Unraveling molecular mechanisms underlying plant defense in response to dual insect attack:

Studying density-dependent effects

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This research was conducted under the auspices of the
Graduate School of Experimental Plant Sciences

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Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr APJ Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 17 June 2016
at 4:00 p.m. in the Aula

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Unraveling molecular mechanisms underlying plant defense in response to dual insect attack: Studying density-dependent effects
266 pages

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summary in English

ISBN 978-94-6257-775-6
DOI 10.18174/377943

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Chapter 1

General introduction

Introduction

Plant-feeding insects activate a range of defense responses in plants (Heidel-Fischer et al., 2014). Not only do these plant responses reduce performance of the feeding herbivore, they also result in the attraction of natural enemies of herbivores (Kessler and Baldwin, 2002; Howe and Jander, 2008; Heidel-Fischer et al., 2014).
The majority of studies investigated the effects of attack by a single herbivore species on induced plant defenses. However, plants exhibit complex molecular defense response mechanisms to cope with attack by multiple herbivore species (Heidel and Baldwin, 2004; Voelckel and Baldwin, 2004; Dicke et al., 2009). A new area of research is aimed at a better understanding of the complexity of regulatory mechanisms underlying defenses to multiple attackers. The aim of this thesis was to investigate plant-mediated interactions between multiple attacking herbivores. Therefore, I combined studies of transcription of defense genes, photochemistry, insect performance and behavior to unravel mechanisms underlying interactions between plants and simultaneous attacking aphids and caterpillars.

Plant defense against herbivores

The induction of plant defenses in response to insect attack depends on the feeding guild of the attacking insect (*e.g.* leaf-chewing caterpillars or phloem-feeding aphids) (De Vos et al., 2005; Bidart-Bouzat and Kliebenstein, 2011). For instance, De Vos et al. (2005) showed that attack by single insect species belonging to different feeding guilds results in the activation of specific sets of defense-related genes in *Arabidopsis thaliana*, and thus to a different regulation of plant defenses.

Direct defense responses

Plant defense responses can directly affect the attacking herbivore, for example, by producing compounds that repel or reduce the performance of the attacker (Schoonhoven et al., 2005). Several phytohormone signaling pathways, including jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) regulate the induction of direct defense responses through synthesis of proteinase inhibitors, volatiles and secondary metabolites (Howe and Jander, 2008; Pieterse et al., 2012; War et al., 2012; Zhu-Salzman and Zeng, 2015). The JA signaling pathway mainly regulates plant defense responses to attack by chewing insects (Reymond et al., 2004; De Vos et al., 2005; Ehlting et al., 2008; Rehrig et al., 2014). Accordingly,

Arabidopsis mutants impaired in JA-regulated defenses are more susceptible to caterpillars (Stotz et al., 2002; Cipollini et al., 2004). The MYC2 branch of the JA signaling pathway positively regulates the expression of wound-inducible JA-responsive marker genes, such as *VSP2* (Dombrecht et al., 2007; Kazan and Manners, 2013). Whereas the ERF branch of the JA pathway is activated by JA/ET-responsive transcription factors, including ERF1 and ORA59 which up-regulate the expression of *PDF1.2* (Lorenzo et al., 2003; Verhage et al., 2011). In the regulation of defense responses against phloem-feeders, both JA as well as SA signaling are involved (Moran and Thompson, 2001; Moran et al., 2002; De Vos et al., 2005; Kusnierczyk et al., 2008). Activation of the SA signaling pathway leads to the expression of *WRKY* transcription factor genes (Wang et al., 2006) and defense-related genes such as *PR-1* (Durrant and Dong, 2004).

Indirect defense responses

Plant defense responses can also be indirect by the release of a blend of volatiles or by providing food (*e.g*. extrafloral nectar) that attract natural enemies of the attacking herbivores (Kessler and Baldwin, 2002; Dudareva et al., 2006; Dicke and Baldwin, 2010). Herbivore-induced plant volatiles (HIPVs) include terpenes, green leaf volatiles (GLVs) and volatile methyl esters of phytohormones (*e.g.* methyl salicylate, MeSA; and methyl jasmonate) (Arimura et al., 2005; Mumm and Dicke, 2010). The induction of plant volatile biosynthesis is regulated by JA, ET and SA signaling pathways (Ozawa et al., 2000; Arimura et al., 2005; Pieterse et al., 2012). The JA signaling pathway regulates the synthesis of volatile terpenes and GLVs (Dicke and Van Poecke, 2002), whereas the volatile MeSA is synthesized in plants from SA (Chen et al., 2003; Liu et al., 2010).

Phytohormonal signaling pathways and their interaction networks

The three major plant defense hormones JA, SA and ET function in a complex regulatory network that is essential in herbivore-induced defense responses (Pieterse et al., 2012). When a plant has to deal with multiple herbivore attack, regulation of induced defense responses involves crosstalk between defense signaling pathways. Crosstalk between defense signaling pathways has been proposed to allow the plant to fine-tune its defense response to the attacker encountered (Pieterse et al., 2012). Crosstalk between SA and JA signaling is mutually antagonistic, through prioritizing SA-dependent defense responses over JA-dependent responses and vice versa (Pieterse et al., 2012; Caarls et al., 2015). Several WRKY transcription factors and in particular WRKY70 have been

implicated in SA-JA crosstalk (Li et al., 2004; Caarls et al., 2015). Jasmonate and ethylene signaling pathways can interact both synergistically and antagonistically in regulating plant defense responses (Zhu and Lee, 2015). The transcription factor ORA59 is an important mediator in the interactions between JA and ET signaling (Pre et al., 2008; Memelink, 2009).
Crosstalk between phytohormonal signaling pathways also allows herbivores to manipulate plant defenses for their own benefit (Pieterse and Dicke, 2007). It has been proposed that phloem feeders suppress JA-dependent defenses through SA-JA crosstalk by activating the SA signaling pathway (Zhu-Salzman et al., 2004; De Vos et al., 2007; Zarate et al., 2007).

Plant defense to multiple herbivory

Plants grow in a complex environment where they are exposed to different insect attackers at the same time. As a consequence, the diverse defense responses of plants to one attacker species may impact the co-occurring species feeding on the same host plant (Rodriguez-Saona et al., 2010; Soler et al., 2012; Zhang et al., 2013). Interactions between multiple insect attackers affect the induction of JA-, SA-, and ET-dependent defense responses and could lead to positive or negative effects on the performance of the attacking herbivores (Rodriguez-Saona et al., 2005; Soler et al., 2012; Ali et al., 2014; Li et al., 2014). Similarly, multiple insect attack could also affect indirect defense responses through a change in the emission of HIPVs such that it can affect the attraction of parasitoids and predators (Zhang et al., 2009; Erb et al., 2010; Schwartzberg et al., 2011; Zhang et al., 2013; Ponzio et al., 2014; Ponzio et al., 2016).
The regulation of plant defense depends on factors such as the number of insect species attacking simultaneously and insect density. Therefore, the density of the attacking insects may also influence the outcome of interactions between plants and multiple attackers (Zhang et al., 2009). For example, interference of phloem-feeding whiteflies with indirect defenses against spider mites in Lima bean was positively correlated with whitefly density (Zhang et al., 2009). Similarly, *B. brassicae* aphids interfere with caterpillar-induced defenses in plants, which depends on the density of the attacking aphid (Ponzio et al., 2016). Thus, herbivore density may modulate interactions between plants and multiple insect attackers, and therefore needs to be considered as a factor in studies concerning multiple insect-plant interactions.
A plant's response to multiple attackers may cascade through the community and thereby affect insect community composition (Kessler and Halitschke, 2007;

Poelman et al., 2008; Poelman et al., 2012). Effects of early-season herbivores on arthropod community development have indeed been shown several times (Van Zandt and Agrawal, 2004; Viswanathan et al., 2007; Poelman et al., 2010). Consequently, studies investigating molecular mechanisms underlying interference by multiple attacking insects with induced plant defenses will benefit studies on the ecological consequences of induced plant responses (Li et al., 2014).

Study system

This thesis uses a study system consisting of the brassicaceous plants, *Arabidopsis thaliana* and *Brassica oleracea,* the specialist insects, *Plutella xylostella* caterpillars and *Brevicoryne brassicae* aphids, their parasitoids (*Diadegma semiclausum* and *Diaeretiella rapae*, respectively) and the generalist caterpillar, *Mamestra brassicae* (Figure 1). Under natural conditions, brassicaceous plants are commonly attacked by *P. xylostella*, *B. brassicae* and *M. brassicae* (Moyes et al., 2000; Newton et al., 2009b; Poelman et al., 2009). Moreover, valuable fundamental knowledge has been gained on their interactions with both *A. thaliana* and *B. oleracea* plants (Van Poecke and Dicke, 2004; Ehlting et al., 2008; Gols et al., 2008; Kusnierczyk et al., 2008; Zhang et al., 2013; Li et al., 2014).

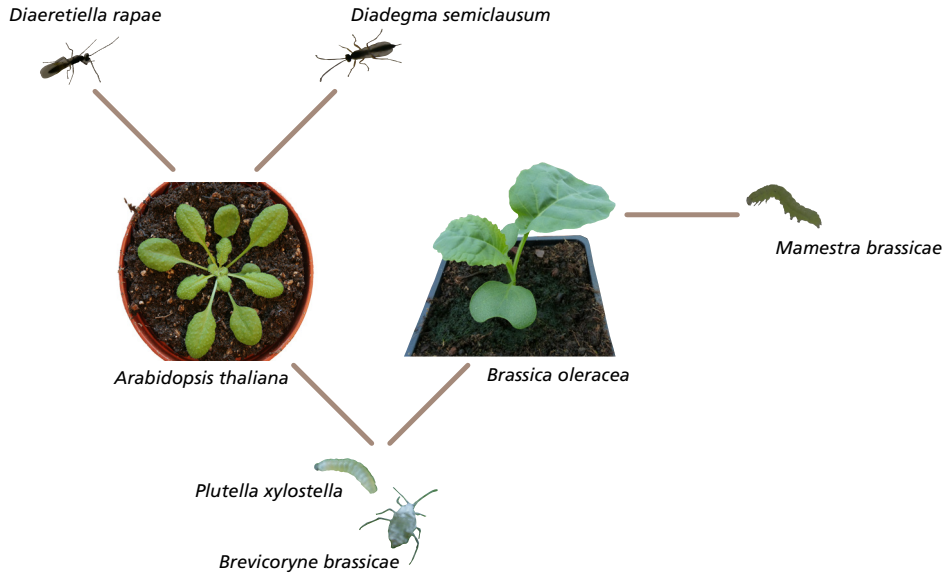

Figure 1. The model system used in this thesis to study effects of plant-mediated interactions between *P. xylostella* caterpillars and *B. brassicae* aphids. Photo of *B. oleracea:* courtesy of Lucille Chrétien.

Plant species

In this thesis both the model plant of molecular genetics, *Arabidopsis thaliana*, and an ecological model plant, wild *Brassica oleracea*, were used. *A. thaliana* is widely used as a model organism for genetic studies in plant biology because of its convenient features such as a small genome, short generation time, small size and high seed production through self-pollination (Koornneef and Meinke, 2010). Research on *A. thaliana* to investigate plant-insect interactions resulted in a detailed model of the mechanisms underlying plant defense (Van Poecke, 2007). Yet, further research is needed to extend beyond responses to single attackers and gain knowledge of the interaction between multiple insects and plants. Wild *B. oleracea* plants occur in natural populations (*e.g.* Kimmeridge, Old Harry and Winspit populations) along the coast of Dorset, UK. The plants from these populations show natural variation in the amount of constitutive and inducible secondary metabolites that act as defense compounds against herbivorous insects (Gols et al., 2008; Newton et al., 2009a).

Plutella xylostella and its parasitoid *Diadegma semiclausum*

Caterpillars of the specialist Diamondback moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) feed on the plants of the family Brassicaceae (Barker et al., 2007). They are one of the most destructive pests that damage Brassicaceae species and cultivars (Sarfraz et al., 2006; Niu et al., 2013).
The parasitoid *Diadegma semiclausum* Hellén (Hymenoptera: Ichneumonidae), a solitary parasitoid of *P. xylostella* larvae, is an important biological control agent of *P. xylostella* (Saucke et al., 2000). It uses HIPVs to locate host-infested plants (Bukovinszky et al., 2005).

Brevicoryne brassicae and its parasitoid *Diaeretiella rapae*

The specialist cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae) only feeds on plants of the Brassicaceae family. The damage caused by *B. brassicae* feeding results in serious losses in yield and marketability of *Brassica* crops (Costello and Altieri, 1995).
The parasitoid *Diaeretiella rapae* (McIntosh) (Hymenoptera: Braconidae) is predominantly specialized on aphids and important for the biological control of cabbage aphids (Nematollahi et al., 2014).

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Mamestra brassicae

The cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) is a generalist herbivore that feeds on many species of plants, of which species from the Brassicaceae family are the most preferred (Ulland et al., 2008). Feeding by the caterpillars causes severe damage to the plants, therefore it is an economically important pest in agriculture (Ulland et al., 2008).Performance of *M. brassicae* caterpillars is negatively affected by high glucosinolate concentrations of their food plants (Poelman et al., 2009).

Outline of thesis

In Chapter 2 the effect of multiple insect attack on plants at different levels of biological organization is reviewed. Feeding by herbivorous insects influences the phenotype of their host plant, which consequently influences the interactions of the plant with its associated community. This chapter focuses on the interactions between plants and multiple insect attackers and their effects on species interactions and community dynamics. Furthermore, links between molecular plant mechanisms underlying inducible plant phenotypes and responses at the community level are discussed.

In Chapter 3, I describe investigations of the effect of *B. brassicae* density on interference with induced defense responses against *P. xylostella* caterpillars in the plant *A. thaliana*. Growth rates of *P. xylostella* when feeding alone or together with aphids at different densities were compared. Furthermore, differences in transcriptional responses to simultaneous attack by caterpillars and aphids at a low or high density were studied.

In Chapter 4 indirect defense responses of *A. thaliana* plants against multiple feeding herbivores at different densities was investigated. Wild-type plants and volatile biosynthesis mutants were dually infested by *P. xylostella* caterpillars and *B. brassicae* aphids at different densities or infested by *P. xylostella* caterpillars alone. The responses of the parasitoid *D. semiclausum* to HIPVs emitted by dually infested plants and by caterpillar-infested plants were assessed. Furthermore, the expression profile of genes important for the biosynthesis of plant volatiles and links between the emitted volatile compounds and the behavioral responses of the parasitoid were investigated.

Chapter 5 focuses on the regulation of direct and indirect plant defense in *A. thaliana* against the aphid *B. brassicae* at different densities, when feeding alone or simultaneously with *P. xylostella* caterpillars. I studied the involvement of JA, SA and ET signaling pathways and their interactions during defense responses against caterpillar and aphid attack. Insect performance and *D. rapae* parasitoid behavior were linked to the expression of JA, SA and ET-responsive genes and JA and SA levels were quantified in *A. thaliana* wild-type plants and mutants deficient in JA, SA or ET biosynthesis/signaling.

Chapter 6 presents the results of a microarray study of *A. thaliana* responses to simultaneous feeding by *P. xylostella* caterpillars and *B. brassicae* aphids compared to plants infested by *P. xylostella* caterpillars alone. I particularly addressed the question whether the transcriptomic response to simultaneously attacking aphids and caterpillars is dependent on the density of aphids and time since the onset of herbivory.

Chapter 7 presents a study of the effect of *B. brassicae* aphids and *P. xylostella* caterpillars feeding alone or simultaneously on insect performance and regulation of *B. oleracea* plant defense responses. As a next step in the study of multiple interacting herbivores on the same host plant, consequences of plant resistance induced by the first two herbivores for subsequently arriving *M. brassicae* caterpillars was studied. This study is the first to evaluate ecological consequences of plant responses to dual herbivory for subsequently arriving herbivores.

Finally, in Chapter 8, I discuss the most important findings of this thesis and position them within a broader framework of studies on interactions between plants and multiple insect herbivores.

Acknowledgments

I would like to thank Marcel Dicke and Joop van Loon for constructive comments on an earlier version of this chapter.

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Chapter 2

Plant interactions with multiple insect herbivores: from community to genes

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Published in slightly modified form in:
Annual Review of Plant Biology 65 (2014) 689-713
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Abstract

Every plant is a member of a complex insect community that consists of tens to hundreds of species that belong to different trophic levels. The dynamics of this community are critically influenced by the plant, which mediates interactions between community members that can occur on the plant simultaneously or at different times. Herbivory results in changes in the plant's morphological or chemical phenotype that affect interactions with subsequently arriving herbivores. Changes in the plant's phenotype are mediated by molecular processes such as phytohormonal signaling networks and transcriptomic rearrangements that are initiated by oral secretions of the herbivore. Processes at different levels of biological complexity occur at timescales ranging from minutes to years. In this review, we address plant-mediated interactions with multiple species of the associated insect community and their effects on community dynamics, and link these to the mechanistic effects that multiple attacks have on plant phenotypes.

Keywords

Phenotypic plasticity, trait-mediated interaction networks, phytohormones, systems biology, species interactions

Plants are members of biodiverse communities consisting of a microbiome (Mendes et al., 2011) and a macrobiome (Dicke and Baldwin, 2010; Whitham et al., 2010). The microbiome consists of, *e.g.*, symbiotic microorganisms such as mycorrhizae, endophytes, and nitrogen-fixing bacteria; plant pathogenic microorganisms; and their antagonists (Hartley and Gange, 2009; Pineda et al., 2010). The macrobiome consists of herbivores and their natural enemies, such as predators and parasitoids, as well as pollinators (Figure 1). For each plant species, the combined macrobiome and microbiome can easily comprise several hundred species that belong to different trophic levels (Harvey et al., 2009) (Figure 1). Moreover, each individual plant is surrounded by a range of other plant individuals of the same or different species, which compete for light and nutrients (Cerrudo et al., 2012) and share members of the microbiome and macrobiome.

Understanding the functioning of this complex of interacting species requires studies of their population dynamics in space and time and the underlying trophic and informational mechanisms. In this review, we focus on plants and their associated insect communities. Insects are the most speciose group of organisms, comprising an estimated 6 million species, of which 50% are herbivorous, and the 300,000 plant species represent the group of organisms with the largest biomass (Schoonhoven et al., 2005). Thus, communities of insects and plants make up a significant proportion of life on Earth.

Feeding by herbivorous insects influences the phenotype of their food plant (Dicke and Baldwin, 2010; Kessler and Baldwin, 2002; Mithöfer and Boland, 2012), which consequently influences the interactions of the plant with its associated community (Ohgushi, 2008; Poelman et al., 2011; Utsumi et al., 2010). Such herbivore-induced effects may last throughout the growing season of the plant or for several years (Haukioja, 1980; Johnson and Agrawal, 2007; Poelman et al., 2008; Thaler et al., 2001).

Research on plant–insect interactions has addressed mainly the effects of interactions between one plant and one insect species. This has yielded important knowledge on how insects find and select their host plants and deal with plant defenses (Schoonhoven et al., 2005) as well as how herbivory modifies plant phenotypes (Dicke and Baldwin, 2010; Kessler and Baldwin, 2002; Mithöfer and Boland, 2012).

However, because plants are members of complex communities, interactions with multiple attackers are the rule rather than an exception (Dicke et al., 2009; Ohgushi, 2005; Utsumi et al., 2010). Moreover, attacks by different organisms interact at different levels of biological organization, ranging from the subcellular

level (Pieterse et al., 2012) to the individual (Kaplan and Denno, 2007) and community levels (Poelman et al., 2009). Studies on the interactions between plants and their associated insect communities have received increasing attention and have addressed effects at the levels of gene expression, phytohormonal crosstalk, metabolomic changes, species interactions, and community dynamics. The current focus in the field of plant–insect interactions is on connecting different levels of biological organization (Baldwin, 2012; Keurentjes et al., 2011), which is already challenging for individual plant–insect interactions and certainly so for multiple attacks on a single plant, and therefore requires a multidisciplinary approach.

In this review, we address the effects of multiple attacks on plants at different levels of biological organization in an integrative way. Although plants are members of plant communities that comprise individuals from different species, we limit this review to individual plants, and particularly interactions with multiple insect species aboveground. We also limit the review to plants in the vegetative stage, because most information is available for this plant stage. We conclude with an outlook on the future of this rapidly developing, multidisciplinary field.

Plants affect insect community composition and dynamics

The composition and dynamics of the insect community that interacts with a plant are influenced by plant traits such as chemistry, physiology, and morphology (Bukovinszky et al., 2008: Harvey et al., 2011; Johnson et al., 2006; Ohgushi, 2005; Whitham et al., 2006), which have a genetic basis. Thus, the genotype of a plant and, consequently, the expressed plant phenotype affect insect community members that interact with the plant and shape the composition of the community (Whitham et al., 2006; Whitham et al., 2012). The insect community together with the plant phenotype gives rise to the community phenotype, and plant individuals with similar traits tend to support similar insect communities (Johnson et al., 2006; Keith et al., 2010; Whitham et al., 2006). A plant's genotype can have size- and density-mediated effects on the associated insect community. For example, plant traits may affect the sizes of herbivores and therefore the sizes of parasitoids (Figure 1D,E) that develop in the herbivores, and even the sizes of hyperparasitoids (Figure 1F,G) that develop in those parasitoids that develop in the herbivores (Bukovinszky et al., 2008). Moreover, plant genotype may affect the density of herbivores, parasitoids, and hyperparasitoids as well as

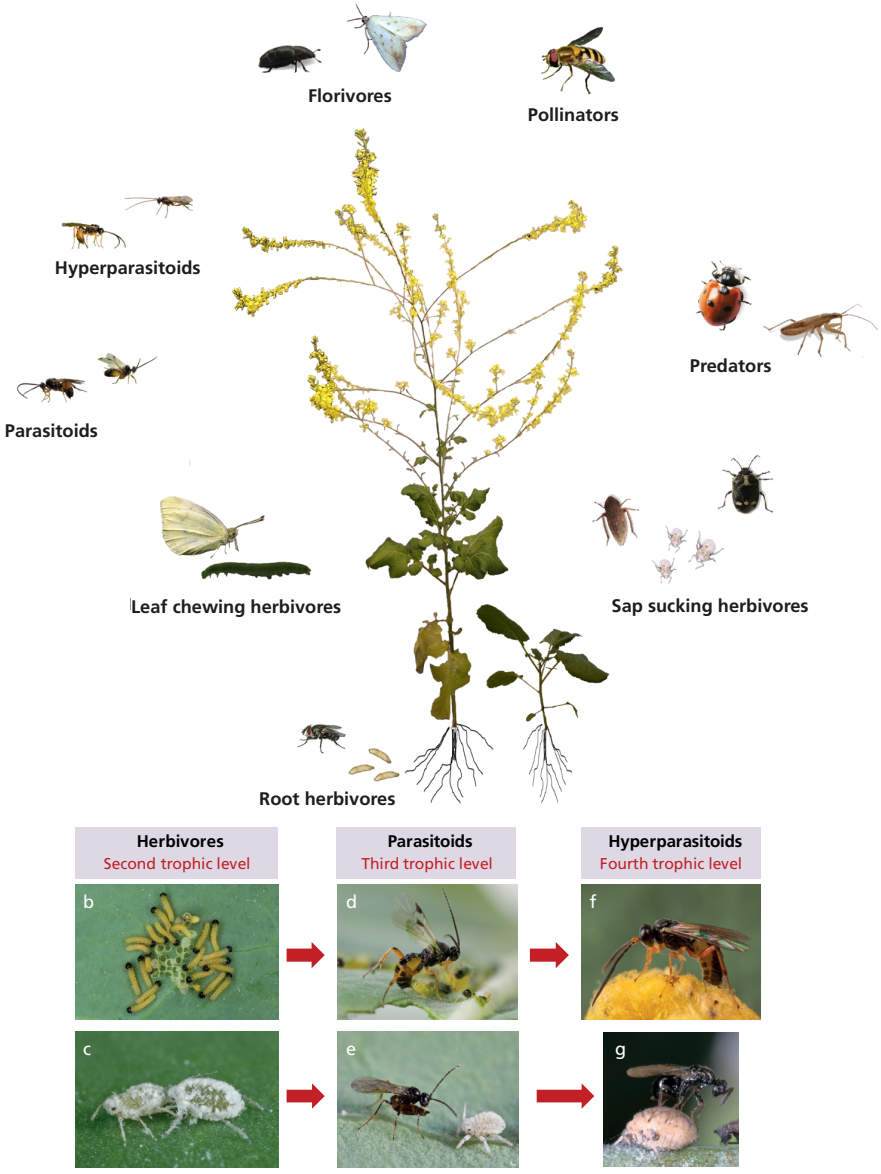

Figure 1. Insect community associated with *Brassica nigra* (black mustard) plants and specific representatives of some members of this community. Community overview (a). Biting/chewing herbivores (*Pieris brassicae* caterpillars) (b). Piercing/sucking herbivores (*Brevicoryne brassicae* aphids) (c). A parasitic wasp (*Cotesia glomerata*) attacking *P. brassicae* caterpillars (d). A parasitic wasp (*Diaeretiella rapae*) attacking a *B. brassicae* aphid (e). A hyperparasitoid (*Lysibia nana*) parasitizing pupae of the parasitoid *Cotesia glomerata* (f). A hyperparasitoid (*Asaphes* sp.) parasitizing a parasitoid that itself has parasitized a *B. brassicae* aphid (g). Photo credits: Tibor Bukovinszky (panels b, c, and g), Hans Smid (panel d), and Nina Fatouros (panels e and f) (http://www.bugsinthepicture.com).

the composition of the herbivore, parasitoid, and hyperparasitoid communities on these plants (Bukovinszky et al., 2008).

Chemical plant traits are well known to be crucial components of the plant phenotype that mediate plant–insect interactions (Schoonhoven et al., 2005). Genotypic variation affects plant chemical traits, which has consequences for species interactions and community dynamics. An example of an extensively studied plant chemical trait that affects insect community composition is condensed tannin concentration, especially in tree species (Schweitzer et al., 2008; Whitham et al., 2006). Tannins are known to negatively influence herbivorous insects (Schoonhoven et al., 2005), and the concentration of tannins in poplar trees indeed affects the composition of insect communities (Whitham et al., 2006). Tannins usually reduce insect growth rate (Schoonhoven et al., 2005), although tannins may also positively affect insect performance or preference; the effects of tannins are likely dependent on species, tissue, and context and influenced by other chemical constituents of plant tissue (Schweitzer et al., 2008). Tannins can also affect community members indirectly through a negative effect on nitrogen mineralization, which subsequently feeds back to root production and consequently to the nutritional value of the tree (Whitham et al., 2006), with long-term effects on herbivorous insects (Schweitzer et al., 2008). Thus, condensed tannin levels affect community phenotypes (Whitham et al., 2006).

In annual or perennial nonwoody plant species, family-specific secondary chemistry can shape the community phenotype. For instance, glucosinolates, which are characteristic secondary metabolites of plants in the Brassicaceae family, have important effects on insect community composition (Hopkins et al., 2009; Newton et al., 2009a; Poelman et al., 2009). The quality and quantity of these compounds are known to deter generalist insect species or hamper their development, whereas they may be used for feeding and or as oviposition stimulants by specialist species (Hopkins et al., 2009). Differences in glucosinolate composition among *Brassica oleracea* cultivars resulted in large differences in herbivore community dynamics (Poelman et al., 2009) that resemble community differences observed in natural populations of *B. oleracea* plants that differ in their chemical profiles (Newton et al., 2009a).

In addition to plant secondary chemistry, many other plant traits can affect insects. These traits include plant biomass and architecture (Andow, 1991; Johnson and Agrawal, 2005; Ohgushi, 2005; Schoonhoven et al., 2005), leaf morphology (Barbour et al., 2009), trichome density (Johnson, 2008), and plant nutritional value in terms of water and nitrogen content (Johnson, 2008; Scriber and Slansky, 1981).

Consequences of plant traits for insect herbivores

To understand how a plant's genotype affects community composition and dynamics, knowledge of the underlying mechanisms is important. Individual plant traits have different effects on different community members.

Among insect herbivores contrasting dietary categories are observed. Generalist species feed on plants belonging to phytochemically unrelated families, whereas specialist species utilize only plant species within a single family or a single genus (Ali and Agrawal, 2012; Schoonhoven et al., 2005). Generalist herbivores are usually more sensitive to plant defense compounds, whereas specialist herbivores may use these same compounds as recognition cues (known as token stimuli) (Gols et al., 2008a; Gols et al., 2008b). Adaptation to plant chemicals specific for certain plant taxa through specialized detoxification or sequestration mechanisms allows specialists to utilize some plants as food and exploit such chemicals for their own defense, whereas generalists are either unable to survive or grow or have a reduced survival or growth rate on such plants (Ali and Agrawal, 2012; Hopkins et al., 2009).

Insect herbivores can also be classified based on feeding guilds - *e.g.*, leaf chewers, phloem feeders, leaf miners, root feeders, and gall-inducing insects - which may differ in their responses to plant traits. Whereas leaf chewers often consume whole leaves and thus are exposed to chemicals in all leaf cells, phloem feeders such as aphids specialize on the phloem. Some secondary compounds that react with each other to form a toxic compound only upon rupture of multiple cells by chewing are thus circumvented by piercing/sucking phloem feeders (Schoonhoven et al., 2005).

Some plant traits are likely to affect all herbivores, whereas others affect only a particular subset, *e.g.*, based on herbivore size. A plant with high leaf toughness will affect many herbivore species, although some species are better able to deal with this than others (Agrawal, 2005). In contrast, a high trichome density particularly affects smaller insects that walk in a forest of leaf hairs (Dussourd, 1995; Schoonhoven et al., 2005), and secondary metabolites particularly affect generalist insects (see above).

Because distinct herbivorous members of a community respond differently to the same plant traits, each trait differentially influences community composition. The many interactions that occur between the various plant traits and the diverse community members, and among herbivore members themselves, potentially increase the complexity of underlying mechanisms that modulate community composition. However, only one or a few so-called foundation species may have

a major effect on the community composition (Whitham et al., 2006). Keith et al. (2010) proposed that a few plant traits particularly affect one or a few foundation herbivore species, which subsequently affect the community. This suggests that effects of plant traits might be passed on not only to single species but also to a whole chain of interacting species.

Consequences of plant traits for insect carnivores

The discussion above considered mainly plant–herbivore interactions, but plant traits also affect organisms at higher trophic levels, such as predators or parasitoids of herbivores as well as carnivorous insects at even higher trophic levels (Bukovinszky et al., 2008; Dicke and Baldwin, 2010; Harvey et al., 2009; Heil, 2008; McCormick et al., 2012; Poelman et al., 2012; Price et al., 1980). Plant traits can directly affect the natural enemies of herbivores, for example, by providing shelter (Romero and Benson, 2005; Schoonhoven et al., 2005) or extrafloral nectar as food (Heil et al., 2010; Schoonhoven et al., 2005). Plant traits can also affect higher trophic levels either directly, through reduced quality of the herbivores (Bukovinszky et al., 2008), or indirectly, through exposure to phytochemicals ingested by the herbivore (Gols and Harvey, 2009). Such indirect interactions with herbivores as a mediator between plant traits and predators or parasitoids can have large effects on the community composition at the second, third, and even higher trophic levels (Bukovinszky et al., 2008; Harvey et al., 2009; Poelman et al., 2012; Smith et al., 2011; Whitham et al., 2006). For example, evening primrose genotype affected aphid population growth rate directly as well as indirectly through effects on the abundance of aphid-tending ants and the diversity of predators (Johnson, 2008). Similar results were found for parasitoids of caterpillars feeding on genetically different willows (Fritz et al., 1997) or cabbage plants (Bukovinszky et al., 2008; Harvey et al., 2011). The adaptation of herbivores to specific plant traits might even affect the evolution of members of higher trophic levels, leading to specialization of parasitoids on herbivores that are adapted to plant traits (Stireman et al., 2006). Plant effects on the composition of the herbivore community can also affect the foraging behavior of carnivores. For example, the foraging success of parasitoids that search for hosts is affected by the presence and identity of additional, nonhost herbivores on the plant (De Rijk et al., 2013). Plant traits may also interfere with the performance of carnivorous insects, thereby providing herbivores with enemy-free space. For instance, in pea plants, a leafless mutation that affects plant architecture hampers the foraging behavior of lady beetles, which results

in enhanced population growth of aphids (Kareiva and Sahakian, 1990).

Top-down effects

The bottom-up effects of plant traits on higher trophic levels (herbivores and their natural enemies) discussed above may be strong predictors of community composition (Kos et al., 2011), but top-down effects of natural enemies on herbivores can have important effects on community composition as well (Hunter and Price, 1992). Predators or parasitoids consume their hosts partly or completely and therefore constrain the population density of herbivores attacking a plant. Parasitoids can exert significant top-down control of herbivore populations (Van Veen et al., 2005), and their activities can influence competition between herbivore species (Van Veen et al., 2006). Interestingly, the elimination of a single parasitoid species from a small community resulted in the extinction of other parasitoid species that were four trophic links away (Sanders et al., 2013). This included effects mediated through herbivores. Thus, top-down effects can be sequentially linked to bottom-up effects (Kareiva and Sahakian, 1990; Sanders et al., 2013).

Insects at the third trophic level do not always have a negative effect on herbivore species: For example, ants may tend aphids and thus protect them from their natural enemies (Johnson, 2008). Although the ants have a positive effect on the aphids, they may also prey on other herbivores that share the plant with the aphids (Vrieling et al., 1991).

In conclusion, plant traits influence members of the associated insect community at different trophic levels, and species at higher trophic levels affect the dynamics of species at lower trophic levels. Many of these plant traits are constitutively expressed. Moreover, community dynamics are also influenced in important ways by the fact that insects modify plant phenotype. The modification of plant phenotype by herbivore attack is the focus of the remainder of this article. We address the effects of phenotypic modification by herbivory on insects at different trophic levels, the molecular mechanisms underlying the phenotypic modification, and how different herbivore species that attack the same plant interfere with one another's effects on the plant's phenotype. Finally, we address the effects of herbivore-induced modification of plant phenotype on community dynamics.

Herbivore-induced change in plant traits

Herbivory or egg deposition by herbivores alters plant phenotype through changes in the production of primary and secondary metabolites, morphological traits, and architecture (Dicke and Baldwin, 2010; Hilker and Meiners, 2010; Howe and Jander, 2008; Kessler and Baldwin, 2002; Mithöfer and Boland, 2012) (Figure 2).

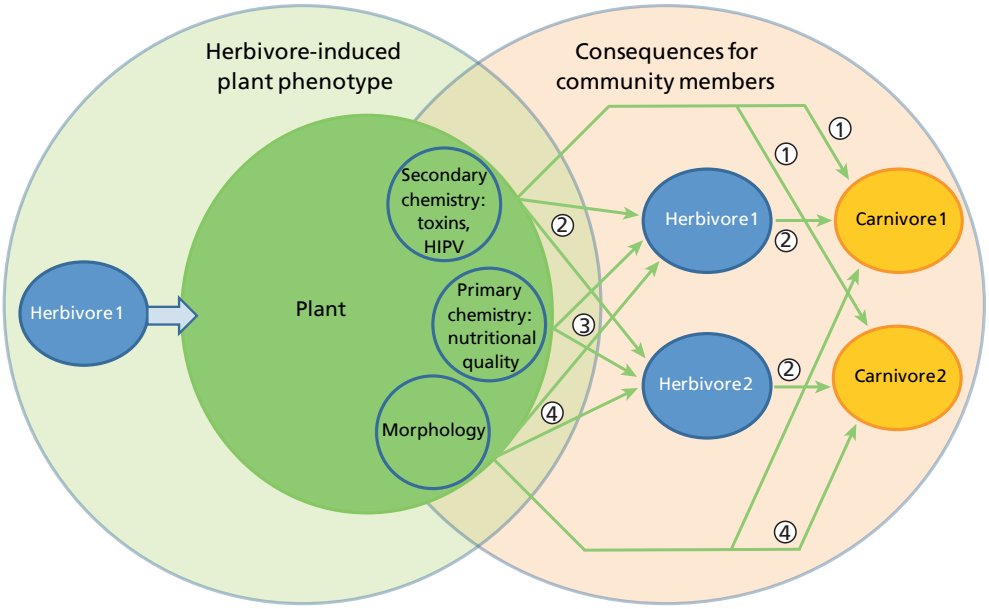

Figure 2. Components of plant phenotypic plasticity in response to herbivore attack and the interactions of these components with other members of the insect community: 1, herbivore-induced plant volatiles that attract carnivorous insects (HIPV); 2, secondary plant metabolites such as toxins and digestibility reducers that affect the performance of herbivores and through herbivores may affect their carnivorous enemies; 3, primary plant metabolites that are used as nutrients by herbivores; 4, morphological characteristics such as trichomes and cuticular wax layers that affect the performance of herbivorous insects and the behavior of their carnivorous enemies.

Such herbivore-induced plant responses may affect the behavior and growth of the initial attacker and may also influence host-plant suitability for other herbivores, even when these are temporally or spatially separated, thus mediating interspecific competition between insect herbivores (Denno et al., 1995; Ohgushi, 2005; Ohgushi, 2008) (Figure 2). Furthermore, the effects of herbivore-induced alterations in plant phenotype are to some extent specific to the attacking herbivores, and they may affect subsequent herbivores either positively or negatively, depending on the characteristics of the responding herbivore species

(Kaplan and Denno, 2007). For example, spider-mite infestation of cotton plants increased resistance against conspecific mites and whiteflies but also enhanced susceptibility to aphids (Agrawal et al., 2000). Willow infestation by leaf rollers enhanced the abundance of aphids and ants but also reduced the abundance of leaf beetles (Ohgushi, 2005).

Herbivore-induced resistance to herbivores

Herbivore-induced resistance of plants to herbivores is a common phenomenon and has been described for many insect herbivores of various feeding guilds (Karban and Baldwin, 1997). For example, through induced changes in plant phenotype, feeding by lepidopteran larvae prolongs immature development of other lepidopteran species that colonize a common host plant later in the season (Agrawal, 2000; Poelman et al., 2008).

A meta-analysis of genetic correlations between plant levels of resistance to multiple enemies revealed positive correlations when the compared species were both generalist herbivores or when they were both specialist herbivores (Leimu and Koricheva, 2006). It also revealed significant positive genetic correlations for plant resistance to herbivores from different feeding guilds, such as miners and gall inducers, miners and leaf folders, and gall inducers and leaf folders (Leimu and Koricheva, 2006). In pairwise comparisons of interactions between herbivores belonging to different feeding guilds, the lowest genetic correlation was recorded for mechanisms of plant resistance to phloem-feeding and leaf-chewing herbivores (Leimu and Koricheva, 2006).

Herbivore-induced susceptibility to herbivores

Herbivore-induced susceptibility seems to be less common than herbivore-induced resistance (Leimu and Koricheva, 2006), and in half of the reported cases it involved interactions between piercing/sucking and biting/chewing herbivores (Denno et al., 1995). Yet 20-40% of the total number of interactions within the herbivore community associated with willow and goldenrod were facilitative (Ohgushi, 2008). Most facilitative interactions were asymmetric, with only one species gaining an advantage (Denno et al., 1995; Kaplan and Denno, 2007).

Different mechanisms may underlie facilitation among different herbivore species. For example, the facilitative interaction between spittlebugs and leaf rollers that was observed on willow was caused by compensatory shoot growth in response to spittlebug infestation; leaf rollers prefer leaves on the new shoots (Ohgushi, 2005). A stem-boring moth induced susceptibility in willow to a specialist leaf

beetle by causing young shoot growth (Utsumi and Ohgushi, 2008). Herbivory by leaf rollers on oak provided shelter and better feeding sites for aphids (Karban and Agrawal, 2002; Karban et al., 1997), and herbivory by aphids interfered with induced defense signaling against caterpillars (Soler et al., 2012).

Herbivore-induced plant responses and carnivorous insects

Herbivore-induced changes in plant secondary chemistry play an important role in habitat and host location of carnivorous insects, mainly via the production of volatiles in response to feeding by their prey or hosts (D'Alessandro and Trulings, 2006; Dicke and Baldwin, 2010; McCormick et al., 2012) (Figure 2). These herbivore-induced plant volatiles attract the carnivorous enemies of herbivores to plants infested with their herbivorous victim. Moreover, even hyperparasitoids at the fourth trophic level may exploit herbivore-induced plant volatiles to find their parasitoid host that feeds within an herbivorous insect (Poelman et al., 2012). However, specific volatile chemicals or mixtures of chemicals may also repel carnivorous insects (Braasch et al., 2012; Snoeren et al., 2010; Webster et al., 2010). Volatile-mediated foraging behavior of carnivores is more difficult to predict when multiple herbivores attack the same host plant (Dicke et al., 2009; Ponzio et al., 2013; Shiojiri et al., 2001). When nonhost herbivores share the same plant individual with hosts, changes in the induced volatile blend can interfere with host location by foraging carnivorous insects (De Rijk et al., 2013; Dicke et al., 2009).

Nonvolatile plant chemistry may also mediate the effects of herbivore-induced changes in plant phenotype on carnivores (reviewed in Gols and Harvey (2009) and Ode (2006)). Herbivore-induced changes in plant chemistry may prolong herbivore development and consequently extend the exposure period of the herbivore to its enemies (Benrey and Denno, 1997). Moreover, some specialist herbivores are able to sequester plant secondary metabolites and exploit these defenses for their own protection from natural enemies (Kazana et al., 2007; Müller, 2009) (Figure 2). Herbivore-induced plants may also influence immune responses of herbivores to parasitoids (Bukovinszky et al., 2009). *Pieris rapae* caterpillars that developed on plants previously damaged by *Pieris brassicae* caterpillars had a reduced ability to encapsulate parasitoid eggs compared with those reared on undamaged plants (Bukovinszky et al., 2009). It is remarkable that herbivory resulted in inferior performance and immune response of the subsequent caterpillars and enhanced their susceptibility to parasitism. However, suppressed performance of host caterpillars on induced plants may also inhibit

parasitoid performance through reduced host nutrient availability (Ode, 2006). Generalist parasitoids tend to be more susceptible to inducible plant metabolites than specialist parasitoids are (Bukovinszky et al., 2012; Gols et al., 2008b).
In conclusion, herbivory alters plant phenotype, which has consequences for the interactions of the plant with herbivorous and carnivorous insects (Figure 2). In the next section, we address the molecular mechanisms underlying the modification of plant phenotype by herbivory and how different herbivores feeding on the same plant affect one another's modifications.

Molecular mechanisms underlying plant phenotypic plasticity under single and multiple attacks

The past decade has brought significant advances in the mechanistic understanding at the (sub)cellular level of induced plant responses that underlie plant–insect interactions (Bonaventure et al., 2011; Felton and Tumlinson, 2008; Howe and Jander, 2008; Kessler and Baldwin, 2002; Maffei et al., 2012; Maffei et al., 2007; Mithöfer and Boland, 2012; Reymond, 2013; Wu and Baldwin, 2010). This relates to the recognition of attackers and the induction of signal transduction pathways, which is followed by transcriptomic changes and the induction of biosynthetic pathways leading to changes in plant phenotype. Most of this research has focused on interactions between a plant and one attacker, but over the past decade, studies of the interactive effects of the combined infestation of a plant by two attackers have been initiated (Dicke et al., 2009; Kessler and Baldwin, 2004; Rodriguez-Saona et al., 2010; Thaler et al., 2012; Voelckel and Baldwin, 2004; Zhang et al., 2013).

Signal transduction pathways

Herbivorous insects produce oral secretions containing compounds that elicit plant responses (Bonaventure et al., 2011). The chemical nature of the active compounds is remarkably diverse and includes small organic compounds such as benzyl cyanide, fatty acid–amino acid conjugates, and proteins such as β-glucosidase (Maffei et al., 2012). The initial step in the elicitation process occurs with considerable specificity for the plant–insect combination studied. The recognition of herbivore elicitors by plant receptors initiates a cascade of responses, including changes in plasma membrane potential and activation of networks of kinases and phytohormones (Maffei et al., 2007). More recently, it has become apparent that insects may also produce so-called effectors that

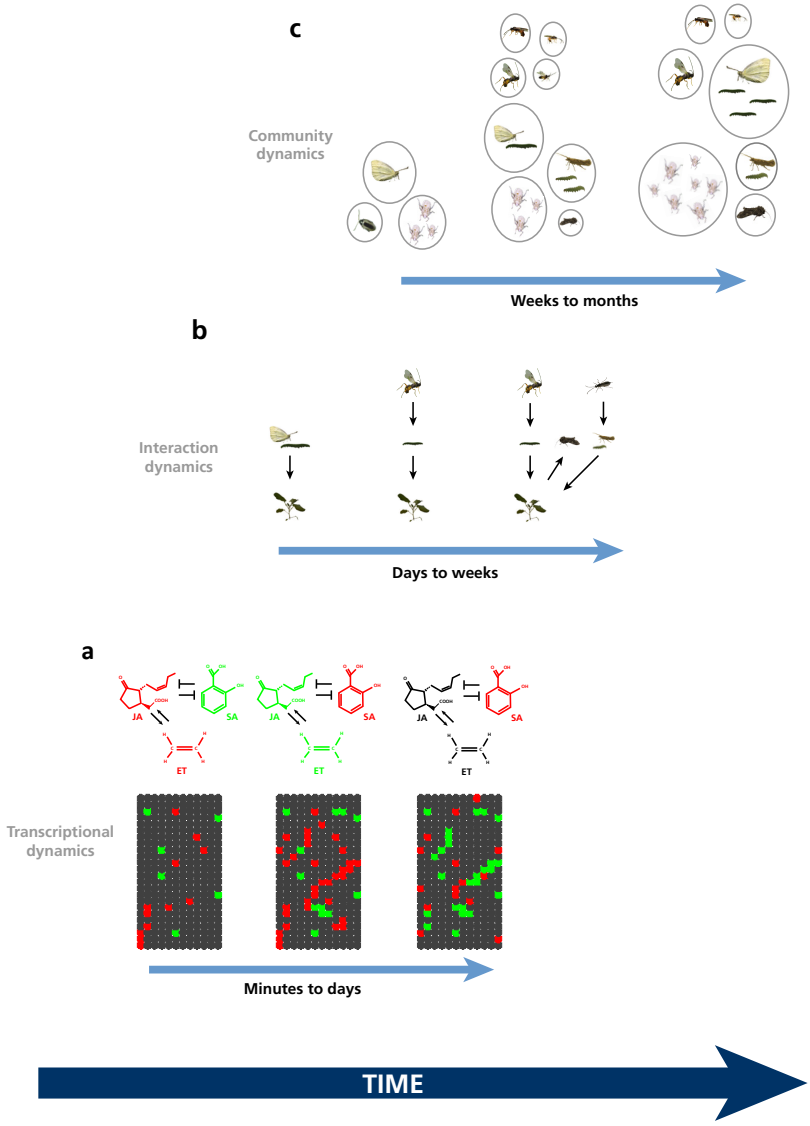

Figure 3. Schematic representation of dynamics at different levels of biological integration, each with its own timescale. Phytohormonal and transcriptional responses to herbivory at a scale of minutes to days (a). The tissue concentrations of the phytohormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), which are involved in defense responses, change dynamically and exhibit crosstalk (arrows); their molecular structures are shown in red when increasing, in green when decreasing, and in black when constant. The dots represent genes in a heat map of gene transcription and are colored red when increasing, green when decreasing, and black when constant. Interactions among individual insects at different trophic levels at a scale of days to weeks (b). Community dynamics at a scale of weeks to years (c).

function to suppress the elicitor-triggered plant defense response, such as glucose oxidase in the interaction between *Helicoverpa zea* caterpillars and tobacco (Felton and Tumlinson, 2008; Maffei et al., 2012). Studies elucidating the regulatory mechanisms underpinning plant defense responses to insect herbivore attack have identified the central role of phytohormones. Three major plant hormones - jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) (Figure 3A) - function in a complex regulatory network that is essential in herbivore-induced defense responses. Other hormones, such as cytokinins, abscisic acid, gibberellins, and auxin, likely also play a role in herbivore-induced defense signaling (Erb et al., 2012; Pieterse et al., 2012).

It is well documented that chewing herbivores and sap feeders induce different plant signaling pathways involving the three major phytohormones, JA, SA, and ET (Pieterse et al., 2012). Much less is known about signaling pathways involved in resistance against insects of other feeding guilds, such as leaf miners, stem borers, leaf folders, and gall-inducing herbivores. SA and ET signaling pathways are involved in the resistance of rice plants to the leaf folder *Cnaphalocrocis medinalis* (Wang et al., 2011). Some leaf miners and gall-inducing insects modulate plant cytokinin levels, probably to manipulate the source–sink status of the infected tissues (reviewed in Erb et al. (2012) and Giron et al. (2013)). Feeding by gall-inducing insects increases auxin level but does not change JA level (Erb et al., 2012; Tooker and De Moraes, 2008). Insect eggs have been reported to induce plant responses via the SA signaling pathway (Reymond, 2013).

The salicylic acid pathway

SA regulates induced plant responses against phloem-feeding insects and biotrophic pathogens (Glazebrook, 2005; Pieterse et al., 2012). In response to phloem-sucking insects, SA can be synthesized from chorismate through the isochorismate pathway (Wildemuth et al., 2001) and the phenylalanine ammonium lyase pathway (Dempsey et al., 2011). Accumulation of SA leads to the translocation of the positive regulatory protein nonexpressor of pathogenesis-related genes 1 (NPR1) to the nucleus. Regulation of the expression of SA-responsive genes occurs downstream of NPR1, which interacts with TGA-type transcription factors and additionally targets *WRKY* transcription factor genes (Wang et al., 2006). This results in the activation of defense gene expression and the production of pathogenesis-related (PR) proteins (Durrant and Dong, 2004).

