et al., 2005). Here, we demonstrate that plants from the nine tested accessions of Arabidopsis, i.e. An-1, C-24, Col-0, Cvi, Eri-1, Kond, Kyo-1, L*er*, and WS, emit different odor blends in response to damage inflicted by the biting-chewing caterpillar *P. rapae* or the herbivory-mimicking JA-treatment. Furthermore, we observed discriminative host-finding behavior for the parasitoid wasp *D. semiclausum* between the odor blends of some of the offered accessions. Variation in HIPV emission was observed for distinct compounds in different chemical classes, e.g. GLVs, terpenoids, and phenolic compounds. HIPVs that were particularly variable among accessions after *P. rapae* herbivory were: (*Z*)-3-hexen-1-ol, ß-myrcene, (*E*)-ß-ocimene, (*Z*)-ß-ocimene, (*E*,*E*)-α-farnesene, (*E*)-ß-farnesene, TMTT and MeSA. These HIPVs contributed to a large extent to the observed variation in the odor blend among accessions An-1, Col-0, C-24, and Cvi. In contrast, the odor blends emitted by Eri-1, Kyo-1, L*er*, and WS plants are more similar, since the total number of HIPV-compounds emitted in different rates between these accessions was relatively small. Table 4 shows the number of compounds that differ between accessions after *P. rapae* herbivory and JA-treatment. Volatiles induced in response to JA-treatment that contributed most to the variation among all tested accessions were: ß-myrcene, ß-sesquiphellandrene, (*E*)-ß-ocimene, (*Z*)-ß-ocimene, TMTT, MeSA, and ethyl salicylate.

Most of the variation found between accessions is quantitative, i.e. all accessions emit the compounds analyzed, but in various amounts, resulting in different odor blends. In contrast, some sesquiterpenes were absent or only present around detection limits in individual samples of accession Cvi, such as α-cuparene and β-bisabolene. In addition to the findings of Tholl and co-workers (Tholl et al., 2005), who reported that the transcript levels of At5g23960 in inflorescence was comparable in both Cvi and Col-0, we do detect sesquiterpenes that have been reported as products of the terpene synthase encoded by At5g23960 (Tholl et al., 2005) such as α-humulene and (*E*)-β-caryophyllene in the headspace of *P. rapae*- infested or JA-treated leaves of Cvi.

Table 4. Total number of compounds that are significantly different between accessions. In bold are the number of compounds depicted that are significantly different after herbivory between two accessions, these numbers are given in italics for the JA-treated plants.

	An-1	**C-24**	**Col-0**	**Cvi**	**Eri-1**	**Kond**	**Kyo-1**	**L*er***	**WS**
An-1		**3**	**5**	**5**	**4**	**3**	**1**	**2**	**1**
C-24	*0*		**2**	**5**	**1**	**6**	**3**	**3**	**4**
Col-0	*1*	*3*		**4**	**2**	**5**	**2**	**1**	**4**
Cvi	*4*	*0*	*3*		**2**	**2**	**3**	**1**	**4**
Eri-1	*1*	*1*	*2*	*1*		**0**	**0**	**0**	**0**
Kond	*2*	*2*	*4*	*4*	*3*		**0**	**1**	**2**
Kyo-1	*2*	*2*	*0*	*4*	*1*	*2*		**1**	**0**
L*er*	*2*	*0*	*0*	*3*	*3*	*3*	*1*		**0**
WS	*0*	*1*	*3*	*1*	*5*	*1*	*2*	*0*	

Up to now, most experiments using Arabidopsis to study induced defenses were conducted with one of the three laboratory lines Col-0, L*er*, or WS. Only few studies compared these accessions for induced defense traits such as volatile production (Duan et al., 2005; Snoeren et al., 2009 chap. 4). The study presented here is an important contribution, as it compares the three above-mentioned accessions together with six other accessions for HIPV emission and its effects on natural enemy behavior. Since laboratory studies are currently mostly conducted with Col-0, L*er* or WS (Van Poecke and Dicke, 2002; Duan et al., 2005; Shiojiri et al., 2006a; Snoeren et al., 2009 chap. 6), we looked more closely at differences among these accessions which may be overlooked when only one accession is used. The consumption of leaf tissue by caterpillars did not differ among Col-0, L*er*, or WS. However, the emitted volatiles of these three accessions do exhibit variation. Yet, variation between these three accessions in HIPV emission is rather moderate when compared to the variation that is present among all nine studied accessions (Fig. 2). For instance Col-0 and L*er* only differed in the presence of (*Z*)-ß-ocimene, and accessions L*er* and WS did not significantly differ in the emission of any of the compounds screened for after *P. rapae* herbivory. In contrast, accessions Col-0 and WS differed significantly for the herbivore-induced compounds (*Z*)-3-hexen-1-ol, (*E*)-nerolidol, (*E*)-ß-ocimene, and (*Z*)-ß-ocimene. The use of JA to induce the emission of volatiles only resulted in differences between Col-0 and WS - for the compounds (*E*)-ß-ocimene, (*Z*)-ß-ocimene, and DMNT - that were all present in higher amounts in the headspace of WS. The induced odor blend of accession L*er* did not differ from that of Col-0, nor from that of WS after JA-treatment. Furthermore, when the overall volatile profile of each accession was visualized

using PCA, accessions Col-0, L*er* and WS partly overlap in both treatments and are separated to some extent from the other accessions (Fig. 2). Thus, when using one of these lines for studying e.g. behavior of parasitoids, it should be noted that these lines do not very well represent the genetic variation in induced volatile formation present in the species *Arabidopsis thaliana*.

The variation found between the induced headspace volatiles of e.g. Col-0 and WS suggests that foraging behavior of carnivorous arthropods might be influenced by these differences. However, these differences did not influence the naïve parasitoid *D. semiclausum* when they were exposed to odors from JA-treated Col-0 versus JA-treated WS plants. As parasitoids did not discriminate between accessions Col-0 and WS, when treated with JA, this might suggest that the compounds (*E*)-ß-ocimene, (*Z*)-ß-ocimene, and DMNT are not of importance for this parasitoid. We, therefore, tried to further identify which compounds in the headspace of JA-treated plants influence the attraction of parasitoid wasps while searching for hosts. The initial assumption was that compounds with a significantly higher emission rate between two accessions could explain the differential attraction. If so, β-myrcene, (*E*)-β-ocimene, (*Z*)-β-ocimene, DMNT and β-sesquiphellandrene, which are emitted in significantly larger amounts in accession Kond than in Col-0 and Kyo-1, would explain the attractiveness of this accession when compared to accessions Col-0 and Eri-1. However, the emission rates of β-myrcene and (*Z*)-β-ocimene are also higher in accession Cvi compared to Col-0, but here this did not result in a stronger attraction of *D. semiclausum* wasps towards Cvi (Fig 5). Our study, in which we compared volatiles from genetically different accessions, therefore does not allow us to allocate individual HIPV compounds to effects on parasitoid behavior. The complex variation in odor blends is likely to interfere with drawing conclusions on the contribution of individual compounds. It is more likely that the contribution of individual compounds to the total headspace composition of the plant after induction is crucial for the parasitoid (Mumm and Hilker, 2005). For instance, TMTT is not attractive to the predatory mite *Phytoseiulus persimilis* when offered as a single compound but when added to a complex blend it does affect the predator's behavior (De Boer et al., 2004). Moreover, we have not included quantitative aspects. Possibly the discrimination between blends is affected by absolute emission rates rather than only the relative emission rates.

At present, only a few genes have been functionally associated to the production of specific plant volatiles in Arabidopsis (e.g. (Bate et al., 1998; Chen et al., 2003; Fäldt et al., 2003). For several other genes, a function has been proposed in plant volatile production, such as *TPS4* and *CYP72A13* genes (Bruce et al., 2008; Herde et al., 2008). Using the co-expression database from RIKEN, we identified that *TPS4* correlated highly with *CYP82G1* (r=0.639). Variation in transcript levels of genes potentially involved in the biosynthesis of volatiles in response to herbivory has already been demonstrated (Arimura et al., 2004b;

Gomez et al., 2005). However, these studies used a single hybrid or plant line. In the present study we screened for variation among nine accessions in stress -induced transcript levels of six genes that are (putatively) involved in the biosynthesis of volatile compounds. We demonstrated differences in transcript levels among the accessions for *BSMT1*, *TPS4*, *CYP72A13*, and *HPL1* after herbivory and/or JA-treatment. Transcript levels of *TPS3* and *CYP82G1,* however, did not vary among accessions after caterpillar feeding or JA treatment**.** *TPS3* has been shown to encode a terpene synthase involved in the biosynthesis of (*E*)-ß-ocimene, (*Z*)-β-ocimene , and ß-myrcene (Fäldt et al., 2003). Although *TPS3* transcript levels did not vary between accessions, volatile analyses showed that ß-myrcene, (*E*)-ß-ocimene, and (*Z*)-ß-ocimene contributed significantly to variation among the induced headspace compositions of the accessions. Possibly, variation among the accessions in substrate availability or the involvement of another TPS responsible for the production of ß-myrcene, (*E*)-ß-ocimene, and (*Z*)-ß-ocimene may explain this discrepancy.

Herbivory induced the emission of TMTT in accessions Col-0, Eri-1, and WS, but not in the other accessions. The *TPS4* gene has been shown to encode a geranyllinalool synthase. Geranyllinalool is an intermediate in the formation of TMTT (Herde et al., 2008). Yet, none of the three mentioned accessions with an herbivore-induced increase in TMTT emission displayed significantly higher *TPS4* transcript levels than the other accessions. Furthermore, for accessions with a high *TPS4* transcript level, i.e. Cvi and Kond, no significantly induced TMTT emission was observed. The results suggest that *TPS4* is not involved in the key regulatory step in TMTT formation or that TMTT emission is not directly correlated with its transcript level. So far it is not known which enzymes convert geranyllinalool into TMTT. As Bruce et al. (2008) postulate a possible involvement of *CYP72A13*, we also monitored transcript levels of this gene after herbivore damage and JA application. We observed that Col-0 and WS, which exhibited a higher emission rate of TMTT compared to other accessions, also showed higher transcript levels of *CYP72A13*. This finding indicates an involvement of *CYP72A13* in the conversion of geranyllinalool into TMTT. The other Cytochrome P450 gene, *CYP82G1,* that we included in our study showed high correlation with *TPS4* transcript levels using the RIKEN Correlated Gene Search Tool and was previously shown to be induced in Arabidopsis by *Botrytis cinerea,* nematodes (www.genevestigator.ethz.ch), *Pseusomonas syringae*, *Frankliniella occidentalis* and to a lesser extent by *P. rapae* (De Vos et al., 2005). In most accessions *CYP82G1* was induced by *P. rapae* infestation and JA treatment, but no differences were found between accessions. Although, we could not link TMTT emission directly to either *CYP72A13* or *CYP82G1* transcription, the results suggest that it is worthwhile to study this more in depth, for example by analyzing the (induced) TMTT emission of knock-out mutants of these genes.

Accessions An-1, Col-0, Eri-1 and Kond showed induced emission of (*Z*)-3-hexen-1-ol after herbivore damage. The GLV (*Z*)-3-hexen-1-ol is one of the

compounds ascribed to result from *HPL1* activity (Bate et al., 1998). Furthermore, most accessions have increased emission of (*E*)-2-hexenal in *P. rapae* infested plants (SOM Table 3), which is another product of the CYP74B enzyme that is encoded by *HPL1*. Transcript levels of *HPL1* were induced by *P. rapae* feeding in most accessions compared to non-treated controls. In contrast, for accession An-1 and L*er* we did not detect induced *HPL1* transcript levels, whereas emission of (*Z*)-3-hexen-1-ol (Fig 3A) and (*E*)-2-hexenal (SOM Table 3) increased in *P. rapae* infested plants. Therefore, our study does not fully support earlier findings (Duan et al., 2005) that only *HPL1* transcript abundance correlates with the emission of GLVs. Moreover, since we observed *HPL1* expression and (*Z*)-3-hexen-1-ol emission in accession Col-0 we cannot confirm the findings of a loss of *HPL1* functioning in Col-0 as a result from a 10-nucleotide deletion in the *HPL1* gene as reported by (Duan et al., 2005). Whether this is specific for our population of Col-0 or for that of Duan *et al.* (2005), remains to be investigated. The involvement of *HPL1* in the biosynthesis of GLVs was originally shown for accession 'Columbia' (Bate and Rothstein, 1998), which really was Col-0 (S. Rothstein, pers. comm.).
All accessions, except Cvi, showed an induced emission of MeSA after *P. rapae* feeding, and also transcript levels of *BSMT1* were significantly higher in all accessions including Cvi after induction when compared to control plants. Overall it can be stated that the emission of MeSA is reflected in the *BSMT1* transcript levels.

In this study we also induced plant volatile emission with the phytohormone JA, since JA is the key hormone involved in volatile induction by caterpillar leaf-tissue-feeding (Dicke et al., 1999; Ozawa et al., 2000). This methodology of mimicking herbivore damage to study several plant defense traits has already been extensively used. However, for several carnivorous arthropods it is known that they prefer the headspace from an herbivore-infested plant over the headspace from a JA-treated plant (Dicke et al., 1999; Van Poecke and Dicke, 2002; Bruinsma et al., 2008). Transcript profiling of Arabidopsis showed that 55% of *P. rapae*-induced genes were also responsive to MeJA (De Vos et al., 2005). We observed that accessions not only differed in the emission of HIPVs and transcript levels of tested genes, but also showed a different induction in response to the two treatments. The emitted headspace differed quantitatively and qualitatively for *P. rapae*-infested plants compared to JA-treated plants. Nevertheless, *D. semiclausum* parasitoids still preferred plants sprayed with JA over non-treated plants. We also observed differences between JA-treated and *P. rapae*-infested plants in transcript levels for the genes that are putatively involved in the emission of a number of volatiles. Therefore, our study confirms that JA-application can only partly mimic herbivory damage.

Chemical ecology addresses the effects of chemical information on interactions within a plant-insect community. Here, we have explored the natural variation in herbivore-induced plant volatiles between nine accessions of *Arabidopsis thaliana* obtained from different geographical origins.

In addition, we investigated whether this variation is also reflected in the transcript levels of the genes that are associated with the formation of some of these volatiles. We demonstrated genotypic variation in indirect defense traits, both at the metabolite level as well as the gene transcript level. This enables the use of newly available marker technologies that allow characterization and positioning of loci that control these types of traits. It would be very interesting to screen recombinant inbred line (RIL) populations of those accessions that have very distinct volatile patterns for their individual volatile compounds and to subsequently perform (expression) quantitative trait locus (e)QTL analysis. These RIL-populations would allow clarification of genetic regulation of HIPV-formation. Finally, we showed that the genetic variation in induced volatile blends indeed also has consequences for the interactions at the third trophic level, i.e. the attraction of carnivorous parasitoids.

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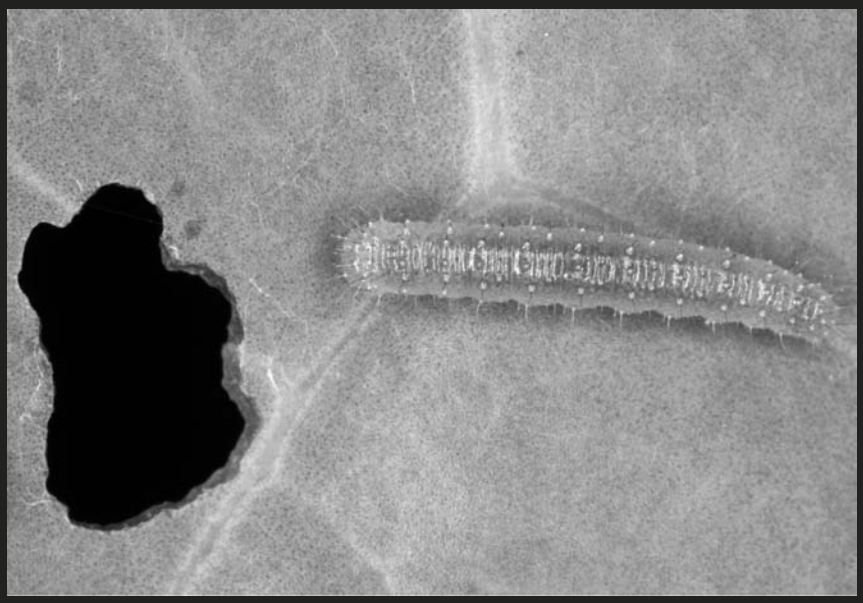

Picture: Tibor Bukovinszky

Chapter 4

Jasmonic acid, not OPDA or dnOPDA, is the major oxylipin involved in indirect defense of *Arabidopsis thaliana*

Tjeerd A.L. Snoeren*, Remco M.P. Van Poecke*, and Marcel Dicke

Submitted in a slightly modified version
*** contributed equally to this work**

Abstract

The jasmonic acid (JA) pathway is commonly involved in induced plant defenses, and is the main signal-transduction pathway induced by insect folivory. JA, as well as pathway intermediates are known to induce plant defenses. Indirect defense against herbivorous insects comprises the production of herbivore-induced plant volatiles (HIPVs). To unravel the precise signal-transduction underlying the production of HIPVs in *Arabidopsis thaliana* and the resulting attraction of parasitoid wasps, we have used a multidisciplinary approach that includes molecular genetics, metabolite analysis and behavioral analysis. Mutant plants affected in the jasmonate pathway (18:0 and/or 16:0 -oxylipin routes; mutants *dde2-2, fad5, opr3*) were studied to assess the effects of JA and its oxylipin intermediates 12-oxo-phytodienoate (OPDA) and dinor-OPDA (dnOPDA) on HIPV emission and parasitoid (*Diadegma semiclausum*) attraction. Interference with the production of the oxylipins JA and OPDA altered the emission of HIPVs, in particular terpenoids and the phenylpropanoid methyl salicylate, which affected parasitoid attraction. Our data show that the herbivore-induced attraction of parasitoid wasps to Arabidopsis plants depends on HIPVs that are induced through the 18:0 oxylipin-derivative JA. Furthermore, our study shows that the 16:0-oxylipin route towards dnOPDA does not play a role in HIPV induction, and that the role of 18:0 derived oxylipin-intermediates, such as OPDA, is either absent or very limited.

Key words
Herbivory, oxylipin, herbivore-induced volatile, metabolite analysis, behavioral analysis, *Pieris rapae*

Introduction

Plants have evolved direct and indirect defenses to effectively combat attack by herbivorous insects (Kessler and Baldwin, 2002). Direct defense mechanisms comprise the production and storage of metabolites that negatively influence herbivore performance (Wittstock and Gershenzon, 2002). In contrast, indirect defense mechanisms encompass the production of metabolites that benefit the natural enemies of herbivores (Dicke et al., 1999; Wäckers et al., 2001; Halitschke et al., 2008; Kost and Heil, 2008). Both defense mechanisms can be constitutively present or induced after herbivore feeding. Induced defenses allow plants to be more cost effective and also to diminish the risk that herbivores adapt to the defenses (Agrawal and Karban, 1999; Pieterse and Dicke, 2007; Steppuhn and Baldwin, 2008). Plant responses to different herbivore species vary as mediated by different feeding modes and defense elicitors in herbivore regurgitant or saliva (Walling, 2000; Voelckel and Baldwin, 2004a; De Vos et al., 2005; Felton and Tumlinson, 2008).
Here, we address induced indirect plant defense at different levels of biological integration. An example of an induced indirect defense mechanism is the production of volatiles by plants in response to herbivory. These volatiles are used by parasitoids or predators to locate their herbivorous victims. Herbivore-induced plant volatiles (HIPVs) mainly comprise green leaf volatiles (GLVs), terpenoids, and phenolics (Dudareva et al., 2006). The composition of induced volatile blends can vary qualitatively or quantitatively (Dicke and Hilker, 2003). With this variation in HIPV composition, the plant can provide the natural enemies of herbivores with detectable and reliable information (Vet and Dicke, 1992; Dicke, 1999a). The induced volatile production is orchestrated by at least three main signal-transduction pathways: the jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) pathways (Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002). These pathways can be differentially induced by different herbivore species (Heidel and Baldwin, 2004; De Vos et al., 2005; Schmidt et al., 2005), leading to the emission of an herbivore-specific volatile blend (Vet and Dicke, 1992; Ozawa et al., 2000; Walling, 2000; Leitner et al., 2005).

Jasmonic acid (JA) is a member of a family of compounds collectively known as jasmonates or oxylipins, produced by the jasmonate pathway (Fig. 1). Tissue-feeding insects, such as the larvae of herbivorous Lepidoptera especially induce the jasmonate pathway (Kessler and Baldwin, 2002; De Vos et al., 2005). The production of jasmonates from linolenic acid (18:3) and linoleic acid (18:2) is initiated in the plastid and completed in the peroxisome and cytosol (Schaller et al., 2005). Lipases that release linolenic acid from membrane lipids, mainly originating from damaged cell walls, are thought to play an important role in regulating the response to herbivore-derived cues (Farmer and Ryan, 1992; Schaller et al., 2005). Linolenic acid and linoleic acid are subsequently converted by lipoxygenase (LOX) (Bell et al., 1995), allene-oxide synthase (AOS) (Laudert and Weiler, 1998) and allene-oxide cyclase (AOC) (Ziegler et al.,

1997) into 12-oxophyto-dienoic acid (OPDA). A parallel cascade converts hexadecatrienoic acid (16:3) to dinor-oxophytodienoic acid (dnOPDA) (Weber et al., 1997). After the activity of 12-oxophytodienoic acid reductase (OPR) (Stintzi and Browse, 2000) and three ß-oxidation steps, the oxylipins OPDA and dnOPDA are metabolized to form JA (Schaller et al., 2005). Another branch, starting at 9- or 13-hydroperoxide formed by the lipoxygenase, leads to the production of GLVs through the action of hydroperoxide lyase (HPL) (Bate et al., 1998).

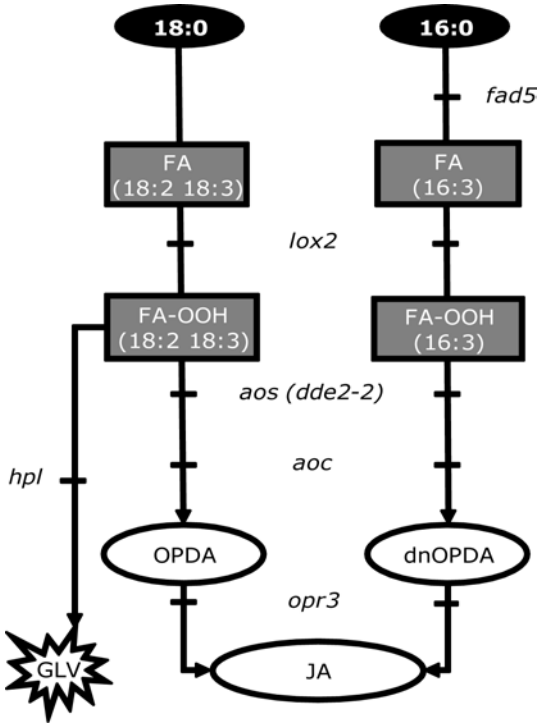

Figure 1. Biosynthetic route of jasmonates in infested *Arabidopsis thaliana* leaves. FA= Fatty Acid; 16:0= hexadecanoic acid; 16:3= 7Z, 10Z,13Z-hexadecatrienoic acid; 18:0= octadecanoic acid; 18:2=9Z,12Z-octadecadienoic acid (linoleic acid); 18:3= 9Z,12Z,15Z-octadecatrienoic acid (linolenic acid); dn-OPDA= dinor-oxo-phytodienoic acid; OPDA= oxo-phytodienoic acid; JA= jasmonic acid; GLVs= green leaf volatiles. Mutations affecting the biosynthesis are indicated in *italics*.

Evidence for the involvement of jasmonates in herbivore-induced responses does not only stem from the induction of JA and its intermediates upon herbivory. Exogenous application of jasmonates mimics the effects of herbivory. For example, exogenous application of methyl jasmonate (MeJA) and caterpillar-feeding induce similar, but not identical, transcriptional responses (Reymond et al., 2004). De Vos *et al.* (2005) demonstrated that there is roughly a 50 percent overlap in gene induction in Arabidopsis after MeJA treatment and herbivory by caterpillars or thrips. Besides MeJA, also other jasmonates trigger transcriptional changes. Interestingly, exogenous treatment with OPDA, JA, or MeJA results in overlapping but not identical gene-expression profiles in Arabidopsis (Taki et al., 2005).

Similar to their effects on gene-expression profiles, jasmonates also influence the emission of volatiles by plants. The production of volatiles by JA-treated plants is quantitatively and qualitatively similar compared to induction by herbivory (Dicke et al., 1999; Ozawa et al., 2000). Application of OPDA has similar effects on secondary metabolite production as applying MeJA (Gundlach and Zenk, 1998). However, exogenously applied OPDA but not JA induces diterpenoids in lima bean plants (Koch et al., 1999).

A third line of evidence for the involvement of jasmonates in herbivore-induced responses comes from mutant analyses. For example, Arabidopsis *opr3* and *aos* mutants show different gene-expression profiles in response to JA, MeJA, OPDA and mechanical damage compared to wild-type plants, indicating distinct signaling roles for dnOPDA, OPDA and JA (Stintzi et al., 2001; Taki et al., 2005). Indeed, *opr3* mutants, lacking JA, still show oxylipin-dependent resistance to pathogens and herbivores, implying a role for jasmonates other than JA in plant defense (Stintzi et al., 2001).
Mutations in the jasmonate pathway also affect indirect defense. In tobacco for example, AOS-silenced plants (as-*aos*), display a reduced JA accumulation and terpenoid emission and antisense-*hpl* mutants release fewer GLVs (Halitschke et al., 2004). Similarly, two antisense-*hpl*-mutants show an altered production of GLVs in Arabidopsis (Shiojiri et al., 2006a). These oxylipin-mediated effects on volatile emissions were accompanied by effects on interactions of plants with carnivorous insects (Shiojiri et al., 2006b; Halitschke et al., 2008).

Thus, several lines of evidence demonstrate the involvement of jasmonic acid in herbivore-induced responses including indirect defense, yet also suggest roles for other intermediates of the jasmonate pathway such as dnOPDA or OPDA. As predators and parasitoids are able to discriminate JA-induced from herbivore-induced volatiles (Dicke et al., 1999; Gols et al., 1999; Van Poecke and Dicke, 2002), other signals besides JA are likely required for the induction of indirect defenses. Mutant plants altered in signaling pathways with a changed volatile emission represent some of the most powerful tools of testing the mechanisms that underlie HIPV production. Investigating the effects of altered HIPV production in behavioral assays is then a valuable key to unravel the ecological relevance of these signaling pathways. Here, we followed a molecular genetic approach to study the involvement of several intermediates from the jasmonate pathway in the induction of plant volatiles by tissue-feeding herbivores. We used *Arabidopsis thaliana* to dissect the jasmonate pathway and analyze the effects on indirect defense after attack by tissue-feeding herbivores. In particular, we are interested in the contribution of the two sub-pathways, originating from galactolipids (16:0) or phospholipids (18:0) (Schaller et al., 2005), with special interest in dnOPDA, OPDA, and JA. For this, we selected mutants with altered production levels of dnOPDA, OPDA, and JA (Weber et al., 1997; Stintzi and Browse, 2000; Stintzi et al., 2001; Von Malek et al., 2002). For caterpillar-infested mutants and their corresponding wild-type plants, the levels of dnOPDA, OPDA and JA were quantified. Subsequently, HIPVs were collected and volatile blend composition was quantitatively analyzed. Finally, we quantified caterpillar-feeding rate and conducted behavioral bioassays with parasitoid wasps to determine the effects of the observed differences in oxylipin profiles and HIPV blend composition after caterpillar-feeding on species interactions.

Materials and methods

Plants and insect material

Arabidopsis seeds (*A. thaliana*; genotypes Columbia (Col-0), Wassilewskija (WS), *fad5*, *opr3*, and *dde2-2*) were germinated on an autoclaved mixture of commercially available potting soil and 33% sand, and cultivated in a growth chamber at 21 ± 2 °C, 50 to 60% relative humidity (RH), and L8:16D photoperiod with 80 to 110 µmol m^{-2} s^{-1} PPF. The selected mutant *fad5* has a Col-0 background and is incapable of biosynthesizing 7Z,10Z,13Z-hexadecatrienoic acid (16:3) (Weber et al., 1997); the mutant *dde2-2* also has a Col-0 background and is defective in allene oxide-synthase (AOS) (Von Malek et al., 2002). The mutant *opr3* has a WS background and lacks the most relevant isozyme of 12-oxo-phytodienoate reductase (OPR) (Schaller et al., 2000; Stintzi and Browse, 2000; Stintzi et al., 2001). Two-week-old seedlings were transferred to plastic cups (5 cm in diameter) filled with the earlier described soil mixture. Plants were watered twice a week. When plants were full-grown vegetative plants, i.e. 6 to 8 weeks after sowing, they were used for experiments.

Herbivore-induced defense responses were initiated by caterpillars from *Pieris rapae*, the small cabbage white. *Pieris rapae* was reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera*, cv Cyrus) in a climatized room (16L:8D; 20 ± 2 °C and 70% RH).
The parasitoid wasp *Diadegma semiclausum* was reared on *Plutella xylostella* caterpillars feeding on Brussels sprouts in a climatized room (16L:8D; 20 ± 2 ° C and 70% RH). Emerging wasp species were provided *ad libitum* with water and honey, and are referred to as 'naïve' wasps as they had received no exposure to plant material, nor an oviposition experience.