The jasmonic acid/ethylene pathway

JA is an important regulator of defense responses against chewing insects, necrotrophic pathogens, and cell content feeders such as spider mites and thrips (De Vos et al., 2005; Glazebrook, 2005; Kant et al., 2008; Pieterse et al., 2012). Upon herbivory, JA is produced via the octadecanoid pathway. In *Arabidopsis*, the enzyme jasmonoyl isoleucine conjugate synthase 1 (JAR1) activates JA by conjugating it to the amino acid isoleucine (Ile) to form JA-Ile (Staswick and Tiryaki, 2004). Binding of JA-Ile to the F-box protein coronatine-insensitive 1 (COI1) mediates the degradation of jasmonate ZIM domain (JAZ) repressor proteins (Thines et al., 2007). These proteins repress JA signaling by binding transcriptional activators such as MYC2. When the repression of JAZ proteins is lifted, JA-responsive genes are activated, including genes encoding JAZ proteins, resulting in a negative-feedback loop (Memelink, 2009). Two branches have been identified within the JA signaling pathway that act antagonistically (Pieterse et al., 2009; Pieterse et al., 2012). The MYC2 branch positively regulates the expression of wound-inducible JA-responsive marker genes such as *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) and *LIPOXYGENASE 2* (*LOX2*). In the ethylene response factor (ERF) branch of the JA pathway, JA and ET synergistically induce the expression of JA/ET-responsive transcription factors, including ERF1 and octadecanoid-responsive *Arabidopsis* 59 (ORA59), which positively regulate JA/ET-responsive genes such as *plant defensin 1.2* (*PDF1.2*) (Dombrecht et al., 2007; Lorenzo et al., 2004). The ERF branch is especially involved in induced defense against necrotrophic pathogens, whereas the MYC2 branch mediates defense against herbivorous insects (Pieterse et al., 2012).

Phytohormonal crosstalk and its molecular mechanisms

When a plant faces multiple herbivore attack, crosstalk may occur between the induced signaling pathways, with consequences for induced defense responses. Crosstalk between signaling pathways allows the plant to fine-tune its defense response to the specific attacker (Pieterse et al., 2012). For instance, induced defense is regulated through interconnection of the JA, SA, and ET signal transduction pathways (Pieterse et al., 2012). Crosstalk between JA and SA signaling is mutually antagonistic, resulting in the prioritization of SA-dependent defense responses over JA-dependent responses or vice versa (Pieterse et al., 2012, Thaler et al., 2012). Molecular players that modulate this JA-SA crosstalk include mitogen-activated protein kinases (MAPKs), WRKY transcription factors, the regulatory protein NPR1, and other phytohormones (Pieterse et al., 2012). NPR1 is a major regulator of JA-SA crosstalk in *Arabidopsis*, and its effect is

mediated by ET, which may have been induced by both biotic and abiotic stresses (Leon-Reyes et al., 2009). In contrast to JA-SA crosstalk, JA- and ET-dependent signaling pathways act synergistically in inducing plant defense responses (Pieterse et al., 2009).

Crosstalk between phytohormonal signaling pathways also allows herbivores to manipulate plant defenses for their own benefit (Pieterse and Dicke, 2007). Feeding by *Manduca sexta* caterpillars induced an ET burst and suppressed nicotine accumulation in tobacco plants (Kahl et al., 2000). It has been hypothesized that by activating the SA signaling pathway, phloem feeders suppress JA-dependent defenses to which phloem feeders are more sensitive (Moran et al., 2002; Zarate et al., 2007). Several recent studies have supported the interference of SA with JA-inducible defenses against chewing insects (Soler et al., 2012; Thaler et al., 2012; Zhang et al., 2013; Zhang et al., 2009), although phloem-feeding insects do not in all cases interfere with the defenses induced by chewing herbivores (Erb et al., 2010), which may be due to density effects or to differences between species.

Transcriptomic changes in response to individual attackers and multiple attacks

Phytohormonal responses to herbivory result in transcriptional responses that have a high degree of specificity. Transcriptional responses depend on the feeding guild of the attacker and the phytohormonal signal signature that the attacker induces. For instance, attack by single insect species belonging to different feeding guilds resulted in the activation of specific sets of defense-related genes in *Arabidopsis* (De Vos et al., 2005). Different species of leaf-chewing herbivores that all induced JA in the plant still induced different transcriptomic changes (Bidart-Bouzat and Kliebenstein, 2011). These induced transcriptomic changes also differed from those induced by JA, most likely because each attacker activates more than one phytohormonal pathway. De Vos et al. (2005) hypothesized that the phytohormonal signal signature regulates the specific transcriptomic changes. Aphid feeding affected the expression of a substantially larger number of genes compared with feeding by caterpillars and thrips, and it tends to induce gene sets more similar to those induced by fungal or bacterial pathogens (De Vos et al., 2005). In *Nicotiana attenuata*, aphids suppressed more genes than chewing herbivores did, and aphids upregulated the expression of SA-dependent genes and suppressed the expression of JA-mediated genes (Heidel and Baldwin, 2004). Similar findings were recorded for the effects of feeding by caterpillars and aphids on tomato (*Solanum lycopersicum*) (Rodriguez-Saona et al., 2010).

Transcriptomic changes in response to phloem-feeding insects

Phloem-feeding insects, such as aphids and whiteflies, cause little damage to the plant tissue because they move their stylets in between plant cells on their way to the phloem, briefly puncturing but not killing cells along the way. SA accumulates in plants upon interactions with aphids and whiteflies, whereas activation of JA leads to resistance to phloem-feeding herbivores. Early transcriptional responses of *Arabidopsis* to *Brevicoryne brassicae* aphids were observed after 6 h, at which point a group of WRKY transcription factors were highly expressed. Genes involved in SA-dependent defense had a peak expression after 24 h of infestation. After 12 h of aphid infestation, the number of inducible genes expressed and the intensity of JA-inducible responses had already decreased (Kusnierczyk et al., 2008).

Transcriptomic changes in response to chewing insects

Plants respond to feeding by chewing insects very differently than they do to feeding by phloem-feeding insects (Bidart-Bouzat and Kliebenstein, 2011). Plant defense responses to chewing insects are regulated mainly by the JA signaling pathway, with ET playing an additional role (De Vos et al., 2005; Ehlting et al., 2008; Heidel and Baldwin, 2004; Reymond et al., 2004). The expression of hundreds of genes changes in response to caterpillar feeding (Ehlting et al., 2008; Reymond et al., 2004; Rodriguez-Saona et al., 2010; Voelckel and Baldwin, 2004; Zhang et al., 2013). Genes involved in signaling and secondary chemistry are commonly upregulated, whereas genes involved in photosynthesis and primary metabolism are often downregulated (Voelckel and Baldwin, 2004). The transcriptional patterns in response to caterpillar feeding are dynamic over time. For instance, a microarray analysis of *Arabidopsis* in response to feeding of *Plutella xylostella* larvae recorded strong upregulation of wound-response genes involved in octadecanoid biosynthesis over a 24-h period (Ehlting et al., 2008). However, SA also seems to be involved in the plant's response to *P. xylostella* feeding, as indicated by upregulation of *PR* genes after 24 h of feeding. Interestingly, *PR* genes are downregulated during early stages of *P. xylostella* feeding (Ehlting et al., 2008). Similar responses have been reported in other plant species as well. For example, in tomato, the transcription of *PR* genes was induced by caterpillar feeding (Kawazu et al., 2012; Rodriguez-Saona et al., 2010). In *N. attenuata*, feeding by various insect herbivores, including the chewing herbivores *Spodoptera exigua, Spodoptera littoralis, Trichoplusia ni and Manduca sexta* larvae resulted in increased SA levels (Diezel et al., 2009; Heidel and Baldwin, 2004). The increased SA levels were consistently correlated with the downregulation of photosynthetic genes (Heidel and Baldwin, 2004).

Transcriptomic response patterns in response to multiple attacks

The transcriptomic response to two attackers is far from an additive response to the two attackers individually (Voelckel and Baldwin, 2004). For instance, in tomato plants infested by aphids (*Macrosiphum euphorbiae*) and caterpillars (*S. exigua*), the aphids suppressed 27% of the genes regulated by caterpillars, whereas the caterpillars suppressed 66% of the genes regulated by aphids (Rodriguez-Saona et al., 2010). In *Arabidopsis*, infestation with the whitefly *Bemisia tabaci* suppresses the upregulation of a large number of genes induced by *P. xylostella* caterpillars (Zhang et al., 2013). The interactive effects of two attackers can uncover novel mechanisms. For instance, infestation of *Arabidopsis* plants by *P. rapae* caterpillars induced JA and ET; ET primed the plant for enhanced SA-dependent gene expression in response to infection by turnip crinkle virus (De Vos et al., 2006). Transcriptional interference is usually asymmetric. For instance, in *N. attenuata*, transcriptional changes induced by the mirid bug *Tupiocoris notatus* are more resistant to erasure by *M. sexta* caterpillars than vice versa (Voelckel and Baldwin, 2004).

Transcriptomic changes occur in distinct patterns and involve large numbers of genes. Analyzing these patterns is usually done with multivariate statistics, but identifying how these transcriptomic changes affect the plant phenotype, especially which genes are responsible for the phenotypic effects and subsequent interactions with members of the insect community, requires a directed approach. In lima bean plants, feeding by *B. tabaci* whiteflies suppressed the induction of the plant's *ocimene synthase* gene, which encodes an enzyme mediating a rate-limiting step in the biosynthesis of the plant volatile (*E*)-β-ocimene in response to spider-mite feeding. (*E*)-β-Ocimene mediates the attraction of a predatory mite that preys on the spider mite, and whitefly feeding resulted in a reduced attraction of the predatory mite to volatiles from spider-mite-infested plants (Zhang et al., 2009).

How to link subcellular mechanisms underlying inducible plant phenotypes to community dynamics

Changes in plant phenotype and their consequences for the plant's interactions with members of the associated insect community take place at very different timescales. Community development takes place on a timescale of weeks to (for perennial woody plants) years, and is based on interactions between individuals that take place on a timescale of days to weeks. These interactions between

individuals are affected by changes in the plant phenotype (timescale of hours to days) that are based on transcriptomic changes at a timescale of minutes to days (Figure 3). The different rates at which changes develop at different levels of biological complexity complicate linking these changes causally. For instance, the transcriptome of *N. attenuata* responds specifically to different herbivore infestations within 24 h, but this difference disappears after 5 days (Voelckel and Baldwin, 2004). Linking the transcriptomic response within the first 24 h to community responses at a timescale of weeks to years requires detailed knowledge of how individual species in the community respond to the plastic plant phenotype. Although understanding how complex molecular changes modulate responses at the community level is a major challenge, detailed knowledge of subcellular mechanisms can provide tools to address this challenge. For instance, knowledge on the involvement of phytohormones can be used to mimic herbivory through the application of a phytohormone. Because JA is one of the major phytohormones involved in plant responses to insect herbivory, it is an interesting initial candidate to manipulate. Pharmacological application of JA to tomato plants has season-long effects on community composition in terms of herbivorous and carnivorous insects. For instance, the abundance of herbivores was reduced and herbivore size was smaller, and these effects on herbivores subsequently affected the performance of predators and parasitoids (Thaler, 1999; Thaler, 2002; Thaler et al., 2001). Applying a single phytohormone at one time point is still a crude method, however, because herbivory results in a dynamic phytohormonal response (Pieterse et al., 2012). Pharmacological applications may be made with different phytohormones at different time points (Koornneef et al., 2008), but we are not aware of any studies that have investigated the effects of such combinations of applications on community development.

A more accurate approach is to use genetic tools, *e.g.*, by using plants that have been silenced in a single gene involved in the plant's induced response. *N. attenuata* plants in which a gene encoding for the enzyme lipoxygenase, which mediates the first rate-limiting step in JA biosynthesis, had been silenced were more susceptible to adapted herbivores and attracted novel herbivore species that normally do not feed or reproduce on this plant (Kessler et al., 2004). Silencing a gene is quite a drastic manipulation. In nature, plant genotypes more likely differ in relative expression of particular genes, so it will be interesting to monitor community development on different genotypes whose genomes have been (partially) genetically characterized. Experiments with genotypes that have not been genetically characterized showed that plant genotypes that differ in secondary metabolites result in considerable variation in community

dynamics (Newton et al., 2009b; Poelman et al., 2009). Community development on different genotypes may converge when the genotypes have been exposed to an early-season specialist herbivore (Poelman et al., 2008; Poelman et al., 2010). Because plants in nature are rarely free of herbivory, community dynamics on plants subjected to herbivory are highly relevant to understanding how plant phenotype affects community dynamics.

Data on community development may be linked to transcriptional responses of plants under field conditions (Broekgaarden et al., 2010), but this is still far from providing information on the causal links because of the different timescales. Transcriptional responses on a timescale of minutes to days result in a cascade of responses that lead to a dynamic change in plant phenotype. Studying the links between transcriptional dynamics, phenotypic dynamics, and community dynamics requires taking a systems approach that includes experiments in combination with modeling to connect the networks at different levels of biological integration, *i.e.*, the transcriptomic network, the metabolomic network, and the species interaction network (Keurentjes et al., 2011).

Sequential changes in herbivore-induced phenotype and community dynamics

Plant–insect interactions represent intricate networks at all levels of biological complexity. These networks consist of hundreds of interacting species at the community level, tens to hundreds of individual insects interacting with a single plant individual, hundreds of plant chemicals that are the product of biosynthetic networks, and hundreds of genes that are regulated by an interacting network of phytohormones. Each of these networks has its own dynamics, and the transcriptomic network that results from herbivore attack affects the biosynthetic network that underlies the change in plant phenotype, which affects interactions with members of the community and consequently community dynamics. Although a systems approach to linking these complex networks at different levels of biological integration will be a major challenge (Keurentjes et al., 2011), interesting building blocks are available at the community level with some initial links to knowledge at the mechanistic level. Community dynamics result from sequential processes in which the first herbivore's modification of the plant's phenotype then has consequences for the interactions of the plant with subsequent herbivores (Erb et al., 2011; Poelman et al., 2008; Poelman et al., 2010; Viswanathan et al., 2007). The interaction of a second herbivore

with the new plant phenotype may modulate processes at the (sub)cellular level in terms of phytohormonal and transcriptional patterns (Poelman et al., 2008; Rodriguez-Saona et al., 2010; Voelckel and Baldwin, 2004; Zhang et al., 2013), further affecting the plant's phenotype and its interactions with subsequent community members (Dicke et al., 2009; Van Zandt and Agrawal, 2004; Zhang et al., 2013; Zhang et al., 2009). The arrival of these new community members, which now also start to interact with the plant, sets a new round in motion, and so on. This set of interactions - an herbivore inducing a phenotypic change that then affects subsequent herbivores on the same plant, mediated by induced plant traits - has been termed a trait-mediated interaction unit (TMIU). A TMIU consists of an inducing insect and a plant that mediates the interaction with a second, responding herbivore (Utsumi et al., 2010). TMIUs are linked sequentially. This is the case when, for example, a responding herbivore itself becomes an inducer (Utsumi et al., 2010), which may happen on both spatial and temporal scales. A spatial chain reaction occurs when the responding herbivore changes its behavior and moves to another plant or plant part (Bukovinszky et al., 2010; Utsumi et al., 2010) or when responses to feeding herbivores affect herbivores elsewhere on the plant through systemic responses (Erb et al., 2011, Utsumi et al., 2010). A temporal chain reaction occurs when the responding herbivore later returns to the same plant as an inducer (Underwood, 2012) or when the altered plant phenotype affects the performance or population density of the responder, thereby affecting the plant it feeds on (Utsumi et al., 2010, Van Zandt and Agrawal, 2004). In fact, several TMIUs might be linked throughout the season, creating a complex indirect interaction web. The resulting cascade shapes the insect community associated with a plant, depending on the first inducing herbivores that arrive on the plant (Poelman et al., 2008; Van Zandt and Agrawal, 2004; Viswanathan et al., 2007). For instance, on milkweed plants, the identity of the first herbivore early in the season has considerable effects on community development throughout the season (Van Zandt and Agrawal, 2004). On *B. oleracea* plants, an early-season, one-week-long infestation by two *P. rapae* caterpillars affected community dynamics throughout the growing season of the plants, with the community on the treated plants comprising more specialist insects than the community on the control plants did (Poelman et al., 2008; Poelman et al., 2010). Such cascades may be caused by direct effects of an inducing herbivore on the suitability of the plant to other herbivores and indirect effects of initiating herbivores on the interaction between two or more subsequent herbivores. Herbivores in a TMIU do not all influence the subsequent interactions in the same way, and this may depend on herbivore traits such as

feeding guild (Bidart-Bouzat and Kliebenstein, 2011; Howe and Jander, 2008). Three mechanisms have been proposed by which a plant's physiological response is directed to (a subset of) certain herbivores: priority effects, overriding effects, and canalization, all of which can be linked to phenomena uncovered at the subcellular level.

Priority effects, overriding effects, and canalization

Priority effects occur when a plant response depends on the order of herbivore arrival on a plant (Miller-Pierce and Preisser, 2012) - for example, when the interaction between two herbivores is asymmetrical (Erb et al., 2011; Miller-Pierce and Preisser, 2012; Poelman et al., 2008; Soler et al., 2012). Asymmetry in these interactions is predominant (Kaplan and Denno, 2007), and priority effects are therefore expected to be important in shaping interaction cascades. These asymmetrical priority effects can have several underlying mechanisms, such as competition between the herbivores (Kaplan and Denno, 2007; Miller-Pierce and Preisser, 2012), which has different outcomes depending on which insect comes first. The kinetics of plant defenses may underlie this. For example, the production of induced plant defense compounds might depend on the sequence of herbivore arrival and can have a larger effect on either the first or the subsequently arriving herbivore (Erb et al., 2011; Viswanathan et al., 2005). Priority effects may also be mediated by crosstalk between different plant defense pathways, such as the JA-SA crosstalk (Pieterse et al., 2012; Thaler et al., 2012).

Overriding effects occur when the inducing effects of one herbivore are overruled by another herbivore on the same plant (Erb et al., 2011; Van Zandt and Agrawal, 2004). For example, the effects of initial damage to a milkweed plant by monarch caterpillars (*Danaus plexippus*) disappeared when the plant was colonized by other herbivores later in the season (Van Zandt and Agrawal, 2004). Moreover, the plant response can also be redirected (Soler et al., 2012; Voelckel and Baldwin, 2004) or enhanced (Poelman et al., 2008) following the arrival of subsequent herbivores. Underlying mechanisms may involve irreversible phenotypic changes, such as morphological changes or overriding effects of one signaling pathway on another (Pieterse et al., 2012).

Canalization occurs when a first herbivore alone determines the plant's response, regardless of subsequently arriving herbivores (Thaler et al., 2002; Utsumi et al. 2010; Viswanathan et al., 2007; Viswanathan et al., 2005). This effect reduces the plant's ability to be flexible in its response to the herbivore community present at any given point in time, and consequently may affect the development of

the herbivore community composition throughout the season. For example, flea beetles affected the number of conspecifics or tortoise beetles throughout the season when arriving first on a plant, irrespective of whether they were followed by tortoise beetles (Viswanathan et al., 2007). Underlying mechanisms may include strong and irreversible effects of the phytohormonal signaling in response to the first herbivore or the rapid induction of biosynthetic pathways that result in persistent changes in the plant's phenotype.

Trait-mediated interaction networks and carnivorous insects

The above discussion of trait-mediated interaction networks considered only herbivores in the ecological interactions. However, the third trophic level, consisting of predators and parasitoids of herbivores, also affects the interaction between inducing and responding herbivores (Utsumi et al., 2010; Van Veen et al., 2006). Combinations of multiple herbivores can induce the emission of different blends of plant volatiles (Dicke et al., 2009) and thus may attract different predators or parasitoids (Schoonhoven et al., 2005). These predators or parasitoids not only decrease the herbivore population by preying on the insects that initially induced the volatiles (Utsumi et al., 2010; Xiao et al., 2012; Zhang et al., 2009) but can also affect other insects, such as herbivores, pollinators, and hyperparasitoids (Dicke and Baldwin, 2010; Poelman et al., 2012). The events at different moments in time may also be linked, for example, when a predator that is attracted to a plant infested by a first herbivore also preys on other herbivores arriving simultaneously or subsequently on the plant. Different interaction units can occur on a spatial scale as well, when predators induce behavioral changes in herbivores, after which the herbivores move to other plants or plant parts (Utsumi et al., 2010). Because herbivores that are affected by predators and parasitoids can influence the subsequent herbivore community in a cascading manner through priority effects, overruling effects, or canalization, the third trophic level greatly increases the complexity of interactions within a plant–insect community. This is particularly the case when considering multiple initiating herbivores (Zhang et al., 2013; Zhang et al., 2009).

In summary, interactions between insects associated with a plant are influenced by several factors. The type and sequence of multiple herbivores determine the plant's response, which consequently affects herbivores that subsequently colonize the plant. These secondary herbivores or attracted predators may become inducers in the next plant-mediated interaction unit, which causes a cascade of interactions throughout the insect community.

Future perspectives

The fact that plants are phenotypically plastic in response to herbivore attack contributes to the complexity of plant–insect interactions. For instance, phenotypic plasticity underlies interspecific competition between herbivores at different temporal and spatial scales (Denno et al., 1995; Kaplan and Denno, 2007). It is important to realize that a plant's genotype determines not only constitutive plant traits but also inducible plant responses, such as the production of metabolites or structural changes. The extent to which constitutive or inducible traits affect plant–insect interactions affects the relative importance of the inducible and the constitutive phenotype for the influence on community dynamics (Poelman et al., 2008; Whitham et al., 2012).

In this review, we have focused on the consequences of direct and indirect effects of inducible plant traits on community processes, with a focus on herbivorous and carnivorous insects. Herbivorous insects are connected by both local and systemic plant-mediated interactions. Systemic effects may involve both roots and shoots (Soler et al., 2013) or leaves and flowers (Kessler et al., 2011; Lucas-Barbosa et al., 2011). We have focused on aboveground plant vegetative tissues because most information on community processes is available for insect communities associated with vegetative plant shoots. However, similar systemic effects are expected when including the belowground tissues (Soler et al., 2013). Including belowground interactions will be important, even when it further increases the complexity of the interactions and therefore the difficulty of understanding the effects of a phenotypically plastic plant on the development of the associated community. The situation is likely to differ between vegetative and flowering plants because of the major physiological changes that occur during the transition from the vegetative to the reproductive stage. A comparison of vegetative and flowering plants and their associated communities will be interesting to address the different selection forces that these different developmental stages are subjected to. For the sake of simplicity, we have limited this review to plant–insect interactions. Although insects are the most speciose group within the macrobiome associated with plants (Schoonhoven et al., 2005), there is also a speciose microbiome associated with plants (Mendes et al., 2011) that represents species with many additional ecological functions, such as pathogens, rhizobia, mycorrhizae, and nonpathogenic rhizobacteria. There is extensive information at the mechanistic, (sub)cellular level for plant–microbe interactions (Pieterse et al., 2012), but knowledge of the community processes of microbes associated with plants is much less developed. Nevertheless, it is becoming clear that pathogenic

and symbiotic microbes can influence and structure insect communities on plants (Pineda et al., 2010; Tack and Dicke, 2013). Thus, involving the microbiome in future studies will significantly enhance our understanding of plant–insect interactions. The extensive information on subcellular processes for plant–microbe interactions provides an excellent starting point to manipulate plants via microbes to study the consequences for insect communities. However, including microbial community processes will provide an important new challenge related to the identification of microbes associated with plants (Mendes et al., 2011). Investigating the effects of plants on community development is already a complex task, and unraveling the mechanisms that underlie the community dynamics throughout the season is a significant challenge as well. With a community that, in the case of long-lived plants, can consist of hundreds of species, the number of species combinations involved in plant-mediated interactions seems too large to handle. However, phenological data and natural history data for the system under study may provide a basis for choices that are relevant to the natural situation. For instance, early-season herbivores that predictably occur in the system may have a prominent effect on plant phenotype that is worth focusing on initially. Furthermore, analyzing community dynamics data through statistical modeling approaches may result in the identification of key species in the community (Keurentjes et al., 2011). Such species and the species they interact with may then be the focus of initial studies on underlying mechanisms. Herbivorous insects will be the first group of insects to focus on. When key herbivore species have been selected for such studies, relevant parasitoids and predators should be included next, because their presence and activities affect herbivore behavior (Thaler et al., 2012), population growth (Van Veen et al., 2005), and interactions with plants (Poelman et al., 2011). Again, natural history data may guide the selection of the first species to include in these studies. The complexity of plant–insect community dynamics and the underlying mechanisms may be overwhelming, and it may seem impossible to understand the processes that shape these speciose and dynamic ecological systems. Rather than stepping back, this complexity should invite directed studies to investigate the ecological processes as well as their underlying mechanisms. Through these studies, we are likely to make small but significant steps toward unraveling how plants influence insect communities. When this has been completed for several different systems, ecological generalities may be identified, and mechanistic knowledge will then allow directed experimental studies to test these generalities. These studies will then enable important progress in understanding interactions between the insects and plants that are so dominant on this planet.

Summary points

1. Plants are members of biodiverse communities consisting of tens to hundreds of species.
2. The insect community associated with plants consists of herbivores at the second trophic level and (hyper)parasitoids and predators at the third, fourth, and higher trophic levels.
3. A plant's phenotypic traits, as determined by its genotype, influence the interactions of the plant with members of the associated community and consequently the community dynamics.
4. A plant's phenotype is highly plastic: Herbivory induces changes in the plant's phenotype, which then influence the plant's interactions with members of the associated community and lead to plant-mediated interactions between community members, such as competition and facilitation.
5. Herbivory induces phytohormonal signaling and transcriptomic rearrangements (timescale of minutes to days) that lead to biosynthetic changes that affect the plant phenotype (timescale of hours to days), with consequences for the plant's interactions with community members (timescale of days to weeks).
6. Plant responses to herbivores exhibit a considerable degree of specificity. Moreover, the response to two attackers is far from an additive response to the two attackers individually; rather, it involves a strong interaction component that leads to suppression or enhancement of the responses to each herbivore alone, *e.g.*, through phytohormonal crosstalk.
7. The first herbivore-induced change in plant phenotype affects the interactions with subsequently arriving herbivores, which then further affect the phenotype in an interactive way. This sequential process determines community dynamics on a timescale of weeks to years. Thus, the first herbivore that attacks a plant can significantly influence the community dynamics on that plant.
8. Linking herbivore-induced changes in plant phenotype to the ecological consequences that occur at very different timescales is an important multidisciplinary challenge that will provide a comprehensive understanding of how plants interact with their associated communities.

Acknowledgments

Our research is funded by a TOP GO grant from the Netherlands Organization for Scientific Research (NWO) (nr 854.10.010 to M.D.).

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Chapter 3

Density-dependent interference of aphids with caterpillar-induced defenses in *Arabidopsis*: involvement of phytohormones and transcription factors

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Published in slightly modified form in:
Plant and Cell Physiology 56 (2015) 98-106

Abstract

In nature, plants are exposed to attacks by multiple herbivore species at the same time. To cope with these attacks plants regulate defenses with the production of hormones such as salicylic acid (SA) and jasmonic acid (JA). Because herbivore densities are dynamic in time, this may affect plant-mediated interactions between different herbivores attacking at the same time. In *Arabidopsis thaliana*, feeding by *Brevicoryne brassicae* aphids interferes with induced defenses against *Plutella xylostella* caterpillars. This is density dependent: at a low aphid density, growth rate of *P. xylostella* was increased, whereas caterpillars feeding on plants colonised by aphids at a high density have a reduced growth rate. Growth of *P. xylostella* larvae was unaffected on *sid2* or on *dde2-2* mutant plants when feeding simultaneously with a low or high aphid density. This shows that aphid interference with caterpillar-induced defenses requires both SA and JA signal-transduction pathways. Transcriptional analysis revealed that simultaneous feeding by caterpillars and aphids at a low density induced the expression of the SA transcription factor *WRKY70* whereas expression of *WRKY70* was lower in plants induced with both caterpillars and a high aphid density. Interestingly, the expression of JA transcription factor *MYC2* was significantly higher in plants simultaneously attacked by aphids at a high density and caterpillars. These results indicate that a lower expression level of *WRKY70* leads to significantly higher *MYC2* expression through SA-JA crosstalk. Thus, plant-mediated interactions between aphids and caterpillars are density-dependent and involve phytohormonal crosstalk and differential activation of transcription factors.

Keywords

Arabidopsis thaliana, *Brevicoryne brassicae*, density dependence, gene expression, hormone crosstalk, *Plutella xylostella*

Introduction

As members of complex communities, plants are exposed to different insect attackers at the same time (Viswanathan et al., 2007). In order to cope with attack by multiple herbivore species, plants have evolved complex molecular defense mechanisms (Dicke et al., 2009; Pieterse and Dicke, 2007). Specific suites of defense response genes are expressed in *Arabidopsis thaliana,* dependent on the type of insect attacker (De Vos et al., 2005). For example, defense responses induced by aphids are clearly distinct from those induced by caterpillars (Bidart-Bouzat and Kliebenstein, 2011; De Vos et al., 2005). Defenses against phloem-feeding insects and chewing herbivores are regulated by signal-transduction pathways in which two plant defense hormones play an important role: salicylic acid (SA) and jasmonic acid (JA) (Pieterse et al., 2012). JA-mediated defenses are mostly induced by chewing insects (Pieterse et al., 2012; Walling, 2000), whereas phloem-feeding insects mainly induce SA-mediated defense responses (Kempema et al., 2007; Kusnierczyk et al., 2008; Moran and Thompson, 2001; Zhang et al., 2013b). Although defense responses of *Arabidopsis* to phloem-feeding insects are regulated by SA signaling, mutations blocking the SA signaling pathway negatively influenced the performance of *Brevicoryne brassicae* and *Myzus persicae* aphids (Mewis et al., 2005) and *Arabidopsis* mutants with enhanced JA responses decreased the fitness of the phloem-feeding silverleaf whitefly *Bemisia tabaci* (Zarate et al., 2007). These findings suggest that JA-regulated defenses are important for resistance against phloem feeders (De Vos et al., 2007; Walling, 2008).

WRKY (a transcription factor containing a highly conserved WRKY domain) genes are important regulators of SA signal-transduction pathways. They control the expression of specific SA-responsive defense genes, and the production of pathogenesis-related proteins (*PR* genes) (Durrant and Dong 2004). *MYC2* (*JASMONATE INSENSITIVE1)* is an important transcriptional activator of JA-responsive genes, such as the marker gene *VSP2* (*VEGETATIVE STORAGE PROTEIN2*) (Kazan and Manners, 2013; Liu et al., 2005).

To further facilitate fine-tuning of defense mechanisms in plants, JA and SA signaling pathways are known to interact, which is also called crosstalk (Pieterse et al., 2012). Crosstalk between SA and JA signaling results into mutual antagonisms between SA-dependent and JA-dependent defense responses (Pieterse et al., 2012). Different regulators of SA-JA crosstalk have been investigated (reviewed by: Koornneef and Pieterse, 2008; Pieterse et al., 2012), and the transcription factor *WRKY70* was identified to have a key role in positively regulating SA

signaling while suppressing JA-responsive genes (Li et al., 2004).
Although SA-JA crosstalk strengthens the coordination of plant defense mechanisms, insects have also been found to induce crosstalk between SA and JA signaling for their own benefit. For example, through SA-JA crosstalk phloem-feeding insects may enhance their survival by activating the SA pathway to weaken effective JA-mediated defenses (Zarate et al., 2007; Zhang et al., 2009; Zhang et al., 2013b).
It has been suggested that simultaneous attack by both phloem-feeding insects and chewing herbivores affects the regulation of JA- and SA-dependent induced defense responses (Stam et al., 2014). This suggests that plants respond differently to multiple insect attack compared to a single insect attack (Rodriguez-Saona et al., 2010; Schwartzberg et al., 2011; Zhang et al., 2013a).
Phloem feeders interfere with caterpillar-induced defense responses by attenuating JA-dependent defense responses, leading to facilitation between these herbivores (Li et al., 2014; Soler et al., 2012) and in suppression of indirect defenses against chewing herbivores (Schwartzberg et al., 2011; Zhang et al., 2013a). Furthermore, interference between caterpillars and phloem feeders may be dependent on the density of the attacking insect (Zhang et al., 2009).
The objective of the present study was to investigate how different densities of the aphid *Brevicoryne brassicae* interfere with induced defenses against *Plutella xylostella* caterpillars in the model plant *Arabidopsis thaliana*. Both *P. xylostella* and *B. brassicae* are specialist feeders on plants in the family Brassicaceae. We compared growth rates of *P. xylostella* when feeding alone or together with a low or high aphid density on wild-type *Arabidopsis thaliana*, a *sid2* mutant (deficient in the induction of SA accumulation) and a *dde2-2* mutant (deficient in JA biosynthesis). Furthermore, we investigated differences in transcriptional responses of *Arabidopsis* plants to simultaneous attack by caterpillars and aphids at a low or high density. Here, we focused on selected JA- and SA-responsive marker genes and genes that are known to be important signaling nodes in SA and JA crosstalk. This study demonstrates that multiple insect attack affected plant defenses in a density-dependent manner. Moreover, our data show that the SA signal-transduction pathway is required for aphid interference with induced defenses against caterpillars.

Materials and methods

Plants and growth conditions

Plants of *Arabidopsis thaliana* accession Columbia-0 (Col-0), the salicylic acid (SA) induction-deficient mutant *sid2-1* and the jasmonate deficient mutant *dde2-2* were grown on autoclaved soil (80 °C for 4 h; Lentse potgrond, Lent, The Netherlands). Plants were cultivated in a growth chamber under an 8L : 16D cycle [200 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR)] light intensity at 21 ± 2 °C and 50-70 % relative humidity. After 10 to 14 days, seedlings with an equal size were transferred to individual pots (5 cm diameter) containing similar soil. Plants were watered three times a week. When plants were five to six weeks old, they were used for experiments. During the experiments all plants remained in the vegetative state.

Insects

The Diamondback moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) and the cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae) were reared on Brussels sprouts plants (*Brassica oleracea* cultivar *gemmifera* cv. Cyrus) in a climate room (21 ± 2 °C, 50-70 % relative humidity, 16L : 8D cycle). For performance studies, Parafilm (M, Bemis Company, Inc. in Neenah, WI, 54956) was presented as oviposition substrate in the rearing cages of adult moths that were allowed to oviposit for 24 h after which the Parafilm was removed from the cages. Naive neonate *Plutella* larvae hatched from the eggs after three days incubation time in a climate cabinet at 22 ± 2 °C with a 16L : 8D cycle.

Caterpillar performance

To assess the effect of aphid density on the performance of caterpillars, plants were infested at the same time with two naive neonate caterpillars and two different densities of aphids. *Arabidopsis* Col-0 (N = 15 replicates), *sid-2* mutant (N = 15 replicates) and *dde2-2* mutant (N = 15 replicates) plants were simultaneously infested with the *P. xylostella* larvae and (a) 5 adult aphids, 'low aphid density' or (b) 25 aphids of mixed life stages distributed over five fully expanded leaves, 'high aphid density'.
Control, *Arabidopsis* Col-0 (N = 15 replicates), *sid-2* mutant (N = 15 replicates), or *dde2-2* mutant plants (N = 15 replicates), were infested with two naive neonate *P. xylostella* larvae to allow for comparison with caterpillars on plants simultaneously infested with both aphids and caterpillars.

Individual plants were placed in cylindrical plastic containers (diameter 8 cm x height 14 cm), and covered with gauze cloth. Containers were randomly distributed in a tray (12-15 containers per tray). Trays were placed in a growth chamber with a 16L : 8D cycle [200 µmol m^{-2} s^{-1} PAR], at 21 ± 2 °C and 50-70 % relative humidity. After five days of feeding, the caterpillars were weighed using a microbalance. Caterpillars were subsequently allowed to feed on the plant until pupation. Pupae were collected, and placed in individual glass vials until adult eclosion. Adults were dried at 80 °C for two days and weighed on a microbalance (accuracy 1 µg; Sartorius AG, Göttingen, Germany). The sex of the adults was recorded.

RNA isolation and quantitative RT-PCR analysis

For gene expression analysis, wild-type *Arabidopsis* Col-0 plants were infested with only caterpillars, only aphids or a combination of both insects at two different aphid densities.

For the 'low aphid density' treatment, plants were simultaneously infested with five *B. brassicae* adults and two second-instar (L2) *P. xylostella* larvae, or five aphids (*B. brassicae* adults) or two L2 *P. xylostella* larvae. Plants that were not infested with caterpillars or aphids were used as control.

For the 'high aphid density' treatment, plants were simultaneously infested with 25 aphids (of mixed life stages distributed over five fully expanded leaves) and two L2 *P. xylostella* larvae, or 25 aphids (of mixed life stages distributed over five fully expanded leaves) or two L2 *P. xylostella* larvae. Plants that were not infested with caterpillars or aphids were used as control.

Individual plants were placed in plastic containers, and covered with gauze cloth and randomly distributed in a tray. Trays were placed in a growth chamber at a 16L : 8D cycle [200 µmol m^{-2} s^{-1} PAR] light intensity at 21 ± 2 °C and 50-70 % relative humidity.

Leaves damaged by caterpillar and/or aphid feeding were harvested at (a) 4 or 6 h, (b) 24 h, (c) 48 h or (d) 72 h since insect infestation. Insects were removed from the leaves before harvesting. For each treatment and time point, five biological replicates were performed each of which consisted of six leaves pooled from three different plants. Leaf samples were snap-frozen in liquid nitrogen and stored at -80 °C prior to analysis.

RNA was isolated from homogenised material with the RNeasy Plant Mini Kit (Qiagen) and treated with DNaseI (Invitrogen) following the manufacturer's instructions. The concentration of RNA obtained from the plant material was

adjusted to 1 µg/µl and subsequently transcribed to cDNA using iScript cDNA synthesis Kit (Bio-Rad).

Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Each reaction contained 12.5 µl SYBER Green Supermix (Bio-Rad), 5 µl cDNA and 10µM of a gene-specific primer pair in a total volume of 25 µl. For each reaction two technical replicates were performed and average values were used in the analyses. The following PCR program was used for all PCR reactions: 3 min 95 °C, followed by 40 cycles of 15 s at 95 °C, and 45 s at 60 °C. At the end of each qPCR, a melting curve analysis was performed. In these reactions, primers for the genes of interest, *PR-1* (At2g14610), *LOX2* (At3g45140), *VSP2* (At5g24770), *WRKY70* (At3g56400) and *MYC2* (At1g64280) were used.

Gene expression was calculated by using the geometric mean of threshold cycle (Ct) values (Vandesompele et al., 2002) from the reference genes *FBOX* and *EF1*-α (*ELONGATION FACTOR 1α*) with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

The statistical analysis of caterpillar performance data was carried out using SPSS v. 19.0 (SPSS Inc., Chicago, IL, USA). To test if there were differences in weight of caterpillars feeding on plants infested with caterpillars only and caterpillars feeding on plants also infested with aphids, a linear mixed model with treatment as fixed factor and individual plant as random factor. Significance of differences in adult dry weight was tested using a linear mixed model with treatment and sex of the insect as fixed factors and individual plant as random factor. The expression of genes between treatments and time points was tested using a generalized linear model with Poisson distribution and log link function in GenStat v. 16.0 (VSN International, Hemel Hempstead, UK). The factors treatment, time point and their interactions were included in the model. When the interaction between both factors was not significant, the overall differences between the four treatments are presented. Post-hoc comparisons between treatments were made using LSD tests.

Results

Effect of different aphid densities on caterpillar growth

To investigate the effect of aphid density on the performance of caterpillars, the growth of *P. xylostella* caterpillars was studied when feeding at the same time with aphids at either of the two different densities. Feeding by the caterpillars over a 5 d-period led to higher weight gain of caterpillars feeding simultaneously with aphids at a low density compared to caterpillars that fed on control plants without aphids ($F = 4.412$, $P = 0.045$; Figure 1A). When caterpillars were feeding simultaneously with a high aphid density, weight gain after 5 days was lower compared to caterpillars feeding alone ($F = 4.431$, $P = 0.044$; Figure 2A). These results indicate that the effect of aphid feeding on caterpillar growth depended on aphid density.

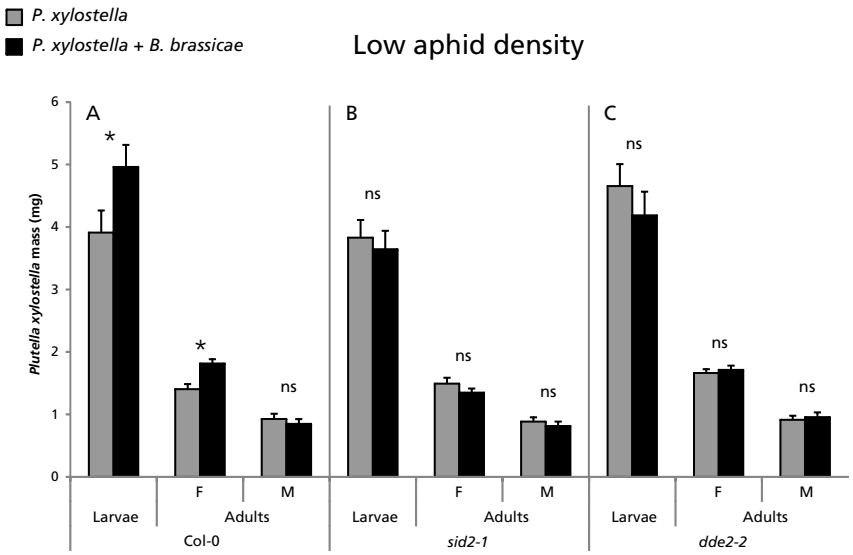

Figure 1. Body mass of *P. xylostella* larvae and female (F) and male (M) adults after feeding alone or simultaneously with *B. brassicae* aphids at a low density on *Arabidopsis* Col-0 or mutant *sid2-1* or mutant *dde2-2* plants. Fresh weight of larvae measured after five days and dry weight of female or male *P. xylostella* adults after they had been feeding alone (*P. xylostella*) or simultaneously (*P. xylostella + B. brassicae*) with a density of five aphids on *Arabidopsis* Col-0 plants (n=15) (A), *Arabidopsis* mutant *sid2-1* plants (n=15) (B) and on *Arabidopsis* mutant *dde2-2* plants (n=15) (C). Bars represent means ±SE. ns, not significant; * $P < 0.05$

To investigate whether aphid feeding interfered with caterpillar-induced defense responses through crosstalk between JA and SA signaling pathways, plants of the *sid2-1* mutant (a SA induction-deficient mutant) and of the *dde2-2* mutant

(a JA-deficient mutant) were infested with only caterpillars or a combination of caterpillars and a low or high aphid density. Growth of *P. xylostella* caterpillars was not affected on *sid2-1* or on *dde2-2* mutant plants: no significant differences in weight gain of caterpillars feeding during 5 d without or with aphids at a low density ($F = 0.205$, $P = 0.653$; Figure 1B and $F = 0.816$, $P = 0.374$; Figure 1C) or at a high density ($F = 0.008$, $P = 0.931$; Figure 2B and $F = 0.820$, $P = 0.373$; Figure 2C) were found. This indicates that intact SA and JA signaling are both required for the interference by aphids.

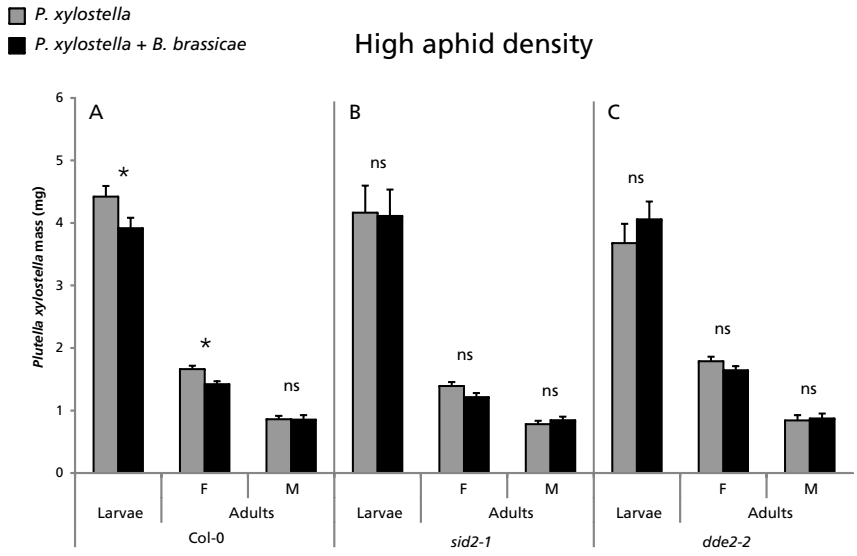

Figure 2. Body mass of *P. xylostella* larvae and female (F) and male (M) adults after feeding alone or simultaneously with *B. brassicae* aphids at a high density on *Arabidopsis* Col-0 or mutant *sid2-1* or mutant *dde2-2* plants. Fresh weight of larvae measured after five days and dry weight of female or male *P. xylostella* adults after they had been feeding alone (*P. xylostella*) or simultaneously (*P. xylostella* + *B. brassicae*) with a density of 25 aphids on *Arabidopsis* Col-0 plants (n=15) (A), *Arabidopsis* mutant *sid2-1* plants (n=15) (B) and on *Arabidopsis* mutant *dde2-2* plants (n=15) (C). Bars represent means ±SE. ns, not significant; * $P < 0.05$

On *Arabidopsis* Col-0 plants, there was a significant difference in weight of *Plutella* adults when the caterpillars had been feeding alone or with aphids at a low density ($F = 4.584$, $P = 0.040$), and this effect was dependent on the sex of the adult moths ($F = 10.201$, $P = 0.003$). However, only adult female moths showed an increased weight gain when they had been feeding in the presence of aphids at a low density compared to only caterpillars feeding ($F = 15.179$, $P = 0.000$; Figure 1A). Furthermore, the presence of aphids at a high density had a significant effect on the dry weight of the *Plutella* adults ($F = 4.727$, $P = 0.037$), which was

dependent on the sex of the adults (F = 4.321, P = 0.045). For adult females, weight gain was decreased when they had been feeding simultaneously with aphids at a high density compared to feeding alone (F = 10.935, P = 0.002; Figure 2A). The results on adult weight are similar to those of caterpillar growth over 5 days confirming that aphid induction affected caterpillar performance depending on the aphid density. As was also found for caterpillar growth, caterpillars that had been feeding alone or together with aphids at a low or high density on the *sid2* mutant (F = 1.857, P = 0.189; Figure 1B and F = 0.895, P = 0.356; Figure 2B, respectively) or on the *dde2-2* mutant (F = 0.434 , P = 0.517; Figure 1C and F = 0.583, P = 0.451; Figure 2C, respectively) showed no significant difference in adult dry weight. This confirms previous results that SA and JA signaling are involved in aphid interference with caterpillar-induced defenses.

Up-regulation of SA-responsive genes underlies the interaction between caterpillars and a low aphid density

To investigate if SA-JA crosstalk underlies the interference with caterpillar growth rate by aphids and if this interference is density-dependent we investigated the expression of known SA- and JA-responsive genes at different time points in *Arabidopsis* Col-0 plants induced by (a) caterpillars only, (b) aphids only or (c) a combination of both insects. In two separate experiments we studied this for plants with low and high aphid densities, while keeping caterpillar density the same.

Figure 3 shows the effect of low aphid density in the interaction with caterpillars on the expression levels of plant defense genes. Activation of the SA pathway is investigated by quantifying the expression of the transcription factor gene *WRKY70* and the marker gene *PR-1*. Expression of *WRKY70* and *PR-1* was significantly affected by treatment, time point and their interaction (Table 1A). Simultaneous feeding by caterpillars and aphids during 24 h and 48 h induced *WRKY70* expression to a significantly higher level compared to undamaged, aphid-infested or caterpillar-infested plants (Figure 3A).

Upon 24 h of simultaneous feeding by caterpillars and aphids, *PR-1* is strongly up-regulated showing that the interaction between both insects results in a synergistic effect on the expression of *PR-1* at this time point (Figure 3B). After 24 h, aphid feeding induced *PR-1* expression in contrast to *P. xylostella* which did not affect the expression of *PR-1* at that time point (Figure 3B). Interestingly, *P. xylostella* caterpillars also induced SA signaling because feeding resulted in significant higher *PR-1* gene expression compared to aphid feeding after 48 h.

Table 1. Effect of the factors treatment and time point and their interaction term on the expression level of *WRKY70*, *PR-1*, *MYC2* and *VSP2* in *Arabidopsis thaliana* after insect only and simultaneous feeding at a low aphid density (A) or a high aphid density (B). Bold typeface indicates significant terms (α=0.05).

A	Generalized Linear Model analysis of deviance table – Low aphid density								
	Factors						Interaction		
	Treatment			Time point			Treatment x Time point		
Gene	d.f.	deviance	*P*	d.f.	deviance	*P*	d.f.	deviance	*P*
WRKY70	3	2.44	**0.048**	3	6.57	**<0.001**	9	7.54	**0.007**
PR-1	3	237.41	**<0.001**	3	382.04	**<0.001**	9	184.76	**0.011**
MYC2	3	75.96	**<0.001**	3	35.93	**<0.001**	9	3.98	0.227
VSP2	3	1322.90	**<0.001**	3	562.47	**<0.001**	9	105.93	0.324
B	Generalized Linear Model analysis of deviance table – High aphid density								
	Factors						Interaction		
	Treatment			Time point			Treatment x Time point		
Gene	d.f.	deviance	*P*	d.f.	deviance	*P*	d.f.	deviance	*P*
WRKY70	3	2.13	**<0.001**	3	0.74	**0.043**	9	1.83	**0.022**
PR-1	3	1102.39	**<0.001**	3	1933.79	**<0.001**	9	592.89	**0.003**
MYC2	3	85.63	**<0.001**	3	84.89	**<0.001**	9	18.88	**<0.001**
VSP2	3	1782.69	**<0.001**	3	77.88	**0.031**	9	49.66	0.734

The main effect of treatment and time point on *MYC2* and *VSP2* expression levels was significant, however, there was no significant interaction between treatment and time point (Table 1A). Caterpillar feeding alone and simultaneous caterpillar and aphid feeding induced significantly higher *MYC2* and *VSP2* transcript levels compared to control plants and plants only infested by aphids. Transcript levels of *MYC2* and marker gene *VSP2* that are involved in JA-regulated responses were not significantly different between plants simultaneously induced by both insects or by caterpillars feeding alone at any time point (Figure 3C and 3D).

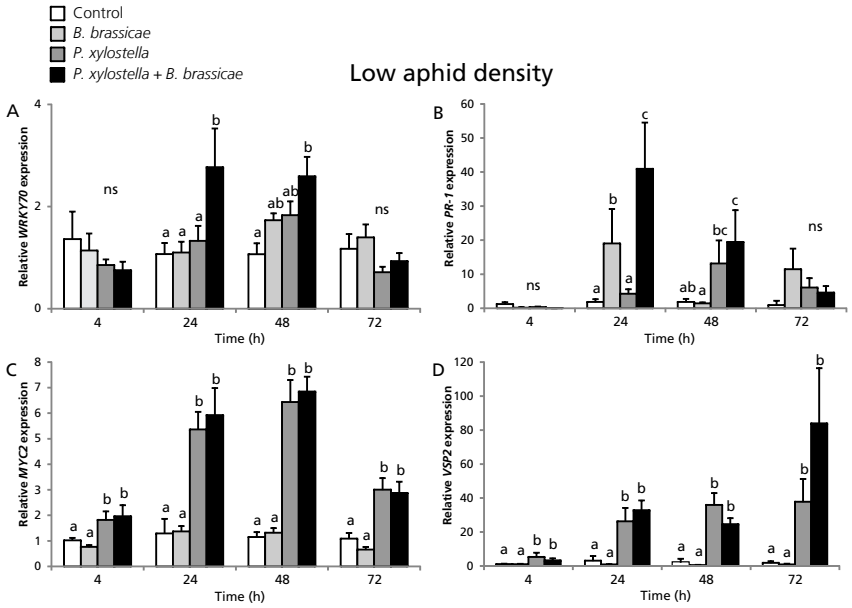

Figure 3. Gene expression in leaves of *Arabidopsis* Col-0 plants at 4, 24, 48 or 72 h after caterpillar only (*P. xylostella*), single aphid (*B. brassicae*), both aphid and caterpillar (*P. xylostella* + *B. brassicae*) and without feeding (Control). Relative expression of *WRKY70* (A), SA-responsive defense marker gene *PR-1* (B), *MYC2* (C), and JA-responsive defense marker gene *VSP2* (D). Bars represent means ±SE (n=5). Bars marked with different letters are significantly different (GLM, $P < 0.05$); ns, not significant. For *MYC2* and *VSP2* expression there was no interaction of the factors time and treatment, therefore treatment effect is presented.

SA-JA crosstalk is involved in the interaction between caterpillars and a high aphid density

Figure 4 shows the expression levels of defense genes in plants infested with caterpillars and a high aphid density. There were significant main and interaction effects of treatment and time point for *WRKY70* and *PR-1* expression levels (Table 1B). Expression of the transcription factor *WRKY70* was lower in plants induced by simultaneous feeding of both insects during 24 h compared to caterpillars feeding alone (Figure 4A). The expression level of *PR-1* was significantly lower in plants simultaneously induced by both insects compared to aphids only at 72 h (Figure 4B). Furthermore, as was also found for caterpillar feeding in the low aphid density study, plants induced with caterpillars activated significantly higher *PR-1* expression levels compared to plants induced with aphids only at 48 h.
Expression of *MYC2* was significantly affected by treatment and time point (Table 1B). Simultaneous feeding of both insects and caterpillar feeding alone resulted

in significant up-regulation of *MYC2* expression compared to control and aphid-infested plants. Significantly higher *MYC2* expression levels were found after 48 h in plants simultaneously induced by caterpillars and aphids. After 72 h of insect feeding, *MYC2* expression is lower in plants induced with caterpillars only, aphids only and induced with both caterpillars and aphids compared to control plants (Figure 4C). Treatment and time point had a significant effect on the expression of *VSP2*, however, there was no significant interaction between treatment and time point (Table 1B). Transcript levels of *VSP2* are significantly higher in plants infested with only caterpillars or in plants infested with both insects compared to undamaged and aphid-infested plants (Figure 4D).

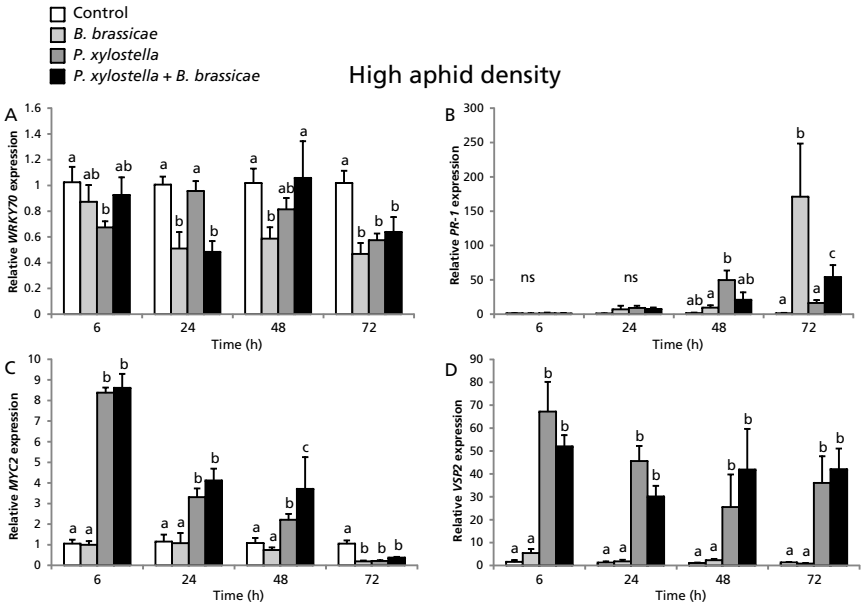

Figure 4. Gene expression in leaves of *Arabidopsis* Col-0 plants at 6, 24, 48 or 72 h after caterpillar only (*P. xylostella*), single aphid (*B. brassicae*), both aphid and caterpillar (*P. xylostella* + *B. brassicae*) and without feeding (Control). Relative expression of *WRKY70* (A), SA-responsive defense marker gene *PR-1* (B), *MYC2* (C), and JA-responsive defense marker gene *VSP2* (D). Bars represent means ±SE (n=5). Bars marked with different letters are significantly different (GLM, $P < 0.05$); ns, not significant. For *VSP2* expression there was no interaction of the factors time and treatment, therefore treatment effect is presented.

Discussion

During an attack, transcriptional plant responses of genes involved in the defense against *Plutella xylostella* caterpillars are entirely different from those to *Brevicoryne brassicae* aphids in *A. thaliana* plants (Bidart-Bouzat and Kliebenstein, 2011). Plant defense to phloem-sucking and leaf-chewing insects is regulated by the interaction between SA and JA signaling pathways (Soler et al., 2012; Zhang et al., 2013a; Thaler et al., 2012). In response to dual insect attack, SA-JA crosstalk may result in facilitation or suppression of caterpillar performance or caterpillar-induced indirect defense by the presence of phloem-feeding insects (Ali and Agrawal, 2014; Li et al., 2014; Rodriguez-Saona et al., 2005; Rodriguez-Saona et al., 2010; Soler et al., 2012; Thaler et al., 2012; Zhang et al., 2009; Zhang et al., 2013a). This has usually been investigated for a single density of the herbivores. However, herbivore densities are dynamic in time and this may affect the plant-mediated interaction between herbivores. Interference by the whitefly *Bemisia tabaci* (also a phloem-feeder) with indirect plant defenses induced by spider mites in Lima bean is density dependent (Zhang et al., 2009). A low *B. tabaci* density did not interfere with indirect plant defenses against spider mites whereas higher *B. tabaci* densities reduced indirect defenses, in terms of attraction of predators, against spider mites. Furthermore, Ponzio et al. (2014) showed that behavioural choices of the parasitoid *Cotesia glomerata* towards herbivore-induced plant volatiles were dependent on the aphid density infesting the plant. The parasitoid was less attracted to *Brassica nigra* plants infested with *B. brassicae* aphids at a high density compared to uninfested control plants, whereas the response of the parasitoid towards plants infested with a low aphid density compared to uninfested control plants was not significantly different. This indicates that aphids at a low or high density differentially influence the expression of indirect plant defense. Our present results add data on the effects on direct defense expression.

Here, we have investigated the effect of aphid density on the interference with defense responses against caterpillars. We show that direct plant defense against *P. xylostella* caterpillars in response to dual insect attack is dependent on the density of the additional herbivorous insect. At a density of five aphids per plant, the growth rate of *P. xylostella* was increased, whereas its growth rate was reduced on plants simultaneously infested with 25 aphids. Also, *P. xylostella* female adults reached higher adult body mass on plants infested with aphids at a low density compared to plants infested with a high aphid density.

Plant yield of the wild tobacco, *Nicotiana attenuata,* attacked by the aphid *Myzus*

persicae Sulz. was affected by aphid density (Donovan et al., 2012). Higher aphid densities reduced yield more compared to low aphid densities. Aphids at higher densities were found to induce higher SA levels in tobacco which probably led to a re-allocation of resources from growth to defense and thus a reduction in yield. Plant defenses in response to aphid feeding are mainly dependent on SA signaling, although also regulated by JA signaling. For instance, the performance of *B. brassicae* aphids was negatively affected on *fou2*, an *A. thaliana* JA-signaling mutant which has constitutively high JA concentrations (Kusnierczyk et al., 2011). Interestingly, caterpillars feeding on the *sid2* (deficient in SA production) and *dde-2* (deficient in JA production) mutants were not affected by the presence of aphids. Our results provide further support for the importance of SA- and JA-mediated defense responses in the aphid density-dependent interference with caterpillar-induced defenses. In transcriptomic analyses, we studied the expression of SA- and JA-inducible genes upon attack by a single species or two species simultaneously at different aphid densities. We show that SA-dependent defense genes are up-regulated in plants simultaneously attacked by caterpillars and aphids at a low density. In contrast, in plants simultaneously infested with caterpillars and aphids at a high density, SA-dependent defense genes are suppressed. Furthermore, simultaneous feeding by caterpillars and aphids at a high density induced the expression of JA-dependent defense genes more compared to plants attacked by either insect species separately.

It has been proposed that aphids manipulate plant defenses by suppressing JA-dependent defenses through SA-JA crosstalk by activating SA signaling (De Vos et al., 2007; Zhu-Salzman et al., 2004). Although this hypothesis of defense manipulation by aphids was only investigated in plants attacked by single aphid species (Mewis et al., 2005; Moran et al., 2002), it may suggest that the facilitation of caterpillar performance when feeding together with low aphid densities is established through aphids suppressing JA defenses by inducing SA-dependent responses. Simultaneous infestation with caterpillars and a low aphid density 24 h after feeding induced the expression of *WRKY70* transcription factor, an activator of SA-dependent defense genes and key-regulator of JA-SA crosstalk, compared to control plants and plants infested with a single species. Furthermore, aphids at low densities simultaneously feeding with caterpillars induced significantly higher levels of *PR-1* expression, an important marker gene of SA signaling, compared to insects feeding alone.

However, we did not detect differences in JA-inducible gene expression levels between caterpillar-infested plants or plants simultaneously infested with

caterpillars and aphids. Because the induction of SA occurs very locally, at the site of aphid feeding (De Vos et al. 2005), it is possible that at a low aphid density changes in transcript levels of JA-mediated defense genes are difficult to detect. Not only aphids but also *Plutella* caterpillars up-regulate *PR-1* expression when feeding alone on *Arabidopsis* plants. This result is consistent with that of Ehlting et al. (2008) who reported that *Arabidopsis* in response to 24 h of *Plutella* feeding up-regulated *PR*-genes. Consequently, by inducing *PR-1* expression caterpillars could interfere with JA-mediated defenses through SA-JA crosstalk. Diezel et al. (2009) found that the generalist *Spodoptera exigua* caterpillar activates SA-dependent responses while inducing low levels of JA in *Nicotiana attenuata*. In *A. thaliana,* herbivory of *Spodoptera* induced the expression of more SA-related genes compared to *Plutella* feeding (Bidart-Bouzat and Kliebenstein, 2011). Moreover, growth of *Spodoptera* caterpillars was increased on *Arabidopsis* plants treated with exogenous SA (Cipollini et al., 2004). Another generalist, the caterpillar *Helicoverpa zea*, was found to overcome plant defenses through salivary components. Glucose oxidase, present in the saliva of *Helicoverpa* caterpillars, induces SA signaling which leads to inhibition of JA signaling and eventually prevents the induction of nicotine in *Nicotiana tabacum* plants (Musser et al. 2005; Musser et al., 2002). These findings suggest that chewing herbivores could be able to manipulate crosstalk between SA and JA for their own benefit. However, it is not yet known if specialist caterpillars, like *P. xylostella*, actively manipulate plant defense signaling to stimulate ineffective SA-regulated defenses.

Growth rate of caterpillars was negatively affected when feeding simultaneously with aphids at a high density compared to caterpillars feeding alone. However, there were no differences found in performance between caterpillars feeding alone or simultaneously with aphids on *sid2* or on *dde2-2* mutant plants. This shows that the effect of aphid feeding is plant-mediated and that it does not reduce plant quality to such an extent that it affects caterpillar performance.

Performance of chewing herbivores is negatively affected when feeding on plants, due to induction of JA-mediated plant defense responses (De Vos et al., 2005; Ehlting et al., 2008; Heidel and Baldwin, 2004). This suggests that aphids at a high density feeding together with caterpillars trigger JA-mediated defenses, even more so compared to caterpillar-infested plants, in which SA signaling may play a role because caterpillar performance is not affected when feeding with aphids at a high density on the *sid2* mutant. Evidence for the involvement of the SA signaling pathway in the interference by aphids at a high density with induced

defenses against caterpillars is found in *WRKY70* transcription levels. Feeding by aphids and caterpillars results in low *WRKY70* expression compared to caterpillars feeding alone. Furthermore, *PR-1* expression is lower in plants simultaneously infested with both insects compared to aphids feeding alone.

Besides SA-mediated plant defenses, *B. brassicae* aphids also induce increased mRNA levels of the JA-responsive defense gene *PDF1.2* upon infestation (Moran et al., 2002), and other JA-dependent genes in *A. thaliana* ecotype Cvi plants (Kusnierczyk et al., 2007). By down-regulating *WRKY70* expression and late up-regulation of *PR-1* expression the plant activates JA-dependent defenses which could lead to a higher resistance against aphids and caterpillars. Interestingly, the expression of *MYC2*, a transcription factor involved in JA-dependent plant defense responses, was significantly higher in plants simultaneously attacked by aphids and caterpillars compared to caterpillar-infested plants 48 h after feeding started.

These results indicate that a lower expression level of *WRKY70* and of *PR-1* expression in plants simultaneously infested with both caterpillars and aphids, acting through SA-JA crosstalk, leads to significantly higher *MYC2* expression.

Crosstalk between defense signaling pathways plays an important role in defense responses against multiple insect attackers. Studies investigating crosstalk between SA and JA implicated *WRKY70* as a molecular player in this interaction. Li et al. (2004) showed that *WRKY70* overexpression in *Arabidopsis* resulted in suppression of JA-responsive gene expression. In addition, in response to *B. brassicae* infestation *Arabidopsis* up-regulated *WRKY* transcription factors (Kusnierczyk et al., 2008). Furthermore, *MYC2* is known to regulate crosstalk between the signaling pathways of JA and SA (Kazan and Manners, 2013). In *Arabidopsis,* Laurie-Berry et al. (2006) showed that the transcription factor *MYC2* negatively regulates the SA pathway because *myc2* mutants are more resistant against infection by *Pseudomonas syringae*, probably due to higher concentrations of SA. Therefore, interactions between aphids and caterpillars feeding on a plant at the same time could affect the timing and intensity of plant defense responses. For example, levels of the defense-related phytohormones JA and SA negatively correlated in milkweed plants when simultaneously attacked by monarch caterpillars *Danaus plexippus* and oleander aphids *Aphis nerii* (Ali and Agrawal, 2014). Our study helps to further resolve the role of SA-JA crosstalk in plant defense responses, and shows how SA- and JA-mediated defense genes are differentially expressed in *Arabidopsis* simultaneous attacked by caterpillars and aphids at different densities. Other recent studies on multiple

insect-plant interactions provide insight in how the sequence of insect infestation differentially affects plant defense responses (Soler et al., 2012; Zhang et al., 2009), and how one herbivore may interfere with the induced defense against another attacking herbivore (Schwartzberg et al., 2011; Zhang et al., 2013a). The outcome of defense responses is also dependent on the density of the attacking insect. Insect infestations at high densities could have different effects on the dynamics of expression of defense genes and their signaling role during multiple insect attack compared to low densities. Here, we show that aphids at different densities interfere in contrasting ways with caterpillar-induced defenses. Aphid densities likely interfere with crosstalk between SA and JA signal-transduction pathways, because caterpillars showed no significant difference in weight when feeding alone or simultaneously with aphids at different densities on the *sid2* or *dde2-2* mutants. Furthermore, *WRKY70* expression is differently affected upon infestation with aphids at low or high densities. Future studies addressing the effects of different aphid densities on the induction of phytohormones and secondary metabolites will be interesting to shed light on the downstream effects of changes in gene transcription. The present study contributes to a better understanding of the regulation of complex defense response networks during multiple insect attack of plants. In the interactions between plants and multiple attackers, insect density needs to be considered as an important factor in orchestrating the complex interactions between induced defense responses.

Acknowledgments

We thank Léon Westerd, André Gidding, Joop Woelke and Frans van Aggelen for rearing the insects; Nawaporn Onkokesung and Ana Pineda for discussions on gene expression analysis; and two anonymous reviewers for providing insightful comments. This work was supported by a TOP GO grant (grant number 854.10.010 to M.D.) from The Netherlands Organization for Scientific Research (NWO).

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Chapter 4

Regulation of terpenoid biosynthesis induced by caterpillars feeding on *Arabidopsis* and parasitoid attraction depend on the density of simultaneously attacking aphids

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Abstract

One of the biochemical responses of plants to insect attack is the production of volatile organic compounds that mediate an indirect defense of plants by attracting natural enemies of the attacking herbivores. Herbivore-induced plant volatiles (HIPVs) include terpenes that play key roles in the attraction of natural enemies. The synthesis of volatile terpenes is regulated by the jasmonic acid (JA) signaling pathway. When caterpillars and aphids simultaneously feed on the same plant, a common event in nature, crosstalk between phytohormonal signaling pathways may affect the regulation of indirect plant defenses which may be dependent on the density of the attacking insects. Consequently, this may alter the emission of HIPVs such that it can affect the attractiveness of the plant to parasitoids compared to single insect attack.

Here, we show that attraction of the parasitoid *Diadegma semiclausum* to volatiles emitted by *Arabidopsis thaliana* plants simultaneously infested by its host, *Plutella xylostella* caterpillars, and by non-host *Brevicoryne brassicae* aphids is influenced by the density of the feeding aphids. Biosynthesis and emission of (*E*,*E*)-α-farnesene could be linked to the observed preference of *D. semiclausum* parasitoids for the HIPV blend emitted by plants dually infested by caterpillars and aphids at a high density compared to dually infested plants with a low aphid density. Natural enemies such as *D. semiclausum* are important biological control agents and a better understanding of how plants regulate indirect defense mechanisms in response to multiple insect attack will enhance pest control strategies.

Keywords

Diadegma semiclausum, herbivore-induced plant volatiles, indirect defense, multiple attack, terpene synthase

Introduction

When facing an attack by herbivorous insects, plants respond through various structural, molecular and biochemical mechanisms of defense (Dicke and Van Poecke, 2002; Kessler and Baldwin, 2002; Schoonhoven et al., 2005; Howe and Jander, 2008). One of the biochemical responses to insect attack is the production of an array of volatile organic compounds that mediate indirect plant defense by attracting natural enemies of the attacking herbivores (Heil, 2008; Dicke and Baldwin, 2010). Herbivore-induced plant volatiles (HIPVs) include terpenes, green leaf volatiles (GLVs) and volatile methyl esters of phytohormones (*e.g.* methyl salicylate and methyl jasmonate) (Arimura et al., 2005; Mumm and Dicke, 2010). The emission of HIPVs can vary depending on the attacking herbivore species, density or developmental stage (Clavijo McCormick et al., 2012; Cai et al., 2013; Pashalidou et al., 2015), and parasitoids and predators use specific blends of HIPVs as cues to locate their herbivore hosts or prey feeding on the plant (De Boer et al., 2004; De Rijk et al., 2013). Therefore, the composition of the HIPV blend plays an important role in the attraction of natural enemies to herbivore-infested plants. For a few herbivore-plant-natural enemy systems particular volatile compounds have been found to affect the attraction of predators and parasitoids to a blend. For instance, both the volatile compounds 4,8,12-trimethyltrideca-1,3,7,11-tetraene ((*E*,*E*)-TMTT) and methyl salicylate (MeSA) increased the preference of the predatory mite *Phytoseiulus persimilis* for prey-infested Lima bean plants (De Boer et al., 2004). Herbivore-induced MeSA decreased attraction of the parasitoid *Diadegma semiclausum* (Snoeren et al., 2010). Other volatile compounds that could play key roles in the attraction of *D. semiclausum*, a parasitoid of the specialist caterpillar *Plutella xylostella* (Ohara et al., 2003), are the terpenes (*E*,*E*)-TMTT and (*E*,*E*)-α-farnesene. These volatiles are released as part of a blend by leaves of *Arabidopsis thaliana* in response to *P. xylostella* feeding (Herde et al., 2008; Huang et al., 2010).

Not only feeding by caterpillars, but also other types of herbivory induce the release of terpenes (Dicke et al., 2003). This is not unexpected because terpenes make up the largest class of plant volatiles and a family of terpene synthase (TPS) genes was identified in the genome of *A. thaliana* (Aubourg et al., 2002; Aharoni et al., 2003). Terpene synthases are important for terpene biosynthesis because these enzymes construct carbon skeletons for terpenes (Tholl and Lee, 2011). Terpene compounds are grouped based on the number of carbon atoms they contain, such as monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20) (Tholl and Lee, 2011). The monoterpene linalool was shown to be

produced by terpene synthase 10 (TPS10) in *A. thaliana* (Ginglinger et al., 2013). Furthermore, two closely related terpene synthase genes, *TPS02* and *TPS03*, are responsible for the formation of the monoterpene (*E*)-β-ocimene in *A. thaliana* ecotype Wassilewskija and the sesquiterpene (*E*,*E*)-α-farnesene in ecotype Col-0, respectively (Fäldt et al., 2003; Huang et al., 2010). The homoterpene volatile, (*E*,*E*)-TMTT, is produced in *A. thaliana* by the (*E*,*E*)-geranyllinalool synthase TPS04 (Herde et al., 2008).

The induction of plant volatile biosynthesis is regulated by two main plant defense signaling pathways, namely, the jasmonic acid (JA) and salicylic acid (SA) pathways (Ozawa et al., 2000; Arimura et al., 2005; Pieterse et al., 2012). The JA signaling pathway regulates the synthesis of volatile terpenes and GLVs (Dicke and Van Poecke, 2002), whereas the volatile MeSA is synthesized in plants from SA (Chen et al., 2003; Liu et al., 2010). It is commonly known that leaf-chewing herbivores, such as caterpillars, induce JA-mediated defense responses, while phloem-feeding insects, such as aphids, trigger the SA- as well as the JA-signaling pathway (De Vos et al., 2005; Stam et al., 2014). When caterpillars and aphids simultaneously feed on the same plant, a common event in nature, crosstalk between both signaling pathways may affect the regulation of plant defenses (Stam et al., 2014). In addition, it is known that phloem-feeding herbivores such as aphids induce lower levels of HIPV emission compared to chewing herbivores (Turlings et al., 1998; Rodriguez-Saona et al., 2003; Ali and Agrawal, 2012; Truong et al., 2014). Consequently, multiple herbivores feeding on plants interact indirectly through plant-mediated effects and this may alter emission of HIPVs (Rodriguez-Saona et al., 2003; Dicke et al., 2009; Ponzio et al., 2013) such that it can affect the attractiveness of the plant to predators and parasitoids compared to single insect attack (Zhang et al., 2009; Erb et al., 2010; Zhang et al., 2013). For example, herbivory by the phloem-feeding whitefly *Bemisia tabaci* interfered with indirect defenses of *A. thaliana* to *P. xylostella* caterpillars. For this interference by *B. tabaci* intact JA- and ethylene signaling was needed (Zhang et al., 2013). In addition, volatile compounds emitted by Lima bean plants simultaneously infested by the whitefly *B. tabaci* and the spider mite *Tetranychus urticae* were less attractive to predatory mites compared to *T. urticae*-infested plants. This effect on the attraction of the predatory mite was the result of a reduction in JA-mediated emission of (*E*)-β-ocimene (Zhang et al., 2009). The same study showed that plant-mediated interference of whiteflies with indirect defenses against spider mites was density dependent (Zhang et al., 2009).

Because herbivores are in close interaction with the plant they are feeding on, their abundance affects the regulation of plant defense responses and also

influences the outcome of multiple insect-plant interactions (Kroes et al., 2015). Thus, herbivore density is important in modulating interactions between plants and multiple insect attacks, and therefore may also influence the attractiveness of herbivore-infested plants to parasitoids and predators. However, still little is known about how multiple herbivory influences the composition of volatile blends and, thus, attractiveness of specific volatile compounds to parasitoids or predators (Ponzio et al., 2013).

This study addressed the effect of dual herbivory on induced indirect plant defenses, by analyzing volatile blend composition and expression of volatile biosynthesis genes of plants attacked by single or multiple herbivores. In addition, effects of differences in HIPV emission on the attraction of parasitoids was assessed. Here, we investigated indirect defense responses of *A. thaliana* wild-type plants and volatile biosynthesis mutants when dually infested by *P. xylostella* caterpillars (inducers of JA-mediated defense responses) and *Brevicoryne brassicae* aphids (inducers of SA- and JA-mediated defense responses) compared to plants infested by *P. xylostella* caterpillars alone. The plants were infested with a low or high aphid density to study density-dependent effects on plant-mediated insect interactions. We assessed the responses of the parasitoid *D. semiclausum* to HIPV emitted by dually infested plants and by caterpillar-infested plants. To better understand the underlying mechanisms of induced indirect defenses to multiple insect attack, the expression profile of genes important for the biosynthesis of plant volatiles and volatile compounds emitted were linked to the behavioral responses of the parasitoid.

Materials & Methods

Plants and growth conditions

Plants of *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) were used as wild-type. Seeds of a mutant defective in the biosynthesis of methyl salicylate, *bsmt1* (*benzoic acid and salicylic acid carboxyl methyltransferase1*; SALK_140496c; Snoeren et al. (2010)) were obtained from the European Arabidopsis Stock Centre (NASC, Nottingham, United Kingdom). Seeds of mutants defective in the biosynthesis of linalool, *tps10* (*terpene synthase10*; Ginglinger et al. (2013)) and of (*E*,*E*)-α-farnesene, *tps03* (*terpene synthase03*; Huang et al. (2010)) were kindly provided by Thierry Delatte (Laboratory of Plant Physiology, Wageningen University, The Netherlands) and Dorothea Tholl (Department of Biological Sciences, Virginia Polytechnic Institute and State University, USA). Seeds of both

wild-type and mutants were sown in autoclaved (80 °C for 4 h) potting soil (Lentse potgrond, Lent, The Netherlands). After 10 to 14 days of growth plants were transferred to individual pots (5 cm diameter) containing similar soil. Plants were cultivated in a growth chamber at 21 ± 2 °C under an 8L : 16D cycle [200 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) light intensity] and 60 % relative humidity (RH). Five-to-six weeks old plants were used in the experiments. During the experiments, all plants remained in the vegetative state.

Insects

Both the Cabbage aphid, *B. brassicae* L. (Hemiptera: Aphididae), and the Diamondback moth, *P. xylostella* L. (Lepidoptera: Yponomeutidae), were reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv Cyrus) at 22 ± 1 °C, 50-70 % RH, 16L : 8D cycle. The parasitoid *D. semiclausum* Hellén (Hymenoptera: Ichneumonidae) was reared on *P. xylostella* feeding on Brussels sprouts plants at 22 ± 1 °C, 60-70 % RH, 16L : 8D cycle. Newly emerged wasps were collected and kept in a cage supplemented with 6-10% sugar water solution in a climate cabinet at 21 ± 1 °C with a 16L : 8D cycle. In all experiments, naive, *i.e.* without oviposition experience, 3-10 days old mated female parasitoids were used.

Olfactory responses of *Diadegma semiclausum*

Preference of *D. semiclausum* parasitoids was analyzed in a dual-choice test performed in a Y-tube olfactometer. The Y-tube olfactometer consisted of two 5 L glass jars which were each connected to one arm of a glass Y-tube. Incoming charcoal-filtered compressed air regulated at a flow of 2 l min^{-1} was led into each of the two glass jars containing an odor source (four *A. thaliana* plants). Prior to placing a plant in one of the jars, the pot of the plant was carefully wrapped in aluminium foil.

At the start of the behavioral assay, a single female parasitoid was released at the base of the Y-tube. Behavior of *D. semiclausum* was observed in the Y-tube olfactometer for 10 min and its choice for either odor source was recorded when the parasitoid spent at least 15 s beyond a line marked 2 cm from the end of each Y-tube arm. Parasitoids that did not choose within the observation period were excluded from the statistical analysis. After five parasitoids were tested, the position of the odor sources was exchanged to exclude positional bias in the set-up. In total 4 sets of plants and 45-60 parasitoids were tested per combination of odor source.

As odor source, four *A. thaliana* plants were subjected to one of the following treatments:
1. Uninfested control (Undamaged)
2. Infested with two second-instar (L2) *P. xylostella* caterpillars (indicated as '*P. xylostella*' infestations)
3. Simultaneously infested with 5 adult *B. brassicae* aphids, 'low density'; and two *P. xylostella* L2 caterpillars (indicated as 'dual' infestations)
4. Simultaneously infested with 25 adult *B. brassicae* aphids, 'high density'; and two *P. xylostella* L2 caterpillars (indicated as 'dual' infestations)

Insects were allowed to feed freely on the plants. Individual plants were placed in a plastic container (diameter 8 cm x height 14 cm), covered with gauze cloth and closed with elastic bands. Containers were randomly distributed in a tray (12-15 containers per tray). Trays were placed in a growth chamber with a 16L : 8D cycle [200 µmol m^{-2} s^{-1} PAR], at 21 ± 2 °C and 50-70% RH. Three days after infestation, plants were used in the behavioral assay. Additionally, after each assay, *P. xylostella* caterpillars were removed from the plants and individually weighed on a microbalance (accuracy 1 µg; CP2P, Sartorius AG, Göttingen, Germany). In four different experiments, the behavioral response of *D. semiclausum* was investigated to the HIPV blend emitted by Col-0 plants, and *tps03*, *tps10* or *bsmt1* mutants.

Gene expression analysis

To link behavioral responses of *D. semiclausum* to the transcription of genes important for the biosynthesis of plant volatiles, we additionally performed a gene-expression analysis on Col-0 plants, *tps03, tps10* and *bsmt1* mutants used to assess parasitoid preference. Before tissue collection, insects were removed from the plants with a fine brush. For each treatment, six leaves pooled from four different plants used as odor source in the Y-tube behavioral assay were pooled to obtain one biological replicate. In total, four biological replicates per genotype per treatment were used. Leaf tissue was snap-frozen in liquid nitrogen and stored at -80 °C prior to analysis.

Finely-ground, frozen plant leaf tissue was used for isolation of total RNA with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Total RNA samples were treated with DNase (Qiagen, Hilden, Germany). With the help of the iScript cDNA synthesis Kit (Bio-Rad), cDNA was synthesised from 1 µg RNA. Quantitative RT-PCR analysis was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Each reaction was performed in a total volume of 25 µl containing

12.5 µl SYBR Green Supermix (Bio-Rad), 5 µl cDNA and 1 µl of 10 µM forward and reverse gene-specific primer pair. For each reaction, two technical replicates were performed and average values were used in the analyses. The studied genes were the terpene synthase (TPS) genes *TPS03* (At4g16740), *TPS04* (At1g61120) and *TPS10* (At2g24210), the salicylic acid methyl transferase gene *BSMT1* (At3g11480) and the two reference genes *ELONGATION FACTOR 1α* (*EF1-α*) (At5g60390) and *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*) (At3g04120). The following thermal profile was used for reactions with *TPS03*, *TPS04* and *BSMT1*: 3 min 95°C, followed by 40 cycles of 15 s at 95 °C, and 45 s at 60 °C. For reactions with *TPS10* thermal conditions consisted of 3 min 95°C, followed by 40 cycles of 15 s at 95 °C, and 45 s at 62 °C. The two reference genes, *GAPDH* and *EF1-α*, were carefully selected after evaluating their expression stability by calculating the geNorm value and coefficient of variation (CV) (qbase+ v. 2.6.1, Biogazelle; Hellemans et al., 2007). Relative expression for each tested gene was calculated by using the geometric mean of threshold cycle (Ct) values (Vandesompele et al., 2002) from the two reference genes with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Headspace collection

Plant volatiles were collected from four *A. thaliana* Col-0 plants subjected to one of the four treatments as described in the 'Olfactory responses of *Diadegma semiclausum*' section above. For each treatment, 7 replicates were sampled. The pots containing the four plants were carefully wrapped in aluminium foil and placed in a clean 5 L glass jar. The jars were sealed with a viton-lined glass lid with an air in- and out-let. Volatile control samples were collected from empty glass jars and from aluminium-wrapped pots filled with soil in order to correct for non-plant related volatiles. Prior to volatile collection, the jars were ventilated for 30 min using charcoal-filtered compressed air. Plant volatiles were collected on 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) in a stainless steel cartridge by drawing air out from the jars using an external pump at 200 ml min^{-1} for 6 h. Immediately after each volatile collection, insects were removed from the plants and plant shoots of each treatment were pooled and weighed on an analytical balance (accuracy 0.1 mg; Mettler Toledo ML54/01). The Tenax TA cartridges were dry-purged under a stream of nitrogen (N_2, 50 ml min^{-1}) for 10 min and stored at room temperature (22 ± 2 °C) until analysis.

Chemical analysis of volatiles

Plant volatiles were identified and quantified as described by Pangesti et al. (2015). Separation and detection of plant volatiles was done using a Thermo Trace Ultra gas chromatograph (GC) coupled to a Thermo Trace DSQ quadrupole mass spectrometer (MS) (Thermo Fisher Scientific, Waltham, USA). The volatiles were thermally released from the Tenax TA cartridges at 250 °C for 10 min with a helium flow of 20 ml min^{-1} on an Ultra 50:50 thermal desorption unit (Markes, Llantrisant, UK), while focused on a cold sorbent trap at 0 °C (Unity, Markes). After completion of the desorption process, volatile compounds were released from the cold trap by ballistic heating at 40 °C s^{-1} to 280 °C, which was maintained for 10 min and were then transferred in a splitless mode to an analytical column [(ZB-5MSi; 30 m x 0.25 mm i.d. x 0.25 µm film thickness with 5 m built-in guard column (Phenomenex, Torrance, CA, USA)] situated inside the GC oven. The temperature of the GC oven was initially held at 40 °C for 2 min, which was then raised at 10 °C min^{-1} to a final temperature of 280 °C and held for 4 min under a helium flow of 1 ml min^{-1}. The DSQ MS was operated in a scan mode with 35 – 350 amu mass range at 5.38 scans s^{-1} and spectra were recorded in electron impact ionisation (EI) mode at 70eV. MS transfer line and ion source were set to 275 and 250 °C, respectively. Volatile compounds were tentatively identified by comparison of mass spectra with those in NIST 2005 and the Wageningen Mass Spectral Database of Natural Products MS libraries, as well as using experimentally obtained linear retention indices (LRI).

Statistical analysis

To determine whether parasitoid preferences and response rates differed between the various odor sources, data on olfactory responses of *D. semiclausum* were analyzed using a χ^2-test in SPSS v. 22.0 (SPSS Inc., Chicago, IL, USA) for each choice situation tested. In addition, data were analyzed using a generalized linear model (GLM) with Poisson distribution and log link function in GenStat v. 17 (VSN International, Hemel Hempstead, UK) to compare choice distributions between choice situations. Genotype and treatment combination (*i.e.* undamaged plants tested in the Y-tube olfactometer against *P. xylostella*-damaged plants or plants infested by both *P. xylostella* and a low density of five aphids per plant (hereafter abbreviated as Dual LD for Dual Low Density) tested against plants infested by both *P. xylostella* and a high density of 25 aphids per plant (abbreviated as Dual HD for Dual High Density)) and the interaction genotype x treatment combination were included as fixed factors for data on proportion of

responsive or non-responsive wasps. In the choice assays involving undamaged plants, the number of wasps choosing the *P. xylostella*-infested plants out of the total number of responding wasps was entered as the response variable. In the choice assays between Dual LD versus Dual HD, the number of wasps choosing the Dual HD plants out of the total number of responding wasps was entered as the response variable. The dispersion parameter was estimated to account for residual variance. Post-hoc comparisons for proportion of responsive or non-responsive wasps were analyzed with an LSD test.

Fisher's exact test (two-tailed) was used to determine whether parasitoid preferences were distributed identically across different days on which the tests were repeated. After each behavioral bioassay, we tested if there were differences in weight of *P. xylostella* caterpillars feeding alone or simultaneously with aphids at low or high density on plants. Data of *P. xylostella* larval weight were analyzed with a linear mixed model with treatment as fixed factor and experimental group (*i.e.* the four *A. thaliana* plants subjected to one of treatments used in the behavioral bioassay) as random factor. Effect of treatment on plant shoot fresh weight was analyzed with an independent samples t-test. The statistical analysis of *P. xylostella* larval weight and plant shoot weight was carried out using SPSS v. 22.0 (SPSS Inc., Chicago, IL, USA).

The expression of genes and the quantity of each volatile emitted by plants on which caterpillars were feeding alone or simultaneously with aphids at either density or left undamaged were compared using a GLM with Poisson distribution and log link function in GenStat v. 17.0 (VSN International, Hemel Hempstead, UK). The factor treatment was included in the model as fixed factor. The dispersion parameter was estimated to account for residual variance. Post-hoc comparisons for gene expression and volatile data were analyzed with an LSD test. Data on volatile emission were also investigated by discriminant analysis. The quantified peak areas of individual volatile compounds were divided by plant shoot fresh mass, log-transformed, univariate scaled and mean-centred prior to subjecting the data to a multivariate data analysis: orthogonal projection to latent structures discriminant analysis (OPLS-DA) using SIMCA-P+ version 14.0 statistical software (Umetrics AB, Umeå, Sweden). The analysis determines whether samples from different treatment groups can be separated on the basis of quantitative and qualitative differences in their volatile blends. The results of the analysis are visualized in score and loading plots. The score plot identifies patterns that discriminate between the sample groups according to the two given model components of OPLS-DA, *i.e.* the predictive and orthogonal component. The

predictive component corresponds to variation between the sample treatments, whereas the orthogonal component corresponds to within sample variation. The loading plot displays the contribution and variable importance in the projection (VIP) of each volatile compound for the discrimination between the sample groups. Volatile compounds with VIP > 1 are considered most influential in the model (Eriksson et al., 2013). Various (pair-wise) OPLS-DA analyses were conducted on the volatile blends of the different treatment groups. The quality of each OPLS-DA model was evaluated using the parameter R^2X, which is used to assess the stability of the model (providing a quantitative measure of the explained variation) and indicates goodness of fit (Eriksson et al., 2013).

Results

Olfactory responses of the caterpillar parasitoid *Diadegma semiclausum* to HIPV blends

Parasitoid preference was studied for caterpillar-infested versus uninfested Col-0 plants and mutants impaired in the biosynthesis of linalool (synthesized by TPS10), MeSA (synthesized by BSMT1) and (*E*,*E*)-α-farnesene (synthesized by TPS03). Female *D. semiclausum* parasitoids preferred volatiles emitted by *P. xylostella*-infested plants of Col-0 wildtype, as well as *tps03* and *bsmt1* mutants over those from undamaged Col-0, *tps03* or *bsmt1* plants (Figure 1A; χ^2-test, Col-0: $\chi^2 = 4.261$, $P = 0.039$; *tps03:* $\chi^2 = 5.828$, $P = 0.016$; *bsmt1*: $\chi^2 = 5.769$, $P = 0.016$). Parasitoids did not discriminate between *P. xylostella*-infested *tps10* mutants and undamaged *tps10* mutants (Figure 1A; χ^2-test, $\chi^2 = 2.462$, $P = 0.117$).
Density-dependent effects of *B. brassicae* aphids on parasitoid attraction was also investigated for Col-0 plants and *tps03*, *tps10* or *bsmt1* mutants. Interestingly, *D. semiclausum* preferred volatiles emitted by Col-0 plants infested by caterpillars and aphids at high aphid density (Dual HD) over volatiles emitted by plants infested by caterpillars and aphids at low density (Dual LD) (Figure 1B; χ^2-test, $\chi^2 = 5.233$, $P = 0.022$). The opposite pattern was recorded for *tps03* plants: parasitoids significantly preferred the volatile blend from Dual LD *tps03* mutants over those from Dual HD *tps03* mutants (Figure 1B; χ^2-test, $\chi^2 = 5.121$, $P = 0.024$). However, wasps did not discriminate between the volatile blend from Dual LD or Dual HD *tps10* and *bsmt1* mutants (Figure 1B; χ^2-test, *tps10*; $\chi^2 = 0.641$, $P = 0.423$; *bsmt1*: $\chi^2 = 0.714$, $P = 0.398$).
Presence of *B. brassicae* at low (LD) or high (HD) density did not interfere with *D. semiclausum* response to volatiles from *P. xylostella*-infested plants, since

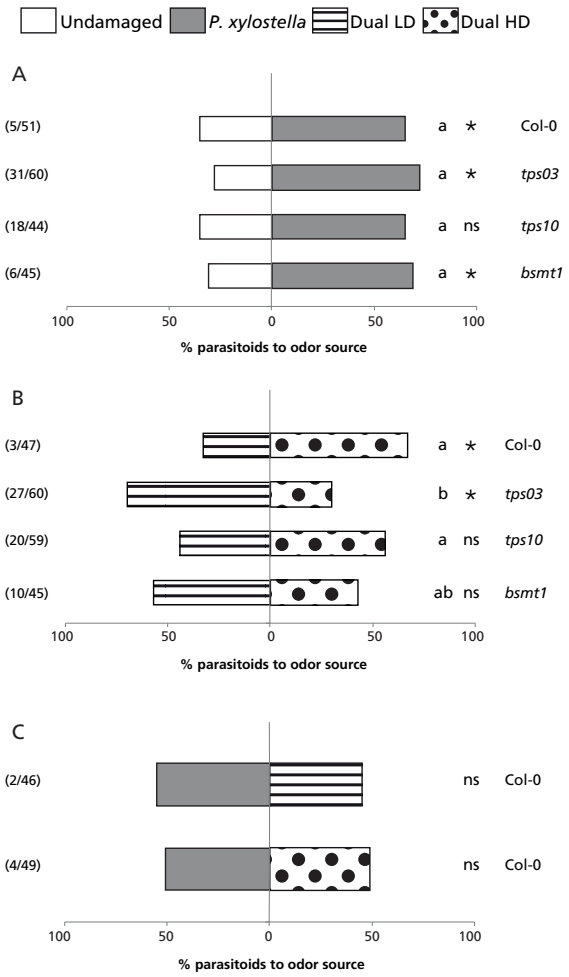

Figure 1. Preference of *D. semiclausum* in a Y-tube olfactometer to volatile blends emitted by *Arabidopsis* Col-0 wild-type, *tps03*, *tps10* or *bsmt1* mutants after three days of insect infestation. Undamaged plants were tested against plants infested by two L2 *P. xylostella* caterpillars (A), plants dually infested by caterpillars and a low density of five *B. brassicae* aphids (Dual LD) were tested against plants dually infested by caterpillars and a high density of 25 *B. brassicae* aphids (Dual HD) (B), or Col-0 plants infested by caterpillars were tested against Col-0 plants dually infested by *P. xylostella* caterpillars and a low aphid density (C), or Col-0 plants infested by caterpillars were tested against plants dually infested by *P. xylostella* caterpillars and aphids at a high density (C). Each bar represents the percentage of wasps choosing for each of the two odor sources, which consisted of four plants per treatment. For each pair-wise comparison, 3-4 sets of plants were tested on different days. An asterisk indicates a significant preference within a dual-choice test: ns, not significant; asterisk, $P < 0.05$ (χ2-test). Parasitoid preference that is significantly different between the different genotypes is indicated with different letters (GLM, $P < 0.05$). Numbers in parentheses represent number of non-responsive wasps and total number of tested wasps, respectively.

parasitoids did not discriminate between the volatile blend from Col-0 plants infested by caterpillars and the volatile blend from Dual LD or Dual HD Col-0 plants (Figure 1C; χ^2-test, Dual LD: $\chi^2 = 0.364$, $P = 0.546$; Dual HD: $\chi^2 = 0.022$, $P = 0.881$).

To further investigate the role of linalool, MeSA and (*E*,*E*)-α-farnesene in mediating parasitoid preference and responsiveness, we compared parasitoid behavior in response to herbivore-infested Col-0 plants to those in response to herbivore-infested volatile biosynthesis mutants. Parasitoid preference was influenced by the different genotypes and treatment combinations tested (Table 1; GLM).

Table 1. Statistical analysis of proportion of responsive *Diadegma semiclausum* parasitoids to volatiles emitted by *Arabidopsis* wild-type Col-0 and mutants *tps10*, *bsmt1* and *tps03* three days after single *Plutella xylostella* infestation, dual *Plutella xylostella* and a low (LD, 5 aphids) or high (HD, 25 aphids) *Brevicoryne brassicae* density infestation and without infestation (undamaged). Generalized Linear Model deviance table for effect of genotype and treatment combination (e.*g.* undamaged versus *P. xylostella* and Dual LD versus Dual HD). Bold number indicate significant effects ($P < 0.05$).

	Factor				Interaction	
	Genotype (1)		Treatment combination (2)		1 x 2	
	d.f. = 3		d.f. = 1		d.f. = 3	
	deviance	*P*	deviance	*P*	deviance	*P*
% responsive wasps	10.50	0.339	63.36	**<0.001**	41.67	**0.021**

There were no significant differences between the different genotypes for the proportion of parasitoids that preferred *P. xylostella*-infested plants over uninfested plants (Figure 1A). Interestingly, volatiles emitted by Dual HD *tps03* mutants significantly affected parasitoid preference compared to Dual HD Col-0 plants (Figure 1B). Preference of the wasps for the volatile blend from Dual HD over Dual LD Col-0 plants was also found for *tps10* or *bsmt1* mutants for the same treatment combination.

Table 2. Statistical analysis of proportion non-responsive *Diadegma semiclausum* parasitoids to volatiles emitted by *Arabidopsis* wild-type Col-0 and mutants *tps10*, *bsmt1* and *tps03* three days after single *Plutella xylostella* infestation, dual *Plutella xylostella* and a low (LD, 5 aphids) or high (HD, 25 aphids) *Brevicoryne brassicae* density infestation and without infestation (undamaged). Generalized Linear Model deviance table for effect of genotype and treatment combination (e.*g.* undamaged versus *P. xylostella* and Dual LD versus Dual HD). Bold numbers indicate significant effects ($P < 0.05$).

	Factor				Interaction	
	Genotype (1)		Treatment combination (2)		1 x 2	
	d.f. = 3		d.f. = 1		d.f. = 3	
	deviance	P	deviance	P	deviance	P
% non-responsive wasps	263.45	**<0.001**	1.30	0.531	10.84	0.361

Analysis of the responsiveness of wasps towards volatile blends emitted by either Col-0 plants or volatile biosynthesis mutants, showed an effect of plant genotype but no effect of the treatment combinations offered (*i.e.* undamaged versus *P. xylostella* or Dual LD versus Dual HD) (Table 2; GLM). Volatiles emitted by *tps10*, *tps03* and *bsmt1* mutants increased the percentage of wasps that did not make a choice compared to Col-0 plants.

Thus, blocking the biosynthesis of linalool, (*E*,*E*)-α-farnesene and MeSA, does not influence preference of *D. semiclausum* parasitoids for plants infested by *P. xylostella* caterpillars versus uninfested plants. On the other hand, mutations in *TPS03, TPS10* and *BSMT1* reduce the responsiveness of the wasps. In addition, (*E*,*E*)-α-farnesene is required for the density-dependent effect on attraction of parasitoids to plants infested by both caterpillars and aphids.