Plant treatments

Defense responses were induced by herbivore feeding, or by spraying the plant with JA. Plants were infested by equally distributing 20 first-instar *P. rapae* larvae over the fully expanded leaves. Herbivore feeding was mimicked by spraying JA. Four plants were sprayed with 5 ml of 1.0 mM (±)-JA (Sigma-Aldrich) aqueous solution.
In all experiments, plants were treated 24 h before the experiments and kept in a climate room (21 ± 2 °C, 50-60% RH; L8:D16 photoperiod and 80 to 110 µmol m^{-2} s^{-1} PPFD).

Quantitative analysis of jasmonate family members

The abundance of dnOPDA, OPDA, and JA, was determined for each of the used Arabidopsis genotypes. After 24 h, *P. rapae* larvae were removed. Leaf rosettes from infested and uninfested control plants were harvested, immediately weighed and frozen in liquid nitrogen for storage. Extraction of the oxylipins was performed according to the protocol described by Weber *et al*., 1997

(Weber et al., 1997). For quantifying the derivatized oxylipins, a gas chromatograph (Hewlett-Packard 5890) equipped with a 30m x 0.25mm HP-MS column (Hewlett-Packard) coupled to a mass-spectrometer (model 5972, Hewlett-Packard) was used. Helium was used as carrier gas with a flow of 1ml/min. The column temperature at the moment of injection was 100 °C. The temperature gradient was 100 °C to 160 °C at 20 °C min^{-1}, 160 °C to 238 °C at 3 °C min^{-1}, and 238 °C to 300 °C at 30 °C min^{-1}. Quantification was done by measuring selective ions (*m/z*=224 for methyl jasmonate, *m/z*=278 for methyl dnOPDA, *m/z*=238 for methyl OPDA, *m/z*=226 for methyl dihydrojasmonate (Internal Standard used for methyl JA) and *m/z*=240 for methyl tetrahydro-OPDA (Internal Standard used for methyl dnOPDA and methyl OPDA).
Titers of dnOPDA, OPDA, and JA were calculated per gram fresh weight and were log10 transformed. The following fixed effects-model was used for each oxylipin to screen for differentiation per genotype: $\log10(O_{ij}) \sim G_i + T_j + G{:}T_{ij} + \varepsilon_{ij}$, where O=oxylipin quantity per gram fresh weight, G=genotype; T=treatment; ε=residual; i=1,…,4; and j=1,2. Subsequently, two-tailed t−tests, for the 2004 data followed by a Benjamini and Hochberg false discovery rate (BH-FDR) multiple comparison correction, were conducted per oxylipin for the genotypes (significance: q/p<0.05) (Benjamini and Hochberg, 1995). All linear models were performed in the R environment (R Development Core Team, 2007) with R packages for linear mixed-effects models.

Headspace collection and volatile analysis

Dynamic headspace sampling was done for sets of four plants in a climate room (20 ± 2 °C, 70% RH; L8:D16 photoperiod and 90 to 110 µmol m^{-2} s^{-1} PPFD). Twenty-four hours before trapping, the pots were removed, soil with the roots were carefully wrapped in aluminum foil, and the four plants were placed together in 2.5 L glass jar. Plants either were left uninfested or were infested with 20 first-instar (L1) *P. rapae* larvae per plant. Just before trapping jars were closed with a Viton-lined inert glass lid having an inlet and outlet. Air was sucked out with a vacuum pump at 100 ml min^{-1} with the incoming air purified through a steel cartridge filled with 200 mg Tenax-TA (20/35-mesh, Grace-Alltech, Deerfield, USA). A same kind of cartridge was used to trap emitted plant volatiles at the outlet. After 3.5 h of trapping at continuous light, fresh weight of the four plants was measured. Headspace collections of uninfested and infested plants, for all the genotypes, were carried out in parallel on one experimental day.

Headspace samples were analyzed with a Thermo TraceGC Ultra™ (Thermo Fisher Scientific, Waltham, USA) connected to a Thermo TraceDSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA). Before desorption of the volatiles, the cartridges were dry-purged with helium at 30 ml min^{-1} for 20 min at ambient temperature to remove moisture. Samples were desorbed from the cartridges using a thermal desorption system at 250 °C for 3 min (Model Ultra Markes Llantrisant, UK) with a helium flow at 30 ml min-1.

Analytes were focused at 0 °C on an electronically-cooled sorbent trap (Unity™, Markes International LTD, Llantrisant, UK). Volatiles were transferred without split to the analytical column (Rtx 5MS, 30 m 0.25 mm i.d., 1.0 µm film thickness, Restek, Bellefonte, USA) by ballistic heating of the cold trap to 300 °C. The GC was held at an initial temperature of 40 °C for 3.5 min followed by a linear thermal gradient of 10 °C min^{-1} to 280 °C and held for 2.5 min with a column flow of 1 ml min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 45-400 m/z with a scan rate of 3 scans s^{-1}.

Compounds were identified by using the deconvolution software (AMDIS version 2.64, NIST, USA) in combination with NIST 98 and Wiley 7th edition spectral libraries and by comparing their retention indices with those from literature (Adams, 1995). Characteristic quantifier ions were selected for each compound of interest. Metalign software (PRI-Rikilt, Wageningen, The Netherlands) was used to align chromatograms of all samples and integrate peak areas for the signals of the quantifier ions. Peak areas were converted to peak area per gram fresh weight of leaf material.

Areas of quantifier ions per gram fresh weight were log10 transformed, and for each HIPV compound the following mixed model was used to screen for HIPV compound differentiation per genotype: $\log10(V_{ijk}) \sim G_i + T_j + G{:}T_{ij} + R_k + \varepsilon_{ijk}$, where V=area of quantifier ions per gram fresh weight; G=genotype; T=treatment; R=replicate; ε=residual; i=1,…,5; j=1,2; and k=1, …,5. Both G and T were used as fixed effects and R as a random effect. Subsequently, two-tailed t-tests followed by a Benjamini and Hochberg false discovery rate (BH-FDR) multiple comparison correction were conducted per compound for the genotypes (significance: $q<0.05$; (Benjamini and Hochberg, 1995)).
Similarities among plant genotypes based on volatile profiles were analyzed using LEGG, a nonlinear dimensionality reduction method based on uncentered Pearson correlations among volatile profiles, which are used to generate 3-dimensional networks, for details see Van Poecke et al. (2007).

Behavioral assays

The effects of HIPV alterations as a result of mutations in the jasmonate pathway on behavioral responses were tested for the parasitoid species *D. semiclausum*. Behavioral assays were carried out in a closed Y-tube olfactometer system as described in detail by Takabayashi and Dicke (1992). To investigate the behavioral responses of 3 to 7 day old mated *D. semiclausum* females, a modified Y-tube olfactometer has been used (Bukovinszky et al., 2005). In short, filtered air was led through activated charcoal and split into two air streams (4 L min^{-1}) that were led through five-liter glass vessels containing the odor sources consisting of four plants each. Plants were infested with 20 L1 *P. rapae* or sprayed with 1.0 mM JA solution 24 h before starting the bioassay. Plants were kept overnight in a climate room (21 ± 2 °C and 50 to 60% RH,

L8:D16 photoperiod and 80 to 110 µmol m^{-2} s^{-1} PPF). The olfactometer was illuminated with 4 high-frequency fluorescent tubes (Philips 840, 36 W) from above at an intensity of 60 ± 5 µmol photons m^{-2} sec^{-1}. All experiments were conducted in a climatized room (20 ± 2 °C).
Individual wasps were transferred into the Y-tube olfactometer, and their behavior was observed and scored as described in detail by Bukovinszky et al. (2005). Odor sources were interchanged to compensate for any unforeseen asymmetry in the set-up after every five wasps tested. Choices between odor sources were statistically analyzed using a chi-square test, with the null-hypothesis that no preference existed.

The total area of consumed leaf-tissue was analyzed for the plants used in the bioassay. Therefore, after an experiment, all individual leaves of each rosette were taped on paper and scanned with a Hewlett-Packard scan jet 3570c. For quantification of the consumed leaf area, analysis was performed with KS400 version 3.0 software service pack 9 (Carl Zeiss Vision, Oberkochen, Germany). The consumed leaf area per genotype was statistically compared using ANOVA (SPSS 15.0, Chicago, USA

Results

Quantitative analysis of jasmonate family members

We quantified the levels of dnOPDA, OPDA and JA in leaf-tissue from the mutants *dde2-2*, *opr3* and *fad5*, after 24h of herbivory by *Pieris rapae,* and from uninfested plants (Fig. 2). As the *dde2-2* and *fad5* mutants have a Col-0 background, while the *opr3* mutant has a WS background, both wild-type accessions were included as well.
Analyses of Col-0 were performed both in 2004 and 2005. Even though plants were grown in a controlled environment, the levels of oxylipins varied between the years (Fig. 2). Therefore, the 2004 dataset was analyzed separately from the 2005 dataset. Constitutive levels of OPDA and JA are similar in both wild-type accessions, whereas constitutive dnOPDA levels are lower in WS compared to Col-0 (Fig. 2). Herbivory by *P. rapae* induced all three oxylipins to similar levels in Col-0 and WS plants (Fig. 2).
Mutations in oxylipin biosynthetic genes clearly affected the oxylipin signatures. In *dde2-2* plants, induction of dnOPDA, OPDA or JA by herbivory was completely abolished. In fact, a decrease in dnOPDA and OPDA levels was observed in *dde2-2* plants in response to herbivory. Moreover, constitutive OPDA levels were lower in the *dde2-2* mutant.
A mutation in *FAD5* resulted in constitutively lower dnOPDA levels that could not be induced by herbivory. However, this mutation did not affect either constitutive or herbivore-induced levels of OPDA or JA.
A mutation in *OPR3* hampered the induction of JA after herbivory; herbivory resulted in a significantly lower induction than in the WS wild-type. Constitutive levels of all three oxylipins were unaffected by *OPR3* mutation.

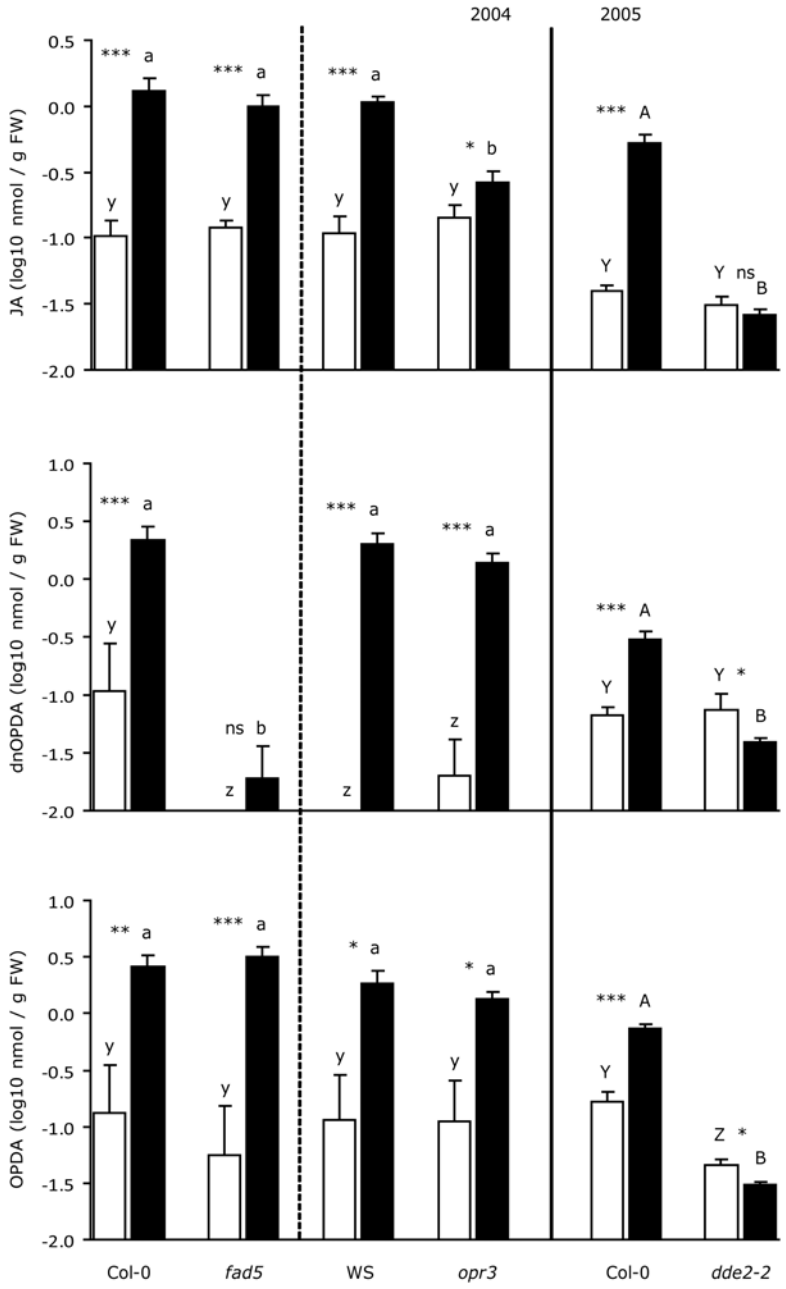

Figure 2. Oxylipin titers in A*rabidopsis thaliana* after 24h of *Pieris rapae* feeding, or left uninfested. Extracted oxylipin concentration for *P. rapae*-infested (black bar) or uninfested (white bar) leaves. Mean oxylipin values + SE are shown (n=4). Dashed line separates wild-type accession backgrounds for 2004. Asterisks indicate significant differences between infested and uninfested plants within a genotype (*P* value: * <0.05; **<0.01; ***<0.001; ns, not significantly different). Bars for infested leaves marked with the same letter are not significantly different (data from 2004 in lower case and data from 2005 in capital letters) (2004: q value > 0.05; 2005: *P* value > 0.05).

In short, the oxylipin mutations have the expected effects on oxylipin production: the *fad5* mutant only affects dnOPDA levels, *opr3* only affects JA levels and *dde2-2* affects the levels of dnOPDA, OPDA and JA. Thus, these three mutants allow us to dissect the roles of dnOPDA, OPDA and JA levels in indirect defense.

Volatile analysis

To assess the effects of altered oxylipin signatures on HIPVs, we measured the volatile emissions in uninfested and *P. rapae*-infested Col-0, WS, *dde2-2, fad5,* and *opr3* plants. Compounds for which an influence on discriminative behavior by carnivorous arthropods is known are selected and presented in Fig. 3 (Dicke et al., 1990c; De Boer et al., 2004; Shimoda et al., 2005). HIPV production in wild-type Col-0 and WS showed both similarities and differences. For example, *P. rapae* feeding induced the emission of methyl salicylate (MeSA), the sesquiterpene (*E,E*)-α-farnesene and the homoterpene (*E,E*) 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) to similar levels in both accessions. However, Col-0, but not WS, showed the induction of the monoterpene linalool, whereas WS, but not Col-0, showed induction of the GLVs (*Z*)-3-hexenal and (*Z*)-3-hexen-1-ol acetate and of the monoterpenes β-myrcene and both stereoisomers of β-ocimene.

A mutation in *FAD5* did not result in an altered HIPV emission, indicating that dnOPDA does not play a role in HIPV induction. In contrast, a mutation in *DDE2-2* abolished the induction of all HIPVs, except for 1-nonanol. A mutation in *OPR3* hampered induction of all HIPVs, except for the GLVs.

To compare total volatile blends instead of single compounds among accessions, the variation in volatile profiles among genotypes was further explored using the algorithm locally linear embedding graph generator (LEGG). LEGG analysis resulted in a network of genotypes, generated by using a non-linear dimensionality reduction method (Fig. 4) (for LEGG details see (Van Poecke et al., 2007)). This demonstrated a strong relationship between volatile profiles emitted by uninfested plants of the various genotypes plus caterpillar-infested plants of the *dde2-2* and *opr3* mutants. In contrast, HIPVs from caterpillar infested Col-0, *fad5* and WS showed a much weaker correlation with the volatile blend from uninfested genotypes. LEGG analysis also showed that the HIPVs from Col-0 and WS show similarities.

Thus, headspace analysis for the mutants *fad5*, *dde2-2*, *opr3* and their respective wild-types pointed out that only hampered JA levels (i.e. the common denominator in *dde2-2* and *opr3* plants) resulted in an altered production of HIPVs.

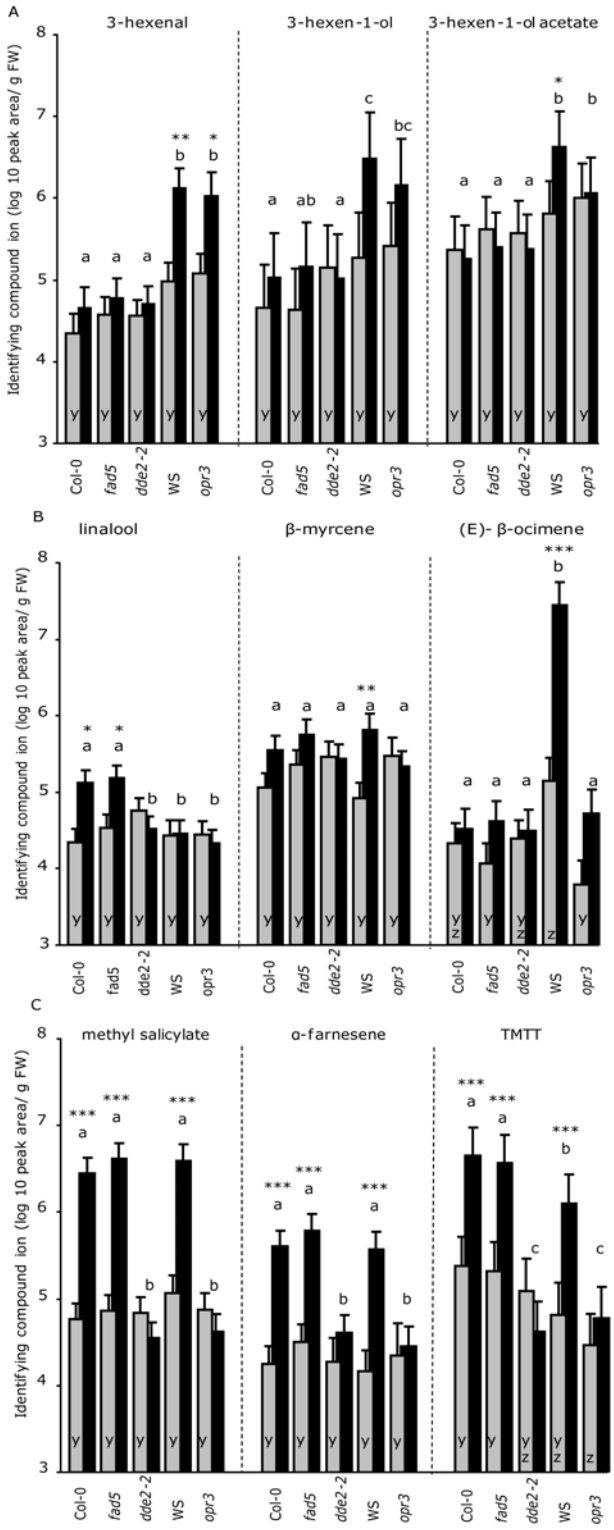

Figure 3. Characteristic *Arabidopsis thaliana* HIPV-compounds per genotype treatment. **A:** GLVs; **B:** monoterpenes **C:** methyl salicylate (MeSA), (*E,E*)-α-farnesene, and (*E,E*) 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT). Given is the mean + SE for compound-representative ions (n=3-5). Asterisks indicate significant differences between uninfested (grey bars) and *Pieris rapae*-infested (black bars) leaves within a genotype (*P* value: *<0.05; **<0.01; ***<0.001). Bars that are marked with the same letter are not significantly different, (y-z=uninfested plants, a-c=infested plants), q value > 0.05. Dashed lines divide separately analyzed compounds.

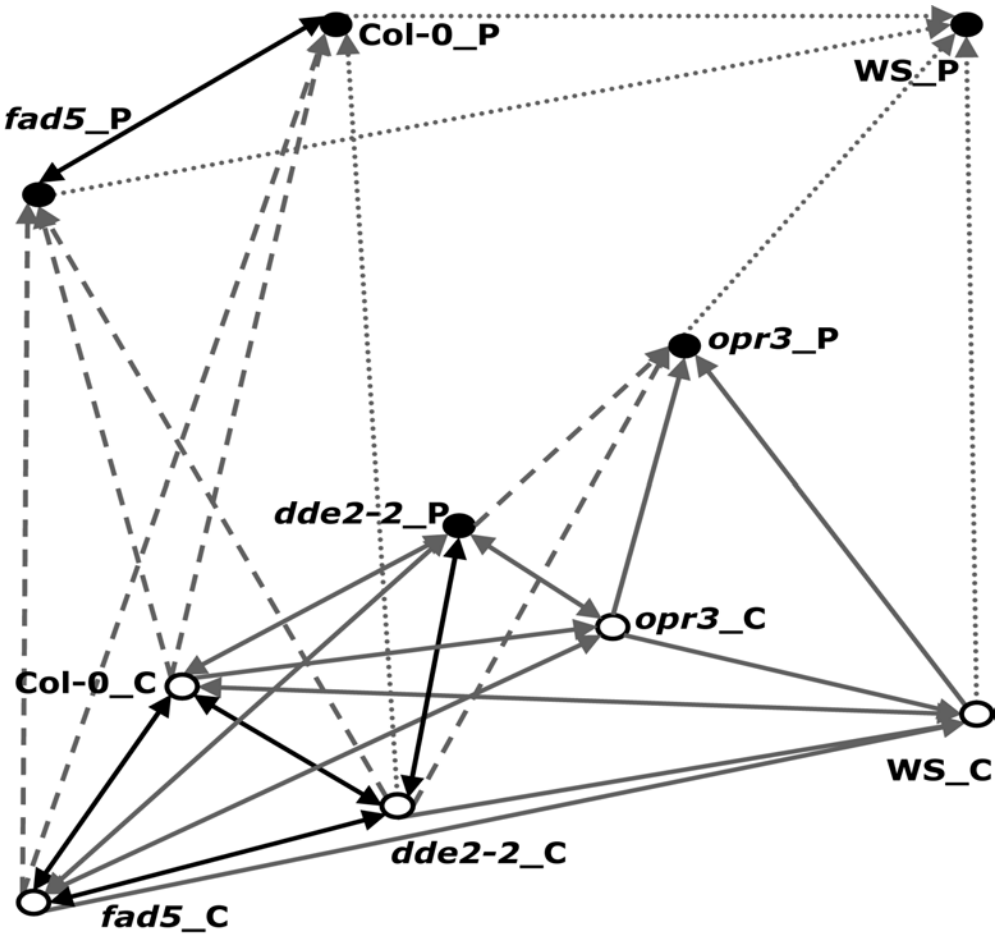

Figure 4. Visualization of the relationships among HIPV blends of different *Arabidopsis thaliana* genotype-treatment combinations. LEGG analysis was applied to the volatile blends of the *Pieris rapae*-infested (_P ; black nodes) and uninfested (_C; white nodes) plants of different genotypes. The peak areas of 22 volatile compounds contribute to the parameter for each volatile blend, indicated as nodes in the graph. Relationships among the profiles that were determined by LEGG are depicted as directed links; genotypes at the base of an arrow represent a near neighbor of the genotype directed at. Arrows that point at two genotypes represent genotypes that are both close neighbors of each other. Directed links can be compared based on their presence, absence or strength. Strength of connections measured by r^2 are indicated as follows: dotted grey arrow, $0.951<r^2<0.964$; dashed grey arrow, $0.964<r^2<0.974$; solid grey arrow, $0.974<r^2<0.984$; and solid black arrow, $0.984<r^2<0.990$ (n=3-5). Arrow lengths do not illustrate strength of connections. Analyzed volatile compounds were: pentan-1-ol; (*E*)-2-penten-1-ol; hexanal; (*Z*)-3-hexenal; (*E*)-3-hexen-1-ol; hexan-1-ol; heptanal; α-pinene; 1-octen-3-ol; 6-methyl-5-hepten-2-one; β-myrcene; (*Z*)-3-hexen-1-ol acetate; (*Z*)-β-ocimene; (*E*)-β-ocimene; (*E*)-2-nonen-1-ol; linalool; (*E*)-4,8-dimethyl-1,3,7-nonatriene; 1-nonanol; methyl salicylate; undecanal; (*E*,*E*)-α-farnesene; (*E*,*E*) 4,8,12-trimethyl-1,3,7,11-tridecatetraene.

Behavioral assays

To investigate whether the changes in HIPV profiles due to the mutations in the jasmonate pathway affect indirect defense, we investigated the behavioral responses of a parasitoid wasp species towards HIPVs originating from *dde2-2*, *opr3, fad-5*, and their wild-types in a Y-tube olfactometer (Fig. 5). *Diadegma semiclausum* females preferred volatiles of infested plants over uninfested plants for all mutants or wild-types ($P<0.001$). However, HIPVs from caterpillar -infested wild-type plants were significantly more attractive than HIPVs from caterpillar-infested mutants *dde2-2* or *opr3* (for both mutants $P<0.05$). The wasps did not discriminate between volatiles of herbivore-infested *fad5* plants and infested wild-type plants.

A possible explanation for the observed differences in HIPV emission and parasitoid attraction among mutants and wild-type plants is that the mutation altered the feeding behavior of the herbivore. A reduced attraction to infested mutant plants might have resulted from a reduced feeding rate on the mutant plants. However, this is not supported by our data on feeding rate: the amount of leaf area consumed did not differ when the caterpillars were feeding on plants of *opr3*, *fad5* or their wild-types ($P>0.05$). Moreover, the caterpillars even consumed more leaf material of *dde2-2* plants compared to wild-type ones ($P<0.05$). Thus, the mutations do not result in reduced feeding and thus altered caterpillar-feeding rates cannot explain the observed changes in headspace composition or parasitoid attraction.

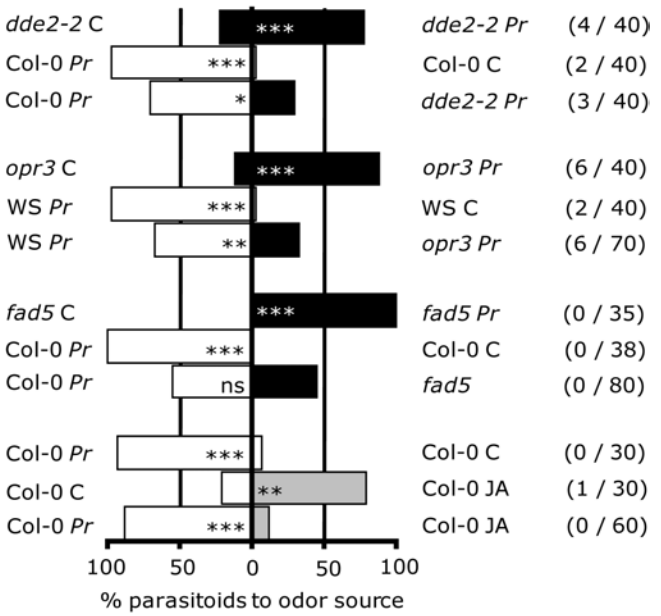

Figure 5. Preference of naïve *Diaegma semiclausum* females to volatiles of differently treated *Arabidopsis thaliana*, as assessed in the Y-tube olfactometer. Plants were infested with *Pieris rapae* (*Pr*) or treated with JA 24h before (JA) or were left untreated (C). Data represent total number of parasitoids that chose for any of the two odor sources as determined in 3-4 replicate experiments, each on a different day with new odor sources. Asterisks indicate a significant difference within a choice test: * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significantly different (X^2 -test). The number of wasps that did not make a choice and the total number of tested parasitoids is given in parenthesis.

We verified the importance of JA signaling in indirect defense by assessing the attractiveness of volatiles induced in the plants by the application of 1 mM JA. Exogenous JA treatment of Col-0 plants made them more attractive than non-treated Col-0 control plants (Fig. 5, $P<0.01$). However, when these JA-sprayed plants were tested against *P. rapae*-induced Col-0, the wasps preferred the latter ($P<0.001$).

Discussion

We used a molecular genetic approach to unravel the involvement of the jasmonate pathway oxylipins dnOPDA, OPDA and JA in caterpillar-induced indirect defense. We selected *Arabidopsis thaliana* mutants, *fad5*, *opr3* and *dde2-2,* for which we quantified oxylipin levels, HIPV emission and ultimately assessed parasitoid attractiveness towards HIPVs.

Based on previous work summarized in figure 1 we expected the *fad5* mutant to show reduced levels of 16:3 derived compounds; the *dde2-2* mutant to show reduced levels of both 16:3 and 18: 3 derived compounds and *opr3* mutants to show reduced levels of JA. Our data confirmed these expectations: after herbivory *fad5* showed no induction of dnOPDA, *opr3* was hampered in the induction of JA, and *dde2-2* showed no induction of dnOPDA, OPDA and JA (Fig. 2). Additionally, these data also demonstrated that 1) the 16:3 pathway does not contribute to constitutive or herbivory-induced levels of JA and 2) that herbivory-induced levels of dnOPDA and OPDA do not depend on JA accumulation through a feedback loop.