Preference of *D. semiclausum* parasitoids was not influenced by the day on which the experiments were performed for Col-0 plants and *tps10*, *bsmt1* or *tps03* mutants (Fisher's exact test, $P > 0.05$). Furthermore, caterpillar body mass reached similar values when feeding on Col-0 plants, *tps10*, *bsmt1* or *tps03* mutants (Supplementary materials Figure S1; LMM, $P > 0.1$) tested during the Y-tube olfactometer bioassays.

Plant volatile emission

Emission of volatiles by undamaged plants, plants infested by *P. xylostella* only, and plants dually infested by caterpillars plus a low or high aphid density was analyzed to study if differences in volatile profile between plant treatments could explain the observed differences in parasitoid preference.

In total, 41 different volatile compounds were detected in the headspace of all

treatments (Supplementary materials Table S1). An OPLS-DA model comparing headspace samples from all four treatments of undamaged plants, *P. xylostella*-infested plants, dually infested by caterpillars and a low aphid density (Dual LD) and caterpillars plus a high aphid density (Dual HD) showed differences in volatile blends based on the presence or absence of herbivores.
The first two components of the OPLS-DA, *i.e.* the predictive and orthogonal component, are plotted in the model (Figure 2A).

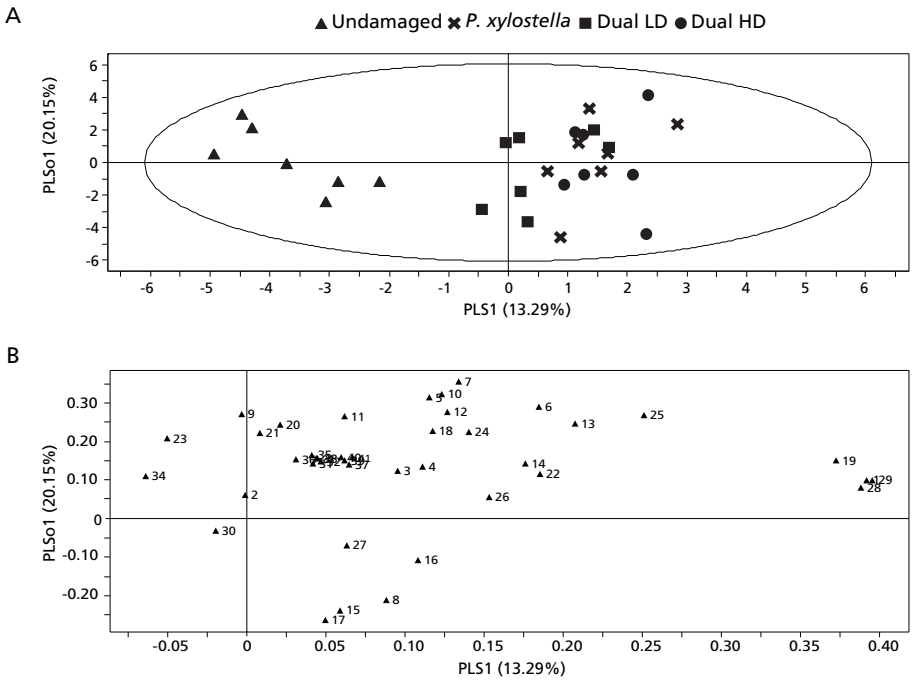

Figure 2. Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) of volatile compounds emitted by *Arabidopsis* wild-type Col-0 plants after three days of insect infestation. Plants were infested by *P. xylostella* alone, dually infested by *P. xylostella* and *B. brassicae* (Dual) or left undamaged. Plants were infested with either a low (LD) or a high (HD) density of *B. brassicae* aphids. (A) Score plot displaying grouping pattern according to the first two model components and the Hotelling's ellipse of the 95% confidence interval for the observations. Each point represents one sample (n = 7 replicates). The OPLS-DA resulted in a model with one significant predictive and two significant orthogonal components with $R^2X = 0.639$. (B) Loading plot of the first two components of OPLS-DA, showing the contribution of each volatile compound to the separation of the four treatments. Numbers refer to the volatile compounds listed in Table S1.

The predictive component explained 13.29 % of the variance, while 20.15 % was explained by the first of two orthogonal components. A group of eight compounds contributed most strongly to the model (VIP > 1), indicating that

these compounds contributed most to the difference between the volatile blends (Supplementary materials Table S2). Based on the three highest VIP-values, 1-penten-3-ol, (*E*,*E)*-TMTT and (*E*,*E*)-α-farnesene influenced the separation of undamaged and herbivore-infested plants the most (Figure 2B). These three compounds were emitted in significantly higher amounts by herbivore-infested plants than by undamaged plants (Supplementary materials Table S1).
Pair-wise comparison by OPLS-DA for volatiles emitted by undamaged plants and plants infested by *P. xylostella* caterpillars shows a clear separation based on the presence or absence of *P. xylostella* caterpillars. The first two components of the OPLS-DA are plotted in the model (Figure 3A).

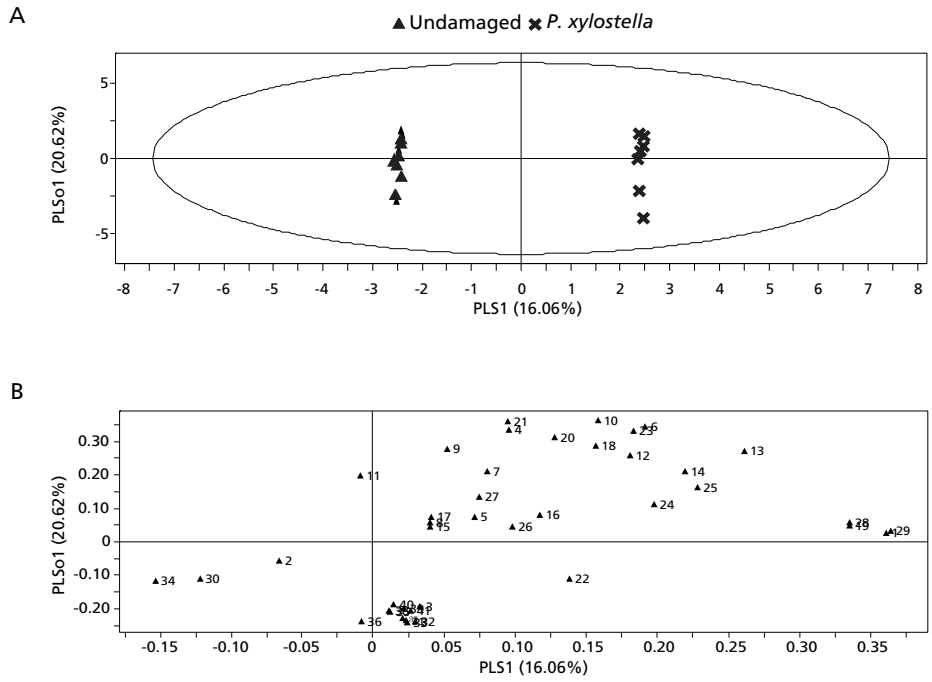

Figure 3. Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) of volatile compounds emitted by *Arabidopsis* wild-type Col-0 plants after three days of insect infestation. Plants were infested by *P. xylostella* caterpillars or left undamaged. (A) Score plot displaying grouping pattern of samples according to the first two model components and the Hotelling's ellipse of the 95% confidence interval for the observations. Each point represents one sample (n = 7 replicates). The OPLS-DA resulted in a model with one significant predictive and seven significant orthogonal components with $R^2X = 0.908$. (B) Loading plot of the first two components of OPLS-DA, showing contribution of each volatile compound to the separation of the two treatments. Numbers refer to the volatile compounds listed in Table S1.

The predictive component explained 16.06 % of the variability, while 20.62 % was explained by the first of seven orthogonal components. A group of 12 plant volatile compounds contributed most strongly to the model (VIP > 1), indicating that these compounds contributed to the difference between the volatile blends (Supplementary materials Table S2). Based on the four highest VIP-values, (*E*,*E)*-TMTT, 1-penten-3-ol, (*E*,*E*)-α-farnesene and MeSA influenced the separation of volatile blends from undamaged and caterpillar-infested plants the most (Figure 3B). These four compounds were emitted in significantly higher amounts by caterpillar-infested plants than by undamaged plants (Supplementary materials Table S1).

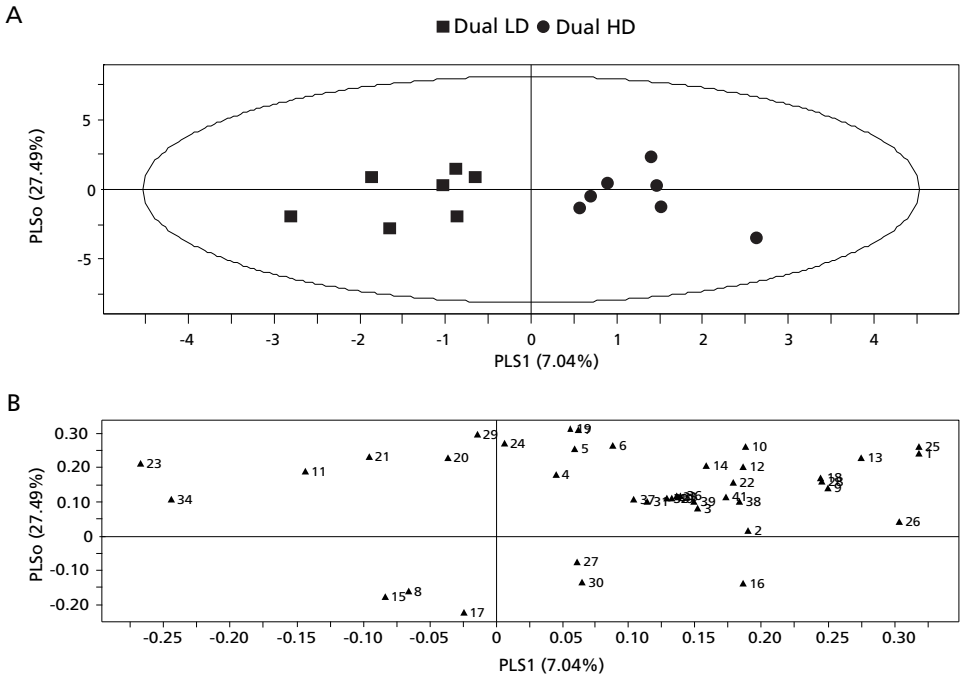

Figure 4. Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) of volatile compounds emitted by *Arabidopsis* wild-type Col-0 plants after three days of insect infestation. Plants were dually infested by *P. xylostella* and a low *B. brassicae* density (Dual LD, 5 aphids) or by *P. xylostella* and a high *B. brassicae* density (Dual HD, 25 aphids). (A) Score plot displaying grouping pattern according to the first two model components and the Hotelling's ellipse of the 95% confidence interval for the observations. Each point represents one sample (n = 7 replicates). The OPLS-DA resulted in a model with one significant predictive and two significant orthogonal components with $R^2X = 0.620$. (B) Loading plot of the first two components of OPLS-DA, showing contribution of each volatile compound to the separation of the two treatments. Numbers refer to the volatile compounds listed in Table S1.

An OPLS-DA model including volatiles emitted by plants dually infested by caterpillars and a low aphid density and plants dually infested by caterpillars and a high aphid density showed a clear separation between the two treatments. The first two components of the OPLS-DA model are plotted in the model (Figure 4A) and explain 34.53 % of the total variance. A group of 13 plant volatile compounds contributed most strongly to the model (VIP > 1) (Supplementary materials Table S2). Based on the three highest VIP-values, 1-penten-3-ol, (*E*,*E*)-α-farnesene and linalool influenced the separation of the two treatments the most (Figure 4B). Volatile blends emitted by plants infested by *P. xylostella* caterpillars and plants dually infested by caterpillars and *B. brassicae* aphids at low or high density were not separated by OPLS-DA. After each collection of volatile compounds plant shoot fresh weight was measured. There was no effect of insect infestation on plant biomass (Supplementary materials Figure S2; ANOVA , $P > 0.1$).

Transcriptional analysis of *TPS03*, *TPS04*, *TPS10* and *BSMT1*

To explain the observed HIPV-profiles, transcript levels of genes important for their biosynthesis, *i.e. TPS03*, *TPS04* and *TPS10* in the terpenoid biosynthesis pathway, and *BSMT1* in the methyl salicylate biosynthesis pathway, were analyzed in Col-0 plants and *tps10, bsmt1* and *tps03* mutants used in the Y-tube behavioral bioassays.

Expression of *TPS03*, *TPS10* and *BSMT1* was verified in the mutants *tps03*, *tps10* and *bsmt1*, respectively. Caterpillar-induced expression of *TPS03*, *TPS10* and *BSMT1* was severely reduced in their corresponding mutants when compared with Col-0 wild-type plants (Supplementary materials Figure S3; GLM, $P < 0.02$). There was a significant effect of treatment on the expression of *TPS03* and *TPS10* in Col-0 plants, of *TPS03* and *BSMT1* in *tps10* mutants, of *TPS10* in *bsmt1* mutants and of *TPS04*, *TPS10* and *BSMT1* in *tps03* mutants (Table 3).However, due to variation in *BSMT1* expression level within treatment type, no significant differences between treatments for *BSMT1* expression level were found for Col-0 plants and *tps10* mutants (Figure 5).

In Col-0 plants, feeding by caterpillars plus aphids at a high density induced *TPS03* expression to a higher lever compared to simultaneous feeding of caterpillars and aphids at low density (Figure 5A).This shows that aphids influence *TPS03* expression level in a density dependent manner. Interestingly, no significant difference was found between *TPS03* expression levels in *tps10* and *bsmt1* mutants infested by caterpillars plus a low or high aphid density (Figure 5A). Furthermore, expression levels of *TPS03* in *P. xylostella*-infested *tps10* and *bsmt1*

mutants remained unchanged compared to uninfested (Control) plants (Figure 5A).

Table 3. Statistical analysis of gene expression in leaves of *A. thaliana* wild-type Col-0 and mutants *tps10*, *bsmt1* and *tps03* at 3 d after single *Plutella xylostella* infestation, dual infestation by *Plutella xylostella* and *Brevicoryne brassicae* aphids at a low or high density and without infestation. Generalized Linear Model deviance table for effect of infestation treatment. Bold numbers indicate significant effects $P < 0.05$.

			Factor	
			Treatment	
			d.f. = 3	
Plant	Product	Gene	deviance	*P*
Col-0	(*E*,*E*)-α-farnesene	*TPS03*	94.98	**0.002**
	(*E*,*E*)-TMTT	*TPS04*	45.45	0.068
	Linalool	*TPS10*	32.48	**0.022**
	MeSA	*BMST1*	450.86	0.114
tps10	(*E*,*E*)-α-farnesene	*TPS03*	24.79	**0.003**
	(*E*,*E*)-TMTT	*TPS04*	3.97	0.473
	MeSA	*BMST1*	418.74	**0.020**
bsmt1	(*E*,*E*)-α-farnesene	*TPS03*	4.10	0.716
	(*E*,*E*)-TMTT	*TPS04*	6.45	0.313
	Linalool	*TPS10*	30.17	**0.027**
tps03	(*E*,*E*)-TMTT	*TPS04*	28.32	**0.010**
	Linalool	*TPS10*	68.05	**0.005**
	MeSA	*BMST1*	199.858	**0.006**

Feeding by *P. xylostella* caterpillars, regardless of whether aphids were present as well, induced the expression of genes important for the biosynthesis of volatiles in plants. Significantly higher expression levels of *TPS04* in *tps03* mutants, of *TPS10* in Col-0 and *bsmt1* plants, and of *BSMT1* in *tps03* mutants were found upon caterpillar feeding compared to undamaged control plants (Figure 5).
In *tps03* mutants, caterpillars feeding alone and simultaneous feeding by caterpillars and aphids at low density induced *TPS10* expression to a significantly higher level compared to uninfested plants (Figure 5). *TPS10* expression was negatively correlated with aphid density on caterpillar-infested *tps03* plants (Figure 5). Expression of *TPS10* is significantly affected by feeding of caterpillars in

combination with aphids at high density in *tps03* mutants compared to uninfested plants. Caterpillars and aphids at high density feeding on *tps03* mutants induced significantly lower levels of *TPS10* expression compared to caterpillars feeding alone, which was not found for Col-0 plants (Figure 5). This indicates that aphids at high density feeding simultaneously with caterpillars interfere with caterpillar-induced *TPS10* expression.

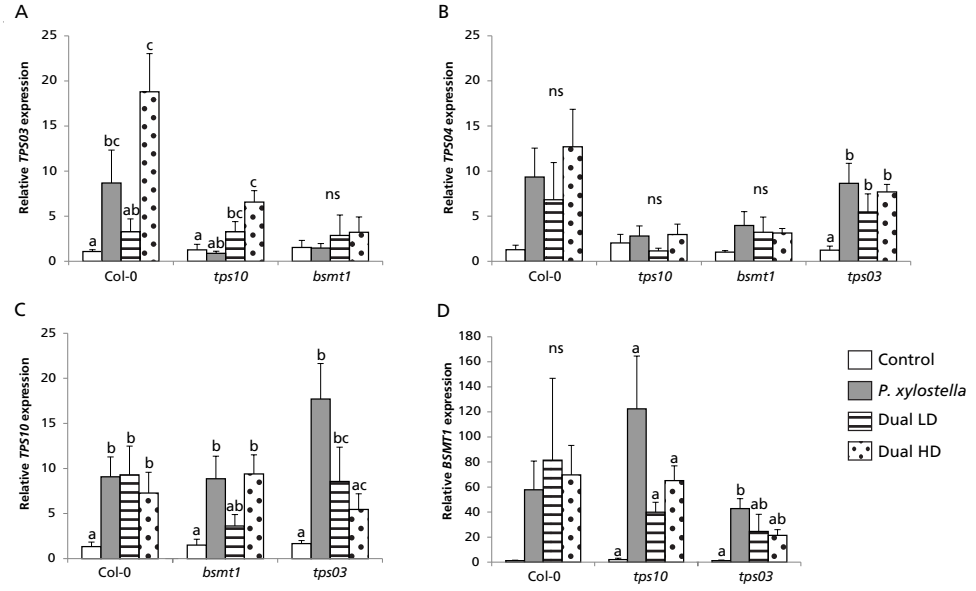

Figure 5. Gene expression in leaves of *A. thaliana* wild-type Col-0 and mutants *tps10*, *bsmt1* and *tps03* used during the Y-tube olfactometer bioassays after single *P. xylostella* and dual *P. xylostella* and *B. brassicae* infestation (Dual) at either a low (LD, 5 aphids) or high (HD, 25 aphids) aphid density and without infestation (control). Bars represent means ± SE (n = 4 biological replications). Bars marked with different letters are significantly different between treatments (GLM, $P < 0.05$; ns, not significant).

Discussion

We investigated the effect of simultaneous feeding by *B. brassicae* aphids and *P. xylostella* caterpillars on induced plant responses and the attraction of the parasitoid *D. semiclausum*, an important natural enemy of *P. xylostella* caterpillars. Our study shows that aphids do not interfere with HIPV-mediated attraction of *D. semiclausum* to caterpillar-infested plants. However, *D. semiclausum* parasitoid attraction is influenced by the density of the aphids. Parasitoids preferred the volatile blend of dually infested plants at high aphid density over those from dually infested plants at low aphid density.

It has been observed before that the level of induced indirect plant defense is influenced by the density of the herbivores feeding on the plant (Dudareva et al., 2006). For instance, attraction of the predatory mite *Phytoseiulus persimilis* to volatiles from Lima bean plants (*Phaseolus lunatus*) infested by spider mites (*Tetranychus urticae*) and attraction of the parasitoid *Cotesia vestalis* to volatile blends emitted by *P. xylostella*-infested cabbage plants (*Brassica oleracea*) are density-dependent (Gols et al., 2003; Girling et al., 2011).

Compared with feeding by only one insect species, herbivory by a second herbivore may influence indirect defense responses (Rodriguez-Saona et al., 2003; Rodriguez-Saona et al., 2005; Heil, 2008; Dicke et al., 2009; Erb et al., 2010; Zhang et al., 2013; Ponzio et al., 2016), which can also be affected by the density of the attacking insects (Zhang et al., 2009; Kroes et al., 2015). Simultaneous feeding by phloem-feeding whiteflies and *Spodoptera exigua* caterpillars on cotton plants (*Gossypium hirsutum*) reduced the emission of the terpenes DMNT ((*E*)-4,-8-dimethyl-1, 3,7-nonatriene), TMTT and mycrene compared to plants infested by only *S. exigua* (Rodriguez-Saona et al., 2003). Zhang et al. (2013) showed that feeding by *B. tabaci* whiteflies significantly reduced the attraction of *D. semiclausum* parasitoids to volatile blends from *A. thaliana* plants simultaneously infested by *P. xylostella*, which was associated with differences in the HIPV blend. Our present results show that feeding by *B. brassicae* aphids does not affect the preference of *D. semiclausum* for *P. xylostella*-infested plants over control uninfested plants. This indicates that aphids and whiteflies, although both phloem feeders, induce different plant responses. Our data indicate that feeding by caterpillars plus aphids at a low density suppressed transcription of *TPS03* (encoding an (*E*,*E*)-α-farnesene synthase) compared to simultaneous feeding by caterpillars and aphids at a high density. This indicates that *TPS03* expression in response to both caterpillar and aphid feeding depends on aphid density. In addition, olfactory responses of *D. semiclausum* to volatiles emitted by dual-infested *tps03* mutants confirmed that *TPS03* expression in *A. thaliana* is required for interference by aphids. We found that *D. semiclausum* preferred volatile blends emitted by Col-0 plants simultaneously infested by caterpillars and aphids at a high density over volatiles from dual-infested plants infested by aphids at low density. However, when *tps03* mutants, lacking (*E*,*E*)-α-farnesene in the HIPV-blend, that were dually infested with caterpillars and a low density of aphids were tested against dually infested *tps03* plants with aphids at high density, the preference was significantly reversed compared to Col-0 plants.

Results from the volatile analysis show that the volatile blends changed depending

on simultaneous feeding by caterpillars and aphids at low or high density and this may explain the behavioral responses by *D. semiclausum*. Here, the same compounds were detected in the headspace of plants infested by caterpillars alone, dually infested by caterpillars and aphids at both densities or undamaged plants. Moreover, four of these compounds (1-penten-3-ol, (*E*,*E)*-TMTT, (*E*,*E*)-α-farnesene and linalool) were found to be important for the separation of the different blends in the multivariate data analysis. Thus, this study underlines the significance of the quantitative composition of volatile blends used by parasitoids to locate host-infested plants. However, it is noteworthy that parasitoids are able to detect very subtle differences in volatile blends which are difficult to identify by chemical analysis (Clavijo McCormick et al., 2014; Ponzio et al., 2016), indicating that other HIPVs may have contributed to the discrimination exhibited by the parasitoids. In addition, no difference in (*E*,*E*)-α-farnesene emission by dual-infested plants at low density and high density were found, while transcript levels of the corresponding *TPS03* gene did differ between treatments. This may be related to substrate availability because many *TPS*-genes vary in substrate specificity, forming different types of terpenoids from a single substrate (Degenhardt et al., 2009; Tholl and Lee, 2011) or through posttranslational protein modifications (Tholl et al., 2005). Furthermore, sesquiterpenes, such as (*E*,*E*)-α-farnesene, are known to be unstable volatile compounds that are rapidly oxidized (Anet, 1969), which may explain why (*E*,*E*)-α-farnesene emission did not differ between dually infested plants at low or high aphid density. Phytohormonal crosstalk between JA- and SA-mediated signaling pathways is thought to underlie plant-mediated interactions with multiple insect species and behavioral responses of parasitoids and predators (Zhang et al., 2013; Stam et al., 2014; Wei et al., 2014). Activation of SA-signaling in response to aphid feeding (Moran et al., 2002; Mewis et al., 2006; Kusnierczyk et al., 2011) may suppress JA-dependent indirect defense responses. This may result in changes in the composition of the volatile blend (Truong et al., 2014). On the other hand, we found that feeding by *P. xylostella* caterpillars alone induced not only JA-regulated terpenoid volatiles in *A. thaliana* but, similar to the finding of Zhang et al. (2013), also relatively high levels of MeSA, a methylated volatile form of SA. This indicates that the general pattern of negative crosstalk between SA- and JA-dependent signaling pathways in the interactions between simultaneous feeding caterpillars and aphids does not always apply. Interestingly, the data show that mutation in *BSMT1* (that catalyzes the synthesis of MeSA from SA) interfered with the responsiveness of *D. semiclausum* to host-infested plants . Similarly, it was shown by Snoeren et al. (2010) that the attraction of *D. semiclausum* was negatively affected by

MeSA. Upon *B. brassicae* infestation, however, also JA-mediated defense marker genes are induced in *A. thaliana* (Moran et al., 2002). To neutralize the crosstalk between JA and SA defense signaling, plants could emit MeSA to discard SA, relieving the suppression of effective JA-mediated defense responses against the attacking aphids.

Induction of linalool and 1-penten-3-ol emission depends on the JA signaling pathway (Fisher et al., 2003; Van Schie et al., 2007; Snoeren et al., 2010), whereas emission of these plant volatiles in response to caterpillar feeding was not affected by simultaneous *B. brassicae* feeding. Since it is known that the emission pattern of HIPVs varies over time and a time lag occurs between gene induction and subsequent volatile emission (Dudareva et al., 2006; Heil, 2008), effects on HIPV emission by simultaneous feeding by caterpillars and aphids might have been found at other time points after induction. For example, an increase in the amount of emitted volatiles was found after 48 h in *A. thaliana* plants simultaneously infested by *P. xylostella* caterpillars and whiteflies compared to plants infested by caterpillars alone (Zhang et al., 2013). Previous studies have also shown that the role of JA-SA crosstalk in interactions between plants and insect attackers varies. It is known that JA signaling is needed for spider mite-induced MeSA emission in tomato plants (*Lycopersicon esculentum*) (Ament et al., 2004). Furthermore, the combination of both JA-induced volatiles and MeSA is important for the attraction of *P. persimilis* predatory mites towards Lima bean plants (De Boer and Dicke, 2004).

In line with Houshyani et al. (2013) and Zhang et al. (2013), we also observed preference of *D. semiclausum* parasitoids for volatile blends from *P. xylostella*-infested *A. thaliana* plants. Mutations in the biosynthesis of linalool influenced the behavioral response of *D. semiclausum* parasitoids to the volatile blend from host-infested plants. Although parasitoids clearly preferred volatiles emitted by Col-0 plants and *tps10* (linalool synthase) and *tps03* ((*E*,*E*)-α-farnesene synthase) mutants infested by *P. xylostella* caterpillars over those from undamaged plants, responsiveness of *D. semiclausum* was influenced by blocking the biosynthesis of linalool and (*E*,*E*)-α-farnesene. Linalool has been reported before as an important attractant for *D. semiclausum* parasitoids (Houshyani et al., 2013) and has been found in *P. xylostella*-induced volatile blends from *A. thaliana* (Zhang et al., 2013; Supplementary materials Table S1). Other volatile compounds induced most strongly by feeding of *P. xylostella* caterpillars were β-mycrene and TMTT and, therefore, may contribute to the attraction of *D. semiclausum* parasitoids (Supplementary materials Table S1). These two specific compounds were also found in the volatile blend emitted by *A. thaliana* plants in response to *P.*

xylostella infestation after 2 d of feeding (Zhang et al., 2013).

In conclusion, we have shown that the behavioral response of parasitoids to HIPVs emitted by plants dually attacked by aphids and caterpillars depends on aphid density and found density associated changes in the HIPV blend. Here, biosynthesis and emission of (*E*,*E*)-α-farnesene could be linked to the observed preference of *D. semiclausum* parasitoids for volatiles emitted by plants dually infested by caterpillars and aphids at a high density. In addition, biosynthesis of linalool and (*E*,*E*)-α-farnesene strongly influenced *D. semiclausum* responsiveness to host-infested plants. Natural enemies of insect pests, such as *D. semiclausum*, are important biological control agents. As plants growing under field conditions are commonly attacked by multiple insect herbivores at the same time, a better understanding of how plants regulate indirect defense mechanisms in response to multiple insect attack will enhance pest control strategies.

Acknowledgments

We thank Léon Westerd, Frans van Aggelen, André Gidding and Joop Woelke for culturing the insects. We would also like to thank Thierry Delatte and Dorothea Tholl for providing seeds of the mutants *tps10* and *tps03*. This work was supported by a TOP GO grant (grant number 854.10.010 to M.D.) from The Netherlands Organization for Scientific Research (NWO).

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Chapter 5

Caterpillar feeding modulates defense against aphids in *Arabidopsis* plants through jasmonate and ethylene signaling

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Abstract

In response to insect feeding, plants activate defense signaling networks regulated by phytohormones such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). Interactions between phytohormonal signaling pathways depend on factors such as the number of insect species attacking simultaneously and insect density. Consequently, this can affect the regulation of plant defenses against attacking herbivores.

Here, we show that *Plutella xylostella* caterpillars affect defenses induced by *Brevicoryne brassicae* aphids in *Arabidopsis thaliana* plants. Simultaneous caterpillar feeding led to increased direct defense that reduced aphid performance, and enhanced attraction of the aphid parasitoid *Diaeretiella rapae* which contributes to indirect plant defense. Analysis of gene expression and phytohormone levels underlying defense responses showed that differential regulation of JA and ET signaling is involved in such interference. The effect of caterpillar infestation on the induction of JA biosynthesis depends on the density of the simultaneously feeding aphids. In addition, plant-mediated effects of aphid density involved SA-JA crosstalk. By demonstrating links between identified molecular defense mechanisms and ecological consequences of plant responses to insect attackers, our study contributes to the understanding of how insect density affects plant defense regulation in response to multiple insect attack.

Keywords

Arabidopsis thaliana, *Brevicoryne brassicae*, density dependence, hormone crosstalk, multiple herbivory, plant defense, *Plutella xylostella*

Introduction

Plant-feeding insects induce specific defense responses in plants (De Vos et al., 2005; Bidart-Bouzat and Kliebenstein, 2011; Kawazu et al., 2012; Stam et al., 2014). Not only do these defense responses directly affect the feeding herbivore (Kessler and Baldwin, 2002; Howe and Jander, 2008), they also act indirectly via the attraction of natural enemies of herbivores through the emission of herbivore-induced plant volatiles (HIPVs) (Dicke and Baldwin, 2010). Insects are members of communities which use the same host plants to feed on. Multiple insect species feeding on the same plant interact through plant-mediated effects (Stam et al., 2014) and affect both direct (Soler et al., 2012; Ali et al., 2014) and indirect (Dicke et al., 2009) plant defense responses. Recent research reported interference by phloem-feeding insects with induced defenses against chewing herbivores (Rodriguez-Saona et al., 2005; Zhang et al., 2009; Erb et al., 2010; Schwartzberg et al., 2011; Zhang et al., 2013; Li et al., 2014; Ponzio et al., 2014; Kroes et al., 2015). However, little is known about caterpillar interference with aphid-induced (in)direct defenses (Agbogba and Powell, 2007; Soler et al., 2012; Ali et al., 2014). The outcome of plant defense in response to multiple insect attack is dependent on the density of the insects. For example, interference of phloem-feeding whiteflies with indirect defenses against spider mites in Lima bean was positively correlated with whitefly density (Zhang et al., 2009). Similarly, *B. brassicae* aphids interfere with caterpillar-induced defenses in plants, which is dependent on the density of the attacking aphid (Kroes et al., 2015; Ponzio et al., 2016). Aphid density may affect the regulation of different signal-transduction pathways underlying defense responses in plants (Koornneef et al., 2008; Smith and Boyko, 2007), which may be important for plant-mediated interactions between multiple attackers and for plant responses to aphids in general.

In response to aphid feeding, plants activate defense signaling networks regulated by phytohormones such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Morkunas et al., 2011; Louis and Shah, 2013). Several genes that function in SA- and ET-dependent signaling as well as genes important for JA biosynthesis were significantly up-regulated after *B. brassicae* infestation (Kusnierczyk et al., 2008). Furthermore, feeding by *B. brassicae* and *Myzus persicae* induced expression of both JA- and SA-mediated defense marker genes in *A. thaliana* (Moran and Thompson, 2001; Moran et al., 2002). Phytohormone signaling pathways are involved in the activation of biosynthesis of secondary metabolites including HIPVs, which are important in indirect defense (Mumm and Dicke, 2010). Studies with the *A. thaliana* mutants *cev1* (which has constitutive expression of JA- and

ET-response genes) and *coi1-16* (which is insensitive to JA) revealed that aphid-induced volatile production requires intact JA and ET signaling pathways (Girling et al., 2008).

However, phytohormones do not act individually, and additional regulation of defense responses is implemented by crosstalk between signaling pathways (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). Jasmonate and ethylene signaling pathways can interact both antagonistically and synergistically (Zhu and Lee, 2015). The AP2/ETHYLENE RESPONSE FACTOR domain transcription factor ORA59 integrates JA and ET signaling leading to induction of specific defense genes, such as *PDF1.2* (Pre et al., 2008; Memelink, 2009). The ET-dependent transcription factors EIN3 and EIL1 regulate *ORA59* expression (Zhu et al., 2011). JA and ET can, therefore, act synergistically through the enhancement of EIN3/EIL1 transcription by JA signaling (Zhu et al., 2011). In contrast, antagonism between JA and ET is mediated by *MYC2* (encoding a transcription factor regulating JA-inducible responses; Kazan and Manners, 2013), that down-regulates *PDF1.2* (Dombrecht et al., 2007) by suppressing the effect of EIN3/EIL1 on ORA59 expression (Song et al., 2014). Aphids also seem to induce an integrated defense signaling response as ethylene response factors like *ERF11* and *ORA59* were induced in aphid-infested *A. thaliana* plants (Kusnierczyk et al., 2008; Kerchev et al., 2013). JA and SA signaling also act antagonistically (Koornneef and Pieterse, 2008; Caarls et al., 2015). In response to SA, *WRKY* genes are induced that regulate SA-dependent gene expression. Moreover, WRKY transcription factors indirectly inhibit transcription of JA-responsive genes by the degradation of ORA59 (Van der Does et al., 2013).

Studies concerning the molecular regulation of plant defenses against multiple herbivory have investigated crosstalk between JA and SA signaling (Rodriguez-Saona et al., 2010; Soler et al., 2012; Ali et al., 2014; Kroes et al., 2015), whereas less is known about the involvement of other key phytohormones such as ET (Zhang et al., 2013). Therefore, investigating the role of the ET signaling pathway and possible crosstalk between ET and JA signaling is warranted to gain more insight into the mechanisms underlying multiple insect-plant interactions.

Combining both gene regulation and biochemical analyses of defense responses mediated by phytohormones to establish causal relationships between the activation of defense signaling pathways and plant resistance will contribute to the understanding of plant defense regulation to multiple insect attack. An important aspect that needs to be addressed is how aphid densities affect regulation between signaling pathways through crosstalk and consequently how

this affects plant defense against multiple feeding herbivores.
Here, we investigated regulation of plant defense in *A. thaliana* against the aphid *B. brassicae* infesting the plant at a low or high density. Furthermore, we studied how *Plutella xylostella* caterpillars interfere with defenses induced by B. brassicae, and if this interference is dependent on aphid density. Both *P. xylostella* and *B. brassicae* are specialist herbivores of plants in the Brassicaceae.
We investigated the involvement of JA, SA and ET signaling pathways and their interactions during defense responses against caterpillars or aphids at the two densities, when feeding alone or simultaneously. We link insect performance as a measure of direct defense and aphid parasitoid behavior (indirect defense) to the expression of JA, SA and ET-responsive genes and quantified JA and SA levels in *A. thaliana* wild-type plants and mutants deficient in JA, SA or ET biosynthesis/ signaling.

Materials & Methods

Plants and growth conditions

Plants of the *A. thaliana* accession Columbia-0 (Col-0) and mutants defective in jasmonic acid (JA)-, ethylene (ET)-, and salicylic acid (SA)-dependent signaling were used. All mutants used were in the Col-0 background. Seeds of Col-0 and the mutants *sid2-1* (*salicylic acid induction-deficient2-1*), *dde2-2* (*delayed-dehiscence2-2*), *myc2* (*jin1-2, jasmonate-insensitive1-2*), *ein2-1* (*ethylene-insensitive2-1*) and *ora59* (*octadecanoid-responsive arabidopsis59*) were sown in autoclaved (80 °C for 4 h) potting soil (Lentse Arabidopsis-potgrond, Lent, The Netherlands). After 10 to 14 days of growth, plants were transferred to individual pots (5 cm diameter) containing similar soil. Plants were cultivated in a growth chamber at 21 ± 2 °C under an 8L : 16D cycle [200 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) light intensity] and 60% relative humidity (RH). Four-to-five-week old plants were used in the experiments. During the experiments, all plants remained in the vegetative state.

Insects

Both the Cabbage aphid, *B. brassicae* L. (Hemiptera: Aphididae), and the Diamondback moth, *P. xylostella* L. (Lepidoptera: Yponomeutidae), were reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv Cyrus) at 22 ± 1 °C, 50-70 % RH, 16L : 8D cycle. The parasitoid *Diaeretiella rapae* (McIntosh)

(Hymenoptera: Braconidae) was reared on *B. brassicae* feeding on Brussels sprouts plants in a climate cabinet at 25 ± 1 °C with a 16L : 8D cycle. Brussels sprouts leaves with aphid mummies attached were placed in a cage supplemented with honey and water. To obtain naïve parasitoids (that had no adult experience with aphids), mummies were removed from the leaves and incubated until emergence in a climate cabinet (25 ± 1 °C with a 16L : 8D cycle). In all experiments, female parasitoids were 2-3 days old.

Performance of *Brevicoryne brassicae* and *Plutella xylostella*

Performance of aphids and caterpillars was assessed after 5 days of feeding on each of the six *A. thaliana* plant genotypes. Per plant genotype, 70-75 plants were infested with adult *B. brassicae* aphids, either 5 aphids ('low density') or 25 aphids ('high density'), or with two second-instar (L2) *P. xylostella* caterpillars (indicated as 'single' infestations) or simultaneously infested with adult *B. brassicae* aphids and two *P. xylostella* L2 caterpillars (indicated as 'dual' infestations). Plants were infested by either 5 aphids ('low density') or 25 aphids ('high density'). Insects were allowed to feed freely on the plants.

Individual plants were placed in a plastic container (diameter 8 cm x height 14 cm), covered with gauze cloth and closed with elastic bands. Containers were randomly distributed in a tray (12-15 containers per tray). Trays were placed in a growth chamber with a 16L : 8D cycle [200 µmol m^{-2} s^{-1} PAR], at 21 ± 2 °C and 50-70% RH. At 5 days after infestation, the number of *B. brassicae* adults and nymphs was recorded and *P. xylostella* caterpillars were individually weighed on a microbalance (accuracy 1 µg; CP2P, Sartorius AG, Göttingen, Germany).

Preference of *Diaeretiella rapae*

Responses of *D. rapae* parasitoids to plant volatiles was analyzed in a dual-choice test performed in a Y-tube olfactometer. The Y-tube olfactometer consisted of two 5-L glass jars which were each connected to one arm of a glass Y-tube. To test if *D. rapae* exhibited a preference for volatile blends emitted by plants subjected to one of the treatments, four plants of a treatment were placed in a glass jar as odor source. Prior to placing a plant in one of the jars, the pot of the plant was carefully wrapped in aluminum foil. Charcoal-filtered air at a flow of 2 L min^{-1} was led through the Y-tube olfactometer to carry plant volatiles from their source into the arms of the Y-tube. A single female *D. rapae* parasitoid was released in the Y-tube and its choice for either odor source was recorded if the parasitoid spent 15 s or more beyond a line marked at 2 cm from the end of each Y-tube

arm. Parasitoids that did not make a choice within 10 min were excluded from statistical analysis. Each parasitoid was used only once. After five parasitoids were tested, the position of the odor sources was exchanged to exclude positional bias in the set-up.

Behavior of *D. rapae* females was observed in the following choice situations:

1. Undamaged plants against plants infested by 5 adult *B. brassicae* aphids
2. Undamaged plants against plants infested by 25 adult *B. brassicae* aphids
3. Plants infested by 5 adult *B. brassicae* aphids against plants infested by 2 *P. xylostella* L2 caterpillars plus 5 adult *B. brassicae* aphids
4. Plants infested by 25 adult B. brassicae aphids against plants infested by 2 *P. xylostella* L2 caterpillars plus 25 adult *B. brassicae* aphids

To test if intact ET signaling is required for plant-mediated effects of caterpillar feeding on *D. rapae* response to plants infested by aphids at different densities, both Col-0 plants and *ein2-1* mutant plants were used in the bioassay. For each of the four choice assays, 4-5 sets of plants and 60-75 *D. rapae* females were tested. Additionally, after each bioassay, insects were removed from the plants and plant shoots of each treatment were pooled and weighed on an analytical balance (accuracy 0.1 mg; Mettler Toledo ML54/01).

Molecular regulation of plant defense

To link the expression of direct plant defense to the underlying molecular mechanisms, we simultaneously performed a gene-expression and phytohormone analysis on Col-0 plants and mutants defective in JA-, ET-, and SA-dependent signaling from the same batch of plants used to assess herbivore performance. For this experiment, 90 plants infested with adult aphids (at low or high density), or with 2 L2 caterpillars, or simultaneously infested with caterpillars and aphids at low or high density were used. Clean uninfested plants were used as control. Individual plants were placed in plastic containers, covered with gauze cloth and closed with elastic bands. Containers were randomly distributed in a tray and placed in a growth chamber at a 16L : 8D cycle [200 µmol m^{-2} s^{-1} PAR light intensity] at 21 ± 2 °C and 50-70% RH. Insect-damaged leaves were collected after 48 h of feeding. Before collection, insects were removed from the leaves with a fine brush. Leaf tissue from three different plants was pooled to obtain one biological replicate for each treatment. In total, three to five biological replicates per genotype per treatment were used for gene expression and phytohormone analysis. Plant material was snap-frozen in liquid nitrogen and stored at -80 °C prior to analysis.

Gene expression analysis

Total RNA was extracted from finely ground plant leaf tissue with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA samples were treated with DNase (Qiagen, Hilden, Germany). cDNA was synthesized from 1 µg RNA using iScript cDNA synthesis Kit (Bio-Rad). Transcript levels of the JA-responsive marker gene *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) (At5g24770), the JA/ET-responsive marker gene *PLANT DEFENSIN 1.2* (*PDF1.2*) (At5g44420), the SA-responsive marker gene *PATHOGENESIS-RELATED PROTEIN 1* (*PR-1*) (At2g14610), the JA/ET-mediated transcription factor *OCTADECANOID-RESPONSIVE ARABIDOPSIS 59* (*ORA59*) (At1g06160), the ET-mediated gene *ETHYLENE INSENSITIVE2* (*EIN2*) (At5g03280) and two references genes *F-BOX FAMILY PROTEIN* (*FBOX*) (At5G15710) and *PEROXIN4* (*PEX4*) (At5g25760) were quantified. Efficiency of each primer was determined before qRT-PCR analysis. Quantitative RT-PCR analysis was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Each reaction was performed in a total volume of 25 µl containing 12.5 µl SYBR Green Supermix (Bio-Rad), 5 µl cDNA and 1 µl of 10 µM forward and reverse gene-specific primer pair. For each reaction, two technical replicates were performed and average values were used in the analyses. The following thermal profile was used for reactions with *VSP2*, *PDF1.2*, *PR-1*, *ORA59*, *FBOX* and *PEX4* : 3 min 95°C, followed by 40 cycles of 15 s at 95 °C, and 45 s at 60 °C. For reactions with *EIN2* thermal conditions consisted of 3 min 95°C, followed by 40 cycles of 15 s at 95 °C, and 45 s at 62 °C.

The two reference genes, *FBOX* and *PEX4*, were carefully selected after evaluating their expression stability by calculating the geNorm value and coefficient of variation (CV) (qbase+ v. 2.6.1, Biogazelle; Hellemans et al., 2007). Relative expression for each tested gene was calculated by using the geometric mean of threshold cycle (Ct) values (Vandesompele et al., 2002) from the two reference genes with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Phytohormone analyses

For quantification, internal standards of JA (d5-JA) and SA (d4-SA) were added to 100 mg finely ground frozen leaf material (see Trapp et al. (2014)). Chromatographic separation was carried out in a Luna Phenyl-Hexyl column (150 × 4.6 mm, 5 µm; Phenomenex, Aschaffenburg, Germany). Formic acid (0.05%, v/v) and methanol with 0.05% (v/v) of formic acid were employed as mobile phases. HPLC-MS/MS analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies, Böblingen, Germany) connected to an LTQ Orbitrap mass

spectrometer (Thermo Scientific, Bremen, Germany). The LTQ mass spectrometer was equipped with an Electrospray ionization source, operating in negative and positive ion modes. Endogenous JA, JA-Ile and SA were quantified as described by Trapp et al. (2014). For quantification of JA, JA-Ile and SA, calibration curves were generated to establish the basal phytohormone level in *A. thaliana* plants. Calibration curves were prepared by adding spiking solutions containing JA, JA-Ile and SA to uninfested (control) *A. thaliana* plant samples. The detection limits for SA, JA and JA-Ile were 25, 12.5 and 0.4 ng/g respectively. In cases where quantification generated negative values for phytohormone levels, they were set 10% below their respective detection limit in the analysis.

Statistical analysis

The effect of aphid density and caterpillar interference on number of *B. brassica* adults and nymphs, gene expression and phytohormone level were analyzed using a generalized linear model (GLM) with Poisson distribution and log link function in GenStat v. 17 (VSN International, Hemel Hempstead, UK). Aphid density, caterpillar presence and their interactions were included as fixed factors. The dispersion parameter was estimated to account for residual variance.
To determine whether parasitoid preferences and proportion of choosing wasps differed between various odor sources, data on olfactory responses of *D. rapae* were analyzed using a χ^2-test in SPSS v. 22.0 (SPSS Inc., Chicago, IL, USA) for each choice situation tested. In addition, preference data were analyzed using a GLM with Poisson distribution and log link function in GenStat v. 17 (VSN International, Hemel Hempstead, UK) to compare choice distributions between choice situations. Genotype, treatment combination (*i.e.* undamaged plants tested in the Y-tube olfactometer against plants infested by *B. brassicae* at a low density of 5 aphids (hereafter abbreviated as Single LD for Single Low Density) or a high density of 25 aphids (abbreviated as Single HD for Single High Density) or plants infested by both *P. xylostella* and a low (abbreviated as Dual LD) or high (abbreviated as Dual HD) aphid density tested against Single LD or Single HD plants) and *B. brassicae* density and the interaction genotype x treatment combination x *B. brassicae* density were included as fixed factors for data on proportion of responsive wasps. In the choice assays involving undamaged plants, the number of wasps choosing the *B. brassicae*-infested plants out of the total number of responding wasps was entered as the response variable. In the choice assays between Single LD versus Dual LD and Single HD versus Dual HD, the number of wasps choosing the dually infested plants out of the total number of

responding wasps was entered as the response variable. The dispersion parameter was estimated to account for residual variance.
Furthermore, Fisher's exact test (two-tailed) was used to determine whether parasitoid preferences were distributed identically across different days on which the tests were repeated. Effect of treatment on plant shoot fresh weight was analyzed with an independent samples t-test. The statistical analysis of parasitoid preference and plant shoot fresh weight was carried out using SPSS v. 22.0 (SPSS Inc., Chicago, IL, USA).
To test if there were differences in weight of caterpillars feeding alone or simultaneously with aphids at low or high density on plants, a linear mixed model with treatment as fixed factor and individual plant as random factor was used in SPSS v. 22.0 (SPSS Inc, Chicago, IL, USA). The expression of genes and phytohormone levels between plants on which caterpillars were feeding alone or simultaneously with aphids at both densities was tested using a GLM with Poisson distribution and log link function in GenStat v. 17.0 (VSN International, Hemel Hempstead, UK). The factor treatment was included in the model as fixed factor. The dispersion parameter was estimated to account for residual variance. Post-hoc comparisons for gene expression and phytohormone levels were analyzed with an LSD test.

Results

Aphid performance

Feeding by *P. xylostella* caterpillars negatively affected aphid population development on Col-0 plants (Table 1). In contrast, on *sid2-1* (deficient in SA production), *dde2-2* (deficient in JA production), *myc2* (defective in JA-responsive transcription factor MYC2) and *ora59* mutants (defective in JA/ET-responsive transcription factor ORA59), aphid numbers were not affected by simultaneous *P. xylostella* feeding. In contrast, caterpillar feeding enhanced aphid performance on *ein2-1* (ET-insensitive) mutant plants (Table 1). Aphid starting density significantly affected *B. brassicae* numbers on all plant genotypes (Table 1). Additionally, growth of *P. xylostella* caterpillars was assessed on Col-0 and mutant plants when feeding alone or simultaneously with aphids at low and high density. Weight gain of the caterpillars was significantly lower only when feeding simultaneously with aphids at high density on *ein2-1* mutant plants compared with caterpillars feeding alone and caterpillars feeding simultaneously with aphids at low density (Supplementary materials Table S1).

Table 1. Population development of *B. brassicae* (mean ± SE) after single *B. brassicae* or dual *P. xylostella* and *B. brassicae* infestation on *A. thaliana* wild-type Col-0 and mutants *sid2-1*, *dde2-2*, *myc2*, *ein2-1* and *ora59* on day 5 since infestation (or day 4 for *myc2* mutant plants). Plants were infested with either a low (LD) or high (HD) density of *B. brassicae* aphids. Generalized Linear Model deviance table for effect of *P. xylostella* presence and *B. brassicae* density and their interaction term. Bold numbers indicate significant effects ($P < 0.05$).

			Aphid number				Factors				Interaction	
							P. xylostella presence (1)		*B. brassicae* density (2)		1 x 2	
							d.f. = 1		d.f. = 1		d.f. = 1	
Plant		*B. brassicae*	Single LD	Dual LD	Single HD	Dual HD	deviance	*P*	deviance	*P*	deviance	*P*
Col-0	Wild-Type	Adults	4 ± 0.2	4 ± 0.1	19 ± 0.9	16 ± 0.9	2.65	**0.013**	243.36	**<0.001**	0.66	0.203
		Nymphs	59 ± 2.9	55 ± 2.9	247 ± 9.8	214 ± 7.4	57.76	**<0.001**	3253.00	**<0.001**	1.91	0.458
sid2-1	SA induction deficient	Adults	4 ± 0.3	4 ± 0.4	18 ± 1.2	17 ± 0.9	1.57	0.141	264.95	**<0.001**	0.05	0.800
		Nymphs	38 ± 3.5	35 ± 3.7	149 ± 7.5	163 ± 10.5	4.47	0.419	2363.91	**<0.001**	7.21	0.305
dde2-2	JA biosynthesis deficient	Adults	4 ± 0.3	3 ± 0.3	17 ± 0.9	16 ± 0.9	0.72	0.242	255.13	**<0.001**	0.27	0.475
		Nymphs	39 ± 3.8	25 ± 2.9	172 ± 9.4	157 ± 13.1	28.49	0.054	2740.31	**<0.001**	18.33	0.120
myc2	defective in MYC2	Adults	4 ± 0.3	5 ± 0.2	18 ± 0.8	19 ± 0.6	0.24	0.387	299.14	**<0.001**	0.14	0.499
		Nymphs	46 ± 3.9	45 ± 3.2	178 ± 6.0	180 ± 8.5	0.04	0.928	2734.82	**<0.001**	0.21	0.831
ein2-1	defective in ET pathway	Adults	3 ± 0.4	3 ± 0.3	11 ± 1.0	14 ± 0.9	4.69	**0.026**	168.86	**<0.001**	0.76	0.362
		Nymphs	42 ± 4.9	35 ± 3.7	146 ± 15.3	170 ± 14.3	7.97	0.437	2179.48	**<0.001**	25.06	0.171
ora59	defective in ET/JA pathway	Adults	5 ± 0.2	4 ± 0.2	18 ± 0.6	17 ± 1.3	0.91	0.124	239.68	**<0.001**	0.01	0.903
		Nymphs	58 ± 3.4	56 ± 5.5	229 ± 11.7	232 ± 11.1	0.03	0.951	3152.37	**<0.001**	0.32	0.838

Preference of the aphid parasitoid *Diaeretiella rapae*

To further investigate if ET also underlies plant-mediated effects of caterpillars on aphid-induced indirect defense responses, parasitoid preference of aphid-infested and dual-infested Col-0 plants and *ein2-1* (ET-insensitive) mutants was studied.

Table 2. Statistical analysis of proportion of responsive *Diaeretiella rapae* parasitoids to volatiles emitted by *A. thaliana* wild-type Col-0 and *ein2-1* mutants three days after single *Brevicoryne brassicae* at a low (LD, 5 aphids) or high (HD, 25 aphids) density infestation, dual *Plutella xylostella* and a low (Dual LD) or high (Dual HD) *Brevicoryne brassicae* density infestation and without infestation (undamaged). Generalized Linear Model deviance table for effect of genotype, treatment combination (e.*g*. undamaged versus *B. brassicae* at a low or high density and *B. brassicae* at a low or high density versus Dual LD or Dual HD). Bold number indicate significant effects ($P < 0.05$).

	Factor							
	Genotype (1)		Treatment combination (2)		Density (3)			
	d.f. = 1		d.f. = 1		d.f. = 1			
	deviance	*P*	deviance	*P*	deviance	*P*		
% responsive wasps	29.94	< **0.001**	15	**0.01**	0.14	0.789		
	Interaction							
	1 x 2		1 x 3		2 x 3		1 x 2 x 3	
	d.f. =1		d.f. = 1		d.f. = 1		d.f. = 1	
	deviance	*P*	deviance	*P*	deviance	*P*	deviance	*P*
% responsive wasps	0.22	0.739	0.02	0.919	0.62	0.575	4.54	0.137

Analysis of the proportion of wasps responsive to volatile blends emitted by either Col-0 plants or *ein2-1* mutants, showed an effect of plant genotype and treatment combinations (*i.e*. undamaged versus *B. brassicae* at a low or high density or *P. xylostella* and *B. brassicae* at a low or high density versus *B. brassicae* at a low or high density) tested (Table 2; GLM).

Female *D. rapae* parasitoids significantly preferred the volatile blend emitted by Col-0 plants infested by aphids only (Single infestation) over those from undamaged plants, which was independent of whether plants were infested with a low (LD) or high (HD) aphid density (Figure 1A; χ^2-test, Single LD: $\chi^2 = 18$, $P < 0.001$; Single HD: $\chi^2 = 28$, $P < 0.001$). Interestingly, feeding by *P. xylostella* influenced the preference of the parasitoid, as the volatile blend from Col-0 plants infested by both caterpillars and aphids (Dual infestation) at low and

high density was significantly preferred by *D. rapae* over the blend emitted by aphid-infested Col-0 plants at both aphid densities (Figure 1A; χ^2-test, Dual LD: $\chi^2 = 0.9$, $P < 0.001$; Dual HD: $\chi^2 = 3.1$, $P < 0.01$).

Parasitoids preferred volatiles emitted by *ein2-1* (ET-insensitive) mutant plants infested by aphids over those from undamaged plants at both aphid densities (Figure 1B; χ^2-test, Single LD: $\chi^2 = 6$, $P < 0.05$; Single HD: $\chi^2 = 4$, $P < 0.05$). However, parasitoids did not discriminate between the volatile blend from *ein2-1* mutants infested with aphids and the blend from *ein2-1* mutants infested with caterpillars and aphids, at both aphid densities (Figure 1B; χ^2-test, Dual LD: $\chi^2 = 0.9$, $P = 0.336$; Dual HD: $\chi^2 = 3$, $P = 0.101$).

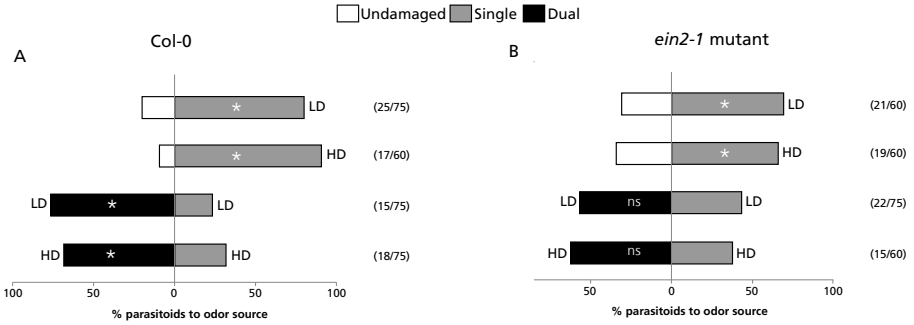

Figure 1. Preference of *D. rapae* in a Y-tube olfactometer to volatile blends emitted by *A. thaliana* Col-0 wild-type (A) or *ein2-1* mutants (B) after 3 days of insect infestation. Undamaged plants were tested against plants infested by five adult *B. brassicae* aphids (low density; LD) or 25 adult *B. brassicae* aphids (high density; HD), plants infested by aphids at a low density were tested against plants dually infested by *P. xylostella* caterpillars and a low density of *B. brassicae* aphids (Dual LD), or plants infested with aphids at a high density were tested against plants dually infested by *P. xylostella* caterpillars and a high density of *B. brassicae* aphids (Dual HD). Each bar represents the percentage of wasps choosing for each of the two odor sources, which consisted of four plants per treatment. For each pair-wise comparison, 4-5 sets of plants were tested, each set on a different day, each parasitoid was tested only once. ns, not significant; asterisk, $P < 0.05$ (χ2-test). Numbers in parentheses represent number of non-responsive wasps and total number of tested wasps.

Thus, ethylene signaling is involved in the interference of *P. xylostella* caterpillar feeding with indirect defenses against *B. brassicae* aphids.

To investigate if shoot fresh weight differed between plant treatments, shoot fresh weight of all treatments was compared for Col-0 and *ein2-1* plants. Weight of the plants used as alternative odor sources per treatment combination was similar in all cases for both Col-0 plants and *ein2-1* mutants (Supplementary materials Figure S1; Independent sample t-test, $P > 0.1$). This indicates that preference of *D. rapae* parasitoids for an odor source was not due to higher

plant biomass. Furthermore, there were no significant associations between the treatment combinations tested and days on which the experiments were repeated for both Col-0 plants and *ein2-1* mutants (Fisher's exact test, $P > 0.1$).

Gene expression analysis

We assessed the transcription of five marker genes for phytohormonal signaling in four plant genotypes upon each of four single or double herbivore infestations. Expression of the marker genes *VSP2* and *PDF1.2* was significantly affected by dual insect infestation compared to aphids feeding alone, on Col-0 and on *myc2*, *ein2-1* or *ora59* mutant plants (Table 3). This indicates that the differences found in defense responses between single or dual insect infestation is not regulated by activation of the transcription factors *MYC2* or *ORA59*, or ET signaling alone. However, only in the *myc2* mutant also *EIN2* expression level was affected by dual insect infestation compared to aphid feeding alone (Table 3), suggesting that JA- and ET-regulated defense responses influence plant-mediated interactions between aphids and caterpillars.

In Col-0 plants, only the expression of *ORA59* is significantly affected by aphid density (Table 3). This indicates that both ET and JA signaling play important roles in responses to attacking aphids at different densities, because the transcription factor ORA59 acts as the integrator of the JA and ET signaling pathways (Pre et al., 2008). The importance of ET and JA signaling in the regulation of responses to low or high aphid density is also shown for the expression of defense genes in *myc2*, *ein2-1* and *ora59* mutant plants. In *myc2* mutants, *PR-1* expression levels are significantly affected by aphid density which was not found for Col-0 plants (Table 3). Aphids feeding at high density on *myc2* plants induced significantly higher levels of *PR-1*. In addition, *VSP2* expression levels are significantly affected by aphid density in *myc2*, *ein2-1* and *ora59* mutant plants (Table 3). Feeding by aphids at high density, both alone and simultaneously with caterpillars, induced *VSP2* expression to a lower level compared to aphids alone or dual aphid and caterpillar infestation at low aphid density.

Table 3. Statistical analysis of gene expression (mean ± SE) in leaves of *A. thaliana* wild-type Col-0 and mutants *myc2*, *ein2-1* and *ora59* at 48 h after single *B. brassicae* or dual *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (LD, 5 aphids) or high (HD, 25 aphids) density of *B. brassicae* aphids. Generalized Linear Model deviance table for effect of *P. xylostella* presence and *B. brassicae* density and their interaction term. Bold numbers indicate significant effects ($P < 0.05$). Gene expression was measured relative to untreated control samples (plants without herbivore infestation).

						Factors				Interaction	
						P. xylostella presence (1)		*B. brassicae* density (2)		1 x 2	
Plant	Gene	Relative expression level				d.f. = 1		d.f. = 1		d.f. = 1	
		Single LD	Dual LD	Single HD	Dual HD	deviance	*P*	deviance	*P*	deviance	*P*
Col-0	*PR-1*	21.5 ± 9.7	14.7 ± 4.2	37.3 ± 12.3	26.9 ± 10.7	14.82	0.370	39.58	0.151	0.08	0.948
	VSP2	0.86 ± 0.19	36.1 ± 11.0	1.40 ± 0.49	49.3 ± 21.8	502.55	**<0.001**	10.73	0.439	0.09	0.943
	EIN2	1.35 ± 0.26	1.33 ± 0.03	1.40 ± 0.24	1.58 ± 0.31	0.02	0.749	0.08	0.518	0.04	0.657
	ORA59	3.19 ± 0.33	5.02 ± 0.79	5.13 ± 0.48	7.11 ± 0.45	3.58	**0.003**	4.01	**0.002**	0.10	0.566
	PDF1.2	6.39 ± 1.74	65.4 ± 13.1	10.6 ± 3.0	47.6 ± 7.5	397.19	**<0.001**	7.15	0.318	12.22	0.197
myc2	*PR-1*	86.7 ± 16.3	91.3 ± 30.0	204.7 ± 53.4	296.8 ± 54.0	69.01	0.237	802.39	**<0.001**	16.62	0.555
	VSP2	0.59 ± 0.07	9.86 ± 3.90	0.20 ± 0.02	3.22 ± 0.71	66.04	**<0.001**	18.58	**0.007**	0.00	0.967
	EIN2	1.00 ± 0.16	1.28 ± 0.18	0.85 ± 0.17	1.38 ± 0.17	0.72	**0.034**	0.00	0.900	0.08	0.465
	ORA59	2.95 ± 0.54	8.05 ± 1.56	5.19 ± 0.54	9.05 ± 1.03	16.22	**<0.001**	2.08	0.106	1.32	0.191
	PDF1.2	16.1 ± 6.9	51.7 ± 13.8	22.9 ± 3.0	125.0 ± 33.0	476.24	**<0.001**	152.08	**0.012**	10.34	0.473

Table 3. (Continued)

	PR-1	16.4 ± 3.6	45.2 ± 18.1	50.6 ± 26.0	49.1 ± 9.4	18.49	0.422	36.43	0.266	37.55	0.259
	VSP2	0.54 ± 0.14	34.3 ± 13.7	0.38 ± 0.07	12.5 ± 2.9	222.81	<0.001	42.01	0.026	0.42	0.804
ein2-1	*EIN2*	0.81 ± 0.10	1.16 ± 0.18	0.74 ± 0.12	0.85 ± 0.13	0.22	0.139	0.16	0.200	0.07	0.396
	ORA59	3.05 ± 0.75	4.59 ± 1.79	1.79 ± 0.32	0.91 ± 0.09	6.08	0.034	0.27	0.621	1.09	0.330
	PDF1.2	2.81 ± 0.50	73.2 ± 27.1	6.01 ± 2.54	97.6 ± 16.3	714.31	<0.001	17.04	0.335	1.70	0.757
	PR-1	38.5 ± 8.4	55.9 ± 15.7	113.2 ± 58.4	78.5 ± 27.7	6.53	0.733	118.71	0.162	33.50	0.445
	VSP2	0.60 ± 15.25	116.4 ± 26.6	0.48 ± 11.72	53.6 ± 16.7	821.70	<0.001	157.90	0.009	0.28	0.900
ora59	*EIN2*	1.21 ± 0.27	1.01 ± 0.14	1.05 ± 0.18	1.17 ± 0.08	0.00	0.881	0.00	0.850	0.09	0.353
	ORA59	0.77 ± 0.14	2.15 ± 0.40	0.94 ± 0.14	1.16 ± 0.14	1.84	0.005	1.02	0.025	0.72	0.053
	PDF1.2	3.36 ± 1.26	415.2 ± 93.3	1.09 ± 0.72	2.40 ± 0.97	1822.37	<0.001	2974.28	<0.001	23.55	0.401

In addition, SA-, JA-, and ET-mediated gene expression were compared between plants infested with *P. xylostella* alone and with both *P. xylostella* and a low or high *B. brassicae* density (Table 4). Feeding by *P. xylostella* caterpillars on Col-0 plants induced significantly higher expression of *VSP2* and *PDF1.2* compared to plants simultaneously infested by caterpillars and aphids at both densities (Table 4). Interestingly, simultaneous feeding by caterpillars and aphids at high density on *myc2* mutants resulted in increased levels of *PR-1* whereas *VSP2* levels were significantly reduced compared to plants with caterpillars feeding alone (Table 4). This indicates that interference of aphids with caterpillar-induced defenses involves SA-JA crosstalk, which is dependent on aphid density.

Table 4. Statistical analysis of gene expression (mean ± SE) in leaves of *A. thaliana* wild-type Col-0 and mutants *myc2*, *ein2-1* and *ora59* at 48 h after single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (LD, 5 aphids) or high (HD, 25 aphids) density of *B. brassicae* aphids. Generalized Linear Model deviance table for effect of treatment. Bold numbers indicate significant effects ($P < 0.05$). Gene expression was measured relative to untreated control samples (plants without herbivore infestation). Relative expression levels indicated with different letters are significantly different between treatments.

					Factor	
					Treatment	
Plant	Gene	Relative expression level			d.f. = 2	
		Single	Dual LD	Dual HD	deviance	*P*
Col-0	*PR-1*	14.4 ± 6.2	14.7 ± 4.2	26.9 ± 10.7	25.84	0.427
	VSP2	337.5^{a} ± 56.4	36.1^{b} ± 11.0	49.3^{b} ± 21.8	1936.91	**<0.001**
	EIN2	1.51 ± 0.16	1.33 ± 0.03	1.58 ± 0.31	0.12	0.644
	ORA59	4.99 ± 0.67	5.02 ± 0.79	7.11 ± 0.45	2.51	0.092
	PDF1.2	371.5^{a} ± 78.7	65.4^{b} ± 13.1	47.6^{b} ± 7.5	1922.15	**<0.001**

Table 4. (Continued)

	PR-1	55.0^{a} ± 7.5	91.3^{a} ± 30.0	296.8^{b} ± 54.0	1088.57	**<0.001**
	VSP2	22.5^{a} ± 6.5	9.86^{ab} ± 3.90	3.22^{b} ± 0.71	83.52	**0.010**
myc2	*EIN2*	0.81^{a} ± 0.10	1.28^{b} ± 0.18	1.38^{b} ± 0.17	0.48	**0.049**
	ORA59	5.93 ± 1.35	8.05 ± 1.56	9.05 ± 1.03	3.39	0.295
	PDF1.2	205.5 ± 91.6	51.7 ± 13.8	125.0 ± 33.0	492.41	0.103
	PR-1	72.1 ± 18.6	45.2 ± 18.1	49.1 ± 9.4	29.47	0.494
	VSP2	124.3^{a} ± 22.5	34.3^{b} ± 13.7	12.5^{b} ± 2.9	483.84	**<0.001**
ein2-1	*EIN2*	0.84 ± 0.03	1.16 ± 0.18	0.85 ± 0.13	0.27	0.212
	ORA59	2.41 ± 0.47	4.59 ± 1.79	0.91 ± 0.09	3.96	0.238
	PDF1.2	105.8 ± 53.5	73.2 ± 27.1	97.6 ± 16.3	25.84	0.799
	PR-1	144.3 ± 75.0	55.9 ± 15.7	78.5 ± 27.7	219.23	0.327
	VSP2	338.4^{a} ± 44.7	116.4^{b} ± 26.6	53.6^{b} ± 16.7	1285.48	**<0.001**
ora59	*EIN2*	0.77 ± 0.22	1.01 ± 0.14	1.17 ± 0.08	0.42	0.242
	ORA59	1.30 ± 0.40	2.15 ± 0.40	1.16 ± 0.14	1.77	0.092
	PDF1.2	26.2^{a} ± 9.1	415.2^{b} ± 93.3	2.40^{a} ± 0.97	3732.90	**<0.001**

Phytohormone analysis

The levels of JA and JA-Ile were significantly affected by *P. xylostella* infestation in Col-0 plants, *myc2* and *ora59* mutants (Figure 2, Table 5). Simultaneous feeding by *P. xylostella* caterpillars plus aphids induced significantly higher levels of JA and JA-Ile compared to aphids feeding alone (Figure 2). This is in line with increased expression of the JA(/ET)-responsive marker genes *VSP2* and *PDF1.2* in all plant genotypes tested after *P. xylostella* infestation (Table 3).

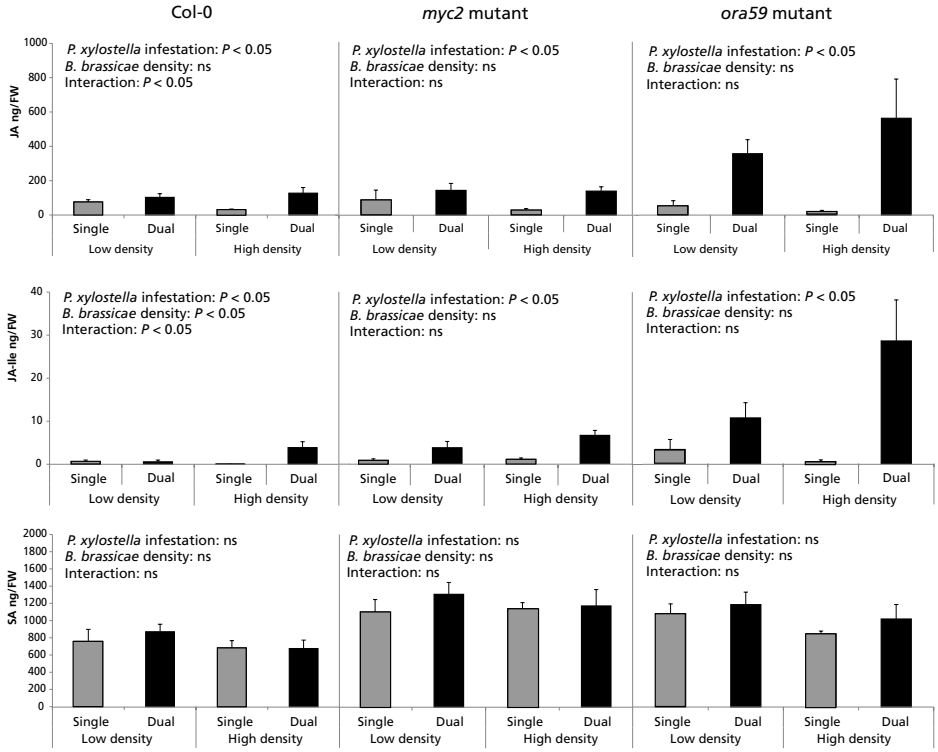

Figure 2. Phytohormone levels in leaves of *A. thaliana* wild-type Col-0 and mutants *myc2* and *ora59* at 48 h after single *B. brassicae* (grey bars) or dual *P. xylostella* and *B. brassicae* (black bars) infestation. Plants were infested with either a low (5 aphids) or high (25 aphids) density of *B. brassicae* aphids. The main effects of *P. xylostella* presence, *B. brassicae* density and the interaction on phytohormone level was examined (GLM). ns, not significant. Bars represent means ± SE (n = 3-5 biological replications). Levels of plant hormones jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile) and salicylic acid (SA) were quantified by HPLC-MS/MS.

Table 5. Statistical analysis of phytohormone level (mean ± SE) in leaves of *A. thaliana* wild-type Col-0 and mutants *myc2* and *ora59* at 48 h after single *B. brassicae* and simultaneous *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (5 aphids) or high (25 aphids) density of *B. brassicae* aphids. Generalized Linear Model deviance table for effect of *P. xylostella* presence and *B. brassicae* density and their interaction term. Bold numbers indicate significant effects ($P < 0.05$).

		Factors				Interaction	
		P. xylostella infestation (1)		*B. brassicae* density (2)		1 x 2	
		d.f. = 1		d.f. = 1		d.f. = 1	
Plant	Phytohormone	deviance	*P*	deviance	*P*	deviance	*P*
Col-0	JA	232.01	**0.003**	6.10	0.582	107.56	**0.031**
	JA-Ile	15.75	**0.005**	7.67	**0.035**	8.95	**0.024**
	SA	18.29	0.624	121.54	0.216	20.40	0.605
myc2	JA	426.78	**0.034**	62.18	0.391	129.02	0.222
	JA-Ile	33.87	**0.002**	4.18	0.213	0.13	0.825
	SA	60.10	0.427	9.97	0.744	30.19	0.572
ora59	JA	3321.00	**0.002**	199.50	0.355	117.40	0.475
	JA-Ile	119.61	**0.004**	32.81	0.082	17.39	0.193
	SA	69.54	0.372	140.75	0.212	7.92	0.760

Interestingly, there was a significant interaction between the effects of *P. xylostella* infestation and *B. brassicae* density in Col-0 plants for both JA and JA-Ile levels (Figure 2, Table 5). At high aphid density, additional *P. xylostella* infestation resulted in higher JA and JA-Ile levels than in response to infestation by aphids alone. At a low aphid density, additional *P. xylostella* infestation had no effect. Furthermore, JA-Ile levels were significantly affected by *B. brassicae* density in Col-0 plants (Table 5). The level of JA-Ile was significantly higher in plants induced by single and dual infestation at high aphid density compared to plants induced by single and dual infestation at low aphid density (Figure 2). Feeding by *P. xylostella* and density of *B. brassicae* did not affect SA levels in Col-0, *myc2* and *ora59* plants (Figure 2, Table 5).

In addition, levels of JA, JA-Ile and SA were compared between plants infested with *P. xylostella* alone and with both *P. xylostella* and either a low or high *B. brassicae* density (Table 6). In Col-0 plants, JA was induced to significantly higher levels after caterpillar feeding compared to plants simultaneously infested by caterpillars and aphids at both densities (Table 6). This is comparable to the

activation of JA signaling in Col-0 plants as seen before (Table 4). Interestingly, the level of SA was significantly affected by *P. xylostella* feeding in Col-0 plants and mutant *ora59* compared with dual-infested plants at low and high density (Table 6). *P. xylostella* feeding induced significantly higher levels of SA compared to the situation when both insect species were feeding simultaneously. As no significant differences in JA and SA levels were found in *myc2* mutants (Table 6), this indicates that for the induction of SA levels by *P. xylostella* feeding intact JA signaling is needed.

Table 6. Statistical analysis of phytohormone levels (mean ± SE) in leaves of *A. thaliana* wild-type Col-0 and mutants *myc2* and *ora59* at 48 h after single *P. xylostella* or simultaneous *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (LD, 5 aphids) or high (HD, 25 aphids) density of *B. brassicae* aphids. Plant hormones jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile) and salicylic acid (SA) were measured by HPLC-MS/MS (n = 3-5 biological replications). Generalized Linear Model deviance table for effect of treatment. Bold numbers indicate significant effects ($P < 0.05$). Hormone levels indicated with different letters are significantly different between treatments.

					Factor	
					Treatment	
Plant	Phytohormone	Phytohormone level			d.f. = 2	
		Single	Dual LD	Dual HD	deviance	*P*
Col-0	JA	291.6^{a} ± 51.4	102.2^{b} ± 13.2	127.3^{b} ± 4.6	573.02	**0.006**
	JA-Ile	4.13 ± 2.48	0.57 ± 0.41	3.84 ± 1.39	17.75	0.167
	SA	1490.8^{a} ± 150.4	869.8^{b} ± 140.1	675.5^{b} ± 84.4	1726.40	**0.001**
myc2	JA	179.6 ± 33.9	174.0 ± 69.4	168.5 ± 10.9	1.79	0.981
	JA-Ile	9.67 ± 4.14	3.62 ± 1.72	6.48 ± 1.42	14.30	0.285
	SA	1437.0 ± 183.4	1313.7 ± 144.8	1178.1 ± 70.7	128.50	0.620
ora59	JA	281.4 ± 91.9	396.6 ± 33.7	625.6 ± 10.3	470.60	0.460
	JA-Ile	8.81 ± 4.22	10.7 ± 3.6	28.8 ± 9.7	56.09	0.136
	SA	1884.8^{a} ± 356.7	1188.3^{b} ± 113.8	1021.0^{b} ± 33.7	1145.90	**0.024**

Discussion

Interactions between induced plant defense signaling pathways are dependent on different factors, such as the number of insect species attacking simultaneously (De Rijk et al., 2013), their feeding guilds (Bidart-Bouzat and Kliebenstein, 2011; Appel et al., 2014) and densities (Dicke et al., 2009; Zhang et al., 2009; Soler et al., 2012; Li et al., 2014; Kroes et al., 2015). Here, we show that enhanced

levels of direct and indirect defense against *B. brassicae* aphids, when feeding simultaneously with *P. xylostella* caterpillars depend on the induction of both ET- and JA-mediated defense responses (Figure 3). Moreover, aphid density plays an important role in this interference: *P. xylostella* caterpillars induce changes in JA and JA-Ile phytohormone levels only when feeding simultaneously with aphids at a high density. In addition, feeding by aphids at low or high density differentially affects activation of the JA-mediated transcription factor *MYC2,* and consequently the regulation of JA/SA crosstalk in *A. thaliana* plants.

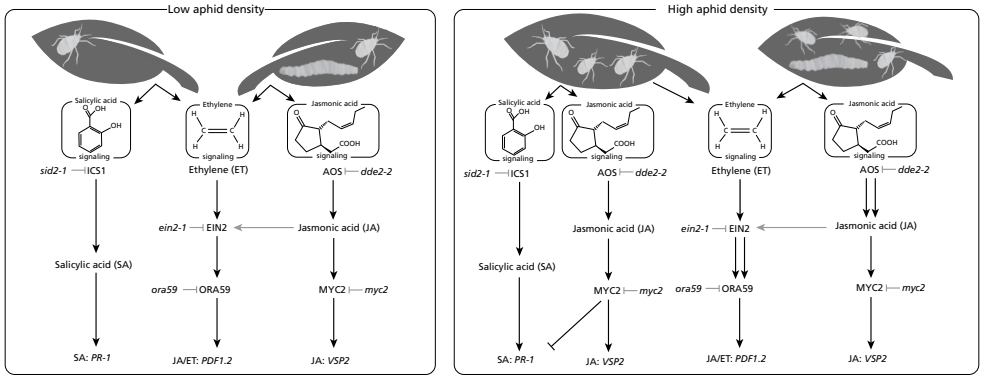

Figure 3. Working model of SA-, JA- and ET-mediated defense regulation in response to infestation by *B. brassicae* aphids alone or infestation by both *P. xylostella* caterpillars and *B. brassicae* aphids in *A. thaliana* plants. Defense signaling is shown for both a low and a high aphid density. Arrows indicate induction, whereas blocked lines indicate suppression. Double arrows indicate a significantly higher level of plant gene transcription or phytohormone biosynthesis relative to undamaged plants, compared to the low aphid density situation. Grey lines represent results from literature (Wildermuth et al., 2001; Lorenzo et al., 2004; Wang et al., 2007; Leon-Reyes et al., 2010; Verhage et al., 2011; Zhu et al., 2011; Van der Does et al., 2013).

Effects on direct defense

In Col-0 plants, *P. xylostella* caterpillars influence *B. brassicae* aphid-induced defenses which has negative consequences for aphid performance. Interestingly, caterpillar feeding did not affect aphid-induced responses in the defense signaling mutants *sid2-1* (deficient in SA production), *dde2-2* (deficient in JA production), *myc2* (defective in JA-responsive transcription factor MYC2) and *ora59* (defective in JA/ET-responsive transcription factor ORA59). This indicates that JA-, SA- and ET-signaling play important roles in modulation of aphid-induced defenses by *P. xylostella* feeding. In contrast, simultaneous feeding by caterpillars positively influenced aphid population development on *ein2-1* mutants, which further

confirms the requirement of intact ET signaling in *A. thaliana* for plant-mediated effects of caterpillars on aphid-induced defense responses.
On the other hand, growth rate of *P. xylostella* caterpillars was reduced on *ein2-1* mutants simultaneously infested with a high aphid density compared with caterpillars feeding on *ein2-1* mutants infested with a low aphid density and without aphids. This suggests that aphid modulation of *P. xylostella*-induced defenses is dependent on ET signaling and aphid density.
The role of ET signaling in plant defense against aphids has not been fully resolved. Growth of *B. brassicae* aphid populations was not altered on the *A. thaliana* ethylene-insensitive *etr1* mutant compared to performance on Col-0 plants (Mewis et al., 2005). As EIN2 has an important role in callose deposition and regulation of glucosinolate biosynthesis (Lu et al., 2013; Groen and Whiteman, 2014), fecundity of *M. persicae* aphids was increased on *A. thaliana ein2* mutants compared to Col-0 plants (Kettles et al., 2013; Lu et al., 2013). Differences in the response of aphid species to ET-mediated defenses may be explained by degree of host-plant specialization. The aphid *B. brassicae* is a specialist herbivore that is adapted to glucosinolates (Kazana et al., 2007), whereas the generalist *M. persicae* is negatively affected by glucosinolates (Kim et al., 2008). To investigate the hypothesis that aphid specialization affects aphid response to ET-mediated defenses, a comparison of several specialist and generalist species should be made.

Effects on indirect defense

To study the overall effect of caterpillar feeding on induced defenses against aphids, with a focus on the role of ET in plant defense against aphids and dual herbivory, we not only addressed interference with direct but also with indirect aphid-induced defenses in *A. thaliana* Col-0 plants and *ein2-1* (ET-insensitive) mutants. We found that *D. rapae* parasitoids had a significant preference for volatiles from aphid-infested Col-0 plants and *ein2-1* mutants, which confirms previous findings that these parasitoids respond to volatile blends emitted by *B. brassicae*-infested *A. thaliana* plants (Kos et al., 2012). Interestingly, simultaneous feeding by *P. xylostella* caterpillars on Col-0 plants increased *D. rapae*'s preference for odors from aphid-infested plants. Volatiles from plants infested by both caterpillars and aphids attracted more *D. rapae* than those of plants infested by aphids only. Because *D. rapae* females are known to distinguish between plant odor blends induced by either aphids or *P. xylostella* caterpillars (Agbogba and Powell, 2007), the observed positive effect of caterpillar feeding on induced

indirect defenses against aphids likely results from a modified plant response to the simultaneous feeding by caterpillars and aphids. In contrast, Agbogba and Powell (2007) recorded that *D. rapae* parasitoids did not distinguish between volatiles emitted by cabbage plants (*Brassica oleracea)* infested with generalist *M. persicae* aphids and plants infested with *M. persicae* plus *P. xylostella* caterpillars. This indicates that caterpillars do not affect aphid-induced indirect defenses in this case. Whether this is due to the different plant species, aphid species or both, remains to be elucidated.

Development of *D. rapae* parasitoids was significantly faster in *B. brassicae* aphids feeding simultaneously with *Pieris brassicae* caterpillars compared to parasitoids developing in aphids feeding alone on *B. oleracea* (Soler et al., 2012). This result on *D. rapae* development is consistent with our finding of *D. rapae* preference for plants simultaneously infested with aphids and caterpillars. Upon disruption of the ET-signaling pathway, *D. rapae* did no longer distinguish between *ein2-1* (ET-insensitive) mutants infested by aphids or by both aphids and caterpillars. This shows that intact ET signaling is needed for caterpillar modulation of the attraction of *D. rapae* parasitoids. Also for interference by whiteflies with indirect defenses against *P. xylostella* the ET-signaling pathway of *A. thaliana* needs to be intact (Zhang et al., 2013). The ET signaling pathway, in particular *EIN2,* plays an important role in the biosynthesis of glucosinolates (Lu et al., 2013). Moreover, glucosinolate levels increased in Col-0 plants after *B. brassicae* feeding. As a result, disrupted ET signaling in *A. thaliana etr1* mutants reduced glucosinolate level in response to *B. brassicae* feeding (Mewis et al., 2005). Since *D. rapae* parasitoids are attracted to glucosinolate hydrolysis products such as isothiocyanates (Pope et al., 2008; Blande et al., 2007), caterpillars may therefore influence the production of host-finding cues for *D. rapae* parasitoids through ET signaling.

Caterpillar effects on indirect defense to aphids was found to be independent of the aphid densities we tested. Similarly, it has also been recorded that the behavioral response of *D. rapae* parasitoids towards volatiles emitted by *Brassica nigra* L. plants infested with both *P. brassicae* caterpillars and *B. brassicae* aphids was independent of aphid density (Ponzio et al., 2016).

Effects on molecular defense response

The induction of plant responses is regulated by phytohormones among which jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) play central roles (Pieterse et al., 2012). Their signaling pathways cross-communicate in an intricate network allowing the plant to fine-tune its defense (Robert-Seilaniantz et al., 2011;

Derksen et al., 2013; Zhu and Lee, 2015). We hypothesized that differential regulation of defense signaling pathways in response to feeding by both caterpillars and aphids is dependent on aphid density, and can thus affect the outcome of plant defense.

Involvement of JA and ET signaling

We show that expression levels of both *VSP2* and *PDF1.2* and levels of the phytohormones JA and JA-Ile are significantly higher when Col-0 plants are infested by both caterpillars and aphids than by aphids only, which could lead to a higher direct defense against aphids when feeding simultaneously with caterpillars (Figure 3). Aphid performance is known to be negatively influenced by JA-mediated defense responses (Ellis et al., 2002; Kusnierczyk et al., 2011). Furthermore, the effects of *P. xylostella* caterpillars on aphid-induced defenses was aphid density-dependent in Col-0 plants. At high aphid density, *P. xylostella* feeding led to higher JA and JA-Ile levels than infestation by aphids alone. In addition, JA-mediated signaling is not the only factor underlying this effect. An enhanced expression of *VSP2* and *PDF1.2* by caterpillar infestation of aphid-infested plants in *myc2*, *ora59* and *ein2-1* mutants indicates a role for both JA- and ET-mediated defense responses (Table 3). Simultaneous feeding by caterpillars and aphids on Col-0 plants resulted in increased *VSP2* levels, whereas levels of *VSP2* were reduced in *myc2* mutants upon feeding by aphids and caterpillars. This indicates that MYC2 is important for the expression of *VSP2* (Verhage et al., 2011).

Caterpillars enhance O*RA59* expression in Col-0 plants and ethylene signaling (*EIN2* expression in *myc2* mutants). Aphids feeding at high density and feeding by both caterpillars and aphids induced significantly higher transcript levels of *ORA59* compared to aphids feeding alone or at low density in Col-0 plants. This suggests the importance of *ORA59* for caterpillar interference with aphid-induced defenses, and further underlines the regulation of JA/ET-mediated defense responses against aphids at different densities. Although *ORA59* expression was affected by simultaneous feeding by caterpillars and aphid density in Col-0 plants, it was not expected for the *ora59* mutant to show differences in *ORA59* expression. This could indicate that this mutant has a leaky *ORA59* mutation. On the other hand, a nucleotide similarity exists between coding regions of *ORA59* and *AtERF15* (members of the AP2/ERF family of transcription factors), making it difficult to design specific primers for reverse transcription-PCR (Pre et al., 2008). Therefore, *ERF15* may be partially expressed in this mutant with the designed

primer pair. Indeed, up-regulated expression level of '*ORA59*' in this mutant is very low.

Involvement of JA and SA signaling

Aphids feeding at high density on *myc2* plants, which are defective in the transcription factor MYC2/JIN1, induced significantly higher levels of *PR-1* compared to aphids feeding at low density (which was not found for Col-0 plants). This shows that in wild-type plants MYC2-mediated suppression of *PR-1* expression levels is induced more strongly at a high density of aphids. Furthermore, this suggests the importance of SA-JA crosstalk in defense responses against aphids attacking at different densities. Aphid interference with direct defenses against *P. xylostella* caterpillars also required both SA and JA signaling and was also aphid density-dependent (Kroes et al., 2015). JA-dependent *MYC2* expression was higher in plants simultaneously attacked by caterpillars and aphids at high density compared to caterpillars feeding alone, which led to down-regulation of SA-mediated defenses (Kroes et al., 2015). This negative correlation between SA- and JA-dependent defenses in response to attack by multiple herbivores was shown before (Rodriguez-Saona et al., 2010; Soler et al., 2012; Ali et al., 2014), although SA-mediated suppression of JA signaling seems to affect interactions between plants and multiple attackers more strongly. The antagonistic effect of JA on SA-mediated defenses or synergistic effects between SA and JA are less often found in studies on plant-insect interactions, therefore the outcome of JA-SA interactions can be specific for the herbivore or plant species. In addition, the outcome of the negative interaction between JA and SA signaling is greatly influenced by the sequence and timing of insect attack and signal molecule production (Mur et al., 2006; Koornneef et al., 2008).

We also observed enhanced resistance to *P. xylostella* caterpillars on plants simultaneously infested by aphids. Caterpillars performed worse when feeding simultaneously with aphids at high density on the *ein2-1* mutant. This was probably due to crosstalk between SA and JA signaling, as increased levels of *PR-1* were associated with reduced *VSP2* levels in *myc2* mutants after simultaneous feeding by caterpillars and aphids at high density compared to caterpillars feeding alone. Recently, Onkokesung et al. (2016) reported that enhanced resistance against *P. brassicae* caterpillars in *A. thaliana* plants previously attacked by *B. brassicae* aphids was dependent on nduction of sinapate malate. Interestingly, the mechanism regulating sinapate malate induction might act independently from JA-SA crosstalk.

Crosstalk between SA and JA signaling may be important for the regulation of defenses against *P. xylostella* caterpillars alone. As shown before, *P. xylostella* feeding activated SA-mediated defense responses in *A. thaliana* plants (Ehlting et al., 2008; Kroes et al., 2015). We also detected significantly higher SA levels in *P. xylostella*-infested plants. It is interesting that *P. xylostella* feeding alone compared to dual insect attack did not result in differences between SA levels in *myc2* mutants. Hence, we speculate that JA acts synergistically on SA biosynthesis in response to *P. xylostella* feeding.

It has been proposed that aphids manipulate plant defenses by suppressing JA-dependent defenses through SA-JA crosstalk by activating SA signaling (De Vos et al., 2007), which could be dependent on the density of the attacking aphid. We show that the effects of caterpillar feeding on aphid-induced defenses are aphid-density dependent and JA-mediated (higher JA and JA-Ile levels in Col-0 plants infested by caterpillars plus aphids compared to infestation by aphids alone). By activating JA-dependent defenses in response to simultaneous feeding of caterpillars and aphids at high density, plants could increase defense against aphids. Interestingly, SA-mediated suppression of ORA59 protein accumulation (Van der Does et al., 2013) could explain manipulation of plant defenses through SA-JA crosstalk by aphids at low density. Another signaling node potentially underlying defense manipulation by aphids via ORA59 regulation, are TGA transcription factors. TGAs regulate SA-induced *PR* expression (Zhang et al., 2003) and expression of JA/ET-dependent genes such as *PDF1.2* (Zander et al., 2010). It was shown by Zander et al. (2014) that TGAs could directly target *ORA59* and regulate SA-mediated suppression of *ORA59.*

Conclusion

In conclusion, by integrating analyses of phytohormone levels and defense gene expression, we show how insect attackers differentially affect molecular integrators of defense signaling pathways depending on insect density and single or multiple attack. Our study implies that crosstalk between JA, SA and ET signaling is essential for plant-mediated interactions between attacking aphids and caterpillars. Induced *VSP2* and *PDF1.2* transcript levels mediated by activation of *ORA59* indicated that *P. xylostella* caterpillars enhance induced defenses against aphids by affecting JA- and JA/ET-signaling. This underlines the importance of studying other signaling molecules in addition to JA and SA and is an important step towards determining regulatory signaling networks that underlie plant defense to multiple herbivory. Moreover, aphid density is

an important factor in mediating plant responses. For example, dependent on aphid density, caterpillar feeding interfered with regulation of JA and ET defense responses. Moreover, induced defenses against aphids feeding at different densities rely on SA-JA crosstalk. We demonstrate links between identified molecular defense mechanisms and ecological consequences of plant defense to insect attackers. This insight contributes to understanding plant defense regulation to multiple insect attack and is important for the development of durable novel methods of crop protection.

Acknowledgments

We thank Léon Westerd, Frans van Aggelen and André Gidding for culturing the insects; Lucille Chrétien for help with culturing *D. rapae* parasitoids; Kerstin Ploss for technical support with the HPLC analysis. This work was supported by a TOP GO grant (grant number 854.10.010 to M.D.) from The Netherlands Organization for Scientific Research (NWO).

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Chapter 6

Effect of different densities of *Brevicoryne brassicae* aphids on whole-genome transcriptional responses of *Arabidopsis thaliana* to feeding by *Plutella xylostella* caterpillars

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Abstract

Plant responses to attack by insect herbivores are regulated at the transcriptional level. The insect species, its feeding guild and the density to which the plant is exposed affect transcriptional regulation. Throughout the growing season plants are commonly attacked by multiple herbivorous species, however, little is known about transcriptional mechanisms that determine plant responses to insect attackers feeding simultaneously on the plant.

We assessed transcriptomic responses of *Arabidopsis thaliana* plants to simultaneous feeding by *Plutella xylostella* caterpillars and *Brevicoryne brassicae* aphids compared to plants infested by *P. xylostella* caterpillars alone, using a microarray analysis. We particularly addressed the question how aphid feeding interferes with the transcriptomic response to *P. xylostella* caterpillars and whether this interference is dependent on aphid density and time since the start of herbivory.

Differences in gene expression were found between plants infested by caterpillars and plants infested by caterpillars plus aphids at both densities investigated. Interestingly, some important modulators of plant defense signaling, including *WRKY* transcription factor genes and ABA-dependent genes, were differentially induced in response to simultaneous aphid feeding at low or high density compared with responses to *P. xylostella* caterpillars feeding alone. Furthermore, aphid density was found to affect transcriptomic responses, which caused an acceleration in plant response against dual insect attack at high aphid density compared to dual insect attack at low aphid density.

In conclusion, our study provides understanding of how aphid density affects mechanisms underlying interactions of plants with dual insect infestation. It highlights the importance of addressing insect density as well as time since the onset of herbivory to understand plant responses to dual or single insect attack.

Keywords

Arabidopsis thaliana, *Brevicoryne brassicae*, density dependence, interference, multiple herbivory, *Plutella xylostella*

Introduction

Insect herbivores can cause severe damage to plant leaf tissues (Bonaventure, 2014). The combined action of elicitors in insect saliva and physical plant damage results in the induction of a plant defense response (Kessler and Baldwin, 2002; Howe and Jander, 2008; Wu and Baldwin, 2009; Felton et al., 2014). The defense-related phytohormones jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and salicylic acid (SA) have been recognized to play key roles in mediating plant defense responses (Erb et al., 2012; Broekgaarden et al., 2015). The different phytohormone signaling pathways operate in a network, and are known to interact with each other which allows the plant to activate an adequate defense response (Robert-Seilaniantz et al., 2011; Derksen et al., 2013; Pieterse et al., 2012). Specific nodes in this network, such as the transcriptional regulators WRKY70 and ORA59, integrate phytohormonal signaling to regulate plant defenses in response to herbivory (Caarls et al., 2015).

Depending on the feeding guild and species identity of the attacking insects, specific plant defense responses are induced (De Vos et al., 2005; Bidart-Bouzat and Kliebenstein, 2011; Appel et al., 2014). For instance, *Brassica rapa* plants respond to feeding by *Pieris brassicae* caterpillars or *Brevicoryne brassicae* aphids by differential induction of glucosinolates (Sotelo et al., 2014).

Plant defenses induced by leaf-chewers such as caterpillars are mainly regulated by the phytohormone jasmonic acid (JA) and its derivatives such as methyl jasmonate (MeJA) and jasmonic acid-isoleucine (JA-Ile) (Thaler et al., 2002; Turner et al., 2002; Halitschke and Baldwin, 2003; Koo and Howe, 2009; Verhage et al., 2011; Rehrig et al., 2014). In *Arabidopsis thaliana,* JA-Ile binds to the CORONATINE INSENSITIVE 1 (COI1) receptor which mediates the degradation of JAZ repressor proteins (Thines et al., 2007). These proteins repress JA signaling by binding to the transcription factor MYC2 that regulates JA-responsive genes such as *PLANT DEFENSIN 1.2* (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) (Lorenzo et al., 2004; Memelink, 2009; Kazan and Manners, 2013). Responses to caterpillars are mediated by two branches of the JA signaling pathway, the MYC2-regulated and the ERF-regulated branch (Lorenzo et al., 2003), and both branches cross-communicate with the ET and ABA pathways (Lorenzo and Solano, 2005; Pieterse *et al.*, 2012; Kazan and Manners, 2013). In addition, MYC2 regulates the biosynthesis of defensive secondary metabolites such as glucosinolates (Dombrecht et al., 2007; Kazan and Manners, 2013) and terpenoids (Hong et al., 2012; Kazan and Manners, 2013).

Different from leaf-chewing by caterpillars, aphids feed on the plant's phloem, by inserting their stylets into the sieve elements (De Vos et al., 2007; Stam et al., 2014). Regulation of plant defenses to aphid feeding has been investigated in *A. thaliana* plants (Moran et al., 2002; De Vos et al., 2005; Couldridge et al., 2007; Kusnierczyk et al., 2008; Barah et al., 2013; Appel et al., 2014; Hillwig et al., 2016). In response to infestation by *B. brassicae* aphids, specialised on Brassicaceae, expression of genes involved in SA-dependent defenses was induced (Moran et al., 2002; Kusnierczyk et al., 2008; Barah et al., 2013). In addition, Barah et al. (2013) found induction of genes related to the biosynthesis of tryptophan-derived secondary metabolites, the ET signaling pathway, as well as to cell wall metabolism (Kusnierczyk et al., 2008). Moreover, also JA-regulated defenses are found to be involved in responses to aphid feeding (Moran et al., 2002; Kusnierczyk et al., 2008; Morkunas et al., 2011).