Mutation of the *FAD5* gene did not result in an altered emission of HIPVs, showing that dnOPDA does not play a role in HIPV-mediated indirect defense. On the other hand, mutations in *DDE2-2* or *OPR3* did show clear effects on HIPV production, resulting in reduced induction of many volatiles, especially the terpenoids, indicating roles of OPDA and/or JA in HIPV-indirect defense. As knocking out *DDE2-2* or *OPR3* has very similar effects on HIPV production, we conclude that JA, and not OPDA, is the most important oxylipin in HIPV production. A minor role for OPDA cannot be excluded as *opr3* plants, showing moderate JA induction, still showed induction of a few volatile compounds, such as GLVs while *dde2-2* plants, lacking OPDA and JA induction, did not. However, it is likely that this difference between *opr3* and *dde2-2* is caused by differences in the genetic background: all three genotypes with a Col-0 background (Col-0, *fad5* and *dde2-2*) did not show induction of GLVs, whereas WS does show induction of GLVs. The lower GLV-levels produced by Col-0, *fad5* and *dde2-2* plants, are most likely caused by a dysfunctional HPL1 enzyme in the Col-0 background (Duan et al., 2005). In any case, the role of OPDA or earlier intermediates is minor, as LEGG analyses show that the composition of the overall volatile blend of *opr3* and *dde2* plants is very similar (Fig. 4). Moreover, the blends of infested *dde2-2* and *opr3* plants were very similar to the blends of uninfested controls while they were different from the blends of infested wild-type plants (Fig. 4).

Parasitoid behavioral assays reflected the results obtained by headspace analyses: mutation of *FAD5* did not affect parasitoid behavior, whereas infested plants of both *opr3* and *dde2-2* showed reduced parasitoid attraction compared to wild-type plants (Fig. 5). Some compounds were still induced in the mutants *dde2-2* and *opr3*: 1-nonanol and (*Z*)-3-hexenal, respectively. These compounds might explain the attractiveness of infested mutants *dde2-2* and *opr3* over uninfested controls. These results indicate that other signals besides JA or its intermediates are involved in HIPV production. This is also illustrated by the observation that JA-treated Col-0 plants were less attractive to the wasps than caterpillar-infested Col-0 (Fig. 5; see also Van Poecke and Dicke (2002)). Similar results have also been obtained for other plant-herbivore-carnivore systems (Dicke et al., 1999; Bruinsma, 2008).

The combination of biochemical and behavioral analyses of Arabidopsis wild-type and mutant plants not only gives insight in which plant hormones influence indirect defense but also through which volatile compounds these hormones exert their effects. Inhibition of JA production influenced mono-, sesqui- and homoterpene volatile emissions as well as the emission of the shikimate pathway derived methyl salicylate (MeSA). Even though exogenous JA is known to induce GLV production in Arabidopsis (Snoeren et al., 2009 chap. 3), we did not find any effect of the lack of JA on *P. rapae*-induced GLV emissions in Arabidopsis.

The importance of monoterpenes in plant-insect interactions is well studied (Dicke et al., 1990a; Rose et al., 1998; Shimoda et al., 2005; Mumm et al., 2008b; Opitz et al., 2008). Even though Col-0 and WS differed in the identity of monoterpenes induced by *P. rapae* feeding, with Col-0 showing mainly induction of linalool and WS showing mainly induction of (*Z*)- and (*E*)-β-ocimene, lack of JA had a similar inhibitory effect on monoterpene induction in both accessions. Lack of JA also affected emission rates of the sesquiterpene (*E,E*)-α-farnesene and the diterpene-derivative (*E,E*) 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), demonstrating that JA influences not only the plastid-localized mono- and diterpene production, but also the sesquiterpene production located in the cytosol. Both (*E,E*)-α-farnesene and TMTT are known to influence plant-insect interactions (Van Poecke, 2002; De Boer et al., 2004; Ibrahim et al., 2005).

Besides terpenoids, the induction of MeSA was also impaired in JA-lacking mutants. This indicates that hampering the JA pathway consequently hampers some step in the biosynthesis of MeSA. Previous studies using Arabidopsis demonstrated that hampering the SA pathway affects the emission of both MeSA and TMTT and resulted in decreased parasitoid attraction (Van Poecke, 2002). Thus, both JA and SA are required for herbivore-induced emissions of the parasitoid attractants MeSA and TMTT. Such synergism has also been recorded for spider-mite induced volatile emissions of tomato plants (Ament et al., 2004).

In conclusion, the present study has shown the value of using a molecular ecological approach to elucidating the importance of jasmonic acid and its intermediates in indirect defense. By dissecting the jasmonate pathway through the use of selected mutants, we have gained a better understanding of the ecological role of oxylipins in HIPV-mediated indirect plant defense. Even though the importance of the octadecanoid pathway in indirect defense has been demonstrated before (Thaler et al., 2002; Halitschke et al., 2004; Kessler et al., 2004; Shiojiri et al., 2006a; Shiojiri et al., 2006b), the relative contribution of the different oxylipin compounds to indirect defense has remained unclear. Here, we demonstrate that in Arabidopsis the 16:0 branch of the oxylipin pathway does not play a role in HIPV-mediated indirect defense. Moreover, we demonstrate that from the 18:0 pathway, jasmonic acid is the main actor in HIPV-mediated indirect defense, with limited or no contribution from pathway intermediates such as OPDA. The data strengthen the value of using a molecular ecological approach in advancing our understanding of multi-trophic plant-insect interactions.

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Chapter 5

Picture: Tjeerd Snoeren

Jasmonates differentially affect interconnected signal-transduction pathways of induced defenses in *Arabidopsis thaliana*

Tjeerd A.L. Snoeren, Colette Broekgaarden, and Marcel Dicke

Abstract

The jasmonic acid (JA) pathway is the main signal-transduction pathway induced by insect folivory. Mutant plants affected in the jasmonate pathway (18:0 and/or 16:0-oxylipin routes) were studied to assess the effects of JA and its oxylipin intermediates 12-oxophytodienoic acid (OPDA) and dinor-OPDA (dnOPDA) on interconnected signal-transduction pathways that underlie induced defenses in Arabidopsis. Our data show that the oxylipins dnOPDA, OPDA, and JA have different roles in defense signaling induced by caterpillar-feeding. Jasmonic acid is the major signaling compound required for the induction of the defense-related genes *LOX2* (*Lipoxygenase 2*), *OPR3* (*12-Oxophytodienoate reductase 3*), *ACX1* (*Acyl-CoA oxidase 1*), and *PAL1* (*Phenylalanine ammonia-lyase 1*). The herbivore-induced oxylipin JA and not dnOPDA or OPDA is essential for *PAL1* activity. Mutant screenings of *PAL1* transcript levels clearly showed that the biting-chewing herbivore *Pieris rapae* induces the shikimate pathway by means of JA induction. Analysis of mutants that lack JA, or JA and OPDA, further indicated more involvement of the oxylipin OPDA than of JA in the induction of the defense-related gene *HPL1* (*Hydroperoxide lyase 1*). The oxylipin dnOPDA influences the induction of the HPL-branch as well, yet antagonistically to the effects of OPDA and JA. Here we demonstrate that dnOPDA and OPDA may be used to fine-tune Arabidopsis' herbivore-induced responses in terms of the HPL-branch from the oxylipin pathway.

Key words:
Herbivory, oxylipin, qRT-PCR, gene transcript level, *fad5, dde2-2, opr3*

Introduction

Plants possess a broad range of defense mechanisms to effectively combat biotic stresses caused by microbial pathogens and herbivorous insects. These mechanisms include pre-existing physical and chemical barriers, as well as induced defense responses that are activated upon attack (Pieterse and Dicke, 2007). The induced defense responses can be subdivided into direct defense, e.g. production of anti-digestive proteins and toxic secondary compounds (Karban and Baldwin, 1997; Walling, 2000), and indirect defense, i.e. the production of herbivore-induced plant volatiles (HIPV) that attract the natural enemies of the herbivore (Dicke and Hilker, 2003). HIPVs mainly comprise green leaf volatiles (GLVs), terpenoids, and phenolics (Paré and Tumlinson, 1997; Dicke, 1999b), which are products of different biosynthetic routes (reviewed in e.g. (Dudareva et al., 2006). The induced nature of these responses to herbivore attack allows plants to be cost-effective and also to diminish the risk that herbivores adapt to the defenses (Agrawal and Karban, 1999; Heil, 2008; Steppuhn and Baldwin, 2008).

Induced defense responses are orchestrated by a network of interconnecting signal-transduction pathways in which jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) play key roles (Pieterse and Dicke, 2007; Kazan and Manners, 2008). Empirical evidence for the significance of the phytohormones JA, SA, and ET in plant defense came from using mutant plants altered in these pathways (reviewed in e.g. Dong, 2004; Pozo et al., 2004; Schaller et al., 2005; Fujita et al., 2006).
The signal-transduction pathways are differentially induced depending on the herbivore species (Heidel and Baldwin, 2004; Voelckel and Baldwin, 2004b; De Vos et al., 2005; Schmidt et al., 2005). Tissue-feeding insects, e.g. larvae of herbivorous Lepidoptera, and cell-content feeders, e.g. thrips, especially induce JA and related compounds from the same signal-transduction pathway, collectively known as jasmonates or oxylipins (Kessler and Baldwin, 2002; Weber, 2002; De Vos et al., 2005). Phloem feeders, such as aphids and whiteflies, especially induce the SA pathway (Heidel and Baldwin, 2004; De Vos et al., 2005; Zarate et al., 2007). However, the JA pathway seems to be the most important in induced defenses in plant-insect interactions (Kessler and Baldwin, 2002).

In brief, the biosynthesis of oxylipins is as follows (Fig. 1). After herbivore damage of the cell walls, lipases release linoleic acid (18:2) and linolenic acid (18:3) from the chloroplast membrane lipids (Schaller et al., 2005; Bargmann and Munnik, 2006). Linoleic acid and linolenic acid are subsequently converted by lipoxygenase (LOX) into 9(S)-hydroperoxylinoleic acid and 13(S)-hydroperoxylinolenic acid (Bell et al., 1995).
One branch of the oxylipin pathway, starting at the fatty acid 9- or 13-hydroperoxides formed by the lipoxygenase, cleaves the hydroperoxides

through the action of hydroperoxide lyase (HPL) forming 12-oxo-trans-10-dodecenoic acid and subsequently the GLVs hexanal or cis-3-hexenal (Bate et al., 1998). Both these GLVs can be converted into other GLVs such as C6 aldehydes, alcohols, and derivatives (Bate and Rothstein, 1998). At present, besides attracting natural enemies GLVs also have ascribed plant-signaling effects (reviewed in e.g. Matsui, 2006; Choudhary et al., 2008).
Another branch converts the fatty acid 9- or 13-hydroperoxide by allene-oxide synthase (AOS) (Laudert and Weiler, 1998) and allene-oxide cyclase (AOC) activities (Ziegler et al., 1997) into 12-oxophyto-dienoic acid (OPDA). The activity of 12-oxophytodienoic acid reductase (OPR) (Stintzi and Browse, 2000) transforms OPDA to 3-oxo-2(2′[*Z*]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0). OPC-8:0 is then converted to its CoA derivative by OPC-8:0-CoA ligase1 (Koo et al., 2006). OPC-8:0-CoA is the starting point for three consecutive ß-oxidation steps, catalyzed by several enzyme groups (e.g. acyl-CoA oxidases (ACX)), to form JA (Goepfert and Poirier, 2007). A parallel JA biosynthetic pathway starts from chloroplastic pools of hexadecatrienoic acid (16:3) that leads to dinor-oxophytodienoic acid (dnOPDA) (Weber et al., 1997). Subsequently, dnOPDA is also converted into JA. The production of JA is followed by an increase in the titer of its conjugate jasmonoyl-L-isoleucine (JA-Ile) through the JAR1 enzyme (Staswick and Tiryaki, 2004). Subsequently, JA-Ile promotes an interaction between an F-box protein (SCF^{COI1}), which is encoded by *Coronatine insensitive 1* (*COI1*), and the jasmonate ZIM-domain (JAZ) transcriptional repressors, resulting in the degradation of JAZ proteins. As a result of this, the basic helix-loop-helix transcription factor MYC2 is now free, and can upregulate the transcript level of a variety of wound- and/or insect-responsive genes such as *Lipoxygenase 2 (LOX2),* or can repress pathogen-responsive genes such as *Plant defensin 1.2* (*PDF1.*2) and *Pathogenisis-related gene 1* (*PR1*) (Lorenzo and Solano, 2005; Kazan and Manners, 2008; Staswick, 2008) (Fig. 1).

After caterpillar-feeding no accumulation of SA has been observed in Arabidopsis (De Vos et al., 2005). Yet, SA induction by other organisms or external application does interfere with caterpillar-induced responses (Cui et al., 2002; Stotz et al., 2002; Cipollini et al., 2004). In addition, caterpillar-feeding induces the emission of the methylated form of SA, i.e. methyl salicylate from Arabidopsis plants (Van Poecke et al., 2001; Loivamäki et al., 2008; Snoeren et al., 2009 chap. 3, 4, and 6). Therefore, the SA pathway appears to be involved in the defense of Arabidopsis against tissue-feeding caterpillars.
In short, the SA biosynthetic pathway, also called the shikimate pathway, uses chorismate to produce SA. The SA-formation was initially thought to be synthesized exclusively through the phenylalanine ammonium lyase (PAL) pathway. Yet, recently the SA biosynthetic route through isochorismate synthase was proposed to be involved as well (Wildermuth et al., 2001)(Fig. 1).
Increasing evidence showed that the JA pathway and SA pathway can be mutually synergistic or antagonistic, and that this crosstalk fine-tunes induced

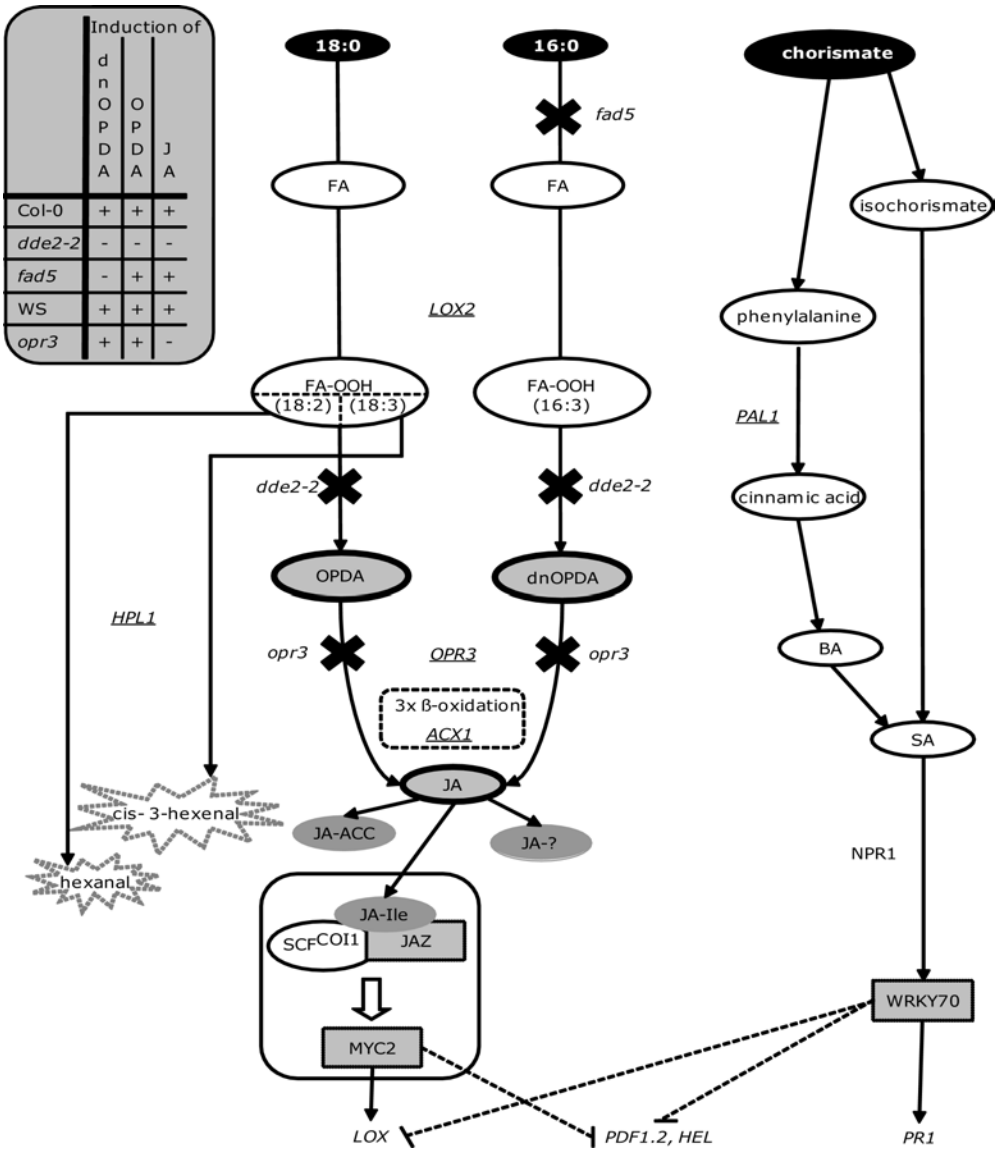

Figure 1. Biosynthetic scheme of defense signaling in caterpillar-infested Arabidopsis leaves. FA= Fatty Acid; 16:0= hexadecanoic acid; 16:3= 7Z,10Z,13Z-hexadecatrienoic acid; 18:0= octadecanoic acid; 18:2= 9Z,12Z-octadecadienoic acid (linoleic acid); 18:3= 9Z,12Z,15Z-octadecatrienoic acid (linolenic acid); dnOPDA= dinor-oxo-phytodienoic acid; OPDA= oxo-phytodienoic acid; JA= jasmonic acid; JA-ACC, JA-Ile, JA-?= jasmonate conjugates; MYC2, WRKY70= transcription factors; SCF^{COI1}= E3 ubiquitin ligase complex; NPR1= regulatory protein; JAZ= jasmonate ZIM-domain; hexanal and cis 3-hexenal= green leaf volatiles; BA= benzoic acid; SA= salicylic acid; Genes investigated in this study are underlined, in *italics*, and in CAPITALS. Mutations affecting the biosynthesis are indicated in *italics.* Arrows reflect stimulating activities; dashed lines represent inhibiting activities. Constructed after: (Wildermuth et al., 2001; Lorenzo and Solano, 2005; Schaller et al., 2005; Staswick, 2008; Snoeren et al., 2009 chap. 4).

defenses of plants (reviewed in e.g. Lorenzo and Solano, 2005; Pieterse and Dicke, 2007; Koornneef and Pieterse, 2008). For this interaction a crucial role was demonstrated for the regulatory protein NONEXPRESSOR OF PR GENES1 (NPR1) (Spoel et al., 2003), which is required for the transduction of the SA signal (Dong, 2004). However, the actual role of mediator between the JA and SA pathways is ascribed to the transcription factor WRKY70 (Li et al., 2006), which is, besides NPR1, also a positive regulator for SA-dependent responses (Lorenzo and Solano, 2005)(Fig. 1).

Here, we study intra-pathway interactions of oxylipins as well as their effects on the SA pathway. For this we focus on caterpillar-induced transcript levels of genes that are involved in the signal-transduction of underlying defense responses in brassicaceous plants. Therefore, our study especially addresses the role of jasmonates in orchestrating induced plant defense mechanisms. We used the brassicaceous plant *Arabidopsis thaliana*, as this species has proven to be a suitable plant for this ecogenomic approach (Van Poecke and Dicke, 2004; Snoeren et al., 2007). For Arabidopsis, several biologically active oxylipins with distinct roles in herbivore-induced defense responses have previously been described, i.e. OPDA and JA (Stintzi et al., 2001; Taki et al., 2005). Furthermore, mutant plants lacking JA and/or its intermediates showed an altered HIPV production and resulted in altered behavioral responses of carnivorous enemies of herbivorous insects (Snoeren et al., 2009 chap. 4). These observed differences in HIPV emission may be connected to biosynthetic pathways, orchestrated by the SA signaling pathway and by both oxylipin signaling pathway branches i.e. the HPL-branch and AOS-branch (Dicke et al., 1999; Van Poecke and Dicke, 2002; Kessler et al., 2004). In addition, analysis of Arabidopsis leaf-tissue consumption by caterpillars, a proxy for direct defense, suggested different roles for the oxylipins dnOPDA and OPDA versus JA in direct defense regulation (Snoeren et al., 2009 chap. 4). Thus, several lines of research suggested that different jasmonates, i.e. JA and its intermediates dnOPDA and OPDA, have distinct effects on induced indirect and induced direct defenses in Arabidopsis.

We used Arabidopsis mutants with altered levels of herbivore-induced dnOPDA, OPDA, and JA (Weber et al., 1997; Stintzi and Browse, 2000; Von Malek et al., 2002; Snoeren et al., 2009 chap. 4), to study the effects of these oxylipins on the activation of SA signaling and of both oxylipin branches, i.e. the HPL-branch and the AOS-branch. Changes in transcript levels of genes involved in signal-transduction pathways (i.e. *LOX2*, *OPR3*, *ACX1*, *HPL1*, and *PAL1*) were analyzed after caterpillar herbivory in mutant and wild-type plants. This will allow us to unravel the relative contribution of the jasmonates dnOPDA, OPDA, and JA to the interconnected signal-transduction pathways that underlie induced defenses in Arabidopsis.

Materials and methods

Plants and Insect Material

Arabidopsis seeds (*A. thaliana*; accessions Columbia (Col-0) and Wassilewskija (WS), and mutants *fad5*, *opr3,* and *dde2-2*) were germinated in preheated (90 °C) sandy Arabidopsis soil (Lentse potgrond BV, Lent, Netherlands), and cultivated in a growth chamber at 21 ± 2 °C, 50-60% relative humidity (RH), and L8:16D photoperiod with 80-110 µmol m^{-2} s^{-1} PPF. The selected mutant *fad5* has a Col-0 background and is incapable of biosynthesizing 7*Z*,10*Z*,13*Z*-hexadecatrienoic acid (16:3) (Weber et al., 1997); the mutant *dde2-2* also has a Col-0 background and is deficient in functional allene oxide-synthase (AOS) (Von Malek et al., 2002). Mutant *opr3* has a WS background and lacks the most relevant isoform of 12-oxo-phytodienoate reductase (OPR) (Schaller et al., 2000). Two-week-old seedlings were transferred to plastic pots (5 cm in diameter) filled with similar soil. To prevent infestation by sciarid larvae, the soil was treated weekly with entomopathogenic nematodes, *Steinernema feltiae* (Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands).
The plants were watered twice a week. When plants were full-grown vegetative plants, i.e. after 6 to 8 weeks since sowing, they were used for experiments.
The herbivore *Pieris rapae* (Lepidoptera: Pieridae), the small cabbage white, was reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera*, cv Cyrus) in a growth chamber (16L:8D; 20 ± 2 °C and 70% RH).

Plant treatment

Twenty first-instar *P. rapae* larvae were equally distributed over the fully expanded leaves of a plant. Plants were infested 24 h before harvesting of leaf material and were kept overnight in a growth chamber at 21 ± 2 °C, 50-60% relative humidity (RH), and L8:16D photoperiod with 80-110 µmol m^{-2} s^{-1} PPF.
Leaf material was collected by selecting two not yet fully expanded leaves with local feeding damage, from which caterpillars and excrements were removed. Collected leaf material was immediately flash frozen in liquid nitrogen and stored at -80 °C. As control plants we used uninfested plants that were otherwise treated similar to the infested plants.

Quantitative RT-PCR analysis

A qRT-PCR analysis was used to screen for differences in transcript levels of *P. rapae*-induced defense-related genes in the JA and SA signaling pathways of Arabidopsis. One µg of total RNA was treated with DNaseI (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA-free total RNA was converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Gene-specific primers were designed using Primer3 (Rozen and Skaletsky, 2000) for five Arabidopsis genes based on sequences obtained from a Basic Local Alignment Search Tool (BLAST) search in the TIGR Arabidopsis database. The primer sequences are shown in table 1.
The primers were tested for gene specificity by performing melt curve analysis

Table 1. Sequences of *Arabidopsis thaliana* primers used in the qRT-PCR analysis.

Gene	AGI-codes	Forward primer (5′ à 3′)	Reverse primer (5′ à 3′)
GADPH	At3g04120	GTGTTCACGGTCAATGGAAAC	ACCACCCTTCAAGTGAGCTG
LOX2	At3g45140	ACAACTAAGTGCCATGGATCC	GTAAGCCTTCCTGGTCAAACC
OPR3	At2g06050	CCCACATGTGCCTGGAATCTATTCAG	AGCCCGAGTGATAGTGGGTCAGAAT
ACX1	At4g16760	TGGAGCAAGACATAGGTGGC	TACGAAGTTGCTGCTGAAGC
HPL1	At4g15440	GGCGTTCGTGTTGGAGTTTATC	GGATTCGATTGTTCCCCAGAA
PAL1	At2g37040	TGTAGCGCAACGTACC	GTTCGGGATAGCCGATG

on a MyIQ Single-Color Real-Time PCR Detection System (BioRad). PCR products were sequenced to confirm the amplification of the gene of interest. Sequence results were checked by a BLAST search in the Arabidopsis TIGR database (data not shown).

Quantitative RT-PCR analysis was carried out in optical 96-well plates with a MyIQ Single-Color Real-Time PCR Detection System (BioRad), using SYBR Green to monitor dsDNA synthesis. Each reaction contained 10 µl 2 x SYBR Green Supermix Reagent (BioRad), 10 ng cDNA and 300 nM of each gene-specific primer in a final volume of 20 µl. All qRT-PCR analyses were performed in duplicate. The following PCR programme was used for all PCR analyses: 95 ° C for 3 min; 40 cycles of 95 °C for 30 s and 60 °C for 45 s. Threshold cycle (Ct) values were calculated using the Optical System software, version 2.0 for MyIQ (BioRad). Subsequently, Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of the constitutively expressed gene *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) from the Ct value of the gene of interest.

The normalized transcript abundance was then calculated as $2^{-\Delta Ct}$. Subsequently, the significant induction of the normalized gene transcript levels after treatment was tested with a one-way ANOVA (α=0.05). Additionally, normalized gene transcript levels were used to calculate $\log_2$-transformed transcript level ratios for each experimental condition that were statistically compared using a one-way ANOVA followed by a Least Significant Difference (LSD) post-hoc test.

Results

Quantitative RT-PCR analysis was used to study *P. rapae*-induced transcript levels of genes of defense-related signal-transduction pathways in Arabidopsis, with special focus on JA signaling. Mutant plants with altered JA signaling (i.e. *dde2-2*, *fad5*, and *opr3*) and their corresponding wild-types (i.e. Col-0 or WS) were tested for the induced transcript levels of defense-related genes: *LOX2* (*Lipoxygenase 2*), *OPR3* (*12-Oxophytodienoate reductase 3*), *ACX1* (*Acyl-CoA oxidase 1*), *HPL1* (*Hydroperoxide lyase 1*), and *PAL1* (*Phenylalanine ammonia-lyase 1*)(Fig. 2).

For both ecotypes, Col-0 and WS, *LOX2* was significantly induced after *P. rapae* herbivory ($P<0.001$, Fig. 2A). Mutant *fad5* showed a similar level of *LOX2* induction as its corresponding wild-type Col-0, whereas mutants *dde2-2* and *opr3* showed lower levels of *LOX2* induction than their corresponding wild-types Col-0 and WS, respectively.

Herbivory by *P. rapae* significantly induced transcript levels of *OPR3* in Col-0, *fad5*, and WS compared to uninfested plants ($P<0.01$, Fig. 2B). The level of *OPR3* induction in mutant *fad5* was similar to that in wild-type Col-0. In contrast, the abundance of the *OPR3* transcript was not induced after *P. rapae* herbivory in *dde2-2* and *opr3* plants (Fig. 2B).

Genotypes Col-0 and *fad5* showed significant induction of *ACX1* after *P. rapae* herbivory ($P<0.01$), whereas the transcript level of *ACX1* in WS plants was marginally increased ($P=0.050$) compared to uninfested WS. Mutants *dde2-2* and *opr3* did not show induced transcript levels of *ACX1* after herbivory. The induction level of *ACX1* transcripts in *fad5* was similar to that in the wild-type (Fig. 2C).

Col-0, *fad5*, WS, and *opr3* showed significantly induced transcript levels of *HPL1* after herbivory ($P<0.01$; Fig. 2D), whereas *dde2-2* plants did not show an herbivory-related induction of the *HPL1* transcript level. The induced transcript abundance of *HPL1* in *fad5* plants was significantly higher compared to the corresponding wild-type Col-0. The induced transcript level of *HPL1* in *opr3* plants was significantly lower than in the relevant wild-type WS (Fig. 2D).

Herbivory induced the transcript level of the *PAL1* gene in both Col-0 and *fad5* plants ($P<0.05$). WS plants demonstrated a marginally insignificant induced abundance of the *PAL1* transcript after *P. rapae*-feeding ($P=0.067$). Mutants *dde2-2* and *opr3* did not show induced transcript levels of *PAL1* after herbivory. The level of induction of *PAL1* in infested versus uninfested plants was comparable for Col-0 and *fad5* plants (Fig. 2E).

In conclusion, mutant *fad5*, which has a reduced dnOPDA production (Snoeren et al., 2009 chap. 4), showed a stronger induction of the transcript level after *P. rapae*-feeding than wild-type plants for *HPL1* only. The transcript levels of the other tested genes were similar in this mutant and its wild-type control. The *dde2-2* mutation that hampered dnOPDA, OPDA, and JA induction

(Snoeren et al., 2009 chap. 4), affected the transcription of all genes studied. The *opr3* mutation, which only resulted in hampered JA levels (Snoeren et al., 2009 chap. 4), negatively affected the transcription of the *LOX2*, *OPR3*, *HPL1,* and *PAL1* genes but not that of *ACX1.*

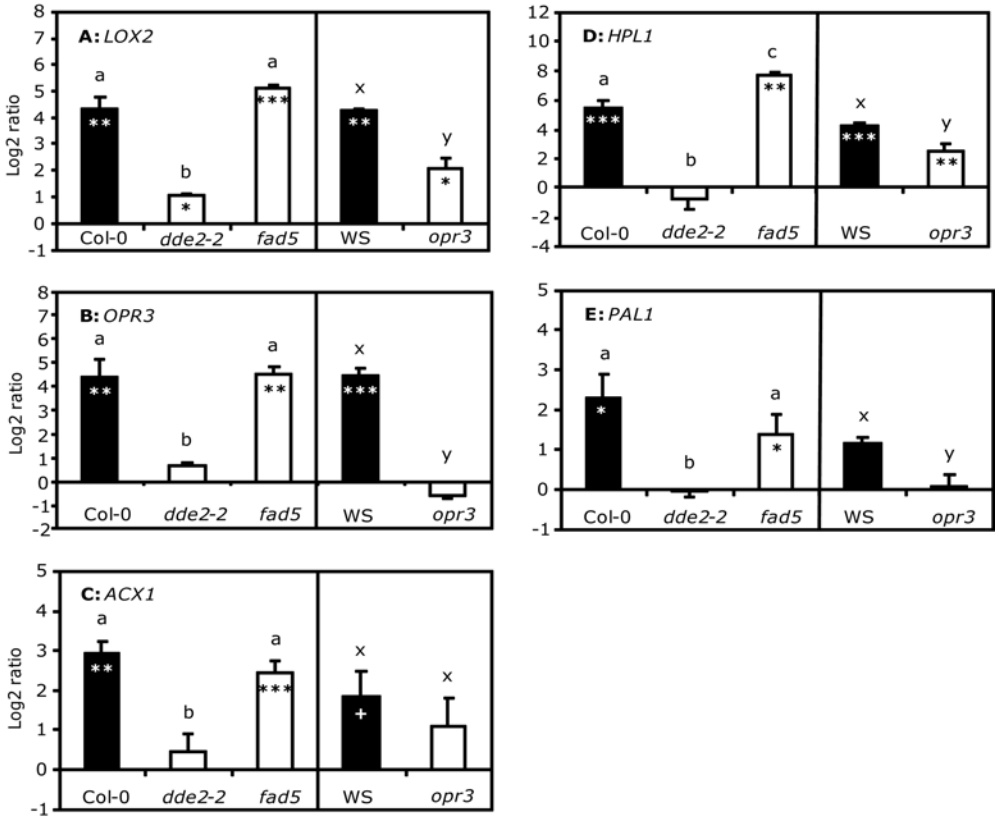

Figure 2. Induced transcript levels of defense genes in the Arabidopsis genotypes Col-0, *dde2-2*, *fad5*, WS, and *opr3* after 24 h of *P. rapae* herbivory quantified with qRT-PCR and presented as $\log_2$ transcript level changes compared to uninfested control plants. Quantitative RT-PCR data are shown for *LOX2* (*Lipoxygenase 2*), *OPR3* (*12-Oxophytodienoate reductase 3*), *ACX1* (*Acyl-CoA oxidase 1*), *HPL1* (*Hydroperoxide lyase 1*), and *PAL1* (*Phenylalanine ammonia-lyase 1*). Values are the mean (+ SE) of four biological replicates and marked with an asterisk when significantly upregulated compared to uninfested controls (one-way ANOVA, + P=0.05, * P<0.05; ** P<0.01; *** P<0.001). Bars marked with different letters are significantly different (one-way ANOVA, LSD, P<0.05).

Discussion

Induced resistance to pathogens or herbivores in plants relies on the ability to recognize a potential attack and trigger an appropriate response. For this, plants employ distinct recognition mechanisms and signaling pathways in response to attacker-specific elicitors. The major endogenous signaling molecules JA, SA and ET play key roles in orchestrating induced defenses. These signaling

pathways are not linear, but appear to form a network of synergistic and antagonistic interactions (Kunkel and Brooks, 2002; Spoel et al., 2003; Kazan and Manners, 2008). Herbivore species from different feeding guilds induce different signaling pathways (Heidel and Baldwin, 2004; Voelckel and Baldwin, 2004b; De Vos et al., 2005; Schmidt et al., 2005). Leaf-tissue consuming herbivores, like the caterpillar *P. rapae* that was used in this study, mainly induce the JA signaling pathway (De Vos et al., 2005). Previous studies indicate that the phytohormone JA may affect the SA pathway (Schenk et al., 2000) and induces GLVs produced by the HPL-branch of the oxylipin pathway (Van Poecke, 2002;
Snoeren et al., 2009 chap. 3). To elucidate the role of the jasmonates dnOPDA, OPDA, and JA, we investigated changes in gene transcript levels in response to herbivory in mutants hampered in the production of these three jasmonates. In this study, we provide strong evidence that the jasmonates dnOPDA, OPDA, and JA have distinct effects on the HPL-branch of the oxylipin pathway and on the SA pathway, or contribute differently to feedback loops within the oxylipin AOS-branch that leads to the final product JA.

Mutant *fad5*, in which the dnOPDA route is blocked, had a higher level of *HPL1* transcripts compared to the wild-type Col-0. This shows that dnOPDA indeed has a biological role in induced plant responses, in particular by repressing the transcription of *HPL1*. To our knowledge this is the first time that the proposed role for dnOPDA, i.e. a biologically active signaling role within induced plant responses, as put forward by Weber et al. (Weber et al., 1997), is confirmed. We hypothesize that dnOPDA, through its effect on the *HPL1* transcription, influences GLV emission and consequently affects the signaling roles of GLVs in plant-carnivore interactions and in inter- and intra-plant communication (Engelberth et al., 2004; Heil and Silva Bueno, 2007; Frost et al., 2008).
The lack of JA, which is the case in the mutants *dde2-2* and *opr3*, results in a reduced induction of *HPL1* transcript level compared to induction observed in wild-types Col-0 and WS, respectively. This effect is much stronger in the *dde2-2* mutant, which lacks not only induced JA but also OPDA, than in the *opr3* mutant, which lacks JA but not OPDA (Snoeren et al., 2009 chap. 4). This indicates the important involvement of OPDA in *HPL1* induction compared to the end product of the AOS-branch, i.e. JA. Together, this indicates that dnOPDA and OPDA have contrasting effects on the HPL-branch of the oxylipin pathway: dnOPDA has a negative effect and OPDA a positive effect on the HPL-branch. Thus, dnOPDA and OPDA may be used to fine-tune Arabidopsis' responses to biotic stress in terms of the HPL-branch and consequently GLV emission.

Our data show that a biting-chewing herbivore like *P. rapae* induces the SA-signaling pathway and that jasmonates are required for observed *PAL1* transcript levels, since *PAL1* induction was not observed in the *dde2-2* mutant, which lacks oxylipins. Furthermore, the absence of dnOPDA in *fad5* mutant plants did not influence *PAL1* induction. In contrast, *opr3* plants that lack JA

production but had no altered dnOPDA and OPDA levels (Snoeren et al., 2009 chap. 4), showed an impaired *PAL1* induction. This indicates that *PAL1* induction is dependent on signaling molecule JA. These observations demonstrate only a biological active role for JA and not for dnOPDA and OPDA in inducing *PAL1* activity after folivory.

Surprisingly, we did not observe a different transcript level of *ACX1* in mutant *opr3,* which lacks JA, when compared to its wild-type WS. Since we observed no transcript induction of *OPR3* in caterpillar-damaged mutant *opr3* plants when compared to the uninfested control *opr3* mutant plants, we conclude that the mutation in *opr3* effectively blocked *OPR3* activity. Therefore, we suggest that the observed comparable *ACX1* transcript abundance in *opr3* and WS plants after herbivory, is provoked by alternative reductases of 12-oxophytodienoic acid. Candidate reductases are OPR1 and OPR2, but enzyme activities of these reductases are poor compared to OPR3 activity (Schaller et al., 2000), though these activities might increase in the absence of OPR3.

The observed lower levels of caterpillar-induced transcription of *LOX2* and *OPR3* in the mutants *dde2-2* and *opr3* compared to their corresponding wild-types*,* demonstrates that the absence of JA results in a lower induction of the first steps of the AOS-branch. Still, to discriminate between roles of OPDA and JA in the activation of upstream genes in the AOS-branch one would ideally require mutant plants having an identical genetic background. In addition, a mutant with a completely blocked conversion of dnOPDA and OPDA into JA is required. For this, comparing mutant *dde2-2* and double mutant *acx1/acx5* (Schilmiller et al., 2007), would allow for the discrimination in terms of biological activity for the jasmonates OPDA and JA.
We demonstrate that dnOPDA has no or a very limited contribution to the induction of genes within the AOS-branch, i.e. *LOX2*, *OPR3*, and *ACX1*. In addition, earlier studies have indicated that *fad5* plants produce wild-type levels of OPDA and JA after 24 h of *P. rapae* herbivory (Snoeren et al., 2009 chap. 4). These two observations together suggest a more prominent contribution of the 18:0 route than the 16:0 route towards the production of JA.

In conclusion, we observed different roles for the oxylipins dnOPDA, OPDA, and JA in caterpillar-feeding-induced defense signaling. In response to folivory jasmonic acid is the major signaling compound required for the induction of genes in both the HPL-branch and the AOS-branch from the oxylipin pathway. Yet, dnOPDA is likely to influence the activity of the HPL-branch, which is antagonistic to the effects of OPDA and JA. Furthermore, the herbivore-induced oxylipin JA and not dnOPDA or OPDA is essential for *PAL1* activity. Thus, our study implicates that in Arabidopsis the biting-chewing herbivore *P. rapae* induces the shikimate pathway by means of the formation of JA, which subsequently induces the shikimate pathway. Studies with pathway-specific mutants in sepa-

rate signal-transduction routes could further distinguish between the roles of different oxylipins within caterpillar-induced plant defenses.

Acknowledgements

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Chapter 6

Picture: Tibor Bukovinszky

Herbivore-induced plant volatile methyl salicylate negatively affects attraction of the parasitoid *Diadegma semiclausum*

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under review

Abstract

The indirect defense mechanisms of plants comprise the production of herbivore-induced plant volatiles that can attract natural enemies of plant attackers. One of the often emitted compounds after herbivory is methyl salicylate (MeSA). Here, we studied the importance of this caterpillar-induced compound in the attraction of parasitoid wasp *Diadegma semiclausum* using a molecular genetic approach. *Pieris rapae* infested *AtBSMT1-KO* mutant Arabidopsis plants, compromised in the biosynthesis of MeSA, were found to be more attractive to parasitoids than infested wild-type plants. This suggests that the presence of MeSA has negative effects on parasitoid host-finding behavior when exposed to wild-type production of herbivore-induced Arabidopsis volatiles. Further, supplementing the headspace of caterpillar-infested mutant plants with synthetic MeSA demonstrated a positive correlation between MeSA-dose and repellence of *D. semiclausum*.

Key words:
Tritrophic interaction, herbivory, infochemical, SABATH methyl transferase, *BSMT1*

Introduction

Herbivores are effectively combated by plants through direct and indirect defenses (Kessler and Baldwin, 2002). Direct defense comprises the production and storage of metabolites that negatively influence herbivore performance (Wittstock and Gershenzon, 2002). In contrast, indirect defense encompasses the production of metabolites that benefit the natural enemies of herbivores (Dicke et al., 1999; Wäckers et al., 2001; Halitschke et al., 2008; Kost and Heil, 2008). These defenses are commonly divided into constitutive and induced defenses. The former include morphological and structural features (e.g. trichomes) as well as constitutively produced defense compounds. Induced defenses are only active in actual threat situations, for instance when plants are under attack by pathogens or insect herbivores. These dedicated responses to an attack allow plants to be more cost effective and also to diminish the risk that herbivores adapt to the defenses (Agrawal and Karban, 1999; Pieterse and Dicke, 2007; Heil, 2008; Steppuhn and Baldwin, 2008).

In this study, we address a component of induced indirect plant defense, i.e. the production of herbivore-induced plant volatiles (HIPVs). HIPVs mainly comprise green leaf volatiles (GLV) (C6 aldehydes, alcohols, and derivatives), terpenoids, and phenolics (Paré and Tumlinson, 1997; Dicke, 1999b). Green leaf volatiles originate from linolenic and linoleic acid, which are released particularly when cells are damaged (Bate and Rothstein, 1998). Terpenoids are synthesized via the mevalonic acid (MVA) or methylerythritol phosphate (MEP) pathway (Dudareva et al., 2006; Gershenzon and Dudareva, 2007). Finally, the aromatic compounds, such as methyl salicylate (MeSA) and indole, are formed via the shikimic acid pathway (Paré and Tumlinson, 1997). The induced volatile production is orchestrated by at least three main signal-transduction pathways: the jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) pathways (Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002). These pathways can be differentially induced by different herbivore species (Heidel and Baldwin, 2004; De Vos et al., 2005; Schmidt et al., 2005), leading to the emission of a volatile blend that is specific for an herbivore species (Vet and Dicke, 1992; Ozawa et al., 2000; Walling, 2000; Leitner et al., 2005). These herbivore-specific volatile blends can provide foraging natural enemies of herbivores, such as predators and parasitoid wasps, with detectable and reliable information to locate their prey or host respectively (Dicke et al., 1990c; Turlings et al., 1991; Vet and Dicke, 1992; Du et al., 1998; Dicke, 1999a).

Variation in attraction of carnivorous arthropods towards host or non-host infested plants is mainly ascribed to the presence and relative abundance of at-

tractive compounds within the HIPV blend (D'Alessandro and Turlings, 2006). An intriguing question is which components of the complex HIPV blend affect parasitoid attraction most. In the past decades, several approaches have been applied to study the relative importance of certain HIPVs in the attraction of carnivorous arthropods, e.g. offering synthetic compounds alone or in mixtures, inducing certain subsets of the HIPV blend with elicitors, or manipulating signal-transduction or biosynthetic pathways through a molecular genetic approach (Snoeren et al., 2007).

One HIPV component for which biological relevance for carnivorous arthropod attraction has been addressed is methyl salicylate (MeSA) (De Boer and Dicke, 2004; De Boer et al., 2004; James and Price, 2004; Zhu and Park, 2005; Ishiwari et al., 2007). This methyl ester of the plant hormone salicylic acid (SA) has been reported in many HIPV blends, e.g. lima bean (Dicke et al., 1990c), tomato (Ament et al., 2004), cabbage (Geervliet, 1997; Poelman et al., in press), and Arabidopsis (Van Poecke et al., 2001; Chen et al., 2003). Its role in the attraction of carnivorous arthropods has been studied by investigating the attraction to synthetic MeSA in field (James and Price, 2004; Zhu and Park, 2005) and laboratory studies (De Boer and Dicke, 2004; De Boer et al., 2004; Ishiwari et al., 2007). However, so far, no studies have addressed the effects of the absence of MeSA from an otherwise complete HIPV blend on the behavior of carnivorous arthropods.

In this study we focus on the function of MeSA within the HIPV blend through a molecular genetic approach that involves the elimination of MeSA. MeSA is synthesized by SA carboxyl methyltransferase (SAMT), a member of the SABATH methyl transferase family, to which also jasmonic acid, indole-acetic acid and cinnamic/*p*-coumaric acid methyltransferases belong (Seo et al., 2001; Chen et al., 2003; Zubieta et al., 2003; Kapteyn et al., 2007). Related enzymes that methylate benzoic acid (BA) to give MeBA have also been reported (Murfitt et al., 2000). Some SABATH enzymes can methylate both SA and BA with roughly equal efficiencies, and have therefore been designated as BSMTs. One such example is the *Arabidopsis thaliana* gene designated *BSMT1* (Chen *et al.*, 2003).

While SAMT or BSMT enzymes have been identified in a number of plant species, including fary fans (*Clarkia breweri*), snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), Arabidopsis and jasmine (*Stephanotis floribunda*), we have selected Arabidopsis for further work on the physiological significance of MeSA emission because of the availability of molecular genetic tools and because this species is a valuable stepping stone towards other brassicaceous

plants for studying the role of HIPVs in plant-insect interactions (Snoeren et al., 2007). We addressed the role of MeSA in the foraging behavior of the parasitoid wasp *Diadegma semiclausum* Hellén (Hymenoptera, Ichneumonidae) that attack caterpillars feeding on brassicaceous plants including Arabidopsis. Caterpillar-feeding is known to induce MeSA in Arabidopsis (Van Poecke et al., 2001; Chen et al., 2003). We used wild-type Arabidopsis plants and a knock-out mutant that does not have functional SAMT and thus no MeSA biosynthesis. We addressed the effects of the mutation on parasitoid- and herbivore behavior, and headspace composition.

Materials and Methods

Plants and Insects

An Arabidopsis line with an insertion in the *AtBSMT1* gene was obtained from the Torrey Mesa Institute collection and the position of the insertion was verified by sequencing (Supplemental Fig. 1). Arabidopsis seeds (*A. thaliana* wild-type Columbia (Col-0) and *AtBSMT1-KO*) were germinated in sandy Arabidopsis soil (Lentse potgrond BV, Lent, Netherlands), and cultivated in a growth chamber at 21 ± 2 °C , 50-60% relative humidity (RH), and L8:16D photoperiod with 80-110 µmol m^{-2} s^{-1} PPF. The soil was heated to 90 °C for at least two hours prior to sowing of the plants. Two-week-old seedlings were transferred to plastic containers (5 cm in diameter) filled with the same soil type. Plants were watered twice a week. To prevent infestation by root-feeding sciarid flies, the soil was treated weekly with the entomopathogenic nematode *Steinernema feltiae* (Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands). Fully grown vegetative plants were used for the experiments, i.e. after 6 to 8 weeks since sowing.

The small cabbage white butterfly, *P. rapae* L. (Lepidoptera, Pieridae), was reared on Brussels sprouts plants (*Brassica oleracea* var. gemmifera, cv Cyrus) in a growth chamber (16L:8D; 20 ± 2 °C and 70% RH) as described in detail in Fatouros et al. (2005).

The parasitoid wasp *D. semiclausum* was reared as described in Bukovinszky et al. (2005). Emerging wasps were provided *ad libitum* with water and honey, and are referred to as 'naïve' wasps as they had neither received exposure to plant material, nor obtained an oviposition experience. This parasitoid is known to be attracted to the volatiles emitted by *P. rapae*-infested Arabidopsis Col-0 plants (Loivamäki et al., 2008; Snoeren et al., 2009 chap. 4).

Plant treatments

Plants were infested by equally distributing 20 first-instar *P. rapae* larvae per plant over the fully expanded leaves. Uninfested plants that otherwise received similar treatment as the infested plants were used as control plants. In all experiments, plants were treated 24 h before the experiments and kept in a climate room (21 ± 2 °C, 50-60% RH; L8:D16 photoperiod, and 80-110 µmol m^{-2} s^{-1} PPFD).

Behavioral assays

Y-tube olfactometer

The effects of HIPVs from wild-type Arabidopsis Col-0 and transgenic *AtBSMT1-KO* plants on parasitoid behavior were tested in a closed Y-tube olfactometer system as described by Bukovinszky et al. (2005). In short, filtered air was led through activated charcoal and split into two air streams (4 L min^{-1}) that were led through five-liter glass vessels containing the odor sources consisting of four plants each. The olfactometer was illuminated with artificial light from above at an intensity of 60 ± 5 µmol m^{-2} sec^{-1} PPFD. All experiments were conducted in a climatized room (20 ± 2 °C).

Naive, 3-7 days-old female *D. semiclausum* were individually transferred from the cage into the Y-tube olfactometer on a plant leaf; this was done by using alternately a Col-0 or *AtBSMT1-KO* leaf, that had been previously infested by *P. rapae* and from which the caterpillars and their products had been carefully removed. Upon release in the olfactometer, parasitoid behavior and parasitoid choice for one of the two odor sources was observed and scored as described in detail by Bukovinszky et al. (2005). Parasitoids that did not make a choice within ten minutes after release or did not choose for one of the two arms of the olfactometer within five minutes were considered as non-responding individuals, and were excluded from preference analysis. After every five parasitoids tested, the odor sources were interchanged to compensate for any unforeseen asymmetry in the set-up.

Experiment 1: effect of AtBSMT knock-out on parasitoid attraction to caterpillar -infested plants

To assess the role of MeSA as a cue for parasitoids *in planta*, we compared parasitoid behavior in response to an HIPV blend that lacked MeSA versus a complete HIPV blend that included MeSA. Caterpillar-infested mutant *AtBSMT1-KO* and wild-type Col-0 plants were offered as odor sources in the Y-tube olfactometer to *D. semiclausum*.

Experiment 2: supplementing headspace of caterpillar-infested *AtBSMT1-KO* plants with synthetic MeSA

To further investigate the role of MeSA, we restored the HIPV blend of *AtBSMT1-KO* plants by adding synthetic MeSA (Merck, 99% pure). To determine if parasitoid behavior to MeSA was dose dependent, different doses of MeSA (0.2 µg, 2 µg, 20 µg, 200 µg) were added downwind to the earlier infested plants. MeSA was diluted in n-hexane (Sigma-Aldrich, 95%). In all experiments 0.1 ml of the MeSA solution was applied on filter paper (15 cm^2) and positioned in the last section of the olfactometer arm. A piece of filter paper with 0.1 ml hexane was placed at a similar position in the other arm as a control treatment. The solvent was allowed to evaporate for 30 − 60 seconds, after which a parasitoid was introduced in the olfactometer. New filter papers with MeSA or hexane were used for each parasitoid tested in the olfactometer.

Caterpillar-feeding

Areas of consumed leaf-tissue were assessed for the caterpillar-infested Col-0 and *AtBSMT1-KO* plants that were used in experiment 1. Immediately after finishing an olfactometer bioassay, individual leaves were taped on paper and scanned with a Hewlett-Packard scan jet 3570c. Original leaf shapes were reconstructed using Paint.NET v3.30, Microsoft Corporation. Quantification of consumed leaf-tissue area was performed using Winfolia pro 2006a, Regent instruments (Québec, Canada).

Headspace collection and volatile analysis

Dynamic headspace sampling was carried out in a climate room (20 ± 2 °C, 70% RH; L8:D16 photoperiod and 90-110 µmol photons m^{-2} s^{-1} PPFD). Twenty-four hours before sampling, the pots were removed, roots and soil were carefully wrapped in aluminum foil, and four plants were placed together in a 2.5 L glass jar. The glass jars were then covered with insect-proof gauze. Just before trapping, the gauze was removed and jars were closed with a Viton-lined glass lid having an inlet and outlet. Inlet air was filtered by passing through tubes filled with 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, USA). Air was sucked out of the jar with 100 ml min^{-1} by passing through a tube filled with 200 mg Tenax TA. Headspace volatiles from different treatments were collected for a period of 3.5 h. Fresh weights of all rosettes were determined immediately after the experiments. On each experimental day, headspace samples for two or three replicates of each treatment were collected simultaneously.

Headspace samples were analyzed with a Thermo TraceGC Ultra (Thermo Fisher Scientific, Waltham, USA) connected to a Thermo TraceDSQ (Thermo

Fisher Scientific, Waltham, USA) quadrupole mass spectrometer. Before desorption of the volatiles, the Tenax cartridges were dry-purged with helium at 30 ml min^{-1} for 20 min at ambient temperature to remove moisture. Samples were desorbed from the cartridges using a thermal desorption system at 250 ° C for 3 min (Model Ultra Markes Llantrisant, UK) with a helium flow at 30 ml min^{-1}. Analytes were focused at 0 °C on an electronically-cooled sorbent trap (Unity, Markes, Llantrisant, UK). Volatiles were transferred without split to the analytical column (Rtx-5ms, 30 m, 0.25 mm i.d., 1.0 µm film thickness, Restek, Bellefonte, USA) by ballistic heating of the cold trap to 250 °C. The GC was held at an initial temperature of 40 ^{o}C for 3.5 min followed by a linear thermal gradient of 10 ^{o}C min^{-1} to 280 ^{o}C and held for 2.5 min with a column flow of 1 ml min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 45-400 m/z with a scan rate of 3 scans s^{-1}.

Compounds were identified by using the deconvolution software AMDIS (version 2.64, NIST, USA) in combination with NIST 98 and Wiley 7th edition spectral libraries and by comparing their retention indices with those from the literature (Adams, 1995). For quantification characteristic quantifier ions were selected for each compound of interest (see Fig. 2). Metalign software (PRI-Rikilt, Wageningen, the Netherlands) was used to align peaks of chromatograms of all samples and integrate peak areas for the quantifier ions. Peak areas of all compounds were corrected for the fresh weight of the leaf rosettes.

Statistical analysis

Bioassay

Parasitoid preference for infested *AtBSMT1-KO* vs. infested Col-0 plants (i.e. experiment 1) were statistically analyzed using a Chi-square test, with the null-hypothesis that parasitoids did not have a preference for any of the two odor sources. Secondly, we analyzed whether the parasitoids exhibited a MeSA-dose -dependent response when the HIPV blend from *AtBSMT1-KO* plants was restored with synthetic MeSA (i.e. experiment 2). We constructed an overall generalized linear model (GLM) including MeSA-dose as a covariate, the tested genotype combination as a fixed factor, and the interaction of the terms. As the null-hypothesis we defined that addition of MeSA did not result in a different attraction of parasitoids. Total number of parasitoids that preferred infested *AtBSMT1-KO* plants with the supplemented MeSA over the control (infested Col -0 or *AtBSMT1-KO*) plants was taken as response variate. Total numbers of parasitoids that made a choice per day were used as the binomial total and we used a logit-link function. We used a Chi-square, with the aforementioned null-hypothesis, to test for a significant preference of each tested MeSA-dose per

genotype combination. Parasitoids that did not make a choice were not included in the test.
Third, we investigated the effects of MeSA-dose on the proportion of parasitoids that made a choice in the Y-tube olfactometer experiments. We used the same overall GLM model as described above, only now with number of non-responding parasitoids as the response variate (SPSS 15.0, Chicago, USA).

A Mann-Whitney-U test was used to test whether the consumed leaf area of Col-0 and *AtBSMT1-KO* plants (experiment 1) was different (SPSS 15.0, Chicago, USA).

Headspace collection
The fresh weight of a corrected peak area for a volatile compound quantifier ions were log10 transformed, and for each HIPV the following mixed model was used to screen for HIPV compound differentiation per genotype: $\log10(V_{ijk}) \sim G_i+T_j+G{:}T_{ij}+R_k+\varepsilon_{ijk}$, where V=area of quantifier ions per gram fresh weight; G=genotype; T=treatment; R=replicate; ε=residual; i=1,2; j=1,2; and k=1,…,5 (see also Snoeren et al., 2009 chap. 4). Both G and T were used as fixed effects and R as a random effect. Subsequently, two-tailed t-tests followed by a Benjamini and Hochberg false discovery rate (BH-FDR) multiple comparison correction were conducted per compound for the genotypes (significance: $q<0.05$; (Benjamini and Hochberg, 1995)).

Results

Parasitoid behavior

Experiment 1: effect of AtBSMT knock out on parasitoid attraction to caterpillar-infested plants
Diadegma semiclausum females are attracted to the headspace of *P. rapae*-infested Arabidopsis Col-0 plants (Loivamäki et al., 2008; Snoeren et al., 2009 chap. 4). To investigate whether changes in this headspace, as a result of a knock-out mutation in the *AtBSMT1* gene, affected indirect defense we investigated the behavioral responses of *D. semiclausum* towards plant volatiles induced by *P. rapae* herbivory in a Y-tube olfactometer. Females of *D. semiclausum* preferred the volatiles emitted by herbivore-infested *AtBSMT1-KO* plants over those emitted by infested Col-0 plants ($P<0.05$; Fig. 1a). The amount of leaf-tissue consumed by the caterpillars did not differ between Col-0 and *AtBSMT1-KO* plants (mean ± SE: Col-0 10.41 ± 0.91 mm^2, *AtBSMT1-KO* 10.88 ± 0.80 mm^2; Mann Whitney U test: U=259.00; $P=0.90$, n=23). Thus, the difference in attraction cannot be explained by a difference in the amount of feeding by the caterpillars.

Experiment 2: supplementing headspace of caterpillar-infested *AtBSMT1-KO* plants with synthetic MeSA

To assess if the absence of MeSA in the HIPV blend can explain parasitoid behavior, the volatile blend of infested *AtBSMT1-KO* plants was restored by adding synthetic MeSA downwind of the plant. Different MeSA-doses were used to test for dose-dependent effects of MeSA presence. The addition of MeSA eliminated the preference for the knock-out plants, as seen in experiment 1, and with increasing MeSA-dose this effect was stronger. Parasitoid preference was not influenced by the different genotype combinations, but solely by the MeSA-dose used (GLM: genotype combination P=0.167, MeSA dose P<0.001, R^2=0.28).