Throughout the growing season plants are commonly attacked by multiple herbivorous species. The responses of plants to herbivory may impact other insects feeding on the same host plant (Rodriguez-Saona et al., 2010; Soler et al., 2012; Zhang et al., 2013; Stam et al., 2014). Interestingly, induced defenses in response to aphid herbivory can interfere with defenses against caterpillars (Rodriguez-Saona et al., 2010; Tzin et al., 2015b; Onkokesung et al., 2016), which can have positive or negative effects on the performance of the attacking herbivores (Soler et al., 2012; Ali et al., 2014; Li et al., 2014; Kroes et al., 2015). For example, while oleander aphids (*Aphis nerii*) developed more slowly on milkweed plants (*Asclepias syriaca*) previously infested by monarch caterpillars (*Danaus plexippus*) (Ali et al., 2014), *B. brassicae* aphids developed faster on cabbage plants previously infested by *Pieris brassicae* caterpillars (Soler et al., 2012). Such a difference in the effect of multiple insect feeding on induced defenses can be explained by dissimilarities between species, but may also be a result of insect density effects (Zhang et al., 2009; Kroes et al., 2015; Stewart et al., 2016). Therefore, an interesting question is whether mechanisms underlying multiple insect-plant interactions are affected by the density of the attacking insects. Investigating effects of insect density on plant defense responses can provide novel insights for studies on plant-mediated interactions between multiple attacking insects. Molecular aspects of plant-mediated interactions among multiple insects have been studied, and the data show that transcriptomic responses to multiple attack is clearly different from responses to single insect attack (Voelckel and Baldwin, 2004; Rodriguez-Saona et al., 2010; Zhang et al., 2013; Davila Olivas et al., 2016). For instance, simultaneous attack by *Spodoptera*

exigua caterpillars and *Macrosiphum euphorbiae* aphids on tomato plants (*Solanum lycopersicum*) induced a different transcriptomic response compared to aphid- or caterpillar-infested plants (Rodriguez-Saona et al., 2010). This study also demonstrated that aphid feeding suppressed caterpillar-induced genes, whereas caterpillar feeding down-regulated the expression of genes up-regulated by aphids (Rodriguez-Saona et al., 2010). In *A. thaliana* plants dually infested by the phloem-feeding whitefly *Bemisia tabaci* and *P. xylostella* caterpillars, whiteflies suppressed the expression of genes up-regulated by *P. xylostella* caterpillars (Zhang et al., 2013). Investigating both the effects of multiple herbivory by insects belonging to different feeding guilds and insect density is an important step towards unravelling how simultaneously feeding herbivores affect transcriptional mechanisms that determine plant responses to insect attackers.

In the present microarray analysis we studied interactive effects of the leaf chewing larvae of *Plutella xylostella* and *B. brassicae* aphids. *Plutella xylostella* caterpillars are one of the most destructive pests that damage brassicaceous species and cultivars (Sarfraz et al., 2006; Barker et al., 2007; Niu et al., 2013). Defense responses induced by *P. xylostella* caterpillars in plants from the Brassicaceae family have also been studied in *A. thaliana* plants (Stotz et al., 2000; Ehlting et al., 2008; Herde et al., 2008; Bidart-Bouzat and Kliebenstein, 2011; Zhang et al., 2013; Kroes et al., 2015).

We assessed transcriptomic responses of *A. thaliana* plants to simultaneous feeding by *P. xylostella* caterpillars and *B. brassicae* aphids compared to plants infested by *P. xylostella* caterpillars alone. We particularly addressed the question whether the transcriptomic response to simultaneous attack by aphids and caterpillars is dependent on the density of aphids and time since initiation of herbivory. To study density-dependent effects on transcriptomic responses, plants were infested with a low or high aphid density. In addition, transcriptional responses were studied at two time points.

Materials & Methods

Plant growth conditions

Seeds of *Arabidopsis thaliana* accession Columbia-0 (Col-0) were sown in autoclaved (80 °C for 4 h) potting soil (Lentse potgrond, Lent, The Netherlands). Plants were cultivated in a growth chamber at 21 ± 2 °C under an 8L : 16D cycle [200 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) light intensity] and 60 ± 10 % relative humidity (RH). Two-week-old seedlings were transferred to

individual pots (5 cm diameter) containing similar soil. Plants were watered three times a week. Five-week-old plants were exposed to different insect-infestation treatments. During the experiments, all plants remained in the vegetative state.

Insects

Both the Cabbage aphid, *B. brassicae* L. (Hemiptera: Aphididae), and the Diamondback moth, *P. xylostella* L. (Lepidoptera: Yponomeutidae), were reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv Cyrus) at 22 ± 1 °C, 50-70 % RH, 16L : 8D cycle.

Insect infestation treatments

Plants were infested with (1) two second-instar (L2) caterpillars (indicated as single infestation), (2) simultaneously infested with two L2 caterpillars and a low density of five adult aphids (abbreviated as Dual LD for Dual Low Density), (3) simultaneously infested with two L2 caterpillars and a high density of 25 adult aphids (abbreviated as Dual HD for Dual High Density), or (4) left uninfested (indicated as control). Insects were allowed to feed freely on the plants.
Individual plants were placed in a plastic container (diameter 8 cm x height 14 cm), covered with gauze cloth and closed with elastic bands. Containers were randomly distributed in a tray (12-15 containers per tray). Trays were placed in a growth chamber with a 16L : 8D cycle [200 µmol m^{-2} s^{-1} PAR], at 21 ± 2 °C and 50-70% RH.
Leaves damaged by insect feeding or control leaves from uninfested plants were collected after 24 or 48 h of infestation. The experiment was performed in two rounds started on two successive days (February 2015). For each treatment and time point, four biological replicates were obtained by performing two biological replicates per round. One biological replicate consisted of six leaves pooled from three different plants. For each time point, a different set of plants was used. Insects were removed from the leaves before harvesting. Leaf samples were flash-frozen in liquid nitrogen and stored at -80 °C prior to analysis.

RNA extraction and microarray hybridization

Total RNA was extracted from finely ground frozen leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA samples were treated with DNase (Qiagen, Hilden, Germany). The concentration and purity of RNA was determined by spectrophotometry and integrity was confirmed using an Agilent

2100 Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). Whole-genome transcriptome analysis was conducted by hybridizing four biological samples of total RNA per treatment to Affymetrix Arabidopsis Gene 1.1 ST Array Strips (Affymetrix, Santa Clara, CA, USA).

Microarray data analysis

The raw data files (CEL files) were normalized using the Robust Multi-array Average (RMA) background correction with quantile normalization, $\log_2$ transformation and mean probe-set summarization with adjustment for GC content. Normalized gene expression data obtained from the microarray experiments were initially statistically analyzed with one-way and two-way ANOVA using the software TIGR MeV version 4.9 (Saeed et al., 2003; Saeed et al., 2006) to study the effects of treatment, time point and their interaction on gene expression levels, with $\alpha = 0.05$. Expression ratios of the genes significantly differentially expressed between the four treatment groups (Control, *P. xylostella*, Dual LD and Dual HD) and time points (24 and 48 h) were then used for further analysis.

Differentially expressed genes

Differentially expressed genes (DEGs) were identified per time point for the different single and dual-infestation treatments. Differential gene expression in caterpillar- or dual-infested plants was determined compared to expression in uninfested control plants, with gene expression in dually infested plants additionally being compared to expression in *P. xylostella*-infested plants.
Genes were considered to be differentially regulated in a given pair of treatments if a *t*-test demonstrated a significant result at $P < 0.05$ (accepting a false discovery rate of up to 0.2; Ehlting et al. (2008)) and a $\log_2$-fold change of ≤-1 or ≥1 (TIGR MeV v4.9).

Functional enrichment

Identification and enrichment of DEGs within functional gene ontology (GO) terms for biological processes was done using the online tool provided by DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/; Huang da et al., 2009)). Only enrichment groups with an enrichment score ≥ 1.3 were examined (Huang da et al., 2009). Genes were considered statistically enriched if Fisher's exact test (EASE score) resulted in $P < 0.05$ and if the Benjamini-Hochberg correction for multiple comparisons returned $P < 0.05$.

Hierarchical clustering

Genes differentially expressed at 24 and 48 h after single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation at low or high aphid density (measured relative to uninfested control samples) were organized further by hierarchical clustering. Hierarchical clustering analysis was performed with the Spearman Rank Correlation using average linkage in the software TIGR MeV version 4.9.

Statistical analysis

Multivariate data analysis

Changes in the expression pattern of genes that were significantly different between treatments were analyzed using projection to latent structures discriminant analysis (PLS-DA; Eriksson et al. (2013)) using SIMCA-P+ version 14.0 statistical software (Umetrics AB, Umeå, Sweden). The analysis determines whether samples from different treatment groups can be separated on the basis of differences in their gene expression patterns. The results of the analysis are visualized in score plots. The score plot identifies patterns that discriminate the treatments according to model components of PLS-DA. The quality of each OPLS-DA model was evaluated using the parameter R^2 (goodness of fit) and Q^2 (predictive value) (Eriksson et al., 2013).

Validation of microarray analysis by quantitative real-time PCR

cDNA was synthesized from the same RNA (1 µg) isolated for the microarray hybridization as described in the 'RNA extraction and microarray hybridization' section using iScript cDNA synthesis Kit (Bio-Rad). Transcript levels of the genes *TERPENE SYNTHASE 04* (*TPS04*) (At1g61120) (Snoeren et al., 2010), *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) (At5g24770) (Anderson et al., 2004) and *PLANT DEFENSIN 1.2* (*PDF1.2*) (At5g44420) (Anderson et al., 2004) and the reference gene *ELONGATION FACTOR 1α* (*EF1-α*) (At5g60390) (Remans et al., 2008) were quantified. Efficiency of each primer was determined before qRT-PCR analysis. Quantitative RT-PCR analysis was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Each reaction was performed in a total volume of 25 µl containing 12.5 µl SYBR Green Supermix (Bio-Rad), 5 µl cDNA and 1 µl of 10 µM forward and reverse gene-specific primer pair. For each reaction, two technical replicates were performed and average values were used in the analyses. The following thermal profile was used: 3 min 95°C, followed by 40 cycles of 15 s at 95 °C, and 45 s at 60 °C.

Relative expression for each tested gene was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and subsequently $\log_2$ transformed. Relative expression levels of *TPS04*, *VSP2* and *PDF1.2* were compared to their respective $\log_2$-expression ratios found using microarray analysis (Supplementary Materials Figure 1).

Results

Transcriptomic changes in plants in response to feeding by *P. xylostella* alone or by both *P. xylostella* and *B. brassicae*

Transcriptional responses in *A. thaliana* after 24 h (Figure 1A) and 48 h (Figure 1B) to feeding by *P. xylostella* only, or dual *P. xylostella* and *B. brassicae* at low and high density and without infestation were analyzed by PLS-DA using expression levels of all the genes that showed significant differences in expression level between treatments (based on one-way ANOVA analysis).

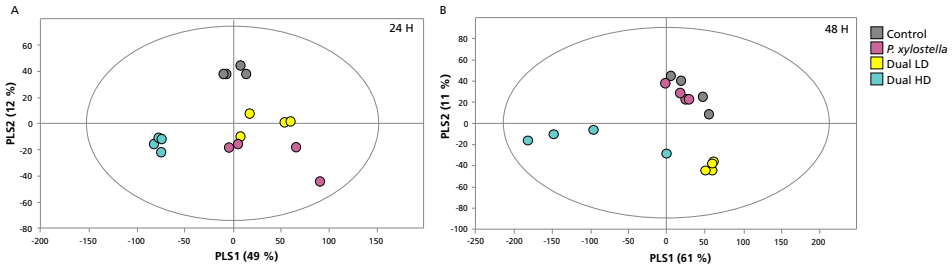

Figure 1. Partial least squares discriminant analysis (PLS-DA) of gene expression levels in *A. thaliana* at 24 h (A) and 48 h (B) after single *P. xylostella*, dual *P. xylostella* and *B. brassicae* and without infestation (control). Plants were infested with either a low (LD, 5 aphids per plant) or high (HD, 25 aphids per plant) density of *B. brassicae* aphids. The PLS-DA resulted in two models with six (24 h; $R^2X = 0.80$, $R^2Y = 0.99$ and $Q^2 = 0.92$) and five (48 h; $R^2X = 0.83$, $R^2Y = 0.98$ and $Q^2 = 0.87$) significant components, respectively. The score plots of the treatment samples at 24 and 48 h, with the percentage of explained variation in parentheses, is shown. The ellipse in the score plots defines the Hotellings's T2 confidence region (95%).

At 24 h, the first two significant principal components (PCs) explain 49 and 12 % of the total variance, respectively (Figure 1A). The first PC shows a clear separation between expression levels of dually infested plants at high density (Dual HD) versus the other three treatments, while the second PC separated expression levels based on the presence or absence of herbivores.

At 48 h, the first two significant PCs of the PLS-DA explain 61 and 11 % of the total variance, respectively (Figure 1B). As was found for the 24-h time point, the first PC shows a clear separation of expression levels between Dual HD plants versus the other three treatments, while the second PC separates transcriptome

responses based on the presence of aphids. Interestingly, when comparing the position of *P. xylostella*-infested treatment samples between the PLS-DA models of 24 and 48 h, the pattern of gene expression in response to caterpillar feeding differs more strongly from that of non-infested plants after 24 h, whereas the patterns have converged after 48 h.

Differentially expressed transcripts in plants infested by *P. xylostella* and plants infested by both *P. xylostella* and *B. brassicae,* compared to expression in uninfested plants

Differential gene expression in *A. thaliana* in response to caterpillars feeding alone or simultaneous feeding of caterpillars and aphids was determined compared to expression in uninfested control plants.
The number of differentially expressed genes (DEGs) up-regulated in response to feeding by *P. xylostella* was larger than the number of repressed genes (Figure 2A). However, the number of up-regulated genes decreased over time. When *P. xylostella* caterpillars were feeding simultaneously with *B. brassicae* aphids, the number of DEGs was higher after 24 and 48 h compared to caterpillars feeding alone (Figure 2A). Interestingly, there was an aphid-density effect on the number of DEGs over time (Figure 2A). Dual HD plants after 24 h showed a larger number of DEGs compared to dual aphid and caterpillar infestation at low aphid density (Dual LD). More repressed DEGs were found after 48 h in Dual HD plants, while the number of up-regulated DEGs was comparable to that in Dual LD plants (Figure 2A).

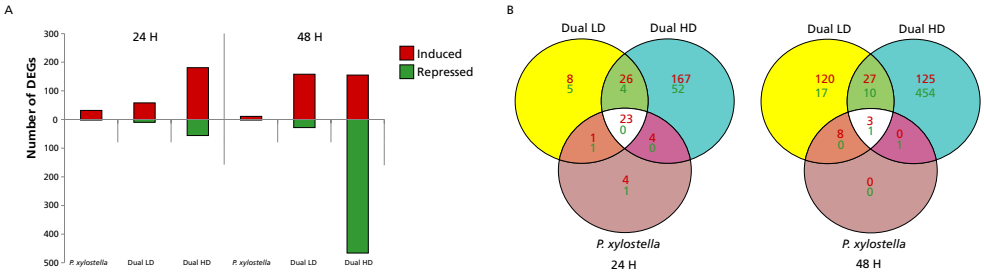

Figure 2. Differentially expressed genes (DEGs) (A) and Venn diagram of number of DEGs (B) in *A. thaliana* at 24 and 48 h after single *P. xylostella*, dual *P. xylostella* and *B. brassicae* and without infestation (control). Plants were infested with either a low (LD, 5 aphids per plant) or high (HD, 25 aphids per plant) density of *B. brassicae* aphids. Number of DEGs in *P. xylostella*-infested, Dual LD and Dual HD plants were compared with non-infested control plants. Red bars or numbers indicate up-regulated genes, while green bars or numbers represent down-regulated genes. Genes were considered to be differentially expressed if they met the criteria of $\log_2$-fold change ≤ -1 or ≥ 1 and a t-test P-value < 0.05.

In total, only 10 % of up-regulated DEGs were shared among the different treatments at 24 h (Figure 2B). Moreover, the treatments only shared 1 % of their up-regulated genes at 48 h (Figure 2B). Thus, *A. thaliana* responses to aphids and caterpillars feeding simultaneously or caterpillars feeding alone are highly dissimilar.

Mainly up-regulated DEGs in response to Dual LD or Dual HD were shared with DEGs up-regulated by *P. xylostella* feeding at both 24 and 48 h (Figure 2B). Respectively, 41 % of up-regulated genes in response to Dual LD and 12 % of up-regulated genes in response to Dual HD were shared with DEGs up-regulated by *P. xylostella* feeding at 24 h. At 48 h, a low proportion of up-regulated DEGs (< 7 %) in response to Dual LD and Dual HD were shared with DEGs up-regulated in response to *P. xylostella* feeding. In conclusion, dual herbivory by aphids and caterpillars resulted in different transcriptional responses compared to those induced by *P. xylostella* caterpillars feeding alone. Furthermore, specificity in transcriptional responses to simultaneous feeding of both herbivores or caterpillars feeding alone increased over time.

Gene clustering and GO terms

To identify biological functions of the DEGs we assigned GO terms for biological processes and performed a functional clustering analysis using the DAVID Functional Clustering Tool (Supplementary materials Tables 1, 2A-B).

For *P. xylostella*-induced genes (at both 24 and 48 h), the clusters mainly relate to responses to biotic stress and jasmonic acid stimuli, including responses to pathogens, wounding and jasmonic acid. However, when caterpillars feed simultaneously with aphids at either of the two densities, clusters also associated with metabolism of organic acids, fatty acids and lipids.

After 48 h in Dual HD plants, repressed DEGs mainly clustered in classes that relate to photosynthesis and carbohydrate metabolism, including genes encoding for thioredoxins and glutaredoxins, Photosystem (PS I and II) proteins, PsbP proteins and proteins involved in the glycolysis pathway.

Differentially expressed transcripts under dual herbivory, compared to expression in caterpillar-infested plants

To investigate how aphid density affects transcriptional responses in *A. thaliana* to dual herbivory, differential gene expression was determined compared to expression in caterpillar-infested plants. We examined differences and overlap in DEGs between Dual LD plants or Dual HD plants during 24 and 48 h (Figure 3A,B).

No DEGs were found in Dual LD plants after 24 h (Figure 3A). However, Dual LD caused changes in responses after 48 h (Figure 3B). At 48 h, a total of 87 up-regulated and 77 down-regulated DEGs were found in Dual LD plants. Of these DEGs, only 2 % up- and down-regulated genes were shared with DEGs in Dual HD plants.

Respectively 236 up-regulated DEGs after 24 h and 113 up-regulated DEGs after 48 h were found in Dual HD plants. In addition, respectively 121 and 360 DEGs were down-regulated in Dual HD plants after 24 and 48 h. In conclusion, when comparing differential expression between the two aphid densities after 48 h, DEGs have very little overlap which indicates that DEGs are highly density-specific.

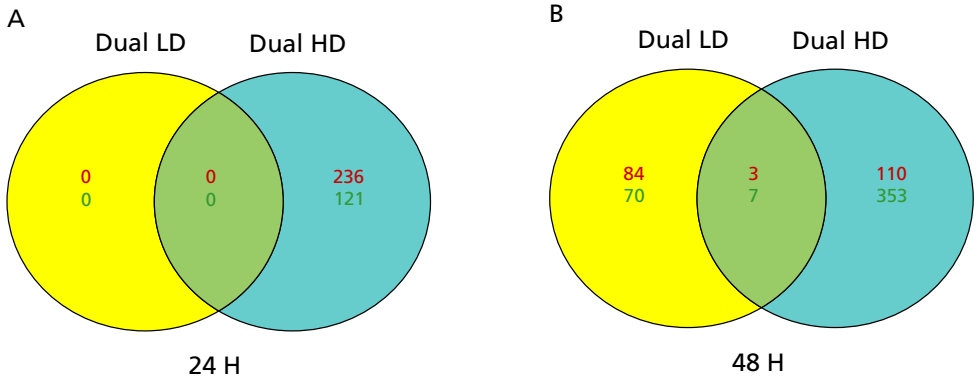

Figure 3. Venn diagram representing numbers of genes differentially expressed (DEGs) in *A. thaliana* at 24 h (A) and 48 h (B) after single *P. xylostella* and dual *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (LD, 5 aphids per plant) or high (HD, 25 aphids per plant) density of *B. brassicae* aphids. Number of DEGs specifically or co-expressed in Dual LD and Dual HD were compared with single *P. xylostella* infestation. Numbers in red indicate up-regulated genes, while numbers in green represent down-regulated genes. Genes were considered differentially expressed if they met the criteria of $\log_2$-fold change ≤ -1 or ≥ 1 and a t-test P-value < 0.05.

Gene clustering and GO terms

To identify biological functions of these genes, we assigned GO terms for biological processes and performed a functional clustering analysis using the DAVID Functional Clustering Tool (Supplementary materials Table 1, 2A-B).

After 48 h in Dual LD plants, up-regulated genes were associated with defense, cell death and auxin signaling. Repressed genes could not be clustered.

In Dual HD plants after 24 h, up-regulated genes were associated with transcriptional responses to hormone signaling (ABA- and auxin-activated signaling pathways) and carbohydrate metabolism. Repressed genes could not be clustered. At 48 h, up-regulated genes could not be clustered. For repressed

genes, clusters relate to carbohydrate metabolism, photosynthesis, responses to bacterium (several *WRKY* transcription factor genes) and biogenesis of cellular components.

Clustering of gene expression levels

We compared gene expression patterns of *A. thaliana* in response to feeding by *P. xylostella* caterpillars alone and simultaneous feeding by caterpillars and aphids at two different densities to further investigate the effect of simultaneous aphid feeding and aphid density on responses to caterpillars.

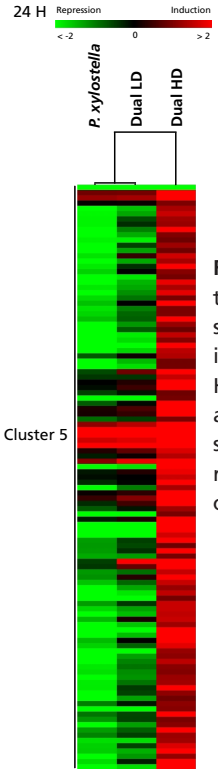

Figure 4. Heat map showing average $\log_2$-fold change ratios (measured relative to non-infested control samples) of genes expressed in *A. thaliana* at 24 h after single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (LD) or high (HD) density of B. brassicae aphids. Hierarchical clustering (HCL) was performed using Spearman correlation with average linkage clustering. Red indicates up-regulated genes, while green shows down-regulated genes. Black represents no change in expression. Each row in the columns corresponds to a single gene. Cluster analysis is shown for cluster 5.

Clustering after 24 h of herbivory

The cluster analysis shows similarities in gene expression levels in response to *P. xylostella* feeding only and to Dual LD because both treatments cluster together and are separate from gene expression levels in response to Dual HD (Supplementary materials Figure 2). Cluster 5 is clearly different across treatments and consists of

111 genes that were more down-regulated in response to *P. xylostella*-feeding and to Dual LD compared to Dual HD (Figure 4). Cluster 5 contains genes involved in defense responses (*MES7*, *PDF1.2B*, *PDF1.2*, *CCR2*, *PGIP2, ERD5*), in responses to phytohormones (*TTL3*, *ACR4*, *BT4*, *PYL4*, *PYL5*) and genes associated with cell-wall remodelling (*PME3*, *EXT3*, *FLA13*, *AGP16*) (Supplementary materials Table 3A).

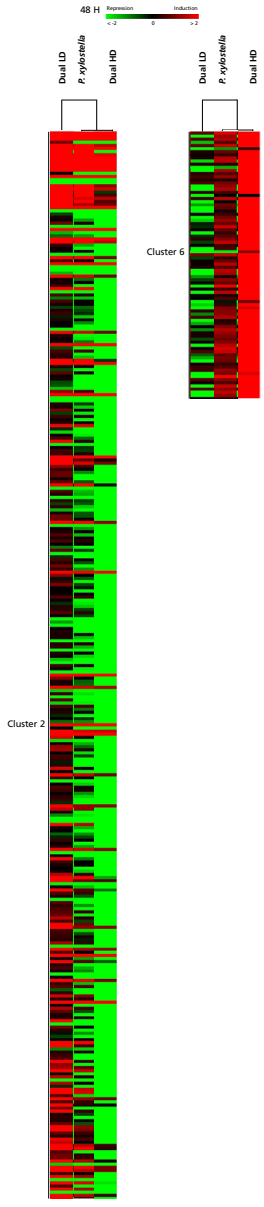

Figure 5. Heat map showing average $\log_2$-fold change ratios (measured relative to non-infested control samples) of genes expressed in *A. thaliana* at 48 h after single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (LD) or high (HD) density of *B. brassicae* aphids. Hierarchical clustering (HCL) was performed using Spearman correlation with average linkage clustering. Red indicates up-regulated genes, while green shows down-regulated genes. Black represents no change in expression. Each row in the columns corresponds to a single gene. Cluster analysis is shown for clusters 2 and 6.

Clustering after 48 h of herbivory

Gene expression levels in response to *P. xylostella* feeding only and to Dual HD cluster separately from those in response to Dual LD. This result shows that aphid density affects gene expression pattern and, moreover, responses induced by *P. xylostella*-feeding are more similar to those induced by Dual HD (Supplementary materials Figure 3).

For example, cluster 2 consists of genes that are more down-regulated in response to Dual HD and *P. xylostella* feeding compared to Dual LD (Figure 5). Cluster 2 consists of 343 genes including genes involved in plant defense signaling (such as genes encoding TIFY protein family, *RIPK*, hevein-like protein, *GLR3.4*, MYB domain proteins and peroxidases), responses to phytohormones (such as genes involved in ABA, auxin and SA signaling), and photosynthesis (such as genes encoding for thioredoxins and glutaredoxins, Photosystem (PS I and II) proteins, PsbP proteins and proteins involved in the glycolysis pathway) (Supplementary materials Table 3B). In addition, cluster 2 consists of genes involved in JA-mediated induced plant defenses (*CHL1*, *JAZ9*, *JR1*, *NATA1*, *CORI3, JAZ1*, *PR4*, *JAZ2*, *PGIP2*, *AOC2*, *PDF1.2b*, *MES18*) and genes involved in the biosynthesis of isopentenyl diphosphate and carotenoid (terpenoid metabolic processes) (Supplementary materials Table S3B). However, cluster 6 consists of 86 genes that were more up-regulated in response to feeding by *P. xylostella* caterpillars and to Dual HD compared to Dual LD (Figure 5). Cluster 6 contains genes involved in secondary metabolism (*CYP706A2*, *CYP71B8*, *CYP710A3*), responses to phytohormones (ethylene and auxin response factors) and genes encoding transcription factors (such as MADS-box and NAC domain proteins) which are involved in controlling all major aspects of development and hormone signaling (Figure 4, Supplementary materials Table 3B).

Clustering over time (based on two-way ANOVA analysis)

When gene expression levels were clustered by time point and treatment, responses induced by Dual LD during 48 h and Dual HD during 24 h clustered together, indicating that insects attacking at high densities cause an acceleration in plant responses compared to insects attacking at low density (Supplementary materials Figure 4). Moreover, Dual LD plants during 24 h and Dual HD plants during 48 h clustered together, suggesting that responses to Dual HD after 48 h diminish to levels found after 24 h of Dual LD (Supplementary materials Figure 4). However, responses to dual infestations at both densities after 24 and 48 h, cluster separately from responses to dual infestations at low and high aphid

density after 48 and 24 h, respectively. This indicates that responses between the two time points are distinctly different (Supplementary materials Figure 4).

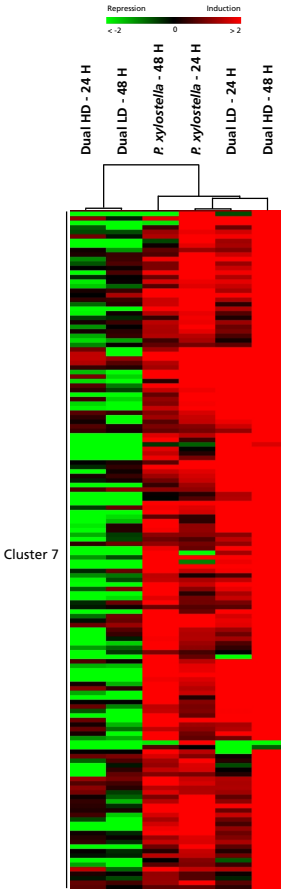

Figure 6. Heat map showing average $\log_2$-fold change ratios (measured relative to non-infested control samples) of genes expressed in *A. thaliana* at 24 and 48 h after single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (LD) or high (HD) density of *B. brassicae* aphids. Hierarchical clustering (HCL) was performed using Spearman correlation with average linkage clustering. Red indicates up-regulated genes, while green shows down-regulated genes. Black represents no change in expression. Each row in the columns corresponds to a single gene. Cluster analysis is shown for cluster 7.

For instance, in cluster 7, 150 genes were found that were more up-regulated by caterpillars feeding during 24 and 48 h, Dual LD during 24 h and Dual HD during 48 h compared to the other treatments (Figure 6). Several regulatory genes involved in defense responses and disease resistance (*WRKY49, WRKY74, WRKY64*,), genes encoding MYB domain proteins and genes involved in secondary metabolism (*CYP706A2*, *CYP71B8*, *CYP710A3*, *CYP71A28*) belong to this cluster (Supplementary materials Table 3C). In addition, cluster 7 consists of genes encoding transcription factors such as MADS-box, genes involved in defense response (such as *PROPEP3*, *MLO5*, *MLP329*, *FRK1* and *LRC29*, *LRC17*, *LRC37*) and phytohormone-mediated signaling (such as *ERF115*, *EIL2* and *ARF23*) (Supplementary materials Table 3C).

Discussion

Plants activate a complex array of defense reactions in response to feeding by insect herbivores (Kessler and Baldwin, 2002; Mithofer and Boland, 2012). Plant responses differ between leaf-chewing and piercing-sucking insects (De Vos et al., 2005; Bidart-Bouzat and Kliebenstein, 2011; Appel et al., 2014). Studying the interactive effects of two insect attackers belonging to different feeding guilds can provide novel insights into plant defense mechanisms underlying responses to dual insect attack.

In the present microarray analysis, we found differences in gene expression in *A. thaliana* plants induced by *P. xylostella* caterpillars alone compared to infestation by a combination of *P. xylostella* caterpillars and *B. brassicae* aphids (Figures 1 and 2). Only a few studies have investigated the molecular mechanisms underlying plant responses to multi-herbivory (Voelckel and Baldwin, 2004; Rodriguez-Saona et al., 2010; Zhang et al., 2013). Voelckel and Baldwin (2004) found that transcriptional responses of *Nicotiana attenuata* plants to simultaneous attack by the sap-feeding insect *Tupiocoris notatus* and the chewing caterpillar *Manduca sexta* were different from those from either herbivore alone. Furthermore, simultaneous feeding by the aphid *Macrosiphum euphorbiae* and the caterpillar *Spodoptera exigua* on tomato plants resulted in a different pattern of gene expression compared to transcriptional responses induced by caterpillars or aphids alone (Rodriguez-Saona et al., 2010). In *A. thaliana* plants, feeding by the whitefly *Bemisia tabaci* suppressed the up-regulation of a large number of genes induced by *P. xylostella* caterpillars (Zhang et al., 2013). Here, we identified a larger number of DEGs in response to simultaneous feeding by *P. xylostella* caterpillars and *B. brassicae* aphids compared to caterpillars feeding alone. This indicates that aphids and whiteflies, although both phloem feeders, interfere in a different way with caterpillar-induced defenses and cautions against generalizations based on feeding guild.

Transcriptional interference between simultaneously feeding insect herbivores can lead to positive or negative effects on the performance of the herbivores. For example, in cabbage (*Brassica oleracea)* positive effects of *B. brassicae* aphid feeding on the performance of *Pieris brassicae* caterpillars were observed (Soler et al., 2012). In addition, caterpillars of the Monarch butterfly *Danaus plexippus* were positively affected on milkweed plants previously infested by oleander aphids *Aphis nerii*, whereas the aphids were negatively affected on milkweed plants previously infested by conspecific caterpillars (Ali et al., 2014). When comparing transcriptional responses of *A. thaliana* plants exposed to

caterpillars feeding alone or to simultaneous feeding by caterpillars and aphids, different plant responses are induced. We observed up-regulation of several JA-responsive genes (*PR4* (*HEL*), *VSP1*, *PDF1.2*, *TPS04*, *CORI3*, *JR1*) and genes involved in JA signal-transduction (*JAZ5*, *JAZ9*) in response to feeding by *P. xylostella* caterpillars (Supplementary Table 2). Several of these genes were also found to be up-regulated by *P. xylostella* in a microarray study (Ehlting et al., 2008), which suggests that JA-mediated responses play an important role in plant defense against *P. xylostella* caterpillars (Zhang et al., 2013). In response to simultaneous aphid feeding, also genes related to metabolism of organic acids, fatty acids and oxylipins were up-regulated, compared to *P. xylostella* caterpillars feeding alone. Oxylipins are involved in plant responses to insect attack (Bostock, 2005). For instance, oxylipin related genes were up-regulated by *P. xylostella* feeding in *Arabidopsis* plants (Ehlting et al., 2008). Interestingly, also in response to aphid feeding oxylipins are induced in *A. thaliana* and maize plants (Kusnierczyk et al., 2008; Tzin et al., 2015a). Therefore, oxylipins may be important for induced defenses in response to dual caterpillar and aphid infestation.

Effect of insect density on transcriptional responses

Induced plant responses to multi-herbivory can be influenced by the density of the attacking insects. For instance, interference of *B. brassicae* aphids with induced defenses against caterpillars depends on the density of the attacking aphids (Kroes et al., 2015; Ponzio et al., 2016). As a next step in the study of density-dependent interference of aphids with caterpillar-induced defenses, we studied the effect of different aphid densities on whole-genome transcriptional responses of *A. thaliana* to feeding by *P. xylostella* caterpillars.

We observed that transcriptional responses of *A. thaliana* were aphid density-dependent. There are differences in the nature of the differentially expressed genes when comparing Dual LD and Dual HD plants with caterpillar-infested plants after 48 h (Figure 3). We found several *WRKY* transcription factor genes (*WRKY33*, *WRKY40* and *WRKY70*) only repressed in response to simultaneous aphid feeding at high density after 48 h. WRKY proteins belong to a large family of transcriptional regulators in *A. thaliana* plants (Rushton et al., 2010) and play an important role in regulating plant responses to pathogens (Pandey and Somssich, 2009). For example, the transcription factor WRKY33 mediates defense responses to the necrotrophic fungus *Botrytis cinerea* in *A. thaliana* (Birkenbihl et al., 2012). Furthermore, *WRKY70* has a key role in regulating interactions between SA- and JA-mediated signaling pathways (Li et al., 2004;

Pieterse et al., 2012). Overexpression of *WRKY70* induced the expression of SA-mediated *PR* genes, while it suppressed JA-responsive *PDF1.2* expression in *A. thaliana* plants (Li et al., 2004). It has been suggested that by activating the SA signaling pathway, aphids could interfere with JA-dependent defenses against caterpillars (Stam et al., 2014). Differential expression of *WRKY70* may underlie plant-mediated interactions between simultaneously attacking aphids and caterpillars. A negative correlation between SA-mediated *WRKY70* expression and JA-dependent *MYC2* expression in *A. thaliana* plants infested by both caterpillars and aphids was shown before and was also aphid-density dependent (Kroes et al., 2015). Expression of *WRKY70* was down-regulated in Dual HD plants, which led to the induction of JA-mediated defenses (Kroes et al., 2015). By activating JA-dependent defenses in response to simultaneous feeding of caterpillars and aphids at high density, plants could increase defense against aphids and caterpillars.

Also *WRKY40* is involved in the crosstalk between JA and SA signaling (Xu et al., 2006), moreover, *WRKY40* negatively regulates ABA-responsive gene expression (Chen et al., 2010). The plant hormone ABA is an important modulator of plant defense responses (Morkunas et al., 2011; Lee and Luan, 2012). Here, we detected ABA-dependent genes that were differentially induced in response to Dual HD after 24 h, compared to genes expressed in caterpillar-induced plants, but not in response to Dual LD (*e.g. ABF1*, *PYR1*, *PLC1*, *SRK2D* and *AHK2*). In addition, we found a group of genes (Cluster 5) that were more strongly up-regulated at 24 h in response to Dual HD compared to *P. xylostella* caterpillars feeding alone and to Dual LD (Figure 4; Supplementary materials Table 3A). Cluster 5 contains the ABA receptors PYL4 and PYL5. These receptors inactivate plant PP2Cs, such as *ABI1* and *ABI2*, which are known to suppress ABA signaling (Ma et al., 2009; Park et al., 2009). Recently, it was shown that aphids feeding on *A. thaliana* and the legume *Medicago truncatula* increase ABA content in the plants (Guo et al., 2016; Hillwig et al., 2016). Furthermore, *M. persicae* aphid population development was negatively affected on ABA-deficient mutants compared to wild-type *A. thaliana* plants (Kerchev et al., 2013; Hillwig et al., 2016). Thus, these results indicate that plant responses to simultaneous caterpillar and aphid feeding involves ABA signaling, which is dependent on aphid density and decreases defense responses against the attacking aphids. However, to support this hypothesis, performance of aphids at different densities feeding simultaneously with caterpillars on ABA-deficient mutants should be studied.

Activation of plant defenses in response to herbivory is costly, and requires the

diversion of resources away from plant growth (Huot et al., 2014). Consequently, herbivory suppresses photosynthesis (Zangerl et al., 2002; Voelckel and Baldwin, 2004; Appel et al., 2014; Huot et al., 2014; Zhu et al., 2015). In response to *P. xylostella* feeding only, or to Dual HD, we found a group of genes (Cluster 2) that were more strongly down-regulated after 48 h compared to Dual LD (Figure 5; Supplementary materials Table 3B). This cluster contains genes associated with photosynthesis and indicates that simultaneous feeding by caterpillars and aphids at a low or a high density has a different impact on the expression of photosynthesis-related genes. As a consequence, induction of plant defenses may be differently affected in response to aphid feeding at low or high densities.

Effect of the time since the start of herbivory on transcriptional responses

Transcriptional responses to feeding by a single herbivore species are highly dynamic over time (Ehlting et al., 2008; Kusnierczyk et al., 2008; Appel et al., 2014; Tzin et al., 2015a; Davila Olivas et al., 2016). Transcriptional responses to caterpillars feeding alone and feeding by both caterpillars and aphids changed over time. Similar to the finding of Ehlting et al. (2008) that more genes are differentially expressed at early time points in response to *P. xylostella* feeding, we found that a higher number of DEGs was up-regulated by *P. xylostella* caterpillar feeding after 24 h as compared to *P. xylostella*-induced DEGs after 48 h. In addition, Voelckel and Baldwin, (2004) showed that specific transcriptional changes to *M. sexta* caterpillar infestation on tobacco plants occur after 24 h but these disappeared after five days of feeding.

Our cluster analysis showed that time-dependent transcripts in response to dual infestation were affected by the density of the attacking aphids. In response to Dual LD during 48 h, a similar transcriptional pattern was expressed as that found in response to Dual HD during 24 h. This indicates that insects attacking at a high density cause an acceleration in plant responses compared to insects attacking at a low density. Furthermore, we found that plant responses to Dual HD during 48 h clustered together with responses found after 24 h of Dual LD. Interestingly, many of the up-regulated genes from Cluster 7 in response to the two aphid densities at both time points are known to be involved in plant defenses (*e.g. LCR17*, *LCR29*, *FRK1*, *PROPEP3*, *MYB78*, *LCR37*, *MLP329* and different cytochrome P450 genes) (Figure 6; Supplementary materials Table 3C). For example, *MYB78* belongs to the R2R3-type *MYB* genes which are involved in plant defense responses (Stracke et al., 2001; Dubos et al., 2010). In *A. thaliana*

plants, *MYB78* was shown to play a role in the defense response regulated by JA against pathogen infection (Mengiste et al., 2003). Another example is *PROPEP3* which encodes precursor proteins that, upon perception by two closely related receptor kinases, PEPR1 and PEPR2, activate plant defense (Bartels et al., 2013). Upon feeding by *Spodoptera littoralis* caterpillars, *PROPEP3* is up-regulated in *A. thaliana* plants (Klauser et al., 2015). Furthermore, performance of *S. littoralis* was positively affected when feeding on *pepr1 pepr2* double mutants (Klauser et al., 2015). Also, *FRK1* (flg22-induced receptor-like kinase 1) expression was shown to be up-regulated to a significantly higher level by *B. brassicae*-derived elicitors compared to water infiltrated *A. thaliana* leaves (Prince et al., 2014). In addition, SA signaling is involved in the regulation of *FRK1* expression (Yi et al., 2014). This may indicate that *FRK1* is involved in defense signaling *B. brassicae* feeding, likely because SA-mediated signaling is the main pathway regulating plant defense against aphids.

Conclusion

We determined if aphids interfere with transcriptional responses of *A. thaliana* plants to *P. xylostella* caterpillars and whether this interference was dependent on aphid density and time since the start of herbivory. We show that the density of simultaneously feeding aphids has a large effect on transcriptional responses in *A. thaliana* plants attacked by *P. xylostella* caterpillars. In addition, transcriptomic responses are dynamic over time since the start of herbivory. In response to *P. xylostella* feeding alone, transcriptional changes were strongest after 24 h and mostly involved JA-responsive genes. When comparing gene expression patterns between time point and insect treatment, transcriptional patterns were similar between infestation at low density during 48 h and dual infestation at high density during 24 h. This indicates that insects attacking at a high density cause an acceleration in plant responses compared to insects attacking at low density. Furthermore, response to dual infestation at low density during 24 h and dual infestation at high density during 48 h mainly involved plant defense genes.
This study highlights the importance of addressing insect density as well as time since the onset of herbivory to understand plant responses to dual or single insect attack. Mutant analysis studies are needed to confirm the function of genes involved in plant responses to single or dual insect attack.

Acknowledgments

We thank Léon Westerd, Frans van Aggelen and André Gidding for culturing the insects; Rieta Gols for assistance with the multivariate data analysis; Sander Weerheim for help with sample collection. This work was supported by a TOP GO grant (grant number 854.10.010 to M.D.) from The Netherlands Organization for Scientific Research (NWO).

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Chapter 7

Plant-mediated interactions between two herbivores differentially affect a subsequently arriving third herbivore in populations of wild cabbage

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Abstract

Plants are part of biodiverse communities, consisting of numerous organisms. Consequently, plants suffer from attack by multiple herbivorous insects. Depending on the insect feeding guild specific changes in the plants' phenotype are induced. Moreover, plants respond differentially to single or dual herbivory, which may cascade into a chain of interactions in terms of resistance to other community members. Whether differential responses to single or dual herbivory have consequences for plant resistance to yet a third herbivore is unknown.

We assessed the effects of single or dual herbivory by *Brevicoryne brassicae* aphids and/or *Plutella xylostella* caterpillars on resistance of wild cabbage plants from three different natural populations, and studied the performance of a subsequently arriving herbivore, *Mamestra brassicae* caterpillars.

Performance of both *B. brassicae* and *P. xylostella* was reduced when feeding simultaneously with the other herbivore, compared to feeding alone. Gene expression and phytohormone levels in plants exposed to dual herbivory were different from those found in plants exposed to herbivory by either *B. brassicae* or *P. xylostella* alone. Plants previously induced by both *P. xylostella* and *B. brassicae* negatively affected growth of the subsequently arriving *M. brassicae*. Furthermore, induced responses varied between wild cabbage populations. Feeding by multiple herbivores differentially activates plant defenses, which has plant-mediated negative consequences for a subsequently arriving herbivore. Plant population-specific responses suggests that plant populations adapt to the specific communities of insect herbivores. Our study contributes to the understanding of herbivore community development in the context of plant-mediated species interactions.

Keywords

Brassica oleracea, *Brevicoryne brassicae*, *Mamestra brassicae*, multiple herbivory, phytohormones, plant defence, *Plutella xylostella*

Introduction

Throughout the growing season, plants suffer from attack by multiple herbivorous insects. To reduce insect attack, plants protect themselves with constitutive defenses like thick cell walls, a waxy epidermal cuticle, or toxins (Schoonhoven et al., 2005). Furthermore, plants show defense responses induced by herbivores, for example by producing compounds that deter or repel the attackers (Schoonhoven et al., 2005), which may also affect subsequently feeding herbivores (Kessler and Halitschke, 2007). Depending on the feeding guild of the attacking insect, changes in phytohormone production, gene transcription and protein production can occur, which lead to a different regulation of plant defenses (Bidart-Bouzat and Kliebenstein, 2011; de Vos et al., 2005; Heidel and Baldwin, 2004; Koo et al., 2013) and, thus, to expression of a different phenotype.

Closely related plant species differ in responses to herbivore attack which may affect interactions between two or more insect species associated with the plant (Agrawal et al., 2014; Johnson and Agrawal, 2005). Even within plant species, populations may differ in the amount of secondary metabolites they produce (Gols et al., 2008), which has consequences for the insect communities on those populations (Li et al., 2014; Newton et al., 2009b; Poelman et al., 2009).

From molecular studies of *Arabidopsis thaliana*, it is known that signaling networks underlying herbivore-induced defense responses involve, amongst others, two major phytohormones: jasmonic acid (JA) and salicylic acid (SA) (Pieterse et al., 2012). In general, JA-mediated signaling underlies plant defense responses against chewing herbivores (Stam et al., 2014). Lipoxygenases (LOXs) are important enzymes involved in JA biosynthesis (Turner et al., 2002). In cabbage, insect herbivory by *Pieris rapae*, *Pieris brassicae* or *Mamestra brassicae* caterpillars induced high transcript levels of *BoLOX* (Broekgaarden et al., 2007). The importance of JA in defense responses against insects was also shown in other plant species such as tomato (*Solanum lycopersicum*) (Thaler et al., 2002), milkweed (*Asclepsias syriaca*) (Ali and Agrawal, 2014) and tobacco (*Nicotiana attenuata*) (reviewed by (Kessler and Baldwin, 2004; Wang and Wu, 2013). Phloem feeders induce SA-regulated defenses (de Vos et al., 2005). Regulation of SA-mediated defenses involves the expression of pathogenesis-related (*PR*) genes. For example, Kusnierczyk et al. (2008) investigated transcriptomic changes of *Arabidopsis* in response to feeding by *Brevicoryne brassicae* aphids and found that the expression of *PR* genes was significantly induced.

Plants regulate induced defenses against attacking herbivores through crosstalk between JA and SA signaling pathways (Pieterse et al., 2009). For example, in

Nicotiana attenuata plants crosstalk between the JA and SA signaling pathways resulted in optimization of defense responses (Rayapuram and Baldwin, 2007). However, insect herbivores can also interfere with JA- and SA-induced defenses, which can affect the outcome of interactions between plants and multiple attackers (Mathur et al., 2013; Rodriguez-Saona et al., 2010; Voelckel and Baldwin, 2004). Through these indirect plant-mediated interactions, competition between attacking herbivores is commonly found in nature (Denno et al., 1995; Kaplan and Denno, 2007). Asymmetric interactions between herbivores seem to be the rule rather than the exception (Kaplan and Denno, 2007), which could lead to positive (*i.e.* facilitation) or negative (*i.e.* antagonism) effects on the performance or preference of the competing herbivore species (Ali and Agrawal, 2014; Erb et al., 2011; Li et al., 2014; Soler et al., 2012; Viswanathan et al., 2007). Induced plant responses involved in herbivore resistance do not only have consequences for the inducing herbivores but also for subsequently arriving feeders on that plant (Erb et al., 2011; Kessler and Halitschke, 2007). For instance, monarch caterpillars developed faster on milkweed plants previously infested by oleander aphids, whereas the aphids developed more slowly on milkweed plants previously infested by caterpillars, which might have been JA-mediated (Ali and Agrawal, 2014).

Next to the effect of one feeding herbivore on induced defenses against a subsequently arriving herbivore, plant responses to dual stresses can have further ecological consequences for interactions with other community members (Utsumi et al., 2010). Interspecific competition which involves plant phenotypic changes (Kaplan and Denno, 2007), may result in altered interactions of the first herbivore with subsequently arriving insects. However, this is not yet studied in great detail. Recently, it has been shown that the specialist caterpillar *Plutella xylostella* gained more weight when feeding on plants previously attacked by both *P. xylostella* and *Spodoptera litura* caterpillars compared with plants previously infested by only *P. xylostella*. In contrast, *S. litura* was negatively affected when feeding on plants previously attacked by both *P. xylostella* and *S. litura,* compared with plants previously infested by only *P. xylostella* (Mathur et al., 2013). The effect of dual herbivory on a third herbivore suggests that interspecific competition between multiple herbivores can have consequences for the composition of whole arthropod communities assembling on the induced plant. Consequently, interactions between plants and co-occurring insects likely play important roles in natural ecosystems (Ali and Agrawal, 2014; Van Zandt and Agrawal, 2004). Effects of early-season herbivores on arthropod community development have indeed been shown several times (Poelman et al., 2010; Van Zandt and Agrawal, 2004;

Viswanathan et al., 2007). Studies on temporal dynamics of plant defenses in response to herbivory indicate that plants may remain induced by herbivory up to several days (Mathur et al., 2013; Underwood, 2012; Voelckel and Baldwin, 2004). However, induced defenses were investigated after short periods of herbivory, after which the herbivore had been removed. Under natural conditions, insects likely arrive at different times on a plant, and more research is needed to study underlying molecular mechanisms in plants induced during different durations of continuous feeding and consequences for subsequent herbivores.