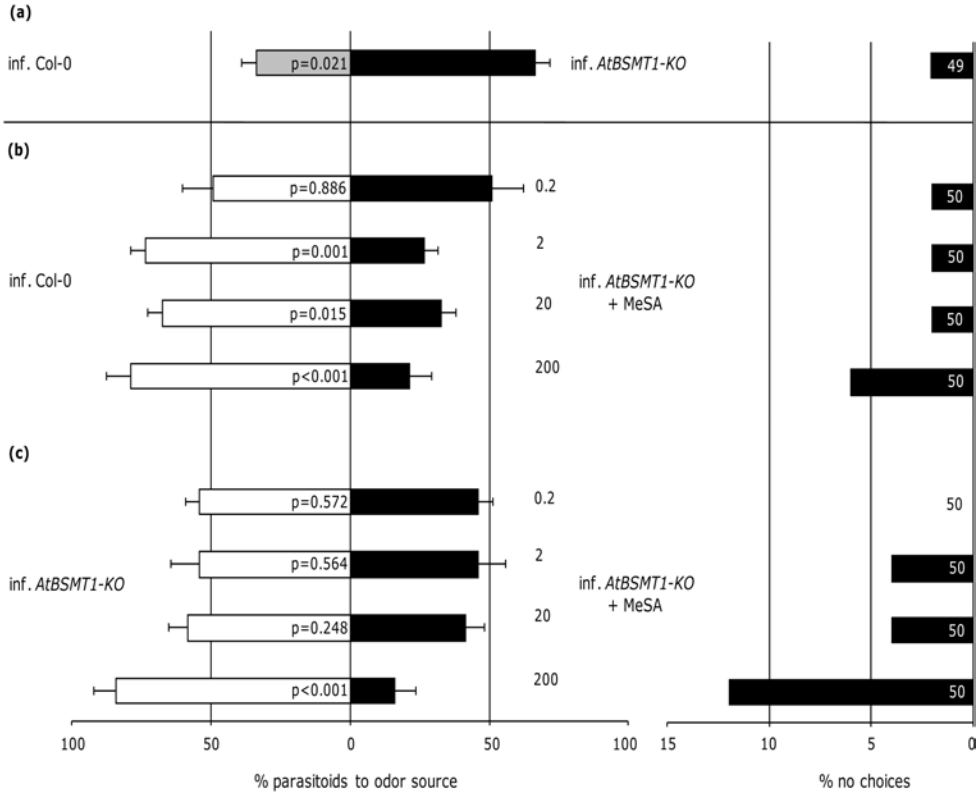

Figure 1. Behavioral responses of naïve *Diadegma semiclausum* females to the volatiles of two sets of *Arabidopsis thaliana* plants, as assessed in the Y-tube olfactometer. All plants were infested (inf.) with 20 *Pieris rapae* caterpillars and in some cases the headspace was supplemented with synthetic MeSA, added downwind from the plants. Added MeSA-doses (µg) are indicated to the right of the bars in the left bar plot. Each bar represents the percentage of choices for each of the two odor sources as determined in 5 replicate experiments; on each replicate day 10 parasitoids were tested per odor source (X^2 test, P values). Error bars indicate SE. GLM analysis for experiments B and C, demonstrated that MeSA dosage (P<0.001) and not the offered genotype (P=0.167) explained parasitoid behavior. Parasitoid MeSA dosage responses between two tested genotype combinations did not differ (P=0.270). The right bar plot indicate the percentage of no choice in each experiment; total number of tested parasitoids are given in these bars.

Analysis of parasitoid choices for each tested MeSA-dose within a tested genotype combination demonstrated that parasitoid preferences slightly varied among the MeSA doses used. Yet, for both genotype combinations tested, *AtBSMT1-KO* HIPV complementation with 0.2 µg MeSA did not result in discrimination between the two odor sources and a complementation with 200 µg MeSA resulted in significant preference for the odor without supplemented MeSA (Fig. 1b, 1c). When parasitoids were offered a choice between volatiles from infested *AtBSMT1-KO* plants supplemented with 2 or 20 µg MeSA versus infested Col-0 HIPVs, they preferred the latter. In contrast, no discrimination between odors from infested *AtBSMT1-KO* plants supplemented with 2 or 20 µg MeSA versus infested *AtBSMT1-KO* plants was observed.

The addition of MeSA negatively influenced the proportion of parasitoids that made a choice for one of the two odor sources. Analysis of the number of wasps that did not make a choice for one of the two odor sources, showed no effect of the offered genotype combination, but only an effect of the MeSA-dose used (GLM: genotype combination P=0.75, MeSA dose P=0.010, R^2=0.15).

Headspace volatile analysis

To evaluate the effects of the SAMT knock-out mutation, we analyzed the headspace of uninfested and *P. rapae*-infested plants of wild-type Col-0 and *AtBSMT1-KO* mutant plants. For the analysis, we selected compounds in the HIPV blend that are known to influence the behavior of carnivorous arthropods or compounds predicted to be affected by the KO mutation, i.e. methyl salicylate and methyl benzoate (MeBA) (Fig. 2) (Dicke et al., 1990c; Turlings and Fritzsche, 1999; Chen et al., 2003; De Boer et al., 2004; Shimoda et al., 2005).

Infested Col-0 and *AtBSMT1-KO* plants differed significantly in the emission of MeSA, ethyl salicylate (EtSA) and MeBA (q<0.001); these compounds were induced in Col-0 but not in *AtBSMT1-KO*. The Y-axis represents a ^{10}log scale. Thus, the headspace analysis for infested *AtBSMT1-KO* and infested Col-0 showed that the production of MeBA (No. 10), MeSA (No. 12), and ethyl salicylate (EtSA, No. 13) was hampered. These compounds are emitted at 14, 59, and 17 times lower emission rates, respectively (Fig. 2).

The remaining compounds, pentan-1-ol, linalool, indole, (*E,E*)-α-farnesene, and (*E*,*E*)-4,8,12−trimethyltrideca-1,3,7,11−tetraene (TMTT) were similarly induced in infested Col-0 and *AtBSMT1-KO* plants when compared to uninfested plants. The green leaf volatile (*Z*)-3-hexen-1-ol was only induced in the mutant (q=0.005) but not in the wild-type. Uninfested Col-0 and *AtBSMT1-KO* plants did not differ in the emission of volatiles, except for TMTT which was emitted in somewhat larger amounts by uninfested Col-0 plants than by uninfested *AtBSMT1-KO* plants (q=0.011). Uninfested Col-0 plants emitted more 1-octen-3-ol and 1-nonanol than infested plants (Fig. 2).

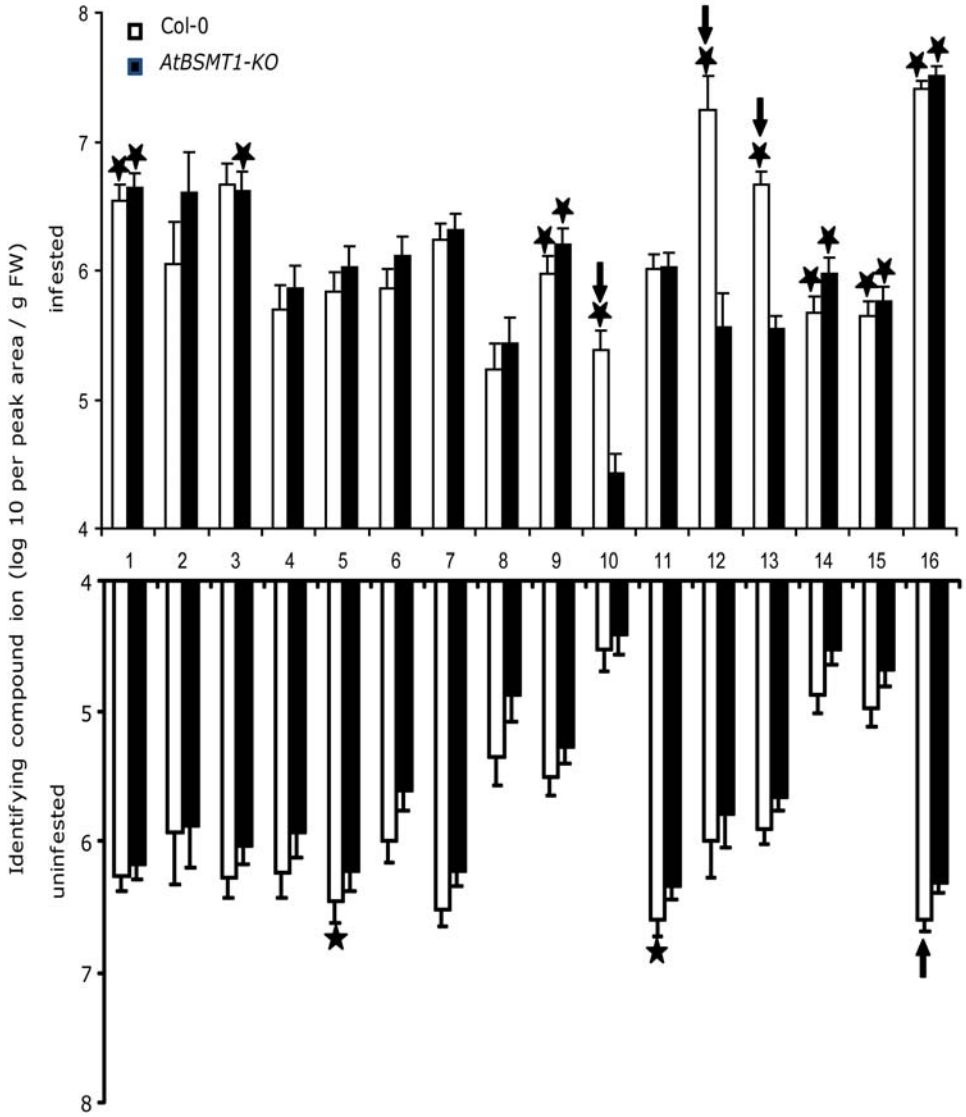

Figure 2. Emission of volatile compounds of Col-0 wild-type and *At-BSMT-KO* plants, either uninfested or infested with 20 *Pieris rapae* caterpillars, expressed as peak area (arbitrary units; mean ± SE; n=5) for the identifying ion per g FW. Compounds: 1=pentan-1-ol (m/z 70); 2= (*Z*)-2-penten-1-ol (m/z 57); 3= (*Z*)-3-hexen-1-ol (m/z 67); 4= α-pinene (m/z 93); 5= 1-octen-3-ol (m/z 57); 6= ß-myrcene (m/z 93); 7= *(Z)*-3-hexen-1-ol acetate (m/z 67); 8= (*E*)-ß-ocimene (m/z 93); 9= linalool (m/z 93); 10= methyl benzoate (m/z 136) 11= 1-nonanol (m/z 56); 12= methyl salicylate (m/z 120); 13= ethyl salicylate (m/z 120); 14= indole (m/z 117); 15= (*E*,*E*)-α-farnesene (m/z 93); 16= (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) (m/z 69).
Bars marked with * indicate a treatment for a genotype significantly emitting more volatiles than its counterpart. Bars marked with arrows represent compounds emitted in significantly different amounts by the two genotypes.

Discussion

Variation in the attraction of carnivorous arthropods towards HIPVs is often ascribed to the relative contribution of attractive compounds within the complex herbivore-induced blend (Van Den Boom et al., 2004; D'Alessandro and Turlings, 2006). Plant species can differ in the relative emission rates of individual attractive compounds after feeding by distinct herbivores. The commonly induced attractive compounds comprise green leaf volatiles, terpenoids and phenolics. Several studies have already shown *in planta* the importance of some green leaf volatiles and terpenoids in attracting carnivorous arthropods (Kappers et al., 2005; Schnee et al., 2006; Shiojiri et al., 2006a). Yet, empirical evidence for the quantitative importance of single compounds in the total HIPV blend of infested plants is still rare.
We used a molecular genetic approach with the crucifer Arabidopsis, to study the importance of the presence-absence of an individual component within the total HIPV blend on parasitoid behavior.

Females of *D. semiclausum* are attracted to the headspace of *P. rapae*-infested Arabidopsis Col-0 plants (Loivamäki et al., 2008; Snoeren et al., 2009 chap. 4). However, so far the attractive compounds of the HIPV blend involved are not known. One of the *P. rapae*-induced volatiles emitted by Arabidopsis Col-0 plants is MeSA (Van Poecke et al., 2001). This compound is only emitted in very low amounts by undamaged Col-0 plants (Van Poecke et al., 2001). New molecular tools allowed us to use the Arabidopsis knock-out mutant for benzoic acid and salicylic acid carboxyl methyltransferase (*AtBSMT1-KO*), to study the ecological effects of the presence of MeSA in the volatile blend induced by herbivory. Our data clearly show that infested *AtBSMT1-KO* plants attract more parasitoids than infested wild-type Col-0 plants (see Fig. 1a). Thus, although the total HIPV blend strongly attracts *D. semiclausum* parasitoids (Loivamäki et al., 2008; Snoeren et al., 2009 chap. 4), a genotype that does not emit MeSA in response to caterpillar infestation is even more attractive to the parasitoids. These data indicate that MeSA does not contribute to the attraction of naïve *D. semiclausum* females but acts as a repellent or masks the attractiveness of other compounds to the parasitoids. This negative effect of MeSA on *D. semiclausum* attraction was not anticipated, as MeSA is commonly induced after herbivory in many plant species, e.g. in lima bean (Dicke et al., 1990c), Brussels sprouts (Geervliet, 1997; Bukovinszky et al., 2005), and Arabidopsis (Van Poecke et al., 2001; Chen et al., 2003). Moreover, MeSA has also been demonstrated to attract other carnivore species (Dicke et al., 1990c; De Boer, 2004; James and Price, 2004).

However, other studies have also recorded repellent or masking effects of HIPV compounds to carnivores. For example, a HIPV fraction of maize, containing (*Z*)-3-hexen-1-ol acetate, linalool and (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was found to be least attractive compared to other tested fractions of the HIPV blend of maize to the parasitoid *Cotesia marginiventris* and might even have a repellent or masking effect on other fractions of the HIPV blend (Turlings and Fritzsche, 1999). Furthermore, for the same plant-herbivore system it was demonstrated that naïve *Microplitis rufiventris* parasitoids preferred HIPV blends lacking the induced compound indole, also indicating a repellent or masking effect of this compound (D'Alessandro et al., 2006). In addition, also isoprenoids were found to interfere with host-finding, as was demonstrated for transgenic Arabidopsis plants emitting isoprene (Loivamäki et al., 2008).
The function of emitting HIPV compounds that do not lure naïve carnivores but repel them may be linked to the compounds' role in other plant defense mechanisms. For example, HIPV compounds can act as repellents towards herbivores (Bruce et al., 2008; Piesik et al., 2008), which also has been demonstrated for MeSA (Hardie et al., 1994; James and Price, 2004; Prinsloo et al., 2007; Ulland et al., 2008). In addition, supporting evidence of a plant-plant signaling role for MeSA has been proposed (Ozawa et al., 2000; James and Price, 2004), suggesting a priming effect of MeSA on plant defense (Turlings and Ton, 2006). Yet, from the perspective of the carnivore, one would expect that any volatile that is correlated with the activity of its herbivorous victim could be exploited in locating the herbivore. It is well known that carnivores can learn to respond to HIPV blends and this has also been demonstrated for the response of a predatory mite to MeSA (De Boer and Dicke, 2004). Whether this also influences the response of *D. semiclausum* remains to be investigated.

To determine if an altered enzyme activity for SAMT and BAMT affected the headspace composition in other respects, we analyzed the headspace of infested knock-out mutant and wild-type plants. The headspace of caterpillar-infested plants differed only in the emission of MeSA and MeBA: the emission rates of these compounds were up to 60 times lower for *AtBSMT1-KO* plants compared to wild-type plants (Fig. 2). This agrees with the reported activity of the enzyme encoded by the *BSMT1* gene (Chen et al., 2003). The very low emission of MeSA that remains may be ascribed to activity of another SABATH enzyme that shows very low levels of activity towards SA and BA (Chen et al., 2003). Eliminating a functional *BSMT1* gene also decreased the emission rate of EtSA. EtSA has been recorded in several plants (Hamilton-Kemp et al., 1988; Scutareanu et al., 1997; Deng et al., 2004) and is known to be perceived by insect chemoreceptors (Ramachandran et al., 1990; Reinecke et al., 2002).

All other investigated HIPVs were emitted at similar rates by infested Col-0 and *AtBSMT1-KO* plants (Fig. 2).

Testing the HIPVs from *AtBSMT1-KO* plants supplemented with synthetic MeSA against HIPVs from Col-0 or *AtBSMT1-KO* demonstrated that MeSA acts as a repellent to naïve *D. semiclausum*. Moreover, the observed dose-dependent repellent effect for MeSA was also reflected in the disturbance of the response level of the parasitoids as fewer wasps chose between odor sources with higher MeSA-doses.
The KO-mutation did not affect the feeding rate by the caterpillars. Thus, the change in volatile emission and parasitoid attraction cannot be attributed to effects on herbivore behavior. Our data from the experiments in which the headspace was supplemented with MeSA show that the altered emission rate of this compound plays an important role in the observed effects. Caterpillar-feeding also induces the emission of EtSA and MeBA (Fig. 2). Whether MeBA and EtSA also affect the attraction of *D. semiclausum* wasps remains to be investigated, though our data suggest an attractive role for these compounds present in the wild-type Col-0.

In conclusion, through a multidisciplinary approach we investigated the role of MeSA by eliminating it from the total HIPV blend rather than investigating the role of MeSA as an isolated compound or as an addition to an artificial blend. We demonstrated that MeSA has a repellent effect on the behavior of naïve *D. semiclausum* parasitoids. This provides a new view on the effects of individual components of herbivore-induced plant volatile blends. The effects on other species in the same community should be investigated to understand the role of MeSA in a community ecological context.

Acknowledgements

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Chapter 7

Picture: Raymond Rutting

Summarizing discussion: ecogenomics used to unravel the mechanisms underlying insect-plant interactions

Tjeerd A.L. Snoeren

Introduction

Plants are sessile organisms at the basis of most food webs where they are under constant threat of possible attackers. Plants have evolved resistance against most of their attackers. A broad range of defense mechanisms allows plants to effectively combat biotic stresses caused by, for instance, herbivorous insects. These mechanisms include constitutively present barriers, as well as induced defense responses that are activated upon attack (Karban and Baldwin, 1997; Pieterse and Dicke, 2007). A constitutive defense involves plant traits that interfere with herbivores and form a pre-existing physical or chemical barrier. Morphological plant traits, such as leaf surface wax layers, thorns or trichomes, form a first physical barrier to the herbivore. Secondary metabolites, such as toxins and digestibility reducers, can form the next barriers that benefit the plant under attack. In addition, plants may also provide shelter to natural enemies of the herbivore (Schoonhoven et al., 2005). The induced defense responses can be subdivided into direct defense, e.g. production of anti-digestive proteins and toxic secondary compounds (Karban and Baldwin, 1997; Walling, 2000), and indirect defense, e.g. by providing the natural enemies with extrafloral nectar (Kost and Heil, 2008) and the production of infochemicals (Dicke and Sabelis, 1988) such as herbivore-induced plant volatiles (HIPV) (Dicke and Hilker, 2003). Chemical cues are a major source of information for interacting plants and insects. Herbivores might use induced changes in infochemicals as information on the status of the plant to determine the suitability of the plant for oviposition or feeding. Carnivorous arthropods can use chemical cues, e.g. HIPVs, to locate their prey or host (Dicke and Hilker, 2003). Furthermore, the induced nature of these defense responses to herbivore attack allows plants to be cost-effective and also to diminish the risk that herbivores adapt to the defenses (Agrawal and Karban, 1999; Heil, 2008; Steppuhn and Baldwin, 2008). Therefore, studying how these HIPVs are involved in indirect defense is a major challenge, requiring a multidisciplinary approach (Baldwin et al., 2002)(chapter 2).

Ecogenomic approach

This PhD project was embedded in an NWO-VICI-project which consisted of two PhD projects and two post-doc projects that addressed the role of infochemicals in insect-plant interactions using an ecogenomic approach. In the VICI project we took a chemical approach to modify the infochemical phenotype and a molecular genetic approach by altering signal-transduction pathways underlying the induction of infochemical production. Both approaches were used to investigate the underlying mechanisms in terms of signal-transduction pathways involved in the induction of infochemicals and to determine whether modified emission rates of HIPVs affect interactions with plant-associated insects. For both approaches crucifer plants were adopted.

Knowledge of mechanisms that underlie the induction of HIPVs is of great importance for studying how these volatiles are involved in indirect defense. Current mechanistic knowledge of the biosynthetic and signaling pathways underlying HIPV production provides interesting tools to modify the emission of plant volatiles through specific elicitors and inhibitors (for review, see Bruinsma and Dicke, 2008). Over the last decade, molecular genetic information on the production of induced plant volatiles has accumulated rapidly. This allowed the use of mutants that are modified in the biosynthesis of volatiles or mutants that have altered signaling defense pathways (for review, see Baldwin et al., 2001; Degenhardt et al., 2003; Dicke et al., 2004). Both a chemical manipulation approach to study HIPV emission and a genotypic approach by studying transgenic plants modified in the underlying pathways have their own advantages and disadvantages (chapter 2).

I used a molecular genetic approach to alter the signaling or biosynthetic pathways involved in the induction or production of HIPVs to study the importance of HIPV blend composition on parasitoid host-finding behavior and to gain insight in the underlying mechanisms. This approach has already been used in several plant species, especially Arabidopsis, tomato, and tobacco (Van Poecke and Dicke, 2002; Ament et al., 2004; Kessler et al., 2004). Although Arabidopsis is not the most interesting plant species from an ecological point of view, it has been proven useful to study underlying mechanisms of herbivore-induced plant defense responses in *Brassica* systems (Mitchell-Olds, 2001; Van Poecke and Dicke, 2004). Compared to other crucifers, Arabidopsis is ideal for a multidisciplinary approach to study the roles of HIPVs in indirect defense by means of transcriptomics, metabolomics and phenomics (Fig. 1). Furthermore, Arabidopsis is a valid stepping stone towards other brassicaceous plants, since, for instance, micro-arrays developed for Arabidopsis can be used for *Brassica* species as well (Lee et al., 2004; Broekgaarden et al., 2007) and HIPVs emitted by Arabidopsis resemble those of other brassicaceous species (Van Poecke and Dicke, 2004). Adopting Arabidopsis to study plant-insect interactions gave me the unique opportunity of combining transcriptomics, metabolomics, and behavioral studies of natural enemies of crucifer pests.

Given the early-season lifecycle of Arabidopsis, under natural growing conditions the plant is not exposed to major crucifer lepidopteran pest species such as *Pieris rapae* and *Plutella xylostella.* Still, I chose to induce defense pathways with *Pieris rapae* larvae that specialize on brassicaceous plants*,* since there are many parallels between Arabidopsis and other brassicaceous species in terms of caterpillar-induced plant volatiles (Van Poecke and Dicke, 2004). Parasitoid behavior towards HIPVs was tested with the specialist Ichneumonid parasitic wasp *Diadegma semiclausum* that uses the specialist herbivore *Plutella xylostella* as host. Although *P. rapae* is not a host species for *D. semiclausum*, bioassays in which the naïve wasps were offered crucifer plants infested with either *P. rapae* or *P. xylostella*, did not show discrimination of the wasps between

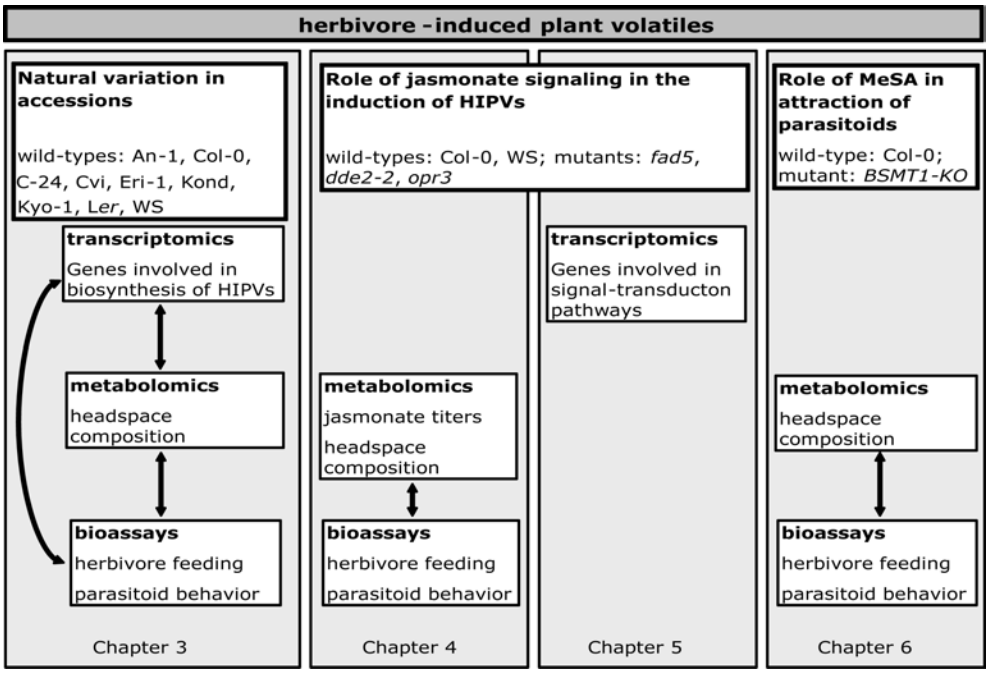

Figure 1. Integrated approach of studying herbivore-induced plant volatiles of *Arabidopsis thaliana* as used in this thesis.

both odor sources (T.A.L. Snoeren, unpublished data). I preferred using *P. rapae* over *P. xylostella* for its higher feeding rate on Arabidopsis, which increased the level of defense induction, and preferred *D. semiclausum* over *Cotesia*-wasp species, since this species was more easy to handle in the chosen experimental set-up.

Herbivore-induced indirect defense

Role of HIPVs

It is well-known that plants increase the production of volatiles after infestation by herbivores. This herbivory-induced volatile blend from the vegetative parts of the plant can vary drastically in the quantity and quality of volatile compounds (Turlings et al., 1995; Tumlinson et al., 1999; Dicke and van Loon, 2000). HIPVs can attract carnivorous arthropods, such as predatory mites and parasitoid wasps, and/or repel herbivores and thus act as a means of plant resistance (Dicke, 1986; Dicke and Dijkman, 1992; Turlings et al., 1995; Dicke, 1999b; Tumlinson et al., 1999; Kessler and Baldwin, 2001; Arimura et al., 2005). Compounds that form the headspace of plants after herbivory comprise alcohols, esters, aldehydes and various terpenoids (Dudareva et al., 2006; Pichersky et al., 2006). Some compounds are released instantly after herbivory, such as green leaf volatiles (GLVs), e.g. (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal or (*Z*)-3-hexen-1-yl acetate. In contrast, the ester methyl salicylate (MeSA), the monoterpenes myrcene and ß-ocimene, the homoterpene (*E*,*E*)-4,8,12-

trimethyltrideca-1,3,7,11-tetraene (TMTT), and the sesquiterpene (*E*,*E*)-α-farnesene are usually not emitted until after some hours after the onset of herbivory (Dudareva et al., 2006; Heil, 2008). Different feeding modes, which can be roughly divided in leaf chewing and piercing-sucking, affect plant defense signaling pathways distinctively and can, therefore, induce insect-plant-interaction specific volatiles (Walling, 2000; Leitner et al., 2005). Numerous carnivorous arthropods can use HIPVs to discriminate between plants attacked by different herbivore species and different plant species infested by the same herbivore species (Du et al., 1996; De Moraes et al., 1998). The attraction of natural enemies that effectively attack the herbivores can result in an increase in plant fitness (Van Loon et al., 2000; Fritzsche-Hoballah and Turlings, 2001). Thus, HIPVs have an important function in insect-plant interactions.

Natural variation in HIPVs among accessions

Carnivorous arthropods are confronted with a plethora of different volatile compounds released by different plant species. Background odors from surrounding plants might interfere with an attractive HIPV blend or mask individual attractive compounds from an HIPV blend (Mumm and Hilker, 2005; Schroeder and Hilker, 2008). Successful localization of a host or prey is therefore dependent on the capacity of carnivores to distinguish the different HIPV blends emitted by distinct plant species (De Moraes et al., 1998). Variation in emission of HIPVs does not only occur between different plant species but also within species. To date, the occurrence of intraspecific variation in headspace composition after herbivory was primarily explored for crop varieties, including cotton (Loughrin et al., 1995), gerbera (Krips et al., 2001), pear (Scutareanu et al., 2003), maize (Hoballah et al., 2004), carrot (Nissinen et al., 2005), rice (Lou et al., 2006), and cruciferous crops (Bukovinszky et al., 2005). So far, no studies were conducted on the occurrence of natural variation in HIPV emission among Arabidopsis accessions. Only few studies compared the herbivore-induced headspace from leaves between Arabidopsis accessions, i.e. Col-0 and WS (chapter 4). In chapter 3 we, therefore, conducted an extensive study on the occurrence of natural variation in HIPV emission. We analyzed nine Arabidopsis accessions from different geographic origins, i.e. An-1, Col-0, C-24, Cvi, Eri-1, Kond, Kyo-1, L*er*, and WS (Table 1). After herbivory by *P. rapae* caterpillars, variation among accessions was detected for distinct HIPV-compound-groups, i.e. GLVs, terpenoids, and phenolics. Accessions also differed in the production of plant volatiles after spraying the plants with the phytohormone jasmonic acid, which is often used to mimic herbivory (chapter 4)(Van Poecke and Dicke, 2002; Loivamaki et al., 2004; Ibrahim et al., 2005; Mewis et al., 2005; Bruinsma et al., 2008). Although we observed that parasitoids discriminated among JA-treated accessions, we were unable to allocate individual compounds to the differences in parasitoid behavior. The complex variation of the volatile headspace is likely to interfere with the postulation of conclusions on the contribution of individual compounds (chapter 3). It is likely that the parasitoid evaluates the HIPV blend more on its total composition or a range of

Table 1. Geographic origins of accessions. (source: http://www.arabidopsis.org and Google Earth for latitude and longitude determination)

Alias	Name	Country	Latitude	Longitude
An-1	Antwerpen	Belgium	N51/N52	E4/E5
Col-0	Columbia[1]	Germany	N52	E15
C24	C-24	Portugal	N40/N41	W8/W9
Cvi	Cape Verde Islands	Cape Verde	N15/N17	W23/W25
Eri-1	Eriengsboda	Sweden	N56	E15
Kond	Kondara	Tadjikistan	'N38'	'E64'
Kyo-1	Kyoto	Japan	'N35'	"E135'
L*er*	Landsberg erecta[1]	Germany	N52	E15
WS	Wassilewskija	Russia	N52/N53	E30

[1] accessions Col-0 and L*er* originate from the same location but are genetically different (see http://arabidopsis.info/)

compounds in the total blend, than on the presence or absence of single compounds (Mumm and Hilker, 2005). For both methods of inducing the production of HIPVs, we also detected variation in the transcript levels of genes that are putatively associated with biosynthetic steps in the formation of volatile compounds, e.g. MeSA, TMTT, (*E*)-ß-ocimene. Furthermore, the expression of some genes could be connected to volatile emission. Although no connection between gene expression and parasitoid behavior was found, this first attempt of combining several disciplines to study the mechanisms underlying the induced production of plant volatiles holds promise for further research on herbivore-induced volatile-regulated indirect defense.

Signal-transduction pathways in HIPV production

Induced defense responses are orchestrated by a network of interconnecting signal-transduction pathways in which jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) play key roles (Kazan and Manners, 2008; Koornneef and Pieterse, 2008). Empirical evidence for the significance of the phytohormones JA, SA, and ET in plant defense came from using mutant plants altered in these pathways (reviewed in e.g. Dong, 2004; Pozo et al., 2004; Schaller et al., 2005; Fujita et al., 2006). These signal-transduction pathways are differentially induced depending on the herbivore species (Heidel and Baldwin, 2004; Voelckel and Baldwin, 2004b; De Vos et al., 2005; Schmidt et al., 2005). Tissue-feeding insects, e.g. larvae of herbivorous Lepidoptera, and cell-content feeders, e.g. thrips, especially induce JA and related compounds from the same signal-transduction pathway, known as jasmonates or oxylipins (Kessler and Baldwin, 2002; Weber, 2002; De Vos et al., 2005). Phloem feeders, such as aphids and whiteflies, especially induce the SA pathway (Heidel and Baldwin, 2004; De Vos et al., 2005; Zarate et al., 2007). However, the JA pathway seems to be the most important in induced defenses in plant-insect interactions

(Kessler and Baldwin, 2002; Liechti and Farmer, 2002). This is supported by the finding that mutants of several plant species, including Arabidopsis, that are modified in the JA pathway are highly susceptible to herbivory (Howe et al., 1996; McConn et al., 1997; Halitschke et al., 2004). Therefore, in this thesis most attention was allocated to studying the effects of the jasmonic acid pathway on induced indirect defense (chapters 4 and 5).

Roles of jasmonates in induced defense

Jasmonates serve a key role in regulating defense responses in various types of herbivore-tissue damage (Kessler and Baldwin, 2002; Howe, 2004). In addition, jasmonates are involved in host-plant resistance to phloem-feeding insects, regulation of tritrophic interactions, trichome development, priming of direct and indirect defenses, and intra-plant transmission of defense signals. (Thaler, 1999; Li et al., 2002; Van Poecke and Dicke, 2002; Engelberth et al., 2004; Li et al., 2004; Mewis et al., 2005; Ton et al., 2007; Zarate et al., 2007). Transcriptional profiling experiments using DNA microarrays show a correlation of herbivory-induced JA signaling with changes in the transcription of hundreds of defense-related genes (Reymond et al., 2000; Halitschke et al., 2003; Reymond et al., 2004; De Vos et al., 2005; Devoto et al., 2005b). Jasmonates generally promote defense- and reproductive processes while growth and photosynthetic processes are inhibited (Devoto et al., 2005a). This suggests, therefore, a role of jasmonates in mediating "the dilemma of plants: to grow or defend" (Herms and Mattson, 1992).

The JA-titer increases rapidly (<30 min) at the site of wounding after herbivory by biting-chewing insects (Reymond et al., 2000). JA appears to be the actual compound required in herbivore-induced plant defense (Schilmiller et al., 2007). Furthermore, only the JA-conjugate jasmonoyl-L-isoleucine (JA-Ile) has been proven to be the active compound inducing JA-dependent responses. JASMONATE ZIM-DOMAIN (JAZ) proteins were discovered as repressors of JA-signaling (Chico et al., 2008). JA-Ile relieves this repression by promoting binding of JAZ1 with the F-Box protein CORONATINE INSENSITIVE1 (COI1), which releases transcription factor MYC2. The JA precursor 12-oxo-phytodienoic acid (OPDA), the methylated form of JA (MeJA), and some other tested JA-conjugates were incapable to promote the JAZ-COI1 interaction (Staswick, 2008). It is surprising that JA, MeJA, and OPDA were not able to promote JAZ-COI interactions as these jasmonates were good potential candidates for jasmonate signaling (Seo et al., 2001; Stintzi et al., 2001; Farmer et al., 2003). Nevertheless, recent studies indicate that OPDA signaling is distinct from the mechanism of JA-derived signals like JA-Ile (Taki et al., 2005; Thines et al., 2007). Earlier studies indicate that OPDA is a biologically active signal in itself for a limited range of direct (Stintzi et al., 2001) and indirect defense (Koch et al., 1999) responses to herbivory. Also, dinor-OPDA (dnOPDA), which originates from a parallel oxylipin cascade, is suggested to be involved in induced plant defense (Weber et al., 1997). Therefore, I was especially interested in

the different roles of the oxylipins OPDA, dnOPDA, and the end-product of the octadecanoid pathway, i.e. JA (chapters 4 and 5).
Headspace compositions of *P. rapae* caterpillar-infested mutants *opr3* (Stintzi and Browse, 2000), *fad5* (Weber et al., 1997), and *dde2-2* (Von Malek et al., 2002) were analyzed. Mutant *fad5*, which lacks dnOPDA-derived products, did not result in hampered production of HIPVs when compared to wild-type plants. In contrast, the absence of OPDA and/or JA in mutants *dde2-2* and *opr3*, did result in reduced induction of many volatiles, especially terpenoids and the aromatic compound MeSA. This implicates that OPDA and/or JA have a role in HIPV-indirect defense. As knocking out *DDE2-2* and *OPR3* has very similar effects on HIPV production, it was concluded that JA, and not OPDA, was the most important oxylipin in HIPV production (chapter 4). Quantitative RT-PCR analysis also demonstrated that, after *P. rapae* feeding, JA was the major signaling compound required for the induction of the defense-related genes *LOX2* (*Lipoxygenase 2*), *OPR3* (*12-Oxophytodienoate reductase 3*) and *ACX1* (*Acyl-CoA oxidase 1*) from the oxylipin pathway (chapter 5). Yet, roles for OPDA and dnOPDA galactolipid conjugates cannot be excluded in plant defense against herbivores, for instance the role of OPDA conjugates such as arabidopsides E and G (Kourtchenko et al., 2007).

Behavioral assays with the parasitoid *D. semiclausum* reflected the results from headspace analyses: both caterpillar-infested *dde2-2* and *opr3* mutants were shown to be less attractive when compared to infested wild-type plants. Mutant *opr3*, which only lacks JA but showed wild-type induced levels of OPDA and dnOPDA, still emitted induced quantities of the GLV (*Z*)-3-hexenal (chapter 4). This indicates that the absence of JA did not influence the emission of GLVs. This finding was further supported by analysis of mutants *dde2-2* and *opr3* for their transcript levels of *HPL1*, which mediates a step in the GLV producing pathway (Bate et al., 1998)(chapter 5). The lack of JA in the mutants *dde2-2* and *opr3* resulted in a reduced induction of *HPL1* transcript levels compared to the wild-types Col-0 and WS, respectively. This effect was much stronger in the *dde2-2* mutant that lacked not only JA but also OPDA, when compared to mutant *opr3* that only lacked JA but showed wild-type OPDA levels. Thus, this finding indicates the important involvement of OPDA compared to JA in *HPL1* induction. Furthermore, transcript levels of the *HPL1* gene in *P. rapae*-infested *fad5* plants were significantly more induced compared to infested Col-0 wild-type plants (chapter 5). This suggests a regulatory role for the products coming from the dnOPDA oxylipin-route in GLV production. Yet, this was not reflected by an increased GLV emission rate in the headspace of *fad5* plants and also not in distinct behavior of parasitoids when offered a choice between caterpillar-infested wild-type and *fad5* plants (chapter 4). This indicates that the emission of GLVs, e.g. (*E*)-3-hexen-1-ol, cannot be directly extrapolated from transcript levels of *HPL1* (chapter 3). Nevertheless, dnOPDA and OPDA may be used to fine-tune Arabidopsis' response to biotic stress in terms of the HPL-branch in other respects.

Besides terpenoids (i.e. (*E*,*E*)-α-farnesene and TMTT), the emission of MeSA was also hampered after caterpillar herbivory in JA-lacking mutants, i.e. *dde2-2* and *opr3*. This indicates that the JA-signaling pathway influences the biosynthesis of MeSA (chapter 4). Furthermore, interference with the SA pathway influenced TMTT- and MeSA-emission as well (Van Poecke, 2002). It is, therefore, assumed that both JA and SA signal-transduction pathways regulate the emission of MeSA and TMTT in response to herbivory. This is indicative of a synergistic interaction for the emission between both pathways, which has also been recorded for the emission of spider-mite induced volatiles in tomato (Ament et al., 2004). In contrast, only JA- and not SA-related signaling pathways are essential for TMTT-emission in Arabidopsis after pathogen-attack (Attaran et al., 2008). The data presented in chapter 4, resulting from a metabolomics approach, that suggest synergism between the JA- and SA-pathways after herbivory, were supplemented with data from a transcriptomics approach (chapter 5). Quantitative RT-PCR analysis indicates that in *opr3* mutant plants transcript levels of *Phenylalanine ammonia-lyase 1* (*PAL1*), a candidate gene for the formation of SA, did not show an induction after *P. rapae* herbivory compared to the herbivore-infested wild-type. Thus, in the presence of OPDA and dnOPDA, the absence or presence of JA can influence *PAL1* expression. This implies a major role of JA or its conjugates in crosstalk between JA and SA signaling transduction pathways after *P. rapae* herbivory. Together with the findings in chapter 4, this suggests that JA influences the emission of MeSA by affecting SA production. Furthermore, the observation that *PAL1* transcript abundance was not induced in mutant plants that were completely hampered in oxylipin production, i.e. no OPDA, dnOPDA, and JA, supports that induction of the SA pathway after *P. rapae* herbivory requires the signaling compound JA (chapter 5).

Role of methyl salicylate in HIPV blend

Attraction of parasitoid wasps towards host or non-host infested plants is mainly ascribed to the presence and relative abundance of attractive volatile compounds in the headspace of plants (Van Den Boom et al., 2004; D'Alessandro and Turlings, 2006). A fascinating question is: what are the compounds in the headspace that affect parasitoid behavior most? Several approaches have been made to study the relative importance of certain HIPVs on carnivorous arthropods, e.g. offering synthetic compounds alone or in mixtures, inducing certain subsets of the HIPV blend with elicitors, or manipulating signal-transduction or biosynthetic pathways through a molecular genetic approach (chapter 2).

In chapters 3 and 4 I reported that MeSA together with terpenoids (e.g. TMTT) and GLVs forms a group of volatiles that were induced after *P. rapae* feeding. Hampering the JA signaling pathway resulted in an altered emission of MeSA, among other HIPVs, that correlated with a reduced attraction of the parasitoid wasp *D. semiclausum* (chapter 4). The initial assumption was, therefore, that MeSA is used by *D. semiclausum* to locate its host, which I tested in chapter 6.

Several studies demonstrated with a molecular genetic approach that some terpenoids and GLVs contribute to the attractiveness of an HIPV-blend to the carnivorous arthropod (Kappers et al., 2005; Schnee et al., 2006; Shiojiri et al., 2006a). Methyl salicylate, the methyl ester of the plant hormone salicylic acid (SA), has been reported in many HIPV blends, e.g., lima bean (Dicke et al., 1990c), tomato (Ament et al., 2004), cabbage (Geervliet, 1997; Poelman et al., in press), and Arabidopsis (chapters 3 and 4)(Van Poecke et al., 2001; Chen et al., 2003). The attractiveness of MeSA to carnivorous arthropods has also been studied by investigating the attraction of synthetic MeSA in the field (James and Price, 2004; Zhu and Park, 2005) and in the laboratory (De Boer and Dicke, 2004; De Boer et al., 2004; Ishiwari et al., 2007). I used a molecular genetic approach to study the effect of the absence of MeSA from an otherwise complete HIPV blend on parasitoid behavior, by using a transgenic Arabidopsis line that was hampered in the methylation of SA. Behavioral experiments with naïve parasitoids resulted in a preference for the headspace of caterpillar-infested plants lacking MeSA over wild-type plants. Subsequently, supplementing the headspace from these transgenic plants with synthetic MeSA resulted in a dose-dependent decrease of attractiveness of the transgenic plants with supplemented MeSA for *D. semiclausum.* This indicates that MeSA acts as a repellent or masks the attractiveness of other compounds to naïve *D. semiclausum* wasps. Masking or repellence effects of HIPVs have also been shown for other HIPV compounds in different systems (Turlings and Fritzsche, 1999; D'Alessandro et al., 2006; Loivamäki et al., 2008). My results were logically followed by the question: "Why do plants allocate resources to the emission of the HIPV MeSA?" Possibly, MeSA has other defense roles than luring parasitoids. One role of MeSA is already described for other insect-plant systems: repelling herbivores (Hardie et al., 1994; James and Price, 2004; Prinsloo et al., 2007; Ulland et al., 2008). Also, it is commonly accepted that plants can perceive volatiles and respond to them. Methyl salicylate can be converted back to salicylic acid, which subsequently has a role in inducing defense against pathogens (Shulaev et al., 1997). MeSA is, therefore, potentially involved in volatile-induced resistance (Choh et al., 2004; Engelberth et al., 2004; Baldwin et al., 2006). This suggests that MeSA could have an intra-plant signaling role as well.

Future perspectives

In this thesis, we explored the natural variation in HIPVs between nine accessions of Arabidopsis obtained from different geographical origins. We demonstrated genotypic variation in indirect defense traits, both at the transcriptional level as well as for the biosynthesis of metabolites (chapter 3). Newly available marker technologies can be used for the characterization and positioning of loci that control these types of traits. It would be very interesting to screen recombinant inbred line (RIL) populations of those accessions that strongly differ quantitatively and qualitatively for their individual volatile compounds and subsequently perform expression quantitative trait locus (e)QTL analysis. Such RIL -populations would enable the unraveling of genetic regulation underlying HIPV -formation.

Recent findings in protein-protein interaction studies in the absence of other plant proteins indicate that JA-Ile is the sole active compound of the JAZ-COI1 interaction (Thines et al., 2007). For Arabidopsis this resulted in the transcriptional activity of MYC2 to regulate genes involved in jasmonate response (Chini et al., 2007). This finding sheds new light on oxylipin signaling. Yet, this does not exclude roles of other jasmonates than JA-Ile, i.e. JA, dnOPDA and OPDA. First, only the JAZ1 protein was tested and other JAZ-proteins might interact with other jasmonates. Second, there are known jasmonate-regulated genes that are COI1-independent and other jasmonates like OPDA might be active in such different defense signaling mechanisms (Taki et al., 2005). Therefore, unraveling the distinct roles of OPDA versus JA and JA-Ile could further elucidate alternate jasmonate-signaling mechanisms. The double Arabidopsis mutant *acx1/acx5* (Schilmiller et al., 2007), which hampers the formation of JA downstream of the precursor OPDA, and the mutant *jar1-1* (Suza and Staswick, 2008), which hampers the conversion of JA to JA-Ile, allow studying of separate effects of both jasmonate-signaling mechanisms and the role of JA-Ile on HIPV production.

From the perspective of the carnivorous arthropod, it is to be expected that any volatile induced by its prey or host is used. Surprisingly, we did not observe this response by the naïve wasp *D. semiclausum* for *P. rapae*-induced MeSA. These findings resulted in a new view on effects of individual HIPV compounds on carnivorous arthropods. Effects on other natural enemies of crucifer pests should be investigated to understand the role of MeSA.
In addition, it should be stressed that I have used naïve wasps in all behavioral studies of this thesis. Yet, it is well-known that carnivores such as parasitoid wasps can learn to respond to volatiles which enables them to cope with variation in HIPV blends during host location (Vet et al., 1998; Drukker et al., 2000; De Boer et al., 2005). The predatory mite *Phytoseiulus persimilis* can learn to respond to HIPVs such as MeSA (De Boer and Dicke, 2004). Whether the innate response of *D. semiclausum* towards MeSA changes after an oviposition experience, remains to be examined.

Many molecular approaches that study the effects of genes on plant defense have used mutants that represent qualitative variation in the functional expression of genes. New molecular tools, such as quantitative RT-PCR, have provided plant scientists with methods that allow comparison of quantitative differences in the transcript levels of genes foremost because of the sensitivity and precision of this new method.
Quantitative RT-PCR can be used to screen segregating populations for genotypes that show quantitative variation in the expression of genes of interest. This can be connected to investigations of the performance of plant phenotypes in interactions with community members. Together, this provides a valuable group of genotypes for addressing questions about the evolutionary ecology of plant-insect interactions (Dicke et al., 2004). Although several genes are likely involved in the regulation of the expression of a trait such as HIPV production (Kant and Baldwin, 2007), studies of transcript levels of regulatory genes that are plant-trait associated enables the correlation of quantitative transcript levels with quantitative variation in the plant's phenotype.
Arabidopsis can be used as a stepping stone for molecular approaches to other crucifer species (Zheng and Dicke, 2008). Mechanistic knowledge gathered by studying Arabidopsis can be used to link to the ecology of ecologically more relevant crucifers, e.g. *Brassica* species. Studies on plant transcriptional responses in the field will further help to understand plant responses to herbivory under ecologically more relevant conditions. An excellent example demonstrating the value of a molecular approach to community ecology in the field is, for instance, work by Broekgaarden and colleagues (in prep.). They investigated whether differences in herbivore community composition between two
B. oleracea cultivars can be correlated to intraspecific transcriptional variation. In chapter 3 we took the first steps to link transcriptomics, metabolomics and insect behavior assays for nine Arabidopsis accessions in a study of plant-insect interactions. This approach can be very helpful when studying the functional expression of genes of interest in plant-insect interactions for other crucifers under field conditions.

The swift development of newly available tools to address the ecological functions of genes provokes ecology by allowing the integration of molecular genetics and community ecology. Conducting multiple research approaches simultaneously, i.e. transcriptomics, metabolomics and phenomics, enables the correlation of molecular genetic plant traits with insect behavior, which will further promote the understanding of the ecology of induced plant-insect interactions.

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Samenvatting

Planten bevinden zich aan de basis van de meeste voedselketens, waar ze continu bedreigd worden door mogelijke aanvallers. Tegen de meeste van deze aanvallers heeft de plant verdedigingsmechanismen ontwikkeld. Er bestaat een breed scala aan verdedigingsmechanismen waarmee de plant zich kan beschermen tegen biologische stressfactoren zoals vraat door plantenetende insecten. Eén van de mogelijke verdedigingsmechanismen van een plant is een altijd aanwezige directe verdedigingsvorm, waardoor de planteneter moeilijker van de plant kan eten. Deze vorm kan bestaan uit eigenschappen als beharing en de aanwezigheid van doorns of een waslaag. Een ander onderdeel van deze permanente directe barrière bestaat uit chemische stoffen die schadelijk zijn voor de planteneter. Sommige plantensoorten hebben een indirect aanwezige verdedigingsvorm door natuurlijke vijanden van planteneters schuilplaatsen aan te bieden. Veel planten beschikken daarnaast ook over een verdedigingsmechanisme dat pas geactiveerd wordt als de plant aangevreten wordt door een insect. Deze geïnduceerde verdediging bestaat ook uit een directe en een indirecte vorm. Door vraat kan de productie van chemische stoffen die schadelijk zijn voor de planteneter omhoog gaan. Van insecten is ook bekend dat ze een indirect verdedigingsmechanisme kunnen induceren dat gericht is op de natuurlijke vijand van de planteneter. Bijvoorbeeld door het aanmaken van suikers die als voedsel kunnen dienen voor de natuurlijke vijanden, of door het maken van geurstoffen die natuurlijke vijanden helpen bij het lokaliseren van de prooi of gastheer. Deze geurstoffen voorzien bijvoorbeeld de natuurlijke vijand van zeer specifieke informatie over de plantensoort, de soort planteneter en soms zelfs over het ontwikkelingsstadium van de planteneter. De door vraat geïnduceerde aanmaak van deze geurstoffen maakt het voor de plant mogelijk om efficiënt om te gaan met de beschikbare energie en de induceerbaarheid verkleint de kans dat de planteneter resistent wordt tegen de verdedigingsmechanismen.
Dit proefschrift richt zich op de rol van herbivoor-geïnduceerde plantengeurstoffen (HIPVs) binnen de indirecte verdediging. Het bestuderen hiervan is een uitdaging die vraagt om een muldisciplinaire aanpak. Tot op heden zijn verschillende onderzoeksmethoden gebruikt om de aanmaak van HIPVs te bestuderen. Gedurende mijn onderzoeksproject heb ik gekeken naar de effecten van het modificeren van de productie van plantengeurstoffen op het gastheerzoekgedrag van een sluipwesp.

Studiesysteem

Mijn onderzoek vond plaats binnen een project waarin de interacties van koolplanten met de daarop voorkomende insecten worden onderzocht. Koolplanten beschikken over directe en indirecte verdedigingsmechanismen tegen planteneters. Om de verdediging van de plant te activeren moet de plant de veranderingen, die veroorzaakt worden door planteneters (signalen), kunnen waarnemen en verwerken. De verwerking van signalen verloopt via verscheidene

signaaltransductie-routes. Om de rol van HIPVs binnen de indirecte verdediging van de plant nauwgezet te bestuderen zijn mutanten met specifieke veranderingen in de signaaltransductie-routes die leiden tot de productie van HIPVs zeer waardevol. Tot op heden zijn dergelijke mutanten voor koolsoorten nog niet beschikbaar. De zandraket behoort tot dezelfde plantenfamilie als kool en blijkt over de verdedigingsmechanismen te beschikken die typerend zijn voor koolsoorten. Ook blijkt uit eerder werk dat de geurprofielen die de zandraket en koolachtigen produceren na vraat door rupsen van bijv. het kleine koolwitje vergelijkbaar zijn. De zandraket wordt binnen de moleculaire genetica veelvuldig gebruikt als modelplant. Hierdoor zijn er veel mutanten beschikbaar die specifieke veranderingen hebben in uiteenlopende planteneigenschappen, waaronder eigenschappen die belangrijk zijn voor de verdediging van de plant. Deze eigenschappen van de zandraket maken haar tot een geschikte plant voor deze studie waarin een moleculair genetische onderzoeksbenadering centraal staat.

Natuurlijk variatie in HIPV productie

Natuurlijke vijanden van planteneters, zoals sluipwespen, worden tijdens het lokaliseren van een plantetende gastheer blootgesteld aan geurstoffen van uiteenlopende bronnen. Plantensoorten die aangevreten worden door verschillende planteneters, produceren elk verschillende mengsels van geurstoffen en veroorzaken hiermee een grote diversiteit aan geurprofielen. Daarnaast produceren planten niet enkel geurstoffen als ze door insecten aangevreten worden, maar ook vanuit de bloemen om bijv. bestuivers aan te trekken. Ook binnen een plantensoort treedt variatie op in de emissie van HIPVs. Dit is reeds onderzocht door HIPVs van verschillende cultivars van cultuurgewassen te bestuderen. Om effectief een gastheer te lokaliseren dient een sluipwesp dus in staat te zijn specifieke geurprofielen, die in dit geval geproduceerd worden door een bepaalde plantensoort die aangevreten wordt door haar gastheer, te onderscheiden van de andere geurbronnen. Eerdere studies hebben aangetoond dat sluipwespen dit onderscheid kunnen maken.

Om te bepalen of er ook natuurlijke variatie bestaat in HIPV productie, heb ik voor negen zandraket populaties met een verschillende geografische herkomst, de HIPV productie na rupsenvraat geanalyseerd (hoofdstuk 3). Dit onderzoek toonde aan dat er binnen de zandraket natuurlijke variatie bestaat in de samenstelling van het HIPV geurprofiel na vraat. Zowel het aantal geurstoffen als de hoeveelheid van de geurstoffen verschilt tussen de verschillende zandraket populaties. De sluipwespen konden de geurprofielen geproduceerd door de zandraketpopulaties onderscheiden.

Signaaltransductie-routes binnen plantenverdediging

Verdedigingsmechanismen tegen planteneters worden voornamelijk gereguleerd door drie signaaltransductie-routes die als eindproduct jasmonzuur (JA), salicylzuur (SA) of ethyleen hebben. Signaaltransductie-routes kunnen door plantenetende insectensoorten verschillend geïnduceerd worden. Insecten die

bladmateriaal eten, bijv. rupsen, of de bladcelinhoud opnemen, bijv. thrips, induceren de jasmonzuurroute. Planteneters die zich voeden met het floëem van de plant, zoals bladluizen en witte vliegen, induceren meer de salicylzuurroute. Maar over het geheel genomen blijkt toch dat de jasmonzuurroute het meest betrokken is bij geïnduceerde verdediging tijdens plant-insect interacties. In mijn proefschrift heb ik me daarom in het bijzonder gericht op het onderzoeken van effecten van deze JA-signaaltransductie-route op de HIPV emissie.

Met drie Arabidopsis mutanten is de rol van de JA-signaaltransductie-route op de HIPV productie bestudeerd. De mutanten zijn geblokkeerd in de JA-signaaltransductie-route, waardoor ze verschillen in de productie van JA of de twee biologisch actieve tussenproducten, nl. de jasmonaten OPDA en dnOPDA. Voor OPDA, dnOPDA en JA is het effect op de inductie van plantengeurstoffen in reactie op vraatschade onderzocht. Van de mutanten en hun bijbehorende wildtype Arabidopsis planten heb ik eerst de geïnduceerde productie van de jasmonaten gekwantificeerd (hoofdstuk 4). Vervolgens zijn de geïnduceerde geurprofielen voor deze planten in reactie op rupsenvraat geanalyseerd. Deze analyse van de geurprofielen toont aan dat enkele mutanten een geurprofiel hebben dat afwijkt van het wildtype. Mutanten die geen OPDA of JA produceren blijken minder geurstoffen te produceren, zoals 'green leaf volatiles', terpeenachtige verbindingen en de gemethyleerde vorm van salicylzuur (MeSA). Planten die geen dnOPDA bezitten, produceren een geurprofiel dat vergelijkbaar is met dat van wildtype planten.

Deze variaties in geïnduceerde geurprofielen van mutanten en wildtypen, heb ik getest op relevantie voor sluipwespen in een biotoets. De gebruikte sluipwespenvrouwtjes zoeken rupsen om hun eieren in te leggen. Bij het lokaliseren van deze gastheer maken ze gebruik van het geurprofiel dat na rupsenvraat door de plant wordt uitgescheiden. In een twee-keuze-experiment heb ik sluipwespenvrouwtjes laten kiezen tussen wildtypen en mutanten, die beiden aangetast werden door rupsen van het kleine koolwitje. Deze experimenten toonden aan dat deze sluipwesp een duidelijke voorkeur heeft voor wildtype planten als het alternatief een mutant is die geen JA kan maken. De afwezigheid van dnOPDA beïnvloedde het gedrag van de sluipwespen niet in deze twee-keuze-experimenten. Hieruit concludeer ik dat het eindproduct van de JA-signaaltransductie-route, JA, betrokken is bij de productie van plantengeurstoffen die het sluipwespenvrouwtje gebruikt om haar gastheer te vinden.

Eén van de geurcomponenten die na vraat niet geïnduceerd werd door mutanten die geen JA kunnen produceren is methyl salicylaat (MeSA). Hierbij is het opmerkelijk dat MeSA een directe afgeleide is van de SA-signaaltransductie-route. Dit doet vermoeden dat er een interactie plaats vindt tussen de twee signaaltransductie-routes, nl. de SA-signaaltransductie-route en de JA-signaaltransductie-route. Dit is vervolgens onderzocht met behulp van dezelfde mutanten uit de JA-signaaltransductie-route, door genactiviteit voor genen uit verschillende signaal-transductie routes te kwantificeren (hoofdstuk 5).

Dit onderzoek toont aan dat de afzonderlijke jasmonaten de signaaltransductie-

routes op verschillende manieren beïnvloeden. Hoewel er geen effect gevonden werd van dnOPDA-afwezigheid op geurstoffenproductie, blijkt er op gen-niveau wel een rol te zijn voor dnOPDA. Het jasmonaat dnOPDA blijkt de expressie van een van de genen die betrokken is bij de aanmaak van 'green leaf volatiles' te reduceren. Dit effect is tegenovergesteld aan het stimulerende effect dat JA en OPDA hebben op dit gen. Verder blijkt dat enkel de aanwezigheid van het jasmonaat JA, en niet van de jasmonaten dnOPDA of OPDA, noodzakelijk is om na rupsenvraat een regulerend gen binnen de SA signaal-transductie route tot expressie te laten komen. Hieruit kan worden afgeleid dat er na rupsenvraat een directe link is van JA-inductie met MeSA productie.

Rol van de geurcomponent MeSA

De emissie van MeSA na rupsenvraat doet vermoeden dat deze component een bruikbare indicator is voor deze sluipwespensoort tijdens het lokaliseren van haar gastheer op een plant. Om dit te onderzoeken is een mutant gebruikt die niet in staat is MeSA te produceren, maar een geurprofiel heeft dat verder gelijk is aan dat van een wildtype plant (hoofdstuk6). Dit resulteerde in het verrassende resultaat dat MeSA niet aantrekkelijk is voor de in mijn onderzoek gebruikte sluipwespensoort. Door ook twee-keuze-experimenten uit te voeren waarbij synthetisch MeSA in toenemende concentratie is toegevoegd aan het geurprofiel van de mutant, heb ik kunnen bevestigen dat MeSA afstotend werkt voor deze sluipwesp.