In the present study, we used wild *B. oleracea* plants that occur in natural populations along the coast of Dorset, UK. These plants show natural variation in the amount of constitutive and inducible secondary metabolites that act as defense compounds against herbivorous insects (Gols et al., 2008; Newton et al., 2009a). Under natural conditions, these plants are attacked by an array of herbivorous insects, amongst others the specialist aphid *Brevicoryne brassicae*, the specialist caterpillar *P. xylostella* and the generalist caterpillar *Mamestra brassicae* (R. Gols, pers. comm.; Moyes et al., 2000). Also in the Netherlands, these insects naturally occur on *B. oleracea* cultivars (Poelman et al., 2009) and wild *B. oleracea* plant populations (J. M. Stam; unpublished data). We investigated whether herbivores from different feeding guilds, namely aphids (*Brevicoryne brassicae*) or caterpillars (*P. xylostella*) feeding alone or simultaneously indeed affected performance of the competing herbivore, and whether this was reflected in expression changes of marker genes involved in defense responses, *i.e. PR-1* and *LOX* (regulated by the phytohormones SA and JA respectively). We quantified JA and SA levels in wild cabbage plants to assess differences in phytohormone levels in response to dual versus single herbivory. By using plants from different wild cabbage populations from the same area, we studied whether plant defense responses are variable across these plant populations. As a next step in the study of multiple interacting herbivores, we studied whether changes in plant resistance induced by the first two herbivores have consequences for the performance of a subsequently arriving generalist herbivore (*M. brassicae*). We discuss the ecological consequences of plant defense to multi-herbivory.

Materials & Methods

Plants and growth conditions

Seeds of wild cabbage *Brassica oleracea* L. (Brassicaceae), from three populations in Dorset, *i.e.* Kimmeridge (50°36'N, 2°07'W), Old Harry (50°38'N, 1°55'W) and

Winspit (50°35'N, 2°02'W) (Gols *et al*., 2008) were sown in potting soil (Lentse potgrond, Lent, The Netherlands). One week later, seedlings were transferred to individual pots (1.54 L) containing similar soil. Plants were cultivated in a glasshouse under a 16L : 8D cycle [500 µmol photons m^{-2} s^{-1}] light intensity at 22 ± 3 °C and 50-70 % relative humidity. Lighting from high-pressure mercury lamps was used in the glasshouse to supplement periods of low natural light. Plants were watered every other day. When plants were four weeks old, they were used for experiments.

Insects

The specialist diamondback moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), the specialist cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae) and the generalist cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) were obtained from stock cultures maintained at the Laboratory of Entomology, Wageningen University. All insects were reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv Cyrus) in a climate room (21±1 °C, 50-70 % relative humidity, 16L : 8D cycle).

Experimental set-up

The experiments were performed in three different rounds (November 2012, January/February 2013 and March/April 2013) to obtain three biological replicates for molecular analyses; for insect performance, individual plants were considered as the unit of biological replication. At time points 3, 7 or 14 d after insect infestation the performance of the insects were assessed, and plant tissue for molecular defense analyses was collected. For each time point, a different set of plants was used to exclude effects of sampling tissue for molecular analyses on insect performance. Therefore, a total of 432 plants (144 plants x 3 rounds) was used over the entire study.

***Plutella xylostella* and *Brevicoryne brassicae* performance**

At each round, 48 plants per *B. oleracea* population were infested with three second-instar (L2) *P. xylostella* caterpillars, or with five adult *B. brassicae* aphids, or simultaneously infested with three L2 *P. xylostella* caterpillars plus five adult *B. brassicae* aphids. Each insect was carefully placed with a small brush on the third fully expanded leaf. Clean uninfested plants were used as control. Insects were caged on the infested leaf for 24 h by using clip cages; upon removal of the

clip cages the insects were allowed to move and feed freely on the plant. Empty clip cages were used for 24 h on leaves of control plants. Individual plants were covered with a gauze net supported by four wooden sticks to prevent insects from escaping. At time points 3, 7 or 14 d after infestation *P. xylostella* caterpillars or pupae were collected per plant and individually weighed (analytical balance: Mettler Toledo ML54/01, accuracy = 0.1 mg), and number of *B. brassicae* aphids per plant was recorded.

Molecular plant defense analyses

For gene expression and phytohormone analysis, one biological replicate consisted of eight leaf discs punched with a cork-borer (diameter = 2.1 cm) and pooled from four different plants. Plant material was collected after 3, 7 or 14 d of infestation from the induced leaf and from the control leaves of uninfested plants. Leaf discs were flash-frozen in liquid nitrogen and stored at -80 °C prior to analysis.

Quantitative real-time PCR

Total RNA was isolated from finely ground and homogenized leaf material with the RNeasy Plant Mini Kit (Qiagen). RNA was treated with DNaseI (Invitrogen) following the manufacturer's instructions. Subsequently cDNA was synthesised from RNA (adjusted to 1 µg/µl) using iScript cDNA synthesis Kit (Bio-Rad). Quantitative RT-PCR analysis was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Each reaction was performed in a total volume of 25 µl containing 12.5 µl SYBR Green Supermix (Bio-Rad), 5 µl cDNA and 1 µl of 10 µM forward and reverse gene specific primer pair. For each reaction, two technical replicates were performed and average values were used in the analyses. The following PCR program including a melting curve analysis was used for all PCR reactions: 3 min 95 °C, followed by 40 cycles of 15 sec 95 °C, and 45 sec 59 °C. Relative expression of a pathogenesis-related protein (*BoPR-1*) and lipoxygenase (*BoLOX*) were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (Broekgaarden et al., 2008) as internal standard.

Table 1. Specific primer sequences used for quantitative RT-PCR analyses.

Gene name	Forward primer	Reverse primer
BoGAPDH	AGAGCCGCTTCCTTCAACATCATT	TGGGCACACGGAAGGACATACC
BoLOX	AAGGCATCGAGCTTCCCAA	TTGCTTTTCAACGGCCACTC
BoPR-1	GTCAACGAGAAGGCTAACTATAACTACG	TTACACCTTGCTTTGCCACATCC

Primer sequences (Table 1) were based on genes of *Brassica oleracea var. gemmifera*, namely *BoLOX* (GenBank accession EF123056)*, BoPR-1* (GenBank accession EF423806) and the reference gene *BoGAPDH* (GenBank accession EF123055).

Phytohormone quantification

JA and SA phytohormone levels were quantified by gas chromatography – mass spectrometry as described by Schulze et al. (2006). Plant material (250 mg) was finely ground and frozen in liquid nitrogen. For quantification, [9,10-$^2H_2$]-9,10-dihydro-JA (250 ng) and [3,4,5,6-$^2H_4$]-SA (500 ng) were added as internal standards. JA levels were quantified by analysing samples on a Finnigan ITQ 900 (Thermo Scientific, Dreiech, Germany) device equipped with an Rtx-200MS column (30 m, 0.25 mm, 0.25 mm; Resteck, Bad Homburg, Germany). Helium (1.5 mL min^{-1}) served as the carrier gas. Mass spectral analysis was carried out in chemical ionization negative (NCI) mode using methane as reagent gas (2.0 mL min^{-1}). Products were eluted under programmed conditions: 100 °C, increase (10 °C min^{-1}) to 210 °C, increase (1 °C min^{-1}) to 227 °C, hold 1 min, increase (40 °C min^{-1}) to 290 °C, hold 2 min. The GC injector (split ratio 1:10), transfer line and ion source were set at 280, 300 and 200 °C, respectively.

SA levels were quantified by analysing samples on a Finnigan Trace MS quadrupole mass spectrometer (Thermo electron) according to Schulze et al. (2006).

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Mamestra brassicae performance

At time points 4, 8 and 15 d after infestation, all 48 plants per population were sampled to investigate the effects of dual herbivory on a subsequently arriving herbivore, *i.e. M. brassicae* caterpillars. Leaves damaged by insect feeding and sampled for gene expression and phytohormone analysis were excised from the plants to ensure *M. brassicae* caterpillars were feeding from locally induced leaves and to arrest further systemic plant resistance response to *M. brassicae* feeding. For each plant, one biological replicate consisted of three leaves placed in a small vial with tap water and sealed with a cotton plug. All insects were removed when

the leaves were excised, to stop further local induction by *P. xylostella* and/or *B. brassicae*. Vials containing the leaves were placed in a plastic container (12 x 18 x 7 cm, L x W x H) covered with a transparent lid pierced with 12 small holes. In each container 10 neonate (L1) *M. brassicae* caterpillars were carefully placed with a small brush on the leaves and allowed to feed for 6 d. Containers were placed in a glasshouse (22 ± 3 °C, 50-70 % relative humidity, 16L : 8D cycle). After 6 d of feeding, caterpillars were individually weighed on an analytical balance (Mettler Toledo ML54/01, accuracy = 0.1 mg). Mortality was calculated as the initial number of larvae placed on the leaves minus the number of larvae that were still alive at the moment of weighing.

Statistical analysis

The effects of herbivore treatments, time points, experimental rounds and plant populations on
(I) *B. brassicae* numbers, gene expression and phytohormone levels were analyzed using a Generalized linear model (forward accumulated analysis of deviance) with Poisson distribution and log link function. Time point was included as covariate, while treatment, plant population and round were included as fixed factors. An estimated dispersion parameter was included to account for residual variance. When interactions between factors were not significant, only main treatment effects are presented.

(II) *P. xylostella* and *M. brassicae* caterpillar and pupal weights were analyzed using a Generalized linear mixed model (sequentially adding terms to fixed model) with normal distribution and identity link function; individual plant identity was included as random factor. An estimated dispersion parameter was included to account for residual variance. In cases where *P. xylostella* caterpillar weight was lower than the accuracy of the balance (n = 31), they were entered with the lowest measurable weight (0.1 mg) in the analysis. Interaction terms between treatments, time points, experimental rounds and plant populations could not be computed for *M. brassicae* data because of an insufficient number of degrees of freedom.

(III) *M. brassicae* mortality and the fraction of *P. xylostella* pupae relative to the total of all *P. xylostella* life stages that were found per plant at each of the time points, were analyzed using a Generalized linear model (forward accumulated analysis of deviance) with binomial distribution and

logit link function. Binomial totals were always 10 *M. brassicae* larvae or were the totals of all *P. xylostella* life stages found per plant. An estimated dispersion parameter was included to account for residual variance. Post-hoc tests for differences between levels of the fixed factors were analyzed with a t-test for pairwise differences of the means for *B. brassicae* numbers and *M. brassicae* mortality. Post-hoc comparisons for *P. xylostella* and *M. brassicae* weights, gene expression and phytohormone levels were analyzed with an LSD test. All statistical analyses were conducted in GenStat software Version 16.2 (VSN International, Hemel Hempstead, UK). Excluded from analysis were all samples (n = 38 plants over the entire study) that had unintended *B. brassicae* infestation.

Results

Performance of *B. brassicae* aphids and *P. xylostella* caterpillars

The number of *B. brassicae* that accumulated per plant was lower when *B. brassicae* aphids were feeding simultaneously with *P. xylostella* on the same plant, compared to *B. brassicae* aphids feeding alone on Kimmeridge plants (Table 2A, Figure 1).

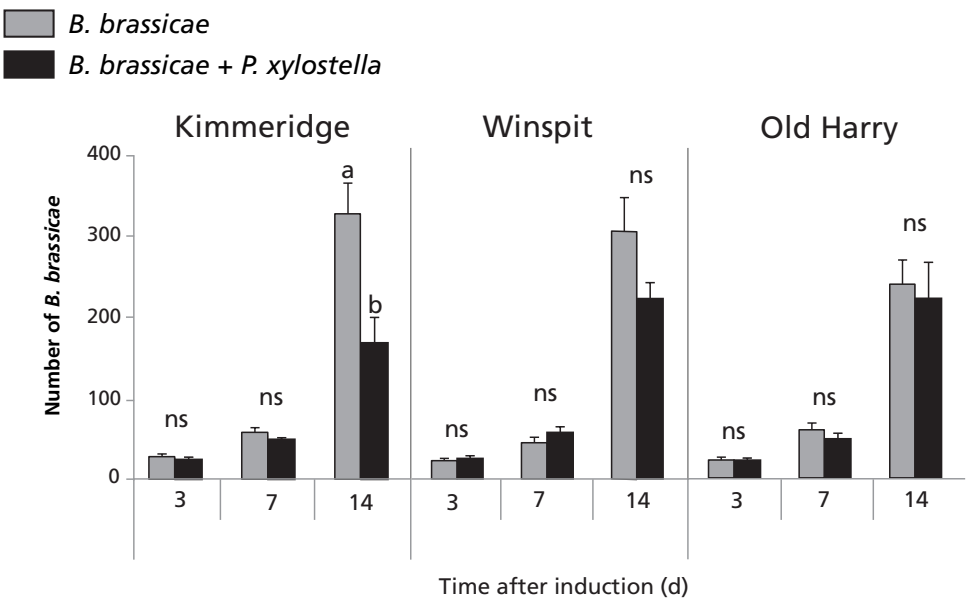

Figure 1. Mean number of *B. brassicae* (± SE) on plants of three wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 3, 7 or 14 days after single *B. brassicae* or simultaneous *P. xylostella* and *B. brassicae* infestation. Bars marked with different letters are significantly different (GLM, $P < 0.05$); ns indicates no significant difference between groups.

Aphid numbers were not affected by plant population and increased significantly with time. The experimental rounds significantly influenced *B. brassicae* numbers (Table 2A). Simultaneous feeding by *B. brassicae* affected *P. xylostella* caterpillar weight negatively, depending on the time point and plant population (Table 2B). Thus, both *B. brassicae* and *P. xylostella* performance were negatively affected by simultaneous feeding by the reciprocal herbivore, and influenced by plant population or time of infestation. At 3 d after induction, there was no difference in caterpillar weights between the treatments, whereas at 7 d after induction, caterpillar weight was lower when *P. xylostella* caterpillars were feeding simultaneously with *B. brassicae* aphids on the same plant, compared to *P. xylostella* caterpillars feeding alone (Figure 2). Plant population affected *P. xylostella* caterpillar weights; highest weight at 7 d after induction was reached on Old Harry plants, lowest weight was reached on Winspit plants (Figure 2).

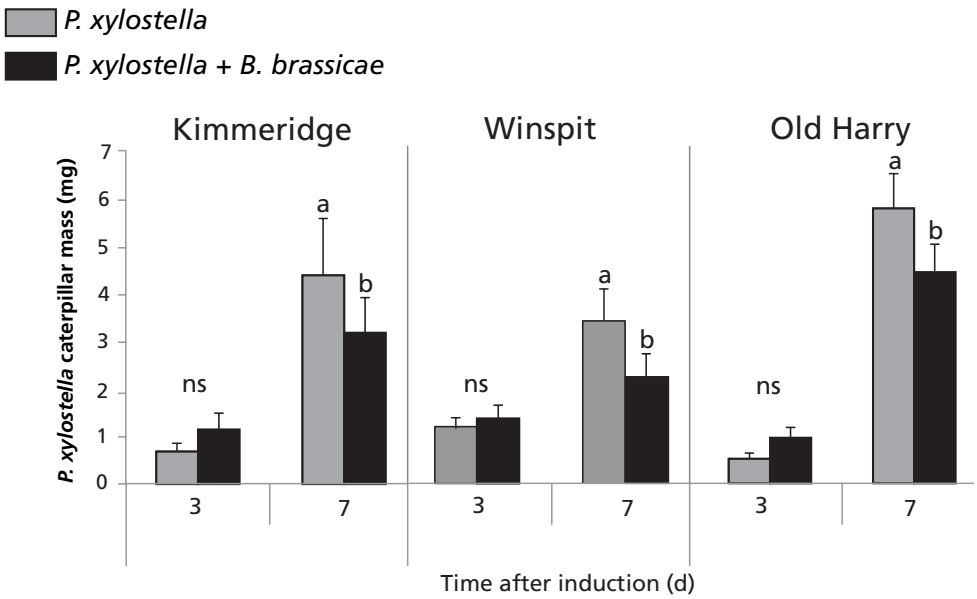

Figure 2. Mean weight (milligrams) of *P. xylostella* caterpillars (± SE) on plants of three wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 3 or 7 days after single *P. xylostella* or simultaneous *P. xylostella* and *B. brassicae* infestation. Data for 14 days after induction are not shown here, because only few *P. xylostella* in the caterpillar stage remained at that time point; the rest pupated. Bars marked with different letters are significantly different (GLMM, $P < 0.05$); ns indicates no significant difference between groups within a time point.

Experimental rounds affected *P. xylostella* caterpillar weight. Pupal weights were neither affected by the presence or absence of *B. brassicae* on the plant (Table 2A),

Table 2. Statistical tests on performance variables of insects feeding on plants from three wild *B. oleracea* plant populations, which were either undamaged or induced by *Plutella xylostella* caterpillars, *Brevicoryne brassicae* aphids, or both. Generalized Linear Model deviance table for *Brevicoryne brassicae* numbers, fraction of *Plutella xylostella* pupae and *Mamestra brassicae* caterpillar mortality (A). Generalized Linear Mixed Model Wald table for *Plutella xylostella* caterpillar weights, *Plutella xylostella* pupal weights, *Mamestra brassicae* caterpillar weights and *Mamestra brassicae* caterpillar weights on control plants only (B). Bold numbers indicate significant effects of the factor on insect performance (α = 0.05).

A	Generalized Linear Model deviance table – Factors												Total Model	
	Treatment			Plant population			Time point			Round				
	d.f. = 1						d.f. = 1			d.f. = 2				
	d.f.	deviance	*P*	d.f.	deviance	*P*	d.f.	deviance	*P*	d.f.	deviance	*P*	d.f.	deviance
B. brassicae number	1	388.54	**<0.001**			ns[a]	1	17811.26	**<0.001**	2	1012.50	**<0.001**	204	23749.81
P. xylostella pupal fraction			ns			ns	6	40.96	**<0.001**			ns	120	217.37
M. brassicae caterpillar mortality			ns			ns	8	157.48	**<0.001**			ns	391	959.05

B	Generalized Linear Mixed Model Wald table – Factors											
	Treatment			Plant population			Time point			Round		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
P. xylostella caterpillar weight	1	8.93	**0.003**	2	5.72	**0.004**	1	545.96	**<0.001**	2	5.59	**0.004**
P. xylostella pupal weight	1	0.29	0.593	2	0.71	0.494	1	2.93	0.091	2	2.92	0.060
M. brassicae caterpillar weight	3	3.72	**0.012**	2	69.07	**<0.001**	1	18.48	**<0.001**	2	54.43	**<0.001**
M. brassicae caterpillar weight control only				2	13.54	**<0.001**	2	3.10	**0.050**	2	24.07	**<0.001**

[a] ns: indicates a non-significant factor that was not included in the model.

nor by plant population, experimental round or time point. At 14 d, the majority of the *P. xylostella* caterpillars had pupated and a small fraction of the pupae had eclosed. Likewise, *P. xylostella* development time until pupation, measured as the fraction of pupation per time point, was not affected by simultaneous feeding by *B. brassicae* on the same plant (Table 2A). Neither differences between plant populations, nor between experimental rounds were significant. The fraction of pupation increased over time.

Transcriptional analyses

Expression of the SA-responsive marker gene *PR-1* was significantly affected by treatment, plant population and time point (Table 3A) as well as their interaction (Table 3B). Treatment, time point, plant population and round also had a significant effect on the expression of the JA-responsive marker gene *LOX* (Table 3A); however, there was no significant interaction between the factors treatment, plant population and time point (Table 3B).
Caterpillars feeding on Kimmeridge plants and simultaneous feeding by caterpillars and aphids on Old Harry plants significantly up-regulated *PR-1* expression 14 d after infestation (Figure 3). At this time point most of the *P. xylostella* caterpillars had pupated. Significantly higher expression levels of *LOX* were found in all three cabbage populations upon aphid feeding alone and simultaneous aphid and caterpillar feeding compared to control plants and plants induced with caterpillars only (Figure 3). The expression level of *LOX* was similar for plants simultaneously infested with caterpillars and aphids and for plants infested with only aphids at all time points. In conclusion, expression of the marker genes *PR-1* and *LOX* differed between single and double herbivory treatments, and was affected by plant population and time of infestation.

Phytohormonal analyses

To further investigate the effect of dual herbivore attack on plant defenses, the levels of the phytohormones JA and SA were assessed. There was a significant interaction between the effects of treatment, time point and plant population on SA level (Table 3B, GLM). The level of JA was significantly affected by time point and experimental round (Table 3A). However, JA levels were similar among treatments for all time points and cabbage populations (Figure 4).
The level of SA was significantly higher in Kimmeridge plants induced by aphids only or by caterpillars only compared to plants simultaneously induced by both insects at 3 d (Figure 4). This indicates that in Kimmeridge plants aphids and

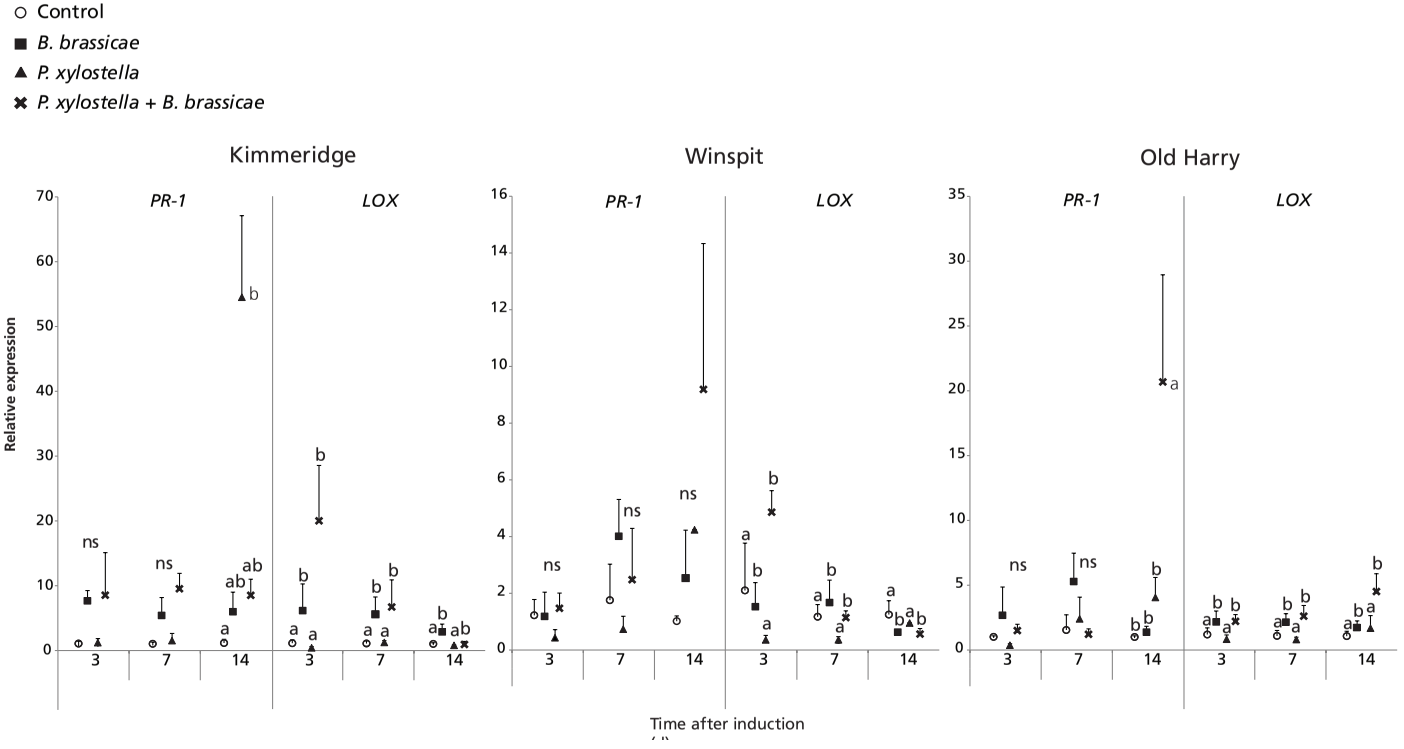

Figure 3. *PR-1* and *LOX* expression in leaves of plants from wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 3, 7 or 14 days after single *P. xylostella*, single *B. brassicae*, simultaneous *P. xylostella* and *B. brassicae* infestation and without feeding (Control). Symbols represent means ± SE (n=3). Symbols marked with different letters are significantly different within a time point (GLM, $P < 0.05$); ns indicates no significant difference between groups within a time point.

Table 3. Statistical tests on gene expression and hormone levels in plants from three wild *B. oleracea* populations, which were either undamaged or induced by *Plutella xylostella* caterpillars, *Brevicoryne brassicae* aphids, or both. Generalized Linear Model deviance table for effect of Time point, Treatment, Round and Population (A) and their interaction term (B). Non-significant interaction terms were not included in the model. Bold numbers indicate significant effects (α = 0.05).

A	Generalized Linear Model deviance table – Factors							
	Treatment (1)		Plant population (2)		Time point (3)		Round (4)	
	d.f. = 3		d.f. = 2		d.f. = 1		d.f. = 2	
	deviance	*P*	deviance	*P*	deviance	*P*	deviance	*P*
PR-1	139.755	**<0.001**	198.65	**<0.001**	168.699	**<0.001**	10.684	0.144
LOX	82.38	**<0.001**	45.01	**<0.001**	64.98	**<0.001**	72.97	**<0.001**
SA	259.82	**0.025**	14.47	0.736	105.78	**0.044**	13.18	0.756
JA	92.02	0.163	24.89	0.481	101.75	**0.020**	401.66	**<0.001**

B	Generalized Linear Model deviance table - Interactions													
	1 x 2		1 x 3		1 x 4		2 x 3		3 x 4		1 x 2 x 3		1 x 3 x 4	
	d.f. = 2		d.f. = 3		d.f. = 6		d.f. = 2		d.f. = 2		d.f. = 6		d.f. = 6	
	deviance	*P*	deviance	*P*	deviance	*P*	deviance	*P*	deviance	*P*	deviance	*P*	deviance	*P*
PR-1			133.71	<0.001							46.902	0.012		
LOX	21.677	**0.005**					30.345	**<0.001**	15.316	**0.001**			14.912	**0.027**
SA	395.60	**0.031**									373.12	0.040		
JA					355.50	**0.011**	173.03	**0.013**						

caterpillars alone induce a different SA-mediated defense response compared to both insects feeding simultaneously. Among the three plant populations, SA levels differed upon insect infestation. Aphid feeding induced significantly higher levels of SA in Winspit plants after 14 d compared to caterpillar-infested plants (Figure 4).
In Kimmeridge plants, 14 d after caterpillar feeding, the SA level was significantly induced to higher levels than in plants simultaneously induced by both insects and in aphid-infested plants (Figure 4). This is a similar activation of the SA pathway (higher *PR-1* expression) as seen before in Kimmeridge plants (Figure 3). For aphid-infested Old Harry plants, SA level was significantly reduced compared to control and caterpillar-infested Old Harry plants 3 d after insect feeding (Figure 4). In conclusion, dual herbivory by aphids and caterpillars resulted in a different phytohormonal response compared to phytohormonal responses induced by aphids or caterpillars alone. Furthermore, in response to herbivory SA levels were different across plant populations.

Performance of third subsequent herbivore *M. brassicae*
Plant resistance was altered by single or simultaneous feeding by the two herbivores *P. xylostella* and *B. brassicae* which negatively affected the third herbivore, *M. brassicae*, subsequently arriving on the same plant. *Mamestra brassicae* performance was lower on plants previously induced by both *P. xylostella* and *B. brassicae* compared to control plants without previous insect feeding (overall treatment effect; Table 2B; Figure 5). The performance of *M. brassicae* on undamaged plants or plants previously induced by either aphids or caterpillars did not differ from each other, indicating that only induction by the two herbivores together negatively affected the performance of *M. brassicae*. *Mamestra brassicae* performance was affected by the length of time previous herbivores had spent feeding, as weight of *M. brassicae* caterpillars differed between the time points, mostly between 4 and 15 d; and 8 and 15 d after the start of previous herbivory. The three plant populations affected *M. brassicae* caterpillar weight differently, with lowest weight obtained on Winspit plants and highest weight on Kimmeridge plants. Also experimental round affected *M. brassicae* weight (Table 2B).
To verify whether the observed differences in *M. brassicae* weight were caused only by previous feeding by the inducing herbivores, or could also have been affected by differences in plant quality between the time points or plant populations, weight of *M. brassicae* caterpillars feeding on the control plants

Figure 4. Salicylic acid (SA) and jasmonic acid (JA) levels expressed as ng per g fresh weight in wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 3, 7 or 14 days after single *P. xylostella*, single *B. brassicae*, simultaneous *P. xylostella* and *B. brassicae* infestation and without feeding (Control). Symbols represent means ± SE (n=3). Symbols marked with different letters are significantly different within a time point (GLM, $P < 0.05$); ns indicates no significant difference between groups within a time point.

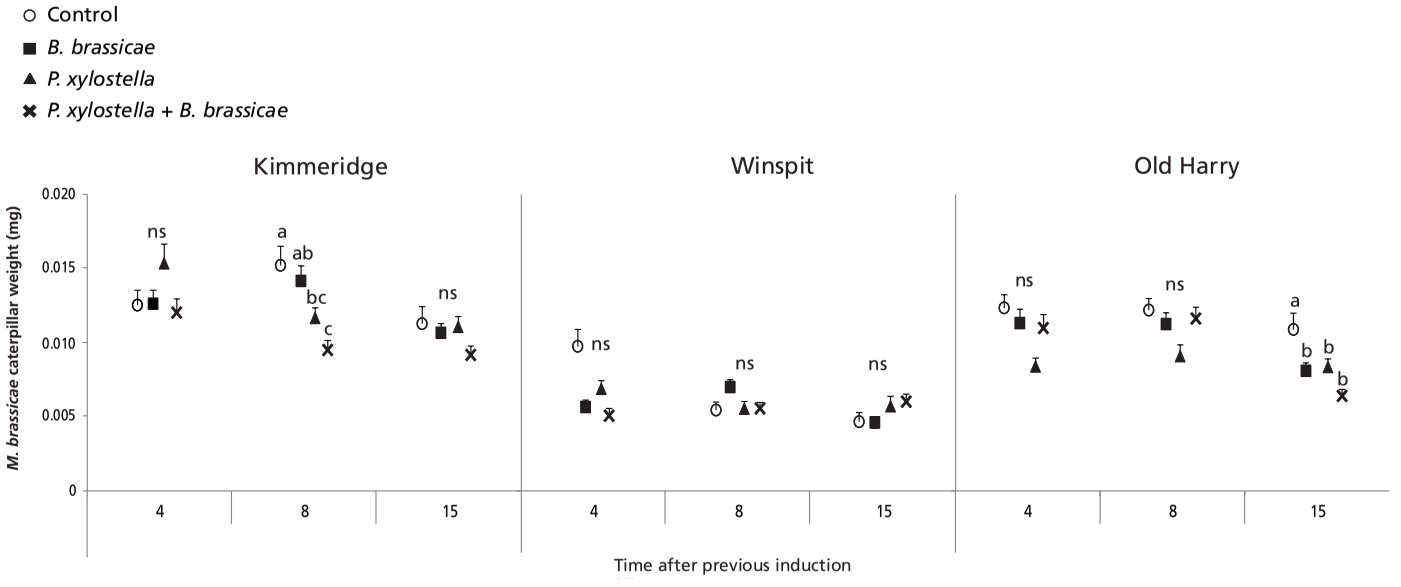

Figure 5. Mean weight (mg) of *M. brassicae* caterpillars (± SE) on plants of wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 4, 8 or 15 days after previous infestation with single *B. brassicae*, single *P. xylostella* or simultaneous *P. xylostella* and *B. brassicae* and undamaged plants (Control). Symbols marked with different letters are significantly different (GLMM, $P < 0.05$); ns indicates no significant difference between groups within a time point.

was analyzed separately. *Mamestra brassicae* caterpillar weight was lower after feeding on control plants 15 d after onset of the experiment compared to *M. brassicae* weight feeding on control plants 4 and 8 d after onset of the experiment (Table 2B). On Winspit control plants, *M. brassicae* caterpillar weight was lower compared to control plants of the two other plant populations. Weight of *M. brassicae* was also affected by the experimental round when feeding from control plants.

Mortality of *M. brassicae* caterpillars was not affected by previous induction by aphids or caterpillars (Table 2A), and was neither affected by plant population nor by experimental round; only at the 14 d time point, mortality was higher than at either of the two other time points.

Discussion

In nature, plants are frequently under attack by multiple insect herbivores. Insects feeding on plants interact indirectly through plant-mediated effects in which initial insect attackers affect plant responses that influence subsequently feeding herbivores (Denno et al., 1995; Kaplan and Denno, 2007). In addition, it is known that induction of plant defense responses differs between dual and single herbivore attack (Voelckel and Baldwin, 2004). Importantly, the majority of herbivores will find themselves feeding on plants previously attacked by multiple insects, but little is known about the effect of multi-herbivore-induced plant phenotypes on resistance to subsequent attackers. We found that simultaneous feeding by *P. xylostella* and *B. brassicae* resulted in different plant defense-related gene expression and differences in plant hormone levels compared to single herbivory, and this had a negative effect on subsequently arriving *M. brassicae* caterpillars, depending on plant population and time point. Furthermore, induced plant responses to herbivory were different across plant populations.

Here, the performance of both *P. xylostella* caterpillars and *B. brassicae* aphids was negatively affected by simultaneous feeding by the reciprocal herbivore. In contrast, in previous studies, positive effects of aphid feeding on caterpillar performance (Agrawal et al., 2014; Li et al., 2014; Soler et al., 2012), or positive effects of caterpillar feeding on the performance of aphids or other insect species (Mathur et al., 2013; Poelman et al., 2008; Rieske and Raffa, 1998; Soler et al., 2012) have been observed. Most of those studies concerned sequential insect infestation (*i.e.* one herbivore after the other). Here, we introduced the two initial herbivore species simultaneously, which might explain the negative reciprocal effects on their performance that we recorded in this study. Sequential

insect infestation causes a time lag between the induction by the first and a second attacker. Because plant defense signaling pathways are known to interact, a time lag could affect the interaction between defense signaling in a different way than when both attackers arrive at the same time (Erb *et al*., 2011; Karban, 2011). In addition, the outcome of interactions between species can be herbivore species-specific (Agrawal, 2000; Uesugi et al., 2013; Van Zandt and Agrawal, 2004). Not only the simultaneously attacking insects but also the subsequently arriving *M. brassicae* caterpillars were negatively affected by dual-herbivore-induced plant resistance. These findings suggest that plant responses to herbivores attacking alone affect an herbivore arriving later in a different way than simultaneously attacking herbivore species do (see also Kaplan and Denno, 2007). Such trait-mediated interaction networks (Utsumi et al., 2010) imply that herbivores can have far-reaching consequences for not only the plant they feed on, but also for all later arriving insects. It has been previously reported that *Spodoptera exigua* caterpillars performed worse when feeding from plants previously attacked by both potato aphids and *S. exigua* or aphids only, compared to plants with previous *S. exigua* attack alone. This coincided with a suppression of genes that were originally upregulated by the reciprocal herbivore and with different regulation of plant biochemistry during dual compared to single insect infestation (Rodriguez-Saona et al., 2010). Furthermore, Mathur et al. (2013) showed that the specialist caterpillar *Plutella xylostella* gained more weight when feeding on plants previously attacked by both *P. xylostella* and *Spodoptera litura* caterpillars than when feeding on plants previously attacked by only *P. xylostella*. However, these studies concerned only two species in conspecific or heterospecific interactions, whereas here we present the effects of two herbivores on responses to a newly arriving third insect species.

Our data provide further insight in how plants physiologically respond to single and dual herbivore attack by analysing the expression of defense genes and levels of the plant hormones SA and JA. We show that simultaneous feeding by the two insect species induced a different plant response compared to responses induced by aphids or caterpillars alone. In Kimmeridge plants, 3 d after herbivory by either aphids or caterpillars, SA levels were induced to significantly higher levels compared to plants simultaneously induced by both caterpillars and aphids. Through antagonistic or synergistic crosstalk between JA and SA, plants are able to fine tune their defenses (Pieterse et al., 2009; Thaler et al., 2012). Although a negative correlation was found between JA and SA levels in milkweed plants after herbivory of both monarch caterpillars and oleander aphids (Ali and Agrawal, 2014), we did not find evidence for overall suppression of JA by SA

or *vice versa* (data not shown). However, simultaneous feeding by aphids and caterpillars resulted in a significant increase of JA-dependent *LOX* expression compared with plants infested by only *P. xylostella* caterpillars or control plants. Therefore, differential induction of JA-regulated transcriptional responses to dual insect attack could have mediated a decrease in *M. brassicae* performance, because resistance to caterpillars (including *M. brassicae* – van Dam and Oomen, 2008) is generally induced by the JA signaling pathway (de Vos et al., 2005; Stam et al., 2014). The induction of plant defense signaling affected both *P. xylostella* and *B. brassicae* performance. Therefore, JA-mediated responses do not only affect caterpillars but also decrease aphid population growth. We showed that *B. brassicae* aphids induced both JA- and SA-mediated resistance (Moran et al., 2002) which may affect aphid performance depending on whether it is feeding alone or simultaneously with caterpillars. Here, we demonstrated that plant resistance changed after multiple insect attack compared to single attack, possibly regulated through JA-SA crosstalk, which subsequently affects the performance of successively arriving herbivores. Direct correlation of gene expression or hormone levels with herbivore performance cannot be done because of the different time scales at which these processes occur (Stam et al., 2014).

Still, relatively little is known about long-term effects of herbivory on the kinetics of defense-related gene expression or hormone levels upon multiple herbivory (de Vos et al., 2005; Kliebenstein, 2014). Underwood (2012) showed that plant resistance responses might last for at least 15 days after herbivory, and had not yet decayed by the time a second herbivore arrived on the plant. However, peaks in defense-related gene expression might decay much earlier (Vos et al., 2013). We observed that 14 d after herbivory a significant up-regulation of *PR-1* expression occurred after feeding by *P. xylostella* caterpillars only in Kimmeridge plants or after simultaneous feeding of both caterpillars and aphids in Old Harry plants. So, after prolonged herbivory, plant defense signaling is still upregulated. Similar to our results, it has been found before that *P. xylostella* feeding activates SA signaling in *Arabidopsis* and Chinese cabbage plants (Ehlting et al., 2008; Koo et al., 2013; Kroes et al., 2015). Interestingly, after 14 d the majority of the caterpillars had pupated and, thus, caterpillar feeding had stopped. Elevated expression of the SA-regulated marker gene *PR-1* in Kimmeridge plants 14 d after feeding by caterpillars, could indicate priming for enhanced defense or a lag in defense response time to caterpillar attack (see Vos et al., 2013). Another possibility could be that an antagonistic effect of JA on SA-mediated *PR-1* expression diminished from the moment the caterpillars stopped feeding upon pupation. Furthermore, the time herbivores spent feeding may differentially

affect defense responses induced by later arriving insects. *Spodoptera litura* was negatively affected by previous dual *P. xylostella* and *S. litura* feeding that started 14 d earlier, but not at earlier or later time points; *P. xylostella* was positively affected by previous dual feeding that had commenced 10 d earlier (Mathur et al., 2013). Similar to the finding of Mathur et al. (2013) that the subsequent herbivore was negatively affected by previous insect feeding depending on the duration of herbivory, we found that *M. brassicae* caterpillars performed worst on plants induced by both *P. xylostella* and *B. brassicae* after 15 d of feeding. This indicates that the length of time first inducers spent on feeding before a subsequent herbivore arrives, has an effect on the latter. However, declining plant quality over time cannot be completely excluded.

Plant species vary in their responses to herbivores, even though plant hormones and their cross-regulation are generally regarded as conserved among most of the Angiosperms (Thaler et al., 2012). Although hormone levels did not differ significantly between plant populations, an interaction effect between plant population and insect treatment was observed. Transcript levels of *LOX* and *PR-1* did differ between plant populations, indicating that regulation of responses to insect feeding varies significantly within the same plant species. Differences in responses to herbivory between plant populations (Li et al., 2014) or closely related plant species (Ali and Agrawal, 2014; Johnson and Agrawal, 2005) have been observed before. That *M. brassicae* caterpillars are differentially affected by plant populations confirms previous work (Gols et al., 2008). Moreover, seasonal changes within controlled climatic conditions in a greenhouse may cause variation in *Brassica* phenotype (Gols et al., 2007), which resembles the variation that we observed among experimental rounds.

In conclusion, we found that plant defense signaling is differentially regulated in response to dual herbivore attack, compared to attack by one herbivore species. These responses differed among wild cabbage populations and negatively affected a subsequently arriving third herbivore. These findings may have long-term consequences for herbivore community development. On hemlock trees, simultaneous infestation in spring by both the hemlock woolly adelgid and the hemlock scale insect affected the number of woolly adelgids in next autumn, compared to previous sequential infestation by the scale followed by the adelgid (Miller-Pierce and Preisser, 2012). Plant-mediated effects of responses to herbivory might cascade through the community and thus affect community composition (Kessler and Baldwin, 2004; Poelman et al., 2010; Van Zandt and Agrawal, 2004; Viswanathan et al., 2007). Therefore, to better predict consequences for

herbivore communities, future studies should aim at a better understanding of how plants regulate their defenses in natural ecosystems (Poelman, 2015). Considering the differences in time scale at which transcriptional changes and insect responses take place (Stam et al., 2014), this poses challenges to experimental design. However, combining ecological and molecular approaches to plant-insect interactions may help to link transcriptomic changes to insect responses. We found that changes in gene expression and phytohormone levels caused by dual herbivory affected a subsequently arriving third herbivore, as an example of trait-mediated interaction networks that are common in insect communities. Such understanding of how plants and insects are able to interact through plant-mediated effects, would help to define herbivore communities and provide insight in how networks of inducers and responders integrate over time (Kliebenstein, 2014; Utsumi et al., 2010). This insight helps to refine the increasingly complex plant-insect interaction models in which factors such as time course, ecological and molecular changes, multiple interacting insect attackers and plant genotype are important.

Acknowledgments

We thank Léon Westerd, Frans van Aggelen, André Gidding and Joop Woelke for culturing the insects and Rieta Gols for providing the *B. oleracea* seeds. This work was supported by a TOP GO grant (grant number 854.10.010 to M.D.) from The Netherlands Organization for Scientific Research (NWO).

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Chapter 8

General discussion

Introduction

Plants are versatile in their ability to adapt to changing environmental conditions. To increase their chances of survival throughout the growing season, plants have numerous adaptations to defend their tissues against the feeding by herbivorous insects (Kessler and Baldwin, 2002; Dicke and Baldwin, 2010). Insect attackers interact with host plants at the levels of gene expression, phytohormone production and biochemical changes (Dicke et al., 2009; Pieterse et al., 2012), resulting in changes in the plant's phenotype. Depending on different factors, such as insect species (Kessler and Baldwin, 2004; Pashalidou et al., 2013), the insect feeding guild (Bidart-Bouzat and Kliebenstein, 2011; Appel et al., 2014) or insect density (Zhang et al., 2009; Ponzio et al., 2016) specific changes in the phenotype of the plant are induced.

The activation of plant defense signaling networks in response to insect attack is regulated by phytohormones such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Arimura et al., 2005; Pieterse et al., 2012). In general, JA-mediated signaling underlies defense responses against leaf-chewing herbivores, such as caterpillars, whereas phloem-feeding insects, such as aphids, mainly induce SA-regulated defenses (De Vos et al., 2005; Stam et al., 2014). When caterpillars and aphids simultaneously feed on the same host plant, crosstalk between phytohormonal signaling pathways may affect the regulation of plant defenses (Stam et al., 2014). Consequently, multiple insect herbivory can interfere with plant defense responses to single herbivore species, and this may affect the outcome of interactions between plants and multiple insect attackers (Voelckel and Baldwin, 2004; Rodriguez-Saona et al., 2010; Soler et al., 2012; Zhang et al., 2013).

As research on consequences of multiple insect infestation on molecular plant responses is in its infancy (Voelckel and Baldwin, 2004; Dicke et al., 2009; Rodriguez-Saona et al., 2010; Zhang et al., 2013), studies investigating molecular mechanisms underlying interference by multiple attacking insects with induced plant defenses will benefit studies on the ecological consequences of induced plant responses (Li et al., 2014). Interestingly, effects of induced defenses against herbivory may cascade through insect communities where they can have large-scale effects on multitrophic insect-plant interactions (Kessler and Halitschke, 2007; Poelman et al., 2008; Poelman et al., 2012).

The aim of this study was to elucidate molecular mechanisms that underlie plant-mediated interactions between attacking aphids and caterpillars. By combining analyses of phytohormone levels, defense gene expression, volatile

emission, insect performance and behavioral responses of parasitoids, I show how simultaneous feeding by *Plutella xylostella* caterpillars and *Brevicoryne brassicae* aphids differs in its effects on defense signaling pathways in *Arabidopsis thaliana* compared to feeding by single insect species. I additionally studied how modulation of induced plant defenses in response to dual insect attack is dependent on insect density. I found that in the interactions between plants and multiple attackers, insect density is an important factor in orchestrating the complex interactions between plant defense responses. As a next step in the research on multiple insect-plant interactions, I studied how plant responses to dual herbivory can have consequences for interactions with a subsequently arriving herbivore (*Mamestra brassicae* caterpillars) on wild cabbage plants (*Brassica oleracea*). The results indicate that simultaneous feeding by caterpillars and aphids has plant-mediated negative consequences for subsequently arriving *M. brassicae* caterpillars.

In this general discussion, I will position important findings of this thesis within a broader framework of studies on plant interactions with multiple insect herbivores and compare them with relevant topics in the current literature. I will discuss the complexity of underlying signaling mechanisms that modulate interactions between insect herbivores when feeding simultaneously on plants. Additionally, insect species specificity of induced defense in the outcome of interactions between herbivores is discussed. Finally, the importance of studying a molecular as well as an ecological model plant system with respect to investigating consequences of plant defense to multiple herbivory will be discussed.

Induced defense in response to multiple insect attack mediated by JA-SA crosstalk

Although plant species vary in their responses to herbivores, phytohormonal signaling and crosstalk between their signaling pathways is evolutionarily highly conserved among most of the Angiosperms (Thaler et al., 2012). The signaling pathways of the two phytohormones salicylic acid (SA) and jasmonic acid (JA) interact antagonistically, most likely as part of the plant's strategy to fine-tune its defense response (Caarls et al., 2015).

It has been proposed that insects can manipulate plant defenses for their own benefit by modulating JA-SA crosstalk (Cipollini et al., 2004; Zhu-Salzman et al., 2004; Zhang et al., 2013). This has also been reported for the tomato russet mite (*Aculops lycopersici*) that suppressed a harmful JA response by inducing

SA signaling in tomato (Glas et al., 2014). A study using tomato plants infested by the mealybug *Phenacoccus solenopsis* also proposed the same mechanism (modification of JA-SA crosstalk) explaining the enhanced performance of *P. solenopsis* nymphs (Zhang et al., 2015). In addition, recent research reported that phloem-feeding insects interfere with JA-mediated defenses against caterpillars by inducing SA-mediated responses (Zhang et al., 2009; Soler et al., 2012; Zhang et al., 2013; Ali and Agrawal, 2014; Chapters 3 and 5). Plant-mediated interactions between herbivores may also involve synergistic effects between SA- and JA-dependent signaling pathways. For example, exogenous application of JA on *A. thaliana* plants induced the emission of methyl salicylate (a volatile derivative of SA) (Van Poecke, 2002). In my study system, *P. xylostella* feeding alone induced significantly higher levels of SA compared to dual insect attack, but no differences were found anymore when insects were feeding on a mutant defective in the JA-pathway (Chapter 5). However, JA-SA crosstalk does not seem to mediate interactions in all insect-plant systems. Crosstalk between SA and JA signaling pathways does not play a major role in the interaction between maize seedlings, *Spodoptera exigua* caterpillars and *Rhopalosiphum maidis* aphids (Tzin et al., 2015).

The crosstalk between JA and SA signal-transduction pathways is greatly influenced by the timing of insect attack and underlying genetic variation (Thaler et al., 2012). The timing of induction of JA and SA, which is dependent on the sequence of arrival of the inducing insects, affects SA-JA crosstalk. When both phytohormones are produced simultaneously, SA is able to suppress JA-mediated defense responses (Koornneef et al., 2008). However, when the SA signal is induced before the activation of the JA signaling pathway, SA suppression of JA-regulated responses is only effective within a short period of time after induction of SA (Koornneef et al., 2008). Patterns of SA- and JA-signaling induction may be shaped by keystone herbivores that through their feeding affect plant-mediated interactions between herbivores (Poelman and Kessler, 2016). Keystone herbivores may affect selection on induced plant defense which could lead to non-additive effects on JA- and SA-signaling crosstalk, and thus to the occurrence of genetic variation in whether and in which direction antagonism between SA- and JA-signaling is expressed. In addition, different stages of co-evolution between plants and insects may lead to less distinct JA-SA crosstalk responses. Insects that are more specialized on their host plants have evolved the ability to interfere with plant defenses through JA-SA crosstalk, while insects that infrequently feed on a wide range of plant species have not (Züst and Agrawal, 2016).

To summarize, investigating the importance of JA-SA crosstalk for plant defense regulation to multiple insect attack will contribute to a better understanding of complex plant-insect interactions. Furthermore, JA and SA are not the only regulators of plant defense. Other mechanisms *e.g.* JA-independent accumulation of the non-protein amino acid GABA, should be considered as well (Scholz et al., 2015).