Conclusie

De snelle ontwikkeling van nieuw beschikbare technieken om de activiteit en het functioneren van genen te bestuderen stelt ecologen in staat om moleculaire genetica te combineren met studies naar de ecologie van insect-plant interacties. Het toepassen van verschillende onderzoeksmethoden stelt ons in staat om planteneigenschappen op het niveau van gen-expressie te koppelen aan insectengedrag. Dit bevordert het verkrijgen van inzicht in de ecologie van plant-insect interacties.

Dankwoord

De laatste pagina's van mijn proefschrift zullen voor veel lezers de eerste zijn die ze lezen. Belangrijke pagina's dus, die ik daarom meteen wil beginnen met het bedanken van de belangrijkste persoon. Eva, jij weet uit eigen ervaring hoe het is om te promoveren, er komt nogal wat bij kijken. Soms moeten er keuzes gemaakt worden waarbij je elkaars steun en begrip nodig hebt. Dit lukt ons goed en ik hoop dat we dat in de toekomst zullen blijven doen. Het laatste half jaar is mede door het door jou voorgedragen 'plan van aanpak' goed verlopen en nu draaien we de rollen om. Natuurlijk is er meer dan promoveren alleen, en dan zijn gedeelde hobbies onmisbaar. Bijvoorbeeld als we in het weekend het 'mogen werken op het NKI' slim koppelen aan het wandelen in een natuurgebied. We hebben beiden een voorliefde voor koken, maar jouw kookkunsten stelen overal de show en daar mag ik, in meer dan een opzicht, erg van genieten. Twee belangrijke eigenschappen van jou, die niet onvermeld mogen blijven zijn je positiviteit en je kracht. Beiden hebben ons erg geholpen na mijn 'akkefietje' op de fiets. Tot slot geef je me steeds de ruimte om mijn overtollige energie kwijt te raken. Kortom, je bent ontzettend belangrijk voor me en ik hoop dat dit nog lang zo blijft.

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Maarten, Renske, Sas, Rolf en Pim D., Dongen city zou Dongen city niet zijn zonder jullie (gelal). Ook mijn studievrienden uit Wageningen wil ik hier niet vergeten. Bram, door al het samen fietsen en schaatsen en je vele telefoontjes zal ik jou niet gauw uit het oog verliezen. Vinz, 'keep da vibes going'. Tiz en Evelien, bedankt voor alle logeerpartijen. Alle anderen, bedankt voor de gezelligheid!

Tot slot gaan we terug naar de roots, de families. Beginnende bij de oorsprong: Oma de Wildt, ondanks de grootte van je familie was je altijd op de hoogte van mijn werk en privé leven. Ik geniet ervan om even wat te eten in Wijchen en lekker bij te kletsen. Ik hoop dat ik dat nog vele jaren mag blijven doen. Dan de schoonfamilie: Oma Aben, de belangstelling die jij toont voor mijn onderzoek heb ik altijd erg bijzonder gevonden. Pierre en Wies, bedankt voor alle steun, belangstelling, (klus)hulp en begrip. Ik voel me in Oisterwijk altijd op mijn gemak en dat beschouw ik als zeer waardevol. Lonneke en Etienne ik heb het met jullie ook getroffen, ik kan altijd met (en om) jullie lachen. Een kleine 25km verderop ligt De Moer, waar mijn ouderlijk huis staat. Dit was en is mijn veilige
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Tjeerd

Curriculum Vitae

Tjeerd Snoeren werd geboren op 18 april 1975 te Tilburg. In 1992 slaagde hij voor de HAVO op het Dr. Schaepmancollege te Dongen, in 1995 haalde hij zijn VWO diploma aan het Dag Avond College te Tilburg. In maart 2000 rondde hij zijn Bachelor van de Tuinbouw opleiding aan de Hogeschool Delft af met als specialisatie onderzoek. Hiervoor doorliep hij een afstudeervak van september 1999 tot februari 2000 onder begeleiding van Dr. F.M. Bakker van MITOX-Trial Management BV (titel: Partneracceptatie van de roofmijt *Typhlodromus pyri* - Effect van gezamenlijk ontwikkelen op verwantenherkenning bij paringsvoorkeur). Vervolgens heeft hij 6 maanden voor MITOX gewerkt in Portugal. In datzelfde jaar begon hij zijn studie Plantenveredeling en Gewasbescherming aan de Wageningen Universiteit (WU). Van september 2001 tot november 2002 doorliep hij een afstudeervak bij de vakgroep Entomologie onder begeleiding van Dr. ir. J.G. de Boer en Prof. dr. M. Dicke (titel: Leren onderscheiden van prooi en niet-prooi geïnduceerde vluchtige plantenstoffen door de roofmijt *Phytoseiulus persimilis*). In februari 2003 haalde hij aan de WU zijn Master met als specialisatie Ecologische Gewasbescherming. De overige maanden van 2003 werkte hij voor MITOX in Zuid-Europa en Nederland. In januari 2004 begon hij aan de vakgroep Entomologie van de WU als assistent in opleiding (AIO). In februari 2009 werd hij aangesteld als Postdoc bij de vakgroep Entomologie van de WU en zal hij verder werken aan insect-plant interacties, waarbij gekeken zal worden naar de mogelijke trade-off tussen geïnduceerde plantenverdediging en bestuiving.

List of publications

Snoeren, T.A.L., De Jong, P.W. & M. Dicke 2007. Ecogenomic approach to the role of herbivore-induced plant volatiles in community ecology. Journal of Ecology, 95: 17-26.

Dicke, M., Bruinsma, M., Bukovinszky, T., Gols, R., De Jong, P.W., Van Loon, J.J.A., **Snoeren, T.A.L.** & S. Zheng 2006. Investigating the ecology of inducible indirect defence by manipulating plant phenotype and genotype. IOBC/wprs Bull. 29: 15-23.

De Boer, J.G., **Snoeren, T.A.L.** & M. Dicke 2005. Predatory mites learn to discriminate between plant volatiles induced by prey and nonprey herbivores. Animal Behaviour, 69: 869-879.

Snoeren, T.A.L., Mumm, R., Poelman, E.H., Yang, Y., Pichersky, E. & M. Dicke. Herbivore-induced plant volatile methyl salicylate negatively affects attraction of the parasitoid *Diadegma semiclausum* (under review)

Zheng, S-J., **Snoeren, T.A.L.**, Hogewoning, S.W., Van Loon, J.J.A. & M. Dicke. Molecular genetic approach to the role of visual cues in butterfly oviposition behavior (under review)

Snoeren, T.A.L., Van Poecke, R.M.P. & M. Dicke. Multidisciplinary approach to unraveling the relative contribution of different oxylipins in indirect defense of *Arabidopsis thaliana* (submitted)

Van Mölken, T., De Caluwe, H., Van Dam, N.M., Hordijk, C.A., Leon-Reyes, A., **Snoeren, T.A.L.**, & J.F. Stuefer. Volatiles emitted by virus-infected plants repel herbivores (to be submitted)

Snoeren, T.A.L., Kappers, I.F., Broekgaarden, C., Mumm, R., Dicke M. & H.J. Bouwmeester. Natural variation in herbivore-induced volatiles in *Arabidopsis thaliana* (to be submitted)

Snoeren, T.A.L., Broekgaarden, C. & M. Dicke. Jasmonates differently affect interconnected signal transduction pathways of induced defenses in *Arabidopsis thaliana* (to be submitted)

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: **T.A.L. (Tjeerd) Snoeren**
Date: **11 May 2009**
Group: **Laboratory of Entomology, Wageningen University**

	1) Start-up phase	*date*
►	**First presentation of your project**	
	Genotypic manipulation of herbivore-induced plant volatiles to study chemical ecology and community ecology	Jun 15, 2004
►	**Writing or rewriting a project proposal**	
	Writing a review or book chapter	
►	Ecogenomic approach to the role of herbivore-induced plant volatiles in community ecology	2008
	MSc courses	
►	**Laboratory use of isotopes**	
	Subtotal Start-up Phase	*7.5 credits**

	2) Scientific Exposure	*date*
►	**EPS PhD student days**	
	PhD-Days MPI-Jena	Mar 20-21, 2006
	EPS PhD student day 2003, Utrecht University	Mar 27, 2003
	EPS PhD student day 2004, Vrije Universiteit Amsterdam	Jun 03, 2004
	EPS PhD student day 2005, Radboud University Nijmegen	Jun 02, 2005
►	**EPS theme symposia**	
	Theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Sep 17, 2004
	Theme 2 symposium 'Interactions between Plants and Biotic Agents', Leiden University	Jun 23, 2005
	NWO Lunteren days and other National Platforms	
	ALW "Exp. Plant Sciences', Lunteren	Apr 04, 2005
►	NERN Annual Meeting, Lunteren	Feb 12-13, 2008
	Annual Entomology Day, Groningen	Dec 19, 2003
	Seminars (series), workshops and symposia	
	Signal transduction in bio-interactions	Dec 12, 2003
	Ecological and evolutionary genomics	Apr 29, 2005
►	Entomology seminar series	2004-2008
	PRI seminar series	2004-2007
	Plant-insect interactions: from molecular biology to ecology	Oct 26, 2007
	Seminar plus	
	International symposia and congresses	
	IOBC-Delemont:workshop on methods in researd on induced resistance against diseases and insects (Neuchatel)	Nov 02-04, 2004
	Regulatory Oxylipins (Lausanne)	Sep 15-16, 2005
	IOBC-Heraklion:Breeding for inducible resistance against pests and diseases	Apr 27-29, 2006
►	SIP 2007, Uppsala, Sweden	Jul 30-Aug 02, 2007
	Presentations	
►	poster IOBC-Delemont	Nov 02-04, 2004
	poster Regulatory Oxylipins	Sep 15-16, 2005
	oral IOBC-Heraklion	Apr 27-29, 2006
	oral SIP Uppsala	Jul 30-Aug 02, 2007
	oral Plant-insect interactions: from molecular biology to ecology	Oct 26, 2007
►	1.5 h lecture: SENSE: Principles of Ecological Genomics	Feb 26 , 2009
	IAB interview	Sep 14, 2007
	Excursions	
	PhD excursion UK	Mar 06-08, 2007
	Subtotal Scientific Exposure	*18.1 credits**

	3) In-Depth Studies	*date*
►	**EPS courses or other PhD courses**	
	Spring School Chemical Communication: from gene to ecosystem	Mar 19-23, 2005
	Summer school Environmental Signalling: Arabidopsis as a model	Aug 22-24, 2005
	Journal club	
	Member of literature discussion groups: Entomology, IPI, and Crucifer	2004-2008
►	**Individual research training**	
	Subtotal In-Depth Studies	*5.4 credits**

	4) Personal development	*date*
►	**Skill training courses**	
	English Scientific Writing	Sep 2007
	NWO talentsday	Sep 2007
	WIAS Course: Introduction to R for statistical analysis	Apr 21-22, 2008
►	**Organisation of PhD students day, course or conference**	
	Organisation of Workshop 'Plant-Interactions from molecular biology to ecology (2006)'	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*4.2 credits**

TOTAL NUMBER OF CREDIT POINTS*	**35.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

SOM Table 1. Characteristics of the identified induced volatile compounds in headspace of *Arabidopsis thaliana* leaves after *Pieris rapae* herbivory or jasmonic acid treatment.

Compound	Retention index	*m/z* fragment used for quantification	Compound	Retention index	*m/z* fragment used for quantification
pentan-2-ol	732	45	acetophenone	1068	105
2-heptene	735	55	hexanoic acid	1085	60
(*E*)-3-penten-2-one	735	69	terpinolene	1093	93
2-pentenal	758	55	linalool	1099	93
pentan-1-ol	767	42	nonanal	1109	57
(*E*)-3-hexenal	770	41	DMNT[1]	1114	69
(*Z*)-2-penten-1-ol	771	57	allo-ocimene	1129	69
(*E*)-3-penten-2-ol	774	71	ß-sesquiphellandrene	1149	69
(*Z*)-3-hexenal	796	41	α-terpineol	1186	59
hexan-2-ol	801	45	methyl salicylate	1193	92+120+152
(*E*)-3-hexen-1-ol	851	41	verbenone	1204	107
(*E*)-2-hexenal	853	41	decanal	1207	57
(*Z*)-3-hexen-1-ol	853	67	nerol	1225	69
hexan-1-ol	868	56	ß-citral	1239	69
hexanal	878	44	pulegone	1240	81
1-nonene	892	56	ethyl salicylate	1248	120
4-methyl-3-penten-2-one	892	83	geraniol	1255	69
heptanal	900	70	indole	1292	117
heptan-2-ol	908	45	α-copaene	1378	161
(*E,E*)-2,4-hexadienal	910	81	ß-elemene	1379	81
α-pinene	939	93	(*E*)-ß-caryophyllene	1420	93
1-octen-3-ol	943	57	thujopsene	1431	119
benzaldehyde	965	77	geranyl acetone	1454	69
trans pinane	974	55	α-himachalene	1454	93
1-octen-1-ol	977	57	α-humulene	1454	107
6-methyl-5-hepten-2-one	989	69	ß-acoradiene	1463	119
ß-myrcene	995	93	(*E*)-ß-farnesene	1465	69
octanal	1000	43	ß-chamigrene	1484	189
(*E*)-3-hexenyl acetate	1006	43	ß-ionone	1486	121
α-phellandrene	1007	93	cuparene	1506	132
(*Z*)-3-hexenyl acetate	1010	43	(*E,E*)-α-farnesene	1509	93
3-carene	1010	93	ß-bisabolene	1510	69
α-terpinene	1017	93	(*Z*)-nerolidol	1535	69
limonene	1033	93	geranyl-isovalerate	1553	85
(*Z*)-ß-ocimene	1040	93	(*E*)-nerolidol	1564	69
(*E*)-ß-ocimene	1052	93	TMTT[2]	1590	69
γ-terpinene	1062	93			

[1], (*E*)-4,8-dimethyl-1,3,7-nonatriene
[2], (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

SOM Table 2. Significant differences in headspace among accessions after either treatment (jasmonic acid or *Pieris rapae*) or left non-treated. One-way ANOVA followed by Dunnett T3 results for 9 *Arabidopsis thaliana* accessions for each volatile compound.

jasmonic acid					
Geno I	**>Geno J**	**Compound**	**Mean[1] Difference (I-J)**	**Std. Error**	**Sign.**
An-1	> Col-0	(*E*)-ß-ocimene	0.957	0.192	0.031
An-1	> Cvi	ß-sesquiphellandrene	1.068	0.224	0.031
An-1	> Cvi	TMTT[1]	0.828	0.148	0.027
An-1	> Eri-1	ß-sesquiphellandrene	0.892	0.192	0.043
An-1	> Kyo-1	(*Z*)-ß-ocimene	1.052	0.143	0.012
An-1	> Kyo-1	(*E*)-ß-ocimene	1.392	0.205	0.004
Col-0	> Cvi	methyl salicylate	1.505	0.573	<0.001
Col-0	> Eri-1	methyl salicylate	1.253	0.180	0.020
C24	> Col-0	(*Z*)-ß-ocimene	0.980	0.229	0.049
C24	> Col-0	DMNT[2]	1.672	0.257	0.004
C24	> Kyo-1	(*Z*)-ß-ocimene	1.082	0.145	0.011
C24	> Kyo-1	(*E*)-ß-ocimene	1.098	0.227	0.037
C24	> WS	ß-bisabolene	5.974	1.154	0.039
Cvi	> An-1	ß-myrcene	0.638	0.126	0.025
Cvi	> An-1	ethyl salicylate	5.564	0.343	0.001
Cvi	> Col-0	ß-myrcene	0.885	0.168	0.014
Cvi	> Col-0	(Z)-ß-ocimene	1.176	0.231	0.017
Cvi	> Eri-1	ethyl salicylate	5.564	0.343	0.001
Cvi	> Kyo-1	(*Z*)-ß-ocimene	1.278	0.148	0.006
Cvi	> Kyo-1	(*E*)-ß-ocimene	1.108	0.199	0.013
Cvi	> Ler	ß-myrcene	0.734	0.147	0.024
Eri-1	> Col-0	ß-myrcene	0.969	0.198	0.022
Eri-1	> C24	α-farnesene	1.478	0.215	0.003
Eri-1	> Kyo-1	(*E*)-ß-ocimene	0.816	0.154	0.023
Eri-1	> Ler	ß-myrcene	0.818	0.180	0.047
Eri-1	> WS	α-farnesene	1.205	0.209	0.007
Eri-1	> WS	ß-bisabolene	7.034	1.140	0.019
Kond	> An-1	α-pinene	0.686	0.115	0.021
Kond	> An-1	ß-myrcene	1.110	0.091	<0.001
Kond	> Col-0	ß-myrcene	1.357	0.144	0.001
Kond	> Col-0	(*Z*)-ß-ocimene	1.682	0.197	0.002
Kond	> Col-0	(*E*)-ß-ocimene	1.531	0.115	<0.001

jasmonic acid

Geno I	>Geno J	Compound	Mean[1] Difference (I-J)	Std. Error	Sign.
Kond	> Col-0	DMNT[2]	0.950	0.189	0.023
Kond	> C24	ß-myrcene	1.250	0.149	0.007
Kond	> C24	α-farnesene	1.266	0.244	0.019
Kond	> Cvi	(*E*)-ß-ocimene	0.858	0.160	0.049
Kond	> Cvi	ß-sesquiphellandrene	1.208	0.240	0.023
Kond	> Cvi	methyl salicylate	1.074	0.099	0.002
Kond	> Cvi	TMTT[1]	1.386	0.179	0.008
Kond	> Eri-1	(*Z*)-ß-ocimene	0.954	0.165	0.024
Kond	> Eri-1	(*E*)-ß-ocimene	1.150	0.099	<0.001
Kond	> Eri-1	ß-sesquiphellandrene	1.032	0.210	0.037
Kond	> Kyo-1	(*Z*)-ß-ocimene	1.784	0.085	<0.001
Kond	> Kyo-1	(*E*)-ß-ocimene	1.966	0.136	<0.001
Kond	> Ler	ß-myrcene	1.206	0.118	0.002
Kond	> Ler	(*Z*)-ß-ocimene	0.962	0.175	0.033
Kond	> WS	ß-myrcene	0.857	0.143	0.017
Kyo-1	> Cvi	methyl salicylate	1.078	0.139	0.012
Kyo-1	> Cvi	TMTT[1]	1.108	0.099	<0.001
Ler	> An-1	α-pinene	0.732	0.134	0.039
Ler	> An-1	ethyl salicylate	6.228	0.153	<0.001
Ler	> Cvi	methyl salicylate	1.900	0.120	<0.001
Ler	> Cvi	TMTT[1]	1.592	0.201	0.009
Ler	> Eri-1	methyl salicylate	1.648	0.208	0.002
Ler	> Eri-1	ethyl salicylate	6.228	0.153	<0.001
Ler	> Kond	methyl salicylate	0.826	0.147	0.013
Ler	> Kyo-1	methyl salicylate	0.822	0.176	0.037
WS	>Col-0	(*Z*)-ß-ocimene	1.257	0.242	0.011
WS	>Col-0	(*E*)-ß-ocimene	1.298	0.154	<0.001
WS	>Col-0	DMNT[2]	1.412	0.196	0.001
WS	> Cvi	methyl salicylate	1.585	0.136	0.001
WS	> Eri-1	(*E*)-ß-ocimene	0.918	0.142	0.003
WS	> Eri-1	methyl salicylate	1.333	0.218	0.007
WS	> Eri-1	geranyl acetone	6.608	1.108	0.039
WS	> Kyo-1	(*Z*)-ß-ocimene	1.359	0.164	0.004
WS	> Kyo-1	(*E*)-ß-ocimene	1.734	0.170	<0.001

Pieris rapae					
Geno I	**>Geno J**	**Compound**	**Mean[1] Difference (I-J)**	**Std. Error**	**Sign.**
An-1	> Col-0	(*Z*)-3-hexen-1-ol	1.945	0.120	<0.001
An-1	> Col-0	(Z)-ß-ocimene	0.789	0.183	0.045
An-1	> Col-0	ß-sesquiphellandrene	1.149	0.256	0.042
An-1	> Col-0	α-farnesene	1.116	0.243	0.038
An-1	> C24	benzaldehyde	1.448	0.226	0.006
An-1	> C24	ß-sesquiphellandrene	1.262	0.285	0.047
An-1	> C24	α-farnesene	1.314	0.297	0.049
An-1	> Cvi	(*Z*)-3-hexen-1-ol	0.900	0.174	0.024
An-1	> Cvi	methyl salicylate	1.704	0.128	<0.001
An-1	> Cvi	α-farnesene	1.724	0.225	0.003
An-1	> Eri-1	ß-sesquiphellandrene	1.280	0.240	0.022
An-1	> Eri-1	methyl salicylate	0.524	0.103	0.026
An-1	> Kond	methyl salicylate	1.152	0.149	0.002
An-1	> Kond	ß-bisabolene	7.448	0.226	<0.001
An-1	> Kyo-1	methyl salicylate	0.808	0.140	0.012
An-1	> Ler	(*E*)-2-hexenal	7.080	1.226	0.045
Col-0	> An-1	decanal	1.352	0.256	0.027
Col-0	> Cvi	methyl salicylate	1.602	0.106	<0.001
Col-0	> Cvi	decanal	1.145	0.285	0.019
Col-0	> Eri-1	methyl salicylate	0.422	0.074	0.013
Col-0	> C24	benzaldehyde	1.155	0.192	0.024
Col-0	> C24	(E)-ß-farnesene	1.043	0.067	<0.001
Col-0	> C24	TMTT[1]	1.454	0.252	0.008
Col-0	> Cvi	TMTT[1]	1.558	0.240	0.005
Col-0	> Kond	methyl salicylate	1.050	0.130	0.008
Col-0	> Kyo-1	methyl salicylate	0.706	0.120	0.030
Col-0	> WS	(*E*)-nerolidol	6.193	1.194	0.047
C24	> Cvi	methyl salicylate	1.182	0.135	0.001
C24	> Kond	ß-bisabolene	7.166	0.312	<0.001
C24	> Kyo-1	α-humulene	1.294	0.170	0.002
C24	> Ler	(*E*)-2-hexenal	7.148	1.202	0.047
Cvi	> An-1	ß-myrcene	0.836	0.160	0.019
Cvi	> An-1	(Z)-3-hexenyl acetate	1.442	0.126	<0.001
Cvi	> Col-0	(*Z*)-3-hexen-1-ol	1.045	0.155	0.012
Cvi	> C24	(*Z*)-3-hexenyl acetate	1.620	0.163	0.001
Cvi	> C24	indole	6.170	0.312	0.001

Pieris rapae

Geno I	>Geno J	Compound	Mean[1] Difference (I-J)	Std. Error	Sign.
Cvi	> C24	(E)-ß-farnesene	1.104	0.144	0.018
Cvi	> C24	(E)-ß-farnesene	1.104	0.144	0.018
Cvi	> Kyo-1	ß-acoradiene	0.564	0.119	0.032
Eri-1	> An-1	decanal	1.066	0.218	0.050
Eri-1	> An-1	ß-myrcene	0.622	0.138	0.044
Eri-1	> Col-0	3-hexen-1-ol	1.899	0.180	0.002
Eri-1	> C24	methyl salicylate	1.180	0.115	0.001
Eri-1	> Cvi	decanal	1.164	0.251	0.040
Eri-1	> Cvi	α-farnesene	1.286	0.183	0.003
Kond	> An-1	ß-myrcene	0.846	0.156	0.015
Kond	> Col-0	3-hexen-1-ol	2.313	0.156	<0.001
Kond	> Col-0	(*Z*)-ß-ocimene	1.005	0.196	0.016
Kond	> Col-0	(*E*)-ß-ocimene	1.202	0.183	0.007
Kond	> Col-0	geranyl acetone	6.275	1.150	0.041
Kond	> C24	3-hexen-1-ol	2.334	0.426	0.041
Kond	> C24	benzaldehyde	1.158	0.213	0.020
Kond	> C24	(*E*)-ß-ocimene	1.274	0.108	<0.001
Kond	> C24	(E)-ß-farnesene	1.096	0.084	0.002
Kond	> C24	TMTT[1]	1.188	0.231	0.022
Kond	> Cvi	3-hexen-1-ol	1.268	0.201	0.005
Kond	> Cvi	TMTT[1]	1.292	0.218	0.012
Kond	> WS	(*E*)-nerolidol	6.190	1.187	0.048
Kyo-1	> Col-0	α-farnesene	1.106	0.205	0.011
Kyo-1	> C24	(E)-ß-farnesene	1.048	0.687	0.001
Kyo-1	> C24	α-farnesene	1.304	0.266	0.040
Kyo-1	> Cvi	methyl salicylate	0.896	0.149	0.008
Kyo-1	> Cvi	α-farnesene	1.714	0.183	<0.001
Kyo-1	> Kond	ß-bisabolene	5.790	0.343	0.001
Kyo-1	> Ler	α-farnesene	1.096	0.233	0.040
Ler	> An-1	ß-myrcene	0.834	0.151	0.013
Ler	> Col-0	(*Z*)-ß-ocimene	0.955	0.157	0.009
Ler	> C24	benzaldehyde	1.380	0.207	0.008
Ler	> Cvi	(Z)-ß-ocimene	0.524	0.096	0.014
Ler	> Cvi	methyl salicylate	1.400	0.128	<0.001
Ler	> Kond	methyl salicylate	0.848	0.149	0.015

Pieris rapae					
Geno I	**>Geno J**	**Compound**	**Mean[1] Difference (I-J)**	**Std. Error**	**Sign.**
WS	> An-1	decanal	1.492	0.129	<0.001
WS	> Col-0	3-hexen-1-ol	1.962	0.169	<0.001
WS	> C24	(Z)-ß-ocimene	0.870	0.149	0.017
WS	> C24	(*E*)-ß-ocimene	1.008	0.210	0.021
WS	> Cvi	(*Z*)-ß-ocimene	0.439	0.082	0.015
WS	> Cvi	(*E*)-ß-ocimene	1.080	0.149	0.002
WS	> Cvi	(E)-ß-farnesene	0.918	0.132	0.014
WS	> Cvi	TMTT[1]	0.917	0.196	0.030
WS	> Cvi	3-hexen-1-ol	0.917	0.211	0.042
WS	> Cvi	methyl salicylate	1.273	0.163	0.001
WS	> Cvi	decanal	1.590	0.179	0.002
Control					
Geno I	**>Geno J**	**Compound**	**Mean[1] Difference (I-J)**	**Std. Error**	**Sign.**
An-1	> Col-0	3-carene	5.740	0.199	<0.001
An-1	> WS	methyl salicylate	1.031	0.201	0.036
Col-0	> Ler	(E)-ß-farnesene	0.922	0.155	0.007
C24	> Col-0	DMNT[2]	1.030	0.155	0.001
C24	> Col-0	(*E*)-nerolidol	5.887	1.125	0.039
C24	> Kyo-1	pentan-2-ol	5.232	1.133	0.042
C24	> Ler	hexanoic acid	6.540	0.087	<0.001
Cvi	> Col-0	α-phellandrene	5.557	1.042	0.045
Cvi	> Kyo-1	pentan-2-ol	5.518	1.122	0.032
Eri-1	> WS	methyl salicylate	0.789	0.171	0.045
Kond	> An-1	(*Z*)-ß-ocimene	1.073	0.175	0.004
Kond	> Col-0	α-phellandrene	5.479	1.042	0.048
Kond	> Col-0	DMNT[2]	0.913	0.133	0.001
Kond	> Cvi	(*Z*)-ß-ocimene	1.620	0.346	0.050
Kond	> Ler	(E)-ß-farnesene	0.748	0.163	0.029
Kyo-1	> WS	methyl salicylate	0.707	0.131	0.005
WS	> Col-0	(*E*)-3-hexenal	1.757	0.280	0.009
WS	> Col-0	(*Z*) 2-penten-1-ol	6.405	1.199	0.036
WS	> Col-0	DMNT[2]	0.902	0.153	0.005
WS	> C24	(*E*)-3-hexenal	1.598	0.278	0.015

[1], (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
[2], (*E*)-4,8-dimethyl-1,3,7-nonatriene

SOM Table 3. Results of t-tests for each volatile compound on differences between non-treated plants and *P. rapae*-infested plants, and non-treated plants and JA-treated plants.

	P. rapae	F	Sig.2tail.		Jasmonic acid	F	Sig.2tail.
An-1	*(Z)-ß*-ocimene	0,023	2,83E-06	**An-1**	*(Z)*-ß-ocimene	0,358	7,96E-06
An-1	methyl salicylate	5,825	4,80E-04	**An-1**	*(E)*-ß-ocimene	0,009	4,28E-04
An-1	α-farnesene	0,014	0,001	**An-1**	α-farnesene	0,000	0,002
An-1	geraniol	2,570	0,003	**An-1**	TMTT	1,503	0,004
An-1	α-terpineol	4,333	0,005	**An-1**	ß-sesquiphellandrene	2,442	0,012
An-1	DMNT	4,379	0,010	**An-1**	geraniol	0,379	0,023
An-1	ß-sesquiphellandrene	1,620	0,011	**An-1**	*(Z)*-nerolidol	2,488	0,044
An-1	*(E)*-ß-ocimene	0,622	0,011	**An-1**	ethyl salicylate	*44,108*	*0,075*
An-1	*(E)*-2-hexenal	0,148	0,015	**An-1**	DMNT	*2,074*	*0,078*
An-1	(*Z*)-3-hexen-1-ol	85,282	0,044				
An-1	octanal	*0,337*	*0,059*				
An-1	*(Z)*-2-penten-1-ol	*44,425*	*0,060*				
An-1	nerol	*4,382*	*0,067*				
An-1	3-carene	*56,757*	*0,072*				
An-1	geranyl-isovalerate	*1,025*	*0,084*				
An-1	ß-elemene	*15,297*	*0,084*				
An-1	allo ocimene	*5,898*	*0,085*				
An-1	α-terpinene	*5,164*	*0,088*				
An-1	ß-bisabolene	*62,390*	*0,089*				
Col-0	methyl salicylate	4,207	2,32E-07	**Col-0**	methyl salicylate	3,887	7,57E-06
Col-0	*(E)*-nerolidol	3,933	2,02E-04	**Col-0**	*(Z)*-2-penten-1-ol	3,806	1,60E-04
Col-0	TMTT	2,574	0,001	**Col-0**	(*E*)-nerolidol	4,810	2,10E-04
Col-0	*(Z)*-ß-ocimene	0,045	0,002	**Col-0**	α-phellandrene	0,000	0,018
Col-0	DMNT	0,471	0,004	**Col-0**	TMTT	1,359	0,045
Col-0	linalool	*1,299*	*0,052*	**Col-0**	α-farnesene	297,446	0,048
Col-0	(Z)-3-hexen-1-ol	*458,342*	*0,069*	**Col-0**	*(Z)*-ß-ocimene	*0,864*	*0,051*
Col-0	decanal	*0,418*	*0,069*	**Col-0**	3-carene	*2.939,156*	*0,076*
Col-0	α-farnesene	*586,641*	*0,072*				
Col-0	*(Z)*-2-penten-1-ol	*2,332*	*0,077*				
Col-0	*(E)*-hexenyl acetate	*25,916*	*0,094*				
C-24	pentan-2-ol	6,816	4,41E-08	**C-24**	*(E)*-ß-ocimene	1,934	8,03E-06
C-24	methyl salicylate	6,603	3,90E-06	**C-24**	*(Z)*-ß-ocimene	0,594	1,49E-04
C-24	(*E*)-ß-ocimene	0,214	8,48E-06	**C-24**	methyl salicylate	0,165	0,002
C-24	hexanoic acid	3,265	5,23E-05	**C-24**	DMNT	0,156	0,014
C-24	(*Z*)-ß-ocimene	3,796	3,48E-04	**C-24**	ethyl salicylate	0,112	0,016
C-24	*(E)*-2-hexenal	0,496	4,28E-04	**C-24**	(*E*)-hexenyl acetate	0,158	0,027
C-24	4-me-3-penten-2-one	3,446	0,002	**C-24**	6-me-5-hepten-2-one	1,724	0,038
C-24	pentan-1-ol	3,614	0,008	**C-24**	(E)-ß-farnesene	*1,500*	*0,061*
C-24	indole	16,298	0,009	**C-24**	α-humulene	*0,296*	*0,098*

	Pieris rapae		F	Sig.2tail.		jasmonic acid		F	Sig.2tail.
C-24	1-octen-1-ol	0,014		0,011					
C-24	α-terpineol	3,710		0,013					
C-24	benzaldehyde	0,103		0,016					
C-24	6-me-5-hepten-2-one	0,082		0,016					
C-24	*(E)*-nerolidol	0,089		0,016					
C-24	α-humulene	0,165		0,024					
C-24	ß-myrcene	0,041		0,027					
C-24	ß-chamigrene	1,980		0,031					
C-24	(*E*)-ß-farnesene	9,643		0,035					
C-24	(*E*)-ß-caryophyllene	0,100		0,040					
C-24	thujopsene	31,742		0,042					
C-24	(*Z*)-3-hexen-1-ol	0,540		0,049					
C-24	(*E*)-hexenyl acetate	*0,205*		*0,064*					
C-24	ß-elemene	*1,867*		*0,076*					
Cvi	*(Z)-3-hexenyl acetate*	1,238		8,15E-07	**Cvi**	(*Z*)-ß-ocimene	1,580		7,33E-05
Cvi	*(Z)*-ß-ocimene	0,957		0,001	**Cvi**	(*E*)-ß-ocimene	0,082		1,17E-04
Cvi	trans pinana	2,702		0,003	**Cvi**	ß-myrcene	2,246		4,22E-04
Cvi	ß-myrcene	0,834		0,004	**Cvi**	linalool	0,365		0,004
Cvi	α-phellandrene	0,097		0,005	**Cvi**	(*Z*)-*3-hexenyl acetate*	1,243		0,005
Cvi	linalool	0,785		0,007	**Cvi**	pentan-1-ol	2,419		0,020
Cvi	pentan-1-ol	0,466		0,014	**Cvi**	(*E*)-nerolidol	133,242		0,029
Cvi	(*E*)-ß-ocimene	1,801		0,016	**Cvi**	α-phellandrene	2,858		0,030
Cvi	ß-acoradiene	2,343		0,028	**Cvi**	acetophenone	1,304		0,035
Cvi	(*E*)-ß-farnesene	0,002		0,037	**Cvi**	(*E*)-3-hexenal	*855,557*		*0,051*
Cvi	geranyl acteone	61,472		0,042	**Cvi**	3-carene	*206,499*		*0,052*
Cvi	(*E*)-ß-caryophyllene	*1,701*		*0,059*	**Cvi**	(*Z*)-2-penten-1-ol	*26,958*		*0,055*
Cvi	benzaldehyde	*3,059*		*0,064*	**Cvi**	γ terpinene	*1,559*		*0,057*
Cvi	α-terpinene	*0,065*		*0,065*	**Cvi**	geranyl acteone	*116,002*		*0,071*
Cvi	methyl salicylate	*2,537*		*0,068*					
Cvi	α-farnesene	*447,986*		*0,069*					
Cvi	hexan-2-ol	*0,202*		*0,074*					
Cvi	pentan-2-ol	*92,570*		*0,085*					
Cvi	(*E*)-2-hexanal	*3,809*		*0,094*					
Cvi	verbenone	*0,148*		*0,096*					
Cvi	*(E)*-3-hexanal	*1.117,670*		*0,096*					
Cvi	(*E*)-nerolidol	*42,628*		*0,100*					
Eri-1	methyl salicylate	2,004		8,59E-05	**Eri-1**	α-farnesene	0,004		4,23E-06
Eri-1	(*Z*)-ß-ocimene	0,895		3,26E-04	**Eri-1**	(*E*)-ß-ocimene	2,622		0,002
Eri-1	(*E*)-ß-ocimene	0,023		0,001	**Eri-1**	(*Z*)-ß-ocimene	0,250		0,003
Eri-1	α-farnesene	0,059		0,001	**Eri-1**	α-humulene	0,336		0,005
Eri-1	DMNT	2,589		0,005	**Eri-1**	DMNT	1,995		0,006

	Pieris rapae		F	Sig.2tail.		jasmonic acid		F	Sig.2tail.
Eri-1	linalool	0,778		0,011	**Eri-1**	TMTT	0,012		0,038
Eri-1	α-humulene	0,088		0,038	**Eri-1**	(E)-ß-farnesene	*0,141*		*0,068*
Eri-1	(Z)-3-hexen-1-ol	*68,315*		*0,050*	**Eri-1**	ethyl salicylate	*60,631*		*0,073*
Eri-1	octanal	*74,681*		*0,071*	**Eri-1**	*(E)*-2-hexenal	*47,663*		*0,081*
Eri-1	heptanal	*0,250*		*0,076*	**Eri-1**	heptanal	*0,018*		*0,087*
					Eri-1	ß-myrcene	*78,719*		*0,093*
Kond	*(E)*-ß-ocimene	1,150		6,73E-06	**Kond**	*(E)*-ß-ocimene	3,553		1,63E-06
Kond	*(Z)*-ß-ocimene	0,022		2,57E-04	**Kond**	*(Z)*-ß-ocimene	2,020		5,03E-06
Kond	*(Z)*-nerolidol	0,220		0,001	**Kond**	*(Z)*-nerolidol	0,100		2,55E-05
Kond	ß-bisabolene	4,718		0,002	**Kond**	ß-myrcene	3,208		4,78E-04
Kond	2-pentanal	0,182		0,007	**Kond**	methyl salicylate	1,399		0,005
Kond	methyl salicylate	0,428		0,007	**Kond**	α-pinene	0,581		0,025
Kond	ß-elemene	0,085		0,017	**Kond**	(E)-ß-farnesene	0,020		0,027
Kond	*(E)*-hexenyl acetate	1,274		0,020	**Kond**	ß-bisabolene	0,011		0,029
Kond	1-nonene	4,542		0,032	**Kond**	*(E)*-3-hexen-1-ol	0,108		0,043
Kond	ß-myrcene	0,966		0,035	**Kond**	ß-sesquiphellandrene	0,020		0,045
Kond	TMTT	*3,853*		*0,065*	**Kond**	α-farnesene	*3,265*		*0,062*
Kond	(Z)-3-hexen-1-ol	*26,140*		*0,065*	**Kond**	TMTT	*3,626*		*0,066*
Kond	A-pinene	*0,004*		*0,073*	**Kond**	α-phellandrene	*0,147*		*0,088*
Kond	*(E)*-3-hexenal	*21,423*		*0,076*					
Kyo-1	methyl salicylate	0,001		5,30E-05	**Kyo-1**	methyl salicylate	0,046		0,003
Kyo-1	α-farnesene	46,460		0,012	**Kyo-1**	*(Z)*-nerolidol	0,420		0,005
Kyo-1	α-himachalene	155,770		0,019	**Kyo-1**	α-farnesene	37,732		0,009
Kyo-1	limonene	39,446		0,020	**Kyo-1**	(*E*,*E*)-2,4-hexadienal	0,902		0,019
Kyo-1	hexanoic acid	76,421		0,041	**Kyo-1**	α-himachalene	116,646		0,031
Kyo-1	trans pinana	422,776		0,048	**Kyo-1**	trans pinana	*1.675,678*		*0,055*
Kyo-1	terpinolene	*0,120*		*0,064*	**Kyo-1**	*(E)*-ß-ocimene	*4,216*		*0,057*
					Kyo-1	ß-myrcene	*8,423*		*0,061*
					Kyo-1	terpinolene	*0,033*		*0,076*
Ler	hexanoic acid	16,710		1,38E-04	**Ler**	methyl salicylate	5,625		0,001
Ler	*(E)*-ß-ocimene	0,800		0,001	**Ler**	(*E*)-ß-ocimene	0,037		0,005
Ler	methyl salicylate	8,118		0,001	**Ler**	ß-sesquiphellandrene	3,207		0,014
Ler	ß-sesquiphellandrene	4,187		0,008	**Ler**	ethyl salicylate	183,936		0,042
Ler	DMNT	9,416		0,015	**Ler**	(*Z*)-ß-ocimene	24,405		0,050
Ler	(E)-ß-farnesene	1,505		0,024	**Ler**	decanal	*1.021,292*		*0,052*
Ler	*(E)*-2-hexenal	0,003		0,026	**Ler**	pulegone	*178,818*		*0,053*
Ler	α-copaene	0,011		0,029	**Ler**	3-carene	*974,396*		*0,076*
Ler	*(Z)*-ß-ocimene	27,146		0,032	**Ler**	geraniol	*352,990*		*0,077*
Ler	acetophenone	178,215		0,047	**Ler**	(*Z*)-3-hexenal	*0,959*		*0,079*
Ler	*(E)-3-hexenal*	*25,167*		*0,057*	**Ler**	allo ocimene	*149,587*		*0,084*
Ler	pulegone	*84,486*		*0,067*	**Ler**	TMTT	*6,776*		*0,089*

	P. rapae	F	Sig.2tail.		jasmonic acid	F	Sig.2tail.
Ler	allo ocimene	*673,440*	*0,074*	***Ler***	(E)-ß-farnesene	*3,541*	*0,090*
Ler	3-carene	*974,396*	*0,076*				
Ler	*(E)*-hexenyl acetate	*22,177*	*0,090*				
WS	methyl salicylate	0,443	2,13E-07	**WS**	methyl salicylate	0,541	5,86E-07
WS	*(Z)*-ß-ocimene	2,285	1,39E-05	**WS**	*(Z)*-ß-ocimene	0,033	5,42E-05
WS	TMTT	2,205	3,09E-05	**WS**	nonanal	0,254	0,009
WS	nonanal	0,520	0,002	**WS**	DMNT	0,011	0,009
WS	*(E)*-hexenyl acetate	3,563	0,003	**WS**	linalool	1,124	0,014
WS	(E)-ß-farnesene	0,148	0,015	**WS**	*(E)*-hexenyl acetate	6,100	0,019
WS	2-pentanal	35,049	0,018	**WS**	(E)-ß-farnesene	0,307	0,025
WS	*(E)*-ß-ocimene	4,715	0,029	**WS**	geranyl acteone	293,039	0,038
WS	ß-myrcene	*33,865*	*0,056*	**WS**	*(E)*-ß-ocimene	5,071	0,045
WS	(*Z*)-3-hexen-1-ol	*4,346*	*0,079*	**WS**	ß-myrcene	30,728	0,050
WS	cuparene	*1,679*	*0,097*	**WS**	ß-sesquiphellandrene	*4,477*	*0,054*
				WS	α-farnesene	*408,025*	*0,060*
				WS	pulegone	*4.725,005*	*0,061*
				WS	hexan-2-ol	*0,491*	*0,063*
				WS	hexanal	*4,296*	*0,063*
				WS	*(E)*-2-hexanal	*2.309,289*	*0,065*
				WS	TMTT	*41,050*	*0,076*
				WS	ethyl salicylate	*4.771,445*	*0,083*

[1], (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
[2], (*E*)-4,8-dimethyl-1,3,7-nonatriene

SOM Table 4. Results of one-way ANOVA for each compound on differences between JA and *Pieris rapae* treatment. For each accession the volatile compounds that are emitted in significantly different (ANOVA) rates between both treatments ((*Pieris rapae* infestation (Pr) and JA-treatment (JA)). F- and *P*-values (Sig.) are given.

	Treatment with higher average emission rate:		F	Sig.
An-1				
(*E*)-2-hexenal	Pr		9.170	0.016
trans pinana	Pr		9.305	0.016
ß-myrcene		JA	11.714	0.009
α-terpineol	Pr		9.712	0.014
ß-acoradiene	Pr		11.409	0.010
(*E*)-nerolidol		JA	9.207	0.016
TMTT[1]		JA	5.632	0.045
Col-0				
DMNT[2]	Pr		10.059	0.010
methyl salicylate	Pr		102.577	0.000
C-24				
pentan-2-ol		JA	15.933	0.004
(*E*)-2-hexenal	Pr		52.706	0.000
4-Me-3-penten-2-one		JA	17.129	0.003
benzaldehyde		JA	8.315	0.020
(*E*)-ß-ocimene		JA	7.592	0.025
ß-sesquiphellandrene		JA	6.925	0.030
α-terpineol		JA	14.910	0.005
methyl salicylate	Pr		10.119	0.013
nerol		JA	50.385	0.000
ethyl salicylate	Pr		12.938	0.007
indole		JA	448.516	0.000
ß-elemene		JA	6.791	0.031
(E)-ß-farnesene		JA	57.984	0.000
(*E*)-nerolidol		JA	10.805	0.011
Cvi				
(*E*)-3-hexenal	Pr		14.663	0.005
(*Z*)-2-penten-1-ol		JA	6.614	0.033
(*Z*)-3-hexenal	Pr		7.115	0.028
(*E*)-2-hexenal	Pr		7.892	0.023
trans-pinana	Pr		5.357	0.049
α-phellandrene	Pr		21.808	0.002
(Z)-3-hexenyl acetate	Pr		10.514	0.012
3-carene		JA	13.407	0.006
limonene	Pr		28.407	0.001

	Treatment with higher average emission rate:		F	Sig.
Cvi				
limonene	Pr		28.407	0.001
(*Z*)-ß-ocimene		JA	8.471	0.020
acetophenone		JA	9.238	0.016
methyl salicylate	Pr		24.951	0.001
(E)-ß-farnesene	Pr		6.445	0.035
(*E*)-nerolidol		JA	7.072	0.029
Eri-1				
2-pentanal	Pr		18.740	0.003
(E)-2-hexenal			14.719	0.005
(*Z*)-3-hexen-1-ol	Pr		5.484	0.047
1-octen-1-ol	Pr		7.716	0.024
ß-myrcene		JA	10.475	0.012
linalool	Pr		8.715	0.018
methyl salicylate	Pr		61.541	0.000
nerol	Pr		6.343	0.036
α-farnesene		JA	30.832	0.001
ß-bisabolene		JA	7.450	0.026
Kond				
(*Z*)-3-hexen-1-ol	Pr		17.445	0.003
hexan-1-ol	Pr		5.420	0.048
ß-myrcene		JA	32.530	0.000
(*Z*)-ß-ocimene		JA	6.367	0.036
verbenone		JA	6.500	0.034
decanal		JA	17.427	0.003
(*Z*)-nerolidol		JA	10.927	0.011
Kyo-1				
(Z)-nerolidol		JA	15.012	0.005
Ler				
ß-myrcene	Pr		9.877	0.014
(*E*)-hexenyl acetate	Pr		19.068	0.002
(*Z*)-ß-ocimene	Pr		8.886	0.018
hexanoic acid	Pr		5.999	0.040
ethyl salicylate		JA	6.290	0.036
WS				
(*E,E*)-2,4-hexadienal		JA	12.120	0.006
(Z)-3-hexenyl acetate		JA	5.309	0.044
linalool		JA	5.361	0.043
geranyl acteone		JA	6.643	0.028
α-farnesene		JA	5.447	0.042

[1], (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
[2], (*E*)-4,8-dimethyl-1,3,7-nonatriene

Supplemental Figure 1.

The AtBSMT1 mutant was obtained from The Torrey Mesa Institute (GARLIC line, 776_B10). To determine the exact lesion (see below) we analyzed the mutant in detail. The insertion caused a deletion of about 200 nucleotides (in bold), and at the same time there are only about 200 nucleotides left from the T-DNA. Therefore, if a Southern blot,is conducted or even a PCR with oligos for the beginning and end of the gene, it is difficult to see the differences. However, we have amplified the gene with the insertion, and sequenced the fragment, so we verified the insertion as shown. Moreover, we noticed that mRNA is still produced from the gene, with a similar size of the WT mRNA. When we cloned this mRNA by RT-PCR and sequenced it, we saw that a new 3' splice site was used (see (▼) in figure showing the position of the new 3′ splice site). This leads to a protein that is much shorter and could not possibly have SAMT activity.
Underlined letters: UTRs
CAPITAL letters: coding regions
Regular letters: introns

At3g11480(AtBSMT1)- MUTANT
gttttttttataaataatgtgctaccttgatgaagttccctctctctataaattgaagtg-
tgtgagatgcatcatccatacacaacatcttccatcttccgataattctcctttagtcttataatcactaataagtacgataattgaa
ATGGATCCAAGATTCATCAACACCATTCCTTCCTTGAGgttcttatctccaattcatgtatcattaatgcaacacat
tcacacacacacacacataaatattctgtatatagagttaatgcttcttaatttgatttagttatttaaatatagatcgatatatatcc
atgcagctgtttatgtaacaatatatgcatgtatgtcgcgatgcagGTATGATGATGATAAGTGTGATGATGAATAT
GCGTTTGTGAAAGCTCTATGTATGAGTGGTGGAGATGGTGCCAACAGTTACTCCGCCAATTCTCG
CCTTCAGgttcttcaatatcttcgcttacttttagggtttaagcagaaacaaattattaatgttcagatttttctctggtgatacaa
acatttttacaatcatttaattatgataaaattgtaaaaactgaacatgaactaactaattgtcaatatcaaatattaaatgaattta
catataagaacataatatttaaatgtctttatatgaattgcaaaaacttcaaatatattatgcatgaagtagaaaaatataaacat
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ctcaaaaagagaagttatgaaagatgcatgatttccatctattttatccaatcaaattcattcatatcatatgttagtttgtttttaag
aagtttacatttgtatttaggaaagtaaaaatatattaaaaatataagtaatttaaaacgaaattataggtttcatttaaaataaa
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attcttaggactagtataaattctctaaattaaaaagggctcattacatcacctataacttataaacctacaagtcattaaagaata
ataaggcatgtacatgcactagtcaaattgtaaactcgaaaatgcataaacactagtaatatataaatgtatataactcaaaatc
atatattgttgttgttatcgtatgaagcatgggtccagtatcacattatcacggatcgacat

T-DNA insertion line Garlic_776_B10: The position of the insertion is indicated by the triangle (▼). Approximately 200 nucleotides of the T-DNA are present, and 328 nucleotides downstream of the insertion site (the nucleotides in bold) are deleted in this GARLIC insertion.

actgtaaa**g▼aaaaaaatcatattttctaaacgaattataaaatccagataataatactatttttttataactaagctt**
cttttttgttgttttttttagAAAAAAGTTTTATCAATGGCCAAACCAGTCTTGGTAAGAAACACA
GAAGAAATGATGATGAACTTAGACTTTCCTACGTACATCAAAGTTGCTGAATTGGGTTGTT
CTTCGGGACAAAACTCTTTTTTGGCTATCTTTGAGATCATCAACACCATTAATGTGTTGTGC
CAACATGTGAACAAAAACTCACCAGAGATCGATTGTTGTCTAAACGATCTCCCGGAAAAT
GATTTCAACACGACCTTTAAGTTCGTACCTTTCTTCAACAAGGAGCTCATGATCACAAACAAATCA
TCATGTTTCGTCTATGGAGCTCCAGGTTCCTTCTATTCCAGGCTCTTCTCTCGCAATAGCCTCCATT
TAATACATTCCTCTTATGCACTCCATTGGCTCTCTAAGgtacttataaataatttcaagatgttgtttatttctatga
aacgttagaggttttgatattgaaacgatggaattgtgtgttcatgattaaagGTTCCCGAGAAACTTGAGAATAATA
AGGGGAATCTGTACATAACAAGTTCAAGTCCTCAAAGTGCATACAAGGCCTACTTGAATCAATTCC
AAAAAGACTTCACCATGTTTCTAAGGTTACGTTCTGAAGAAATTGTCTCTAATGGACGTATGGTTCT
CACCTTCATCGGAAGAAACACTCTTAACGATCCATTGTATAGAGATTGTTGTCACTTTTGGACATTG
CTATCAAACTCTCTCCGTGACCTAGTCTTTGAggtatatcaaatcaacttattcatttcttttgtaaaacttttttaaata
tgggacattcatggtcttcgacatagattaacatttttatatattttactcaaaatataagaccaaacatatatattattgtggaaca
tggtactcattgctatcttcatataaacagGGTCTTGTGAGTGAGTCAAAGCTGGACGCATTCAACATGCCGT
TTTATGATCCGAACGTACAAGAACTCAAAGAAGTGATACAAAAAGAGGGCTCTTTTGAAATCAATG

AATTGGAGTCACATGGATTTGATCTTGGTCACTACTACGAAGAAGATGACTTTGAAGCAGGACGC
AATGAAGCTAATGGCATAAGAGCTGTTAGTGAACCAATGCTCATTGCTCATTTTGGAGAAGAAAT
TATCGATACCTTGTTCGATAAGTATGCATACCATGTGACTCAACATGCCAACTGCAGGAACAAAAC
GACTGTCAGTCTTGTCGTTTCCTTGACTAAGAAGTAAgaagtaatcaacttctgtcatgttgctctatttgtatttta
tttactactgttattt

At3g11480(SAMT1)- WILD-TYPE

gttttttttataaataatgtgctaccttgatgaagttccctctctctataaattgaagtg-
tgtgagatgcatcatccatacacaacatcttccatcttccgataattctcctttagtcttataatcactaataagtacgataattgaa
ATGGATCCAAGATTCATCAACACCATTCCTTCCTTGAGgttcttatctccaattcatgtatcattaatgcaacaca
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ccatgcagctgtttatgtaacaatatatgcatgtatgtcgcgatgcagGTATGATGATGATAAGTGTGATGATGAAT
ATGCGTTTGTGAAAGCTCTATGTATGAGTGGTGGAGATGGTGCCAACAGTTACTCCGCCAATTCT
CGCCTTCAGgttcttcaatatcttcgcttacttttagggtttaagcagaaacaaattattaatgttcagatttttctctggtgata
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TTTCCTACGTACATCAAAGTTGCTGAATTGGGTTGTTCTTCGGGACAAAACTCTTTTTTGGCTATC
TTTGAGATCATCAACACCATTAATGTGTTGTGCCAACATGTGAACAAAAACTCACCAGAGATCGAT
TGTTGTCTAAACGATCTCCCGGAAAATGATTTCAACACGACCTTTAAGTTCGTACCTTTCTTCAAC
AAGGAGCTCATGATCACAAACAAATCATCATGTTTCGTCTATGGAGCTCCAGGTTCCTTCTATTCC
AGGCTCTTCTCTCGCAATAGCCTCCATTTAATACATTCCTCTTATGCACTCCATTGGCTCTCTAAGg
tacttataaataatttcaagatgttgtttatttctatgaaacgttagaggttttgatattgaaacgatggaattgtgtgttcatgatt
aaagGTTCCCGAGAAACTTGAGAATAATAAGGGGAATCTGTACATAACAAGTTCAAGTCCTCAAAG
TGCATACAAGGCCTACTTGAATCAATTCCAAAAAGACTTCACCATGTTTCTAAGGTTACGTTCTGA
AGAAATTGTCTCTAATGGACGTATGGTTCTCACCTTCATCGGAAGAAACACTCTTAACGATCCATT
GTATAGAGATTGTTGTCACTTTTGGACATTGCTATCAAACTCTCTCCGTGACCTAGTCTTTGAGgta
tatcaaatcaacttattcatttcttttgtaaaacttttaaatatgggacattcatggtcttcgacatagattaacatttttatatatttt
actcaaaatataagaccaaacatatatattattgtggaacatggtactcattgctatcttcatataaacagGGTCTTGTGAG
TGAGTCAAAGCTGGACGCATTCAACATGCCGTTTTATGATCCGAACGTACAAGAACTCAAAGAAG
TGATACAAAAAGAGGGCTCTTTTGAAATCAATGAATTGGAGTCACATGGATTTGATCTTGGTCACT
ACTACGAAGAAGATGACTTTGAAGCAGGACGCAATGAAGCTAATGGCATAAGAGCTGTTAGTGAA
CCAATGCTCATTGCTCATTTTGGAGAAGAAATTATCGATACCTTGTTCGATAAGTATGCATACCAT
GTGACTCAACATGCCAACTGCAGGAACAAAACGACTGTCAGTCTTGTCGTTTCCTTGACTAAGAA
GTAAgaagtaatcaacttctgtcatgttgctctatttgtatttatttactactgttattt

At3g11480 cDNA – WILD-TYPE

ATGGATCCAAGATTCATCAACACCATTCCTTCCTTGAGGTATGATGATGATAAGTGTGAT-
GATGAATATGCGTTTGTGAAAGCTCTATGTATGAGTGGTGGAGATGGTGCCAACAGTTACTCCGC
CAATTCTCGCCTTCAGAAAAAAGTTTTATCAATGGCCAAACCAGTCTTGGTAAGAAACACAGAAGA
AATGATGATGAACTTAGACTTTCCTACGTACATCAAAGTTGCTGAATTGGGTTGTTCTTCGGGACA
AAACTCTTTTTTGGCTATCTTTGAGATCATCAACACCATTAATGTGTTGTGCCAACATGTGAACAA
AAACTCACCAGAGATCGATTGTTGTCTAAACGATCTCCCGGAAAATGATTTCAACACGACCTTTAA
GTTCGTACCTTTCTTCAACAAGGAGCTCATGATCACAAACAAATCATCATGTTTCGTCTATGGAGC
TCCAGGTTCCTTCTATTCCAGGCTCTTCTCTCGCAATAGCCTCCATTTAATACATTCCTCTTATGCA
CTCCATTGGCTCTCTAAGGTTCCCGAGAAACTTGAGAATAATAAGGGGAATCTGTACATAACAAG
TTCAAGTCCTCAAAGTGCATACAAGGCCTACTTGAATCAATTCCAAAAAGACTTCACCATGTTTCT
AAGGTTACGTTCTGAAGAAATTGTCTCTAATGGACGTATGGTTCTCACCTTCATCGGAAGAAACAC

TCTTAACGATCCATTGTATAGAGATTGTTGTCACTTTTGGACATTGCTATCAAACTCTCTCCGTGA
CCTAGTCTTTGAGGGTCTTGTGAGTGAGTCAAAGCTGGACGCATTCAACATGCCGTTTTATGATC
CGAACGTACAAGAACTCAAAGAAGTGATACAAAAAGAGGGCTCTTTTGAAATCAATGAATTGGAG
TCACATGGATTTGATCTTGGTCACTACTACGAAGAAGATGACTTTGAAGCAGGACGCAATGAAGC
TAATGGCATAAGAGCTGTTAGTGAACCAATGCTCATTGCTCATTTTGGAGAAGAAATTATCGATAC
CTTGTTCGATAAGTATGCATACCATGTGACTCAACATGCCAACTGCAGGAACAAAACGACTGTCA
GTCTTGTCGTTTCCTTGACTAAGAAGTAA

At3g11480 protein – WILD-TYPE
MDPRFINTIPSLRYDDDKCDDEYAFVKALCMSGGDGANSYSANSRLQKKVLSMAKPV-
LVRNTEEMMMNLDFPTYIKVAELGCSSGQNSFLAIFEIINTINVLCQHVNKNSPEIDCCLNDLPENDF
NTTFKFVPFFNKELMITNKSSCFVYGAPGSFYSRLFSRNSLHLIHSSYALHWLSKVPEKLENNKGNLYI
TSSSPQSAYKAYLNQFQKDFTMFLRLRSEEIVSNGRMVLTFIGRNTLNDPLYRDCCHFWTLLSNSLR
DLVFEGLVSESKLDAFNMPFYDPNVQELKEVIQKEGSFEINELESHGFDLGHYYEEDDFEAGRNEAN
GIRAVSEPMLIAHFGEEIIDTLFDKYAYHVTQHANCRNKTTVSLVVSLTKK

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