Importance of *WRKY* transcription factors as regulators of plant-mediated insect interactions

Transcription factors play an important role in herbivore-induced plant defense responses, as they regulate the expression of responses of hormonal signaling pathways and thereby influence signaling. WRKY transcription factors are known to bind to the W box, an element which is present in the promoters of many plant defense genes (Du and Chen, 2000; Eulgem et al., 2000; Singh et al., 2002). For instance, NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1), a key regulator of SA-mediated responses, is regulated by WRKY transcription factors (Pieterse et al., 2012). The WRKY family of transcription factors also plays a key role in the regulation of crosstalk between JA and SA signaling (Pieterse et al., 2012; Bakshi and Oelmüller, 2014). For instance, Chen and Chen, (2002) showed that WRKY18 is a positive regulator of the expression of *PR* genes, which are SA-responsive marker genes. In addition, WRKY18 (together with WRKY40) contributes to the synergistic interaction between JA and SA signaling pathways by positively regulating JA signaling in response to powdery mildew (*Golovinomyces orontii*) infection (Pandey et al., 2010). WRKY18 expression was also induced upon methyl jasmonate treatment in *A. thaliana* plants (Wang et al., 2008). In Chapter 6, a microarray study was used to study transcriptomic changes in the response of *A. thaliana* wild-type plants to simultaneous feeding of *P. xylostella* caterpillars and *B. brassicae* aphids and to feeding of *P. xylostella* caterpillars alone. After 48 h of simultaneous feeding of caterpillars and aphids, WRKY18 was differentially expressed compared to undamaged control plants. Thus, WRKY18 could play a role in responses to dual insect attack by inducing both JA- and SA-mediated defenses which could lead to a higher resistance against aphids (inducers of SA signaling) and caterpillars (inducers of JA signaling).
The transcription factor WRKY70 is induced by SA, and regulates SA-mediated gene expression. However, WRKY70 negatively regulates JA responses (Caarls et al., 2015). It is commonly known that JA-mediated signaling underlies plant

defense responses against leaf-chewing herbivores, such as caterpillars, while phloem-feeding insects such as aphids, generally induce SA-regulated defenses (Stam et al., 2014). When caterpillars and aphids are feeding simultaneously on the same plant, crosstalk between both signaling pathways may affect the regulation of plant defenses (Stam et al., 2014). An interesting question that remains is whether WRKY70 is part of the molecular mechanism underlying plant-mediated interactions between attacking caterpillars and aphids.

Experimental evidence shows that *B. brassicae* aphids interfere with the expression of the transcription factor WRKY70 in *A. thaliana* plants when feeding simultaneously with *P. xylostella* caterpillars (Chapter 3). SA-dependent WRKY70 expression was lower in plants simultaneously attacked by *P. xylostella* caterpillars and aphids at high density, compared to caterpillars feeding alone, which led to up-regulation of JA-mediated defenses and negatively affected caterpillar performance. Interestingly, WRKY70 was shown to play opposing roles in the induction of defenses against caterpillars in monocot and dicot plants. Onkokesung et al. (2016) demonstrated that suppression of JA-induced responses is mediated by *WRKY70*, which resulted in plant resistance against *P. brassicae* feeding on *A. thaliana* plants. In contrast, *Os*WRKY70 induction positively regulated JA biosynthesis and increased resistance against the chewing herbivore *Chilo suppressalis* feeding on rice (Oryza sativa) (Li et al., 2015). Taken together, the above studies suggest that aphid interference with caterpillar-induced defenses mediated via WRKY70 expression may result in different outcomes depending on whether the insects are feeding on monocot or dicot plants. To investigate the hypothesis that aphids differentially affect induced defenses against caterpillars in dicot or monocot plants, a comparison of several dicot and monocot species should be made.

A recent study indicates that WRKY70 expression is regulated by the transcription factor MYB44 in *A. thaliana* (Shim et al., 2013). Interestingly, Lu et al. (2013) showed that overexpression of MYB44 in *A. thaliana* plants induced resistance to *P. xylostella* caterpillars. MYB44 also appeared to play an important role in *EIN2* expression and biosynthesis of glucosinolates, leading to resistance against *P. xylostella*. In contrast, *P. xylostella* performance was positively affected when feeding simultaneously with aphids at low density on *A. thaliana* plants (Chapter 3), which was associated with induced expression of the transcription factor WRKY70. By inducing SA-mediated defense responses aphids could suppress JA defenses and facilitate caterpillar performance. In addition, results of Chapter 5 of this thesis also suggest that JA- and ET-regulated defense responses influence

plant-mediated interactions between aphids and *P. xylostella* caterpillars. Because JA and ET signaling pathways can interact synergistically, ET-mediated signaling may be dependent on JA signaling. Therefore, induced expression of *WRKY70* (and thus up-regulation of *MYB44*) may also affect the interaction of MYB44 with ET-signaling when aphids and caterpillars are feeding simultaneously (Figure 1).

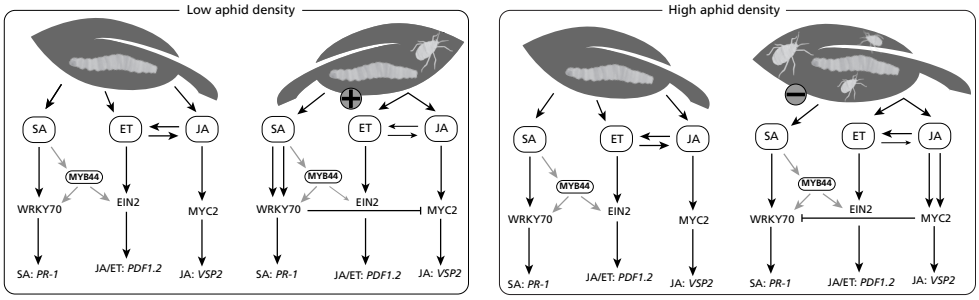

Figure 1. Working model of SA-, JA- and ET-mediated defense regulation in response to infestation by *P. xylostella* caterpillars alone or infestation by both *P. xylostella* caterpillars and *B. brassicae* aphids in *A. thaliana* plants. Defense signaling is shown for both a low and a high aphid density. Arrows indicate induction, whereas blocked lines indicate suppression. Double arrows indicate a significantly higher level of plant gene transcription relative to *P. xylostella*-infested plants and the thickness of an arrow indicates strength of signaling. Plus and minus signs indicate positive and negative effects of simultaneous aphid feeding on caterpillar growth performance compared to caterpillars feeding alone. Grey lines represent results from literature (Lu et al., 2013; Shim et al., 2013).

In addition to effects of simultaneous caterpillar and aphid feeding on induced direct defenses, I also studied effects on induced indirect defenses (Chapter 4). We found that feeding by both caterpillars and aphids at a high density induced transcription of *TPS03* (encoding a sesquiterpene (*E*,*E*)-α-farnesene synthase) compared to simultaneous feeding by caterpillars and aphids at a low density. A study on cotton plants (*Gossypium arboreum*) has identified one of the members of the WRKY transcription family (*Ga*WRKY1) as a regulator of the expression of a sesquiterpene cyclase gene (terpene cyclases are the first key enzymes in pathways leading to the biosynthesis of terpenes) (Xu et al., 2004). Interestingly, a W-box is present in the promoter of sesquiterpene cyclases of other plant species, including *A. thaliana* (Xu et al., 2004). This indicates that WRKY proteins of *A. thaliana* plants could bind to and regulate sesquiterpene cyclase gene expression. It remains to be investigated whether differences in regulation of *WRKY70* in response to simultaneous feeding by caterpillars and aphids at low or high density is also responsible for modulations of herbivore-induced terpene synthases.

Plant-mediated effects of dual insect attack on indirect defenses

Plant-mediated interactions between aphids and caterpillars involve not only direct but also indirect defense responses. Herbivore-induced plant volatile emission mediates an indirect defense of plants by attracting natural enemies of herbivores (Dicke and Baldwin, 2010). The JA- and SA-signaling pathways regulate the biosynthesis of plant volatiles, such as volatile terpenes, green leaf volatiles and the aromatic compound methyl salicylate (MeSA) (Dicke and Van Poecke, 2002; Liu et al., 2010). Interactions between simultaneously feeding caterpillars and aphids may affect the emission of plant volatiles and thus the attractiveness of plants to the herbivores' natural enemies (Dicke et al., 2009; Ponzio et al., 2016). In this thesis, the effects of dual insect attack on the preference of the parasitoid *Diadegma semiclausum*, a specialist parasitoid of *P. xylostella* caterpillars (Chapter 4), and of the parasitoid *Diaeretiella rapae*, a parasitoid of *B. brassicae* aphids (Chapter 5) was studied. Furthermore, effects of aphid density on plant-mediated insect interactions were investigated. These studies indicate how modification of plant defense responses against simultaneously attacking herbivores affect induced indirect defenses. For instance, *D. semiclausum* parasitoids preferred the volatile blend of dually infested plants at high aphid density over those from dually infested plants at low aphid density (Chapter 4). The observed preference can be partially explained by results from the microarray analysis presented in Chapter 6. The microarray data indicated that the linalool synthase *TPS10* gene was differentially expressed in response to feeding of both caterpillars and aphids at high density after 24 h compared to undamaged control plants, but not in response to dual insect infestation at low density (Chapter 6). Linalool has been reported to be an important attractant for *D. semiclausum* parasitoids (Houshyani et al., 2013) and induced biosynthesis of linalool could have influenced *D. semiclausum* preference for plants dually infested by caterpillars and aphids at high density.

Results of Chapter 5 indicate that *D. rapae* parasitoids prefer volatiles emitted by dual-infested plants over plants infested by *B. brassicae* aphids alone, which is noteworthy. Feeding by *B. brassicae* aphids induces the release of allyl isothiocyanate, which is attractive to *D. rapae* parasitoids (Pope et al., 2008). Interestingly, in Brussels sprouts plants, previous infestation of *B. brassicae* aphids led to higher emission of allyl isothiocyanate compared to first-time aphid-infested plants. Furthermore, previous *B. brassicae* infestation also resulted in an increased attraction of the parasitoid *D. rapae* (Najar-Rodriguez et al., 2015).

These results suggest that *B. brassicae* aphids can manipulate emission of allyl isothiocyanate while feeding on the plant, and thereby reduce the attractiveness of the plant to *D. rapae* parasitoids. Allyl isothiocyanate is an hydrolysis product of sinigrin, an aliphatic glucosinolate. In *A. thaliana*, two R2R3-MYB transcription factors (MYB28 and MYB29) positively control the biosynthesis of aliphatic glucosinolates (Hirai et al., 2007). Interestingly, the microarray analysis (presented in Chapter 6) showed that expression of *MYB29* in response to simultaneous feeding by caterpillars and aphids at high density clustered separately from *MYB29* expression in plants induced by simultaneous feeding of both insects at low density during 48 h. This result indicates that expression patterns of *MYB29* differ between plants simultaneously induced by caterpillars and aphids at low or high density, which could also affect the attraction of *D. rapae* parasitoids compared to aphids feeding alone as shown in Chapter 5.

The interaction between *Plutella xylostella* and plants

Defense responses to caterpillar feeding are generally regulated by the JA signaling pathway (Kessler and Baldwin, 2002; De Vos et al., 2005; Stam et al., 2014). On the other hand, we observed that *P. xylostella* caterpillars are also inducers of SA-mediated defenses when feeding alone on *A. thaliana* and *B. oleracea* plants (see Chapters 3, 4, 5, 6 and 7). This is consistent with the findings of Ehlting et al. (2008) and Koo et al. (2013) who reported that *P. xylostella* feeding up-regulated *PR* genes in *A. thaliana* and Chinese cabbage (*B. rapa*) plants. Also, feeding by *P. xylostella* induced the emission of the volatile SA-derivative MeSA in *A. thaliana* plants (Zhang et al., 2013). Wei et al. (2013) found up-regulation of JA and SA signaling pathways, and down-regulation of the ethylene signaling pathway in *Barbarea vulgaris* (Brassicaceae) by *P. xylostella* feeding. Vogel et al. (2007) found that transcriptional responses in *Boechera divaricarpa* (a close relative of *A. thaliana* plants) to *P. xylostella* are influenced by the ET and SA signaling pathways.

Induction of SA-mediated defenses by *P. xylostella* caterpillars could be due to their larval life style, as the feeding habit of first-instar larvae is leaf mining. Leaf-mining insects (*Phyllonorycter blancardella*) on apple leaves induced higher activation of both JA and SA signaling pathways (Zhang et al., 2015; Giron et al., 2016). In addition, effectors from the oral secretion (OS) of *P. xylostella* caterpillars might affect induced defense responses in the plant and thereby influence SA levels (Schäfer et al., 2014). For instance, glucose oxidase has been

identified in the saliva of *Helicoverpa zea* and *Spodoptera exigua* caterpillars and has been shown to induce SA signaling which leads to suppression of JA and ET signaling (Musser et al., 2005; Diezel et al., 2009). However, whether this enzyme is also present in the OS of *P. xylostella* remains to be determined. Finally, *P. xylostella* caterpillars could affect plant defenses through microbial elicitors. Herbivores possess diverse microbes in their digestive systems and these microbial symbionts can modify plant-insect interactions (reviewed in Dillon and Dillon, (2004)). *Plutella xylostella* larvae have rich microbial communities inhabiting the gut, and these bacteria contribute to fitness of the caterpillars (Xia et al., 2013; Lin et al., 2015). A study using tomato plants (*Solanum lycopersicum*) showed that Colorado potato beetle larvae (*Leptinotarsa decemlineata*) exploit bacteria in their OS to induce SA-mediated gene expression which leads to inhibition of JA-mediated defense responses (Chung et al., 2013). More research is needed to investigate the bacteria in *P. xylostella* caterpillar OS.

Although caterpillars are generally thought to induce JA-mediated defenses, herbivores within the same feeding guild and order of Lepidoptera have also been shown to induce distinct defense responses (Bidart-Bouzat and Kliebenstein, 2011). Consequently, plant responses to simultaneous attack by aphids and caterpillars may not be so general. Phytohormonal crosstalk between JA- and SA-regulated signaling pathways is thought to underlie plant-mediated interactions between attacking caterpillars and aphids. However, different caterpillar species may differentially affect the trade-off between defense signaling and thus the regulation of plant defense in response to simultaneous feeding by caterpillars and aphids. To arrive at generalizations on interactions between defense signaling pathways for the outcome of multiple insect-plant interactions various species of caterpillars could be investigated (Li et al., 2016). Species-specific responses to herbivores should be considered to understand the outcome of plant-mediated interactions between multiple attacking herbivores.

Consequences of multiple herbivore-induced *A. thaliana* and *B. oleracea* plant phenotypes

In this thesis both the model plant for molecular genetics, *Arabidopsis thaliana*, and an ecological model plant, wild *Brassica oleracea*, were used to study plant-mediated interactions between caterpillars and aphids. Research on *A. thaliana* to investigate plant responses to herbivory resulted in a detailed explanation of the molecular mechanisms underlying plant defense (Van Poecke, 2007). Therefore, the model plant *A. thaliana* was further exploited in this thesis to gain more

knowledge on underlying mechanisms explaining plant defense against multiple feeding herbivores. To link transcriptomic plant responses to insect responses and better predict consequences of interactions between plants and multiple insect attackers for herbivore communities, plant responses to multiple herbivory were also studied in the ecological model plant wild *Brassica oleracea*.

Experiments with *A. thaliana* (Chapter 3) and *B. oleracea* (Chapter 7) plants showed that both related plant species differentially affected performance of *P. xylostella* when the caterpillar is feeding simultaneously with aphids compared to feeding alone. When *P. xylostella* caterpillars were feeding simultaneously with a density of five aphids on *A. thaliana* plants, their growth rate was increased compared to feeding alone (Chapter 3). In contrast, performance of *P. xylostella* caterpillars was reduced on *B. oleracea* plants when feeding simultaneously with the same density of aphids, compared to caterpillars feeding alone (Chapter 7). Different caterpillar densities used in the experiments on the two host plants could have influenced the outcome of *A. thaliana* or *B. oleracea* plant-mediated interactions between caterpillars and aphids. However, differences in plant nutritional quality or induced plant defenses may also have contributed to the observed effect.

Plant nutritional quality is mainly determined by the concentration of nitrogen-containing nutrients in plant tissues for chewing herbivores such as caterpillars and in phloem sap for aphids, which influences their performance in terms of survival, growth rate and reproduction (Throop and Lerdau, 2004; Ohgushi and Hambäck, 2015). Herbivore-induced defenses are regulated by phytohormonal signaling pathways and their crosstalk (Pieterse et al., 2012; Caarls et al., 2015). Insects may also interfere with induced plant defenses, which could lead to the expression of a different plant defensive phenotype. Simultaneously attacking insect herbivores also compete through these plant-mediated interactions. It was shown, however, that competition is not correlated with levels of defoliation (Kaplan and Denno, 2007). Furthermore, simultaneous feeding by aphids did not affect *P. xylostella* performance on *A. thaliana* mutants defective in SA and JA defense signaling (Chapter 3). This indicates that aphid modulation of caterpillar performance is not due to a reduction in plant nutritional quality but to a reduction in plant defense (Huot et al., 2014).

The outcome of plant-mediated interactions between caterpillars and aphids can be highly asymmetrical, which could lead to positive (*i.e.* facilitation) or negative (*i.e.* antagonism) effects on the performance of the competing herbivore species (Kaplan and Denno, 2007; Ali and Agrawal, 2014). Asymmetry is likely caused

by differences in feeding patterns of the herbivores (Kaplan and Denno, 2007). For instance, unlike chewing caterpillars, aphids feed on plant phloem sap using their stylets, which are specialized mouthparts that do not cause major damage to plant tissues (Züst and Agrawal, 2016). Depending on the insect feeding guild, but also on the sequence of herbivore arrival and insect density, specific changes in plant defense are induced which could underlie asymmetric interactions between herbivores (Kaplan and Denno, 2007; Pieterse et al., 2012; Stam et al., 2014). Moreover, specialized defensive secondary metabolites are unevenly distributed across phylogenetic plant groups and even among plant individuals within a species, and can therefore determine the outcome of plant-herbivore interactions (Schuman and Baldwin, 2015).

Plants that are growing in an unpredictable environment where they are attacked by multiple insect herbivores at the same time, can benefit from a generalized defense response. For plant defense to have a generalized effect, genetic correlation between induced defenses in response to herbivores is needed (Leimu and Koricheva, 2006). Interestingly, differences between life span of the host plants may be an important determinant of genetic correlation between induced defenses in response to herbivores. Annual plants (*e.g. Arabidopsis thaliana*) may be able to respond faster to selection for defenses than biennial or perennial plants (*e.g. Brassica oleracea*). Consequently, annuals are more likely to adopt a defense strategy in which defenses are specifically induced to particular herbivores. Furthermore, *B. oleracea* plants might be more likely to evolve generalized defenses as they interact with many different insect species throughout the growing season (Leimu and Koricheva, 2006).

Outcomes of interactions between plants and herbivores depend on the ecological context (Schuman and Baldwin, 2015). For instance, diurnal timing is an important modifier of plant defense response to the environment (Falk et al., 2014). The study of Falk et al. (2014) indicates an important role for the diurnal regulation of defense metabolites against nocturnal molluscan herbivores in *A. thaliana*, which are proposed as the major herbivores of *A. thaliana* plants. Also, differences in plant traits could affect outcome of competition between multiple feeding herbivores. For example, different from the hairy leaves of *A. thaliana*, the glossy leaves of *B. oleracea* are associated with resistance to *P. xylostella* caterpillars (Ramchiary et al., 2015).

These studies indicate that interspecific competition between caterpillars and aphids is influenced by plant-mediated interactions. However, because of the different time scales at which transcriptional changes and insect responses

take place, causal links are difficult to identify. Translating laboratory results to responses of plants under field conditions is a fundamental step forward in the understanding of how plants influence insect communities (Poelman et al., 2009). To understand the induction of plant defenses and their outcomes in response to multiple insect attackers, plant genetic variation and the ecological context need to be studied as well.

Conclusions

The aim of this thesis was to elucidate plant-mediated interactions between specialist aphids and caterpillars by integrating transcriptional, chemical and behavioral data. In particular, the effect of insect density on the outcome of plant defense in response to dual herbivory was studied. Links between molecular defense mechanisms and ecological consequences of insect attack were identified. The induction of plant defenses in response to dual insect attack is dependent on different factors, such as sequence of herbivore arrival, number of insect species attacking and insect density. Interestingly, in the plant-mediated interactions between caterpillars and aphids, insect density was shown to be an important factor affecting interactions between induced defense signaling pathways. This highlights the importance of considering insect densities when investigating plant defenses in the laboratory. Research into the combination of different factors affecting the induction of plant defense responses might give more insight into the importance of individual factors and how they influence plant-mediated interactions between herbivores. Preferably, this will be done in field situations to determine if insect density-dependent effects as seen in the laboratory are equally important in interactions between multiple herbivores under natural conditions.

Acknowledgments

I would like to thank Marcel Dicke and Joop van Loon for constructive comments on an earlier version of this chapter.

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In the field, plants suffer from attack by herbivorous insects. Plants have numerous adaptations to defend against herbivory. Not only do these defense responses reduce performance of the feeding herbivore, they also result in the attraction of natural enemies of herbivores. The majority of studies investigating plant-insect interactions addressed mainly the effects of attack by a single herbivore species on induced plant defenses. However, because plants are members of complex communities, plants are exposed to different insect attackers at the same time. Moreover, attacks by different herbivores interact at different levels of biological organization, ranging from the level of gene expression, phytohormone production and biochemical changes up to the individual level. Effects of plant responses to feeding by two or more herbivore species simultaneously might cascade through the community and thereby affect insect community composition.

The induction of plant defense responses is regulated by a network of signaling pathways that mainly involve the phytohormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). The signaling pathways of the two phytohormones SA and JA interact antagonistically, whereas JA and ET signaling pathways can interact both synergistically and antagonistically in regulating plant defense responses. In general, JA-mediated signaling underlies defense responses against leaf-chewing herbivores, such as caterpillars, whereas phloem-feeding insects, such as aphids, mainly induce SA-regulated defenses.

When caterpillars and aphids simultaneously feed on the same host plant, crosstalk between phytohormonal signaling pathways may affect the regulation of plant defenses. Consequently, multiple insect herbivores feeding on plants interact indirectly through plant-mediated effects. Studies investigating molecular mechanisms underlying interference by multiple attacking insects with induced plant defenses will benefit studies on the ecological consequences of induced plant responses.

The aim of this thesis was to elucidate molecular mechanisms that underlie plant-mediated interactions between attacking herbivores from different feeding guilds, namely *Brevicoryne brassicae* aphids and *Plutella xylostella* caterpillars.

Because herbivore density affects the regulation of plant defense responses, it may also influence the outcome of multiple insect-plant interactions. To study if modulation of induced plant defenses in response to dual insect attack depends on insect density, plants were infested with two densities of aphids.

Responses of *Arabidopsis thaliana* plants to simultaneous feeding by aphids and

caterpillars were investigated by combining analyses of phytohormone levels, defense gene expression, volatile emission, insect performance and behavioral responses of parasitoids. To better predict consequences of interactions between plants and multiple insect attackers for herbivore communities, the regulation of defense responses against aphids and caterpillars was also studied in the ecological model plant wild *Brassica oleracea*.

In a literature review in Chapter 2, the effect of multiple insect attack on plants at different levels of biological integration is discussed. Transcriptomic changes of plants during multiple insect attack and their consequences for the plant's interactions with members of the associated insect community take place at different time scales. Direct correlation of transcriptomic responses with community development is, therefore, challenging. However, detailed knowledge of subcellular mechanisms can provide tools to address this challenge.

One of the objectives of this thesis, therefore, was to investigate the involvement of phytohormonal signaling pathways and their interactions during defense responses against caterpillars or aphids at different densities, when feeding alone or simultaneously on the model plant *A. thaliana* (Chapters 3 and 5). The studies show that aphids at different densities interfere in contrasting ways with caterpillar-induced defenses, which required both SA- and JA-signal-transduction pathways. Transcriptional analysis revealed that expression of the SA transcription factor gene *WRKY70* was differentially affected upon infestation by aphids at low or high densities. Interestingly, the expression data indicated that a lower expression level of *WRKY70* led to significantly higher *MYC2* expression through SA-JA crosstalk. Based on these findings, it is proposed that by down-regulating *WRKY70* expression, the plant activates JA-dependent defenses which could lead to a higher resistance against aphids and caterpillars.
Plutella xylostella caterpillars also influenced plant defense responses when feeding simultaneously with aphids. Caterpillar feeding affected aphid-induced defenses which had negative consequences for aphid performance. Induction of both ET- and JA-mediated defense responses is required for this interference. Moreover, aphid density also played an important role in the modulation by *P. xylostella* of aphid-induced defenses: *P. xylostella* caterpillars induced changes in levels of JA and its biologically active from, JA-Ile, only when feeding simultaneously with aphids at a high density.

To study the overall effect of dual herbivory on induced plant defenses, not only interference with induced direct defense, but also with induced indirect defenses was addressed in *A. thaliana* (Chapters 4 and 5). We found a significant preference of the aphid parasitoid *Diaeretiella rapae* for volatiles from aphid-infested *A. thaliana* wild-type plants and *ein2-1* (ET-insensitive) mutants. Interestingly, simultaneous feeding by *P. xylostella* caterpillars on wild-type plants increased *D. rapae*'s preference for odors from aphid-infested plants. However, upon disruption of the ET-signaling pathway, *D. rapae* did not distinguish between *ein2-1* mutants infested by aphids or by both aphids and caterpillars. This showed that intact ET signaling is needed for caterpillar modulation of the attraction of *D. rapae* parasitoids.
On the other hand, attraction of the caterpillar parasitoid *Diadegma semiclausum* to volatiles emitted by *A. thaliana* plants simultaneously infested by caterpillars and aphids was influenced by the density of the feeding aphids. Biosynthesis and emission of the terpene (*E*,*E*)-α-farnesene could be linked to the observed preference of *D. semiclausum* parasitoids for the HIPV blend emitted by plants dually infested by caterpillars and aphids at a high density, compared to dually infested plants with a low aphid density.

In Chapter 6, transcriptomic changes in the response of *A. thaliana* wild-type plants to simultaneous feeding by *P. xylostella* caterpillars and *B. brassicae* aphids compared to plants infested by *P. xylostella* caterpillars alone were assessed using a microarray analysis. I particularly addressed the question whether the transcriptomic response to simultaneously attacking aphids and caterpillars was dependent on aphid density and time since initiation of herbivory. The data show that in response to simultaneous feeding by *P. xylostella* caterpillars and *B. brassicae* aphids the number of differentially expressed genes was higher compared to plants on which caterpillars had been feeding alone. Additionally, specific genes were differentially expressed in response to aphids feeding at low or high density. Cluster analysis showed that the pattern of gene expression over the different time points in response to dual infestation was also affected by the density of the attacking aphids. These results suggest that insects attacking at a high density cause an acceleration in plant responses compared to insects attacking at low density.

As a next step in the study of multiple interacting herbivores, I studied whether plant responses to dual herbivory have consequences for the performance of a subsequently arriving herbivore, *Mamestra brassicae* caterpillars (Chapter 7). The ecological consequences of plant responses to dual herbivory cascading into a chain of interactions affecting other community members have remained unstudied so far. We used wild *B. oleracea* plants to evaluate dual herbivore-induced plant adaptations for subsequent herbivory. We found that simultaneous feeding by *P. xylostella* and *B. brassicae* resulted in different plant defense-related gene expression and differences in plant hormone levels compared to single herbivory, and this had a negative effect on subsequently arriving *M. brassicae* caterpillars. Differential induction of JA-regulated transcriptional responses to dual insect attack was observed which could have mediated a decrease in *M. brassicae* performance. The induction of plant defense signaling also affected both *P. xylostella* and *B. brassicae* performance. This study further helps to understand herbivore community build-up in the context of plant-mediated species interactions.

In the general discussion (Chapter 8), I focus on the complexity of underlying signaling mechanisms that modulate interactions between insect herbivores when feeding simultaneously on plants. Furthermore, the specificity of plant-*P. xylostella* caterpillar interactions is addressed. *P. xylostella* caterpillars are not only inducers of JA- but also of SA-mediated defenses, which could affect the regulation of plant defense in response to simultaneous feeding by aphids and caterpillars. Species-specific responses to herbivores should be considered to understand the outcome of plant-mediated interactions between multiple attacking herbivores. Finally, the importance of studying a molecular as well as an ecological model plant system to understand the consequences of plant defense to multiple herbivory is discussed. Interspecific competition between caterpillars and aphids is influenced by plant-mediated interactions. However, to understand the induction of plant defenses and their outcomes in response to multiple insect attackers, plant genetic variation and the ecological context need to be studied as well. In addition, understanding the adaptations of plants to multiple herbivore attack, beyond two herbivore species, will be an important step towards refining complex multiple insect-plant interactions.
Altogether, findings from this thesis reveal a molecular basis underlying plant responses against multiple herbivory and provide insight in plant-mediated interactions between aphids and caterpillars feeding on plants growing in the field or used in agriculture.

Acknowledgments & Dankwoord

De afgelopen 4 jaar waren erg leuk, verrijkend en leerzaam. Zo heb ik me met vol enthousiasme gestort op het wetenschappelijk onderzoek, was er telkens opnieuw de avontuurlijke sensatie van het opzetten en uitdenken van experimenten en het spannende proces van analyseren en schrijven wat uiteindelijk heeft geresulteerd in dit proefschrift. Zeker niet minder verrijkend waren de hulp en inspiratie van vele mensen tijdens mijn PhD project. Daarom wil ik mijn proefschrift niet eindigen zonder een aantal van hen te bedanken.

Als eerste natuurlijk mijn promotors Marcel Dicke en Joop van Loon die mij gedurende het project begeleid hebben. Beste Marcel en Joop, ik weet nog dat ik enorm stond te glunderen toen ik van jullie hoorde dat ik aangenomen was voor deze PhD-positie. Ontzettend bedankt voor de kansen die ik heb gekregen om mij te kunnen ontwikkelen binnen de groep en voor het helpen ontdekken van de insectenwereld. Marcel, ik heb erg veel bewondering voor je betrokkenheid en het belang dat je hecht aan je beschikbaarheid naast je drukke agenda. Ik heb in het bijzonder je snelle reacties, het in de gaten houden van mijn planning en je kritische blik op mijn hoofdstukken erg gewaardeerd. Joop, ik kon altijd bij jou terecht voor goed advies en ondersteuning bij mijn onderzoek. Jouw ervaring, relativeringsvermogen en vooral je vriendelijkheid hebben veel voor mij betekend.

I'm also grateful to my fellow TOP-girls, Yehua and Jeltje, with whom I was very lucky to work together within the project. Thank you for being good friends and brilliant collaborators. I appreciate the inspiring discussions, for helping me out with work and the sometimes much-needed pep talks. It makes me feel confident to have you girls by my side as paranymphs during my defense.

Ik heb mijn werk bij Entomologie niet kunnen doen zonder de steun en hulp van hele fijne collega's. Erik en Rieta, als begeleiders van Jeltje en Yehua waren jullie formeel ook bij het project betrokken. Maar daarnaast stonden jullie altijd open voor een informeel praatje of advies. Ik stel het enorm op prijs dat ik altijd bij jullie binnen kon lopen. I would like to thank the co-authors of my manuscripts, Anja David, Wilhelm Boland, Berhane Weldegergis, Colette Broekgaarden, Sean Mary and Marcos Castellanos Uribe, for their dedication to this project and critical comments. Patrick, bedankt voor je adviezen en nuttige presentaties over qPCRs, primers en andere moleculaire experimenten. Ana, as part of the Utrecht

posse, I could join you in many carpool rides between Utrecht and Wageningen. Eventually I bought my own car but I enjoyed all of our discussions and girl talks, in the car and at work. Dear Nurmi, I really enjoyed being your desk neighbor! Your kindness, patience and understanding makes you a wonderful colleague. Thank you very much for your moral support, help and insightful discussions about molecular work and life in general! Het insectenkweek team, Léon, Joop, Frans en André, jullie waren onmisbaar voor al mijn experimenten. Een heel speciaal dankjewel voor het kweken van de vele rupsen en bladluizen.

I fully enjoyed my time as a PhD at Ento because I could spend it with fantastic colleagues. I'm grateful to all of them for creating such a pleasant atmosphere at work, during coffee breaks, lunches and labuitjes. I would also like to thank the members of the party committee for the fun at meetings (when I was a member of the committee) and great activities. I even discovered a hidden talent: who could have guessed I can sow! Thank you Jenny, Marjolein and Jeltje, for the nice time we had during our Stitch&Bitch afternoons. And I want to thank Jeltje and Camille for being adventurous and hard-working co-organizers of our PhD trip committee. Thanks to everybody who joined for making it a success.

I was fortunate to have the help from amazing master and bachelor students during my project. A well-deserved thank you to all of them! Paulien, jij kwam mij als eerste vergezellen in het project toen alles ook voor mij nog nieuw was. Niet alles verliep daardoor vlekkeloos, maar met jouw geduld en positieve instelling heb je toch veel bereikt. Rinske, jouw project was kort maar krachtig. Bedankt voor je gezelligheid. Ward, bedankt voor je vrolijke enthousiasme en je hulp bij de gedragsonderzoeken. Francesco, thank you for all your hard work and effort with the behavioral assays. You gave me a taste of Italy by bringing delicious goodies, which I much appreciated! Sander, ondanks tegenslagen bleef je doorgaan, ik bewonder je doorzettingsvermogen. Jie, your project included both molecular and ecological aspects but you successfully mastered both. Karen, tijdens mijn schrijffase heb jij bijna geheel zelfstandig een experiment uitgevoerd, maar dat ging je hartstikke goed af. Bedankt daarvoor!
I hope that all of you enjoyed doing your thesis and I wish you all the best in your future career!

Als laatste wil ik mijn vrienden en familie bedanken, jullie steun tijdens en interesse in mijn onderzoek heb ik ontzettend gewaardeerd.
Lieve meiden, Marjolijn, Anique en Marjolein, jullie vriendschap is mij erg waardevol. Als geen ander hebben jullie de ups&downs van een PhD project ervaren. Als ik ergens mee zat waren jullie er voor een kopje thee, goed advies en luisterend oor. Dank jullie wel voor de gezellige etentjes en uitjes.
Mijn oud-huisgenootjes, Gerdienke, Mathilde en Esther, onze vriendschap gaat helemaal terug tot aan het Salamancapad, met Grey's anatomy en veel Ben&Jerry's. Waar we ook zijn, in Bielefeld, Aalten of Utrecht, ik hoop dat we elkaar altijd weer weten te vinden voor fijne avondjes uit. Lieve Eef, ik ben erg dankbaar voor je trouwe vriendschap, eerlijkheid en openheid. Jij hebt mij gesteund om vooral mijn passie te volgen, waaruit ik keer op keer nieuwe energie put om ertegen aan te gaan.

En mijn lieve biologen familie Suzanne&Lucas, mam&pap en Stefan. Pap, ook zonder jouw biologische achtergrond, reken ik je hier natuurlijk gewoon bij. Als ons eeuwige supporter weet ik hoe trots je op ons bent. Jullie staan mij altijd bij met raad en daad, en leven mee met belangrijke en minder belangrijke momenten in mijn leven. Ik ben blij dat ik altijd op jullie kan terugvallen als dat nodig is. Ik bof maar met zo een fijne, gezellige en warme familie. Bedankt voor alle fijne momenten samen!
Lieve Cees en Wilma, ik vind het altijd fijn om jullie te zien en te spreken. Jullie betrokkenheid blijkt wel uit het feit dat jullie zo maar voor mij jullie vakantieplannen hebben verschoven om bij mijn promotie aanwezig te kunnen zijn. Wat leuk, dank jullie wel! Denise, ik wil je ontzettend bedanken voor je prachtige ontwerp voor de cover van dit proefschrift.
Lieve Stefan, wat is het fijn om bij jou te zijn! Samen staan we sterk, zo voel ik dat echt. Samen op vakantie, wandelen, lekker ontspannen en hebben we de grootste lol. Jij bent de beste!

Liefs, Anneke

Curriculum Vitae

Anneke Kroes was born on March 11th, 1987 in Amstelveen, The Netherlands. She followed her secondary education at the Chr. scholengemeenschap Buitenveldert in Amsterdam. In September 2006 she started her BSc study in Biology at Utrecht University, followed by a MSc in Environmental Biology at Utrecht University. During her master she specialized in Plant Biology. As part of her studies she did a major research internship at the Plant Ecophysiology Group of Utrecht University. She investigated how shade avoidance and pathogen defence interact when both stresses are induced simultaneously in the model plant *Arabidopsis thaliana*. She carried out a minor research internship on the topic of root growth and development at the Molecular Genetics Group of Utrecht University. Furthermore, she participated in the MSc talent programme of The Graduate School for Experimental Plant Sciences (EPS) and The Netherlands Organisation for Scientific Research (NWO) where she got the opportunity to write and defend a scientific research proposal.

In January 2012 she started her PhD project with the Laboratory of Entomology at Wageningen University supervised by Prof. dr Marcel Dicke and Prof. dr Joop van Loon. Research on molecular defense mechanisms against dual insect attack in *A. thaliana* is described in this thesis. During the PhD project she participated in the training and supervision programme of The Graduate School EPS. She also organized together with two other PhD-candidates a study trip to Switzerland to discuss research experiences with PhD-students from different research groups. In April 2016 she started a new job as a researcher phytopathology at Bejo Zaden, The Netherlands.

Publications

Published

Stam JM*, Kroes A*, Li Y*, Gols R, Van Loon JJA, Poelman EH, Dicke M (2014) Plant interactions with multiple insect herbivores: from community to genes. Annual Review of Plant Biology 65: 689-713.

Kroes A, Van Loon JJA, Dicke M (2015) Density-dependent interference of aphids with caterpillar-induced defenses in *Arabidopsis*: involvement of phytohormones and transcription factors. Plant and Cell Physiology 56: 98-106.

Submitted

Kroes A, Weldegergis BT, Cappai F, Dicke M, Van Loon JJA (2016) Terpenoid biosynthesis in *Arabidopsis* attacked by caterpillars and aphids: aphid density affects the attraction of a caterpillar parasitoid. Submitted

Li Y, Dicke M, Kroes A, Liu W, Gols R (2016) Interactive effects of aphid and caterpillar herbivory on transcription of plant genes associated with phytohormonal signalling in wild cabbage. Submitted

Kroes A, David A, Boland W, Weerheim S, Van Veen K, Van Loon JJA, Dicke M (2016) Caterpillar feeding modulates defense against aphids attacking at different densities in *Arabidopsis* plants through jasmonate and etlene signaling. Submitted

Kroes A*, Stam JM*, David A, Boland W, Van Loon JJA, Dicke M, Poelman EH (2016) Plant-mediated interactions between two herbivores differentially affect a subsequently arriving third herbivore in populations of wild cabbage. Submitted

In preparation for submission

Kroes A, Broekgaarden C, Castellanos Uribe M, Mary S, Van Loon JJA, Dicke M (2016). Effect of different densities of feeding *Brevicoryne brassicae* aphids on whole-genome transcriptional responses of *Arabidopsis thaliana* to feeding by *Plutella xylostella* caterpillars. In prep.

*Shared first authorship

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: **Anneke Kroes**
Date: **17 June 2016**
Group: **Laboratory of Entomology**
University: **Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Effects of phloem-feeding insects on caterpillar-induced defenses	Sep 11, 2012
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Plant interactions with multiple insect herbivores: from community to genes	Aug 01, 2013
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, University of Amsterdam	Nov 30, 2012
EPS PhD Student Days 'Get2Gether' 2016, Soest, NL	Jan 28-29, 2016
► **EPS theme symposia**	
EPS theme 2 Symposium & Willie Commelin Scholtend day: Interactions between plants and biotic agents, Wageningen University	Feb 10, 2012
EPS theme 2 Symposium & Willie Commelin Scholtend day: Interactions between plants and biotic agents, Utrecht University	Jan 24, 2013
EPS theme 2 Symposium & Willie Commelin Scholtend day: Interactions between plants and biotic agents, University of Amsterdam	Feb 25, 2014
EPS theme 2 Symposium & Willie Commelin Scholtend day: Interactions between plants and biotic agents, Utrecht University	Feb 20, 2015

► **Lunteren days and other National Platforms**	
Netherlands Annual Ecology Meeting, Lunteren	Feb 07-08, 2012
Annual Meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2012
Annual Meeting of the Netherlands Entomological Society	Dec 12, 2012
Annual Meeting 'Experimental Plant Sciences', Lunteren	Apr 22-23, 2013
Annual Meeting of the Netherlands Entomological Society	Dec 13, 2013
Netherlands Annual Ecology Meeting, Lunteren	Feb 11-12, 2014
Annual Meeting 'Experimental Plant Sciences', Lunteren	Apr 14-15, 2014
Annual Meeting of the Netherlands Entomological Society	Dec 19, 2014
Annual Meeting 'Experimental Plant Sciences', Lunteren	Apr 13-14, 2015
► **Seminars (series), workshops and symposia**	
Ento seminar Jacintha Ellers, Evolution of temperature responses: From single species to community dynamics	Jan 31, 2012
Ento seminar Ayco Tack, The importance of pathogens in structuring arthropod communities across multiple spatial scales	Feb 29, 2012
Ento seminar Erik Poelman, Fitness consequences for plants that respond to early-season herbivores	Mar 27, 2012
WEES seminar Michael Strand	Apr 26, 2012
Yearly Entomology Research Exchange Meeting	May 30, 2012
Ento seminar Madeleine Beekman, Parasitism, cloning and other strange things in the Cape honey bee	Sep 04, 2012
Seminar Nancy Schellhorn, Colonization of pests and natural enemies at multiple spatial scales: Does the landscape context matter?	Oct 03, 2012
EPS flying seminar Ruth Finkelstein, ABA signaling networks in Arabidopsis	Nov 14, 2012
WEES seminar Ron Ydenberg, Are top predators important in Dutch ecology?	Nov 22, 2012
Yearly Entomology Research Exchange Meeting	May 17, 2013
WYA public lecture Frans de Waal, The bonobo and the atheist	Jun 26, 2013
EPS symposium, From model system to ecology and evolution	Aug 29, 2013
8th workshop Plant-Insect Interactions	Sep 24, 2013
Mini-symposium:How to write a world class paper	Oct 17, 2013
Yearly Entomology Research Exchange Meeting	May 21, 2014

	Ento seminar: an editor's perspective of the reviewing process	May 27, 2014
	WEES seminar Kevin Foster, The evolution of cooperation and competition in microbes	Jan 22, 2015
	WEES seminar Hanna Kokko, Males exist. Does it matter?	Mar 19, 2015
	WGS PhD workshop carousel	Apr 17, 2015
	EPS flying seminar Alain Goossens, How jasmonates provide the key to harness plant chemistry	Dec 08, 2015
	Wageningen University Career Day	Feb 02, 2016
►	**Seminar plus**	
	WEES seminar and masterclass on Evolution of chemical diversity in plants - Nicole van Dam	Dec 20, 2012
►	**International symposia and congresses**	
	SIP 15, Neuchatel, Switzerland	Aug 17-22, 2014
	PR IR 2015, Aachen, Germany	Sep 6-10, 2015
►	**Presentations**	
	Environmental signaling summer school Utrecht (Poster)	Aug 27, 2013
	NERN, Netherlands Annual Ecology Meeting, Lunteren (Talk)	Feb 12, 2014
	ALW meeting 'Experimental Plant Sciences', Lunteren (Poster)	Apr 14, 2014
	SIP15, Neuchâtel, Switzerland (Poster)	Aug 21, 2014
	EPS theme 2 Symposium & Willie Commelin Scholtend day (Talk)	Feb 20, 2015
	ALW meeting 'Experimental Plant Sciences', Lunteren (Poster)	Apr 13, 2015
	PR IR 2015, Aachen, Germany (Poster)	Sep 08, 2015
	Seminar Laboratory for Molecular & Chemical Ecology, IBED, UVA (Talk)	Sep 21, 2015
►	**IAB interview**	
►	**Excursions**	
	Entomology PhD excursion	Oct 28-Nov 01, 2013
	Subtotal Scientific Exposure	*23.1 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	PhD course, Design of Experiments	Oct 10-12, 2012
	PhD course, Generalized linear models	Jun 13-14, 2013

	Utrecht PhD Summerschool on Environmental Signaling	Aug 26-28, 2013
►	**Journal club**	
	PhD journal club at Entomology (twice a month)	2012-2015
	Insect-Plant Interactions discussion group at Entomology (twice a month)	2012-2015
►	**Individual research training**	
	Subtotal In-Depth Studies	*6.4 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Information Literacy	Oct 30-31, 2012
	Techniques for writing and presenting a scientific paper	Dec 03-05, 2013
	Reviewing a scientific paper	Jun 10, 2014
	Advanced course Guide to Scientific Artwork	March 23-24, 2015
	Adobe InDesign Essential Training	Sep 29-30, 2015
►	**Organisation of PhD students day, course or conference**	
	PhD students excursion to Switzerland	Oct 28-Nov 01, 2013
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*4.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**41,6**

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 ECTS credits

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

Chapter 4

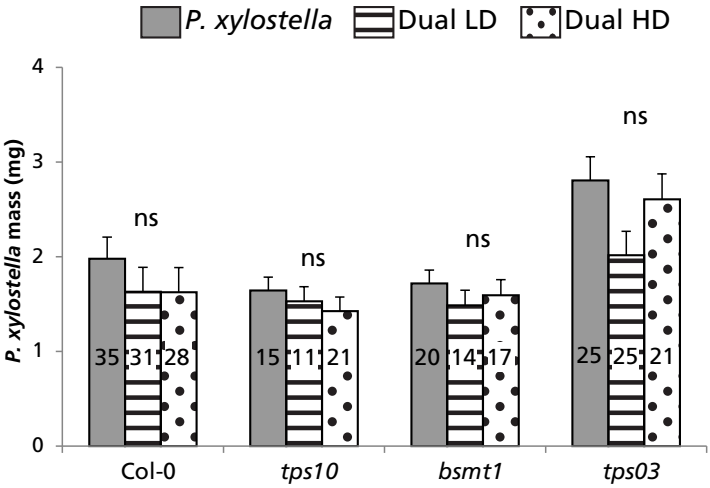

Figure S1. Body mass of *P. xylostella* caterpillars after feeding during three days on *A. thaliana* wild-type Col-0 plants and mutants *tps10*, *bsmt1* and *tps03* used during the Y-tube olfactometer bioassays after single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation (Dual). Plants were infested with either a low (LD, 5 aphids) or high density (HD, 25 aphids) of aphids and two second-instar caterpillars. For each treatment a set of four plants was used. Numbers inside each bar represent the total number of caterpillars weighed. Bars represent means ± SE (Linear Mixed Model; ns, not significant).

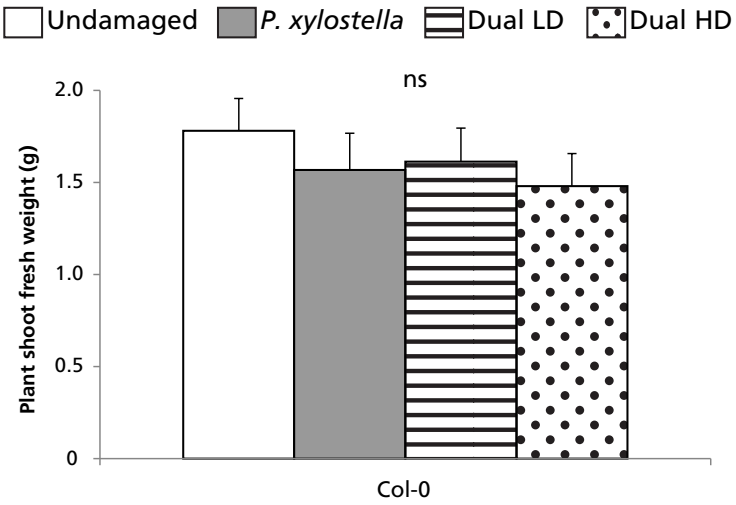

Figure S2. Plant shoot fresh weight of *A. thaliana* Col-0 wild-type plants of all treatments measured after each headspace collection of plant volatiles. Bars represent means ± SE (ANOVA, n = 28 plants, $P < 0.05$); ns, not significant.

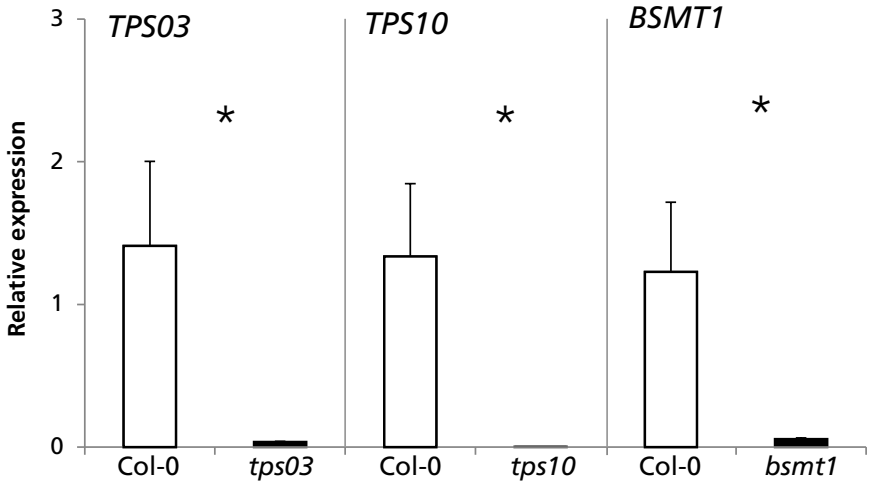

Figure S3. Verification of *TPS03*, *TPS10* and *BSMT1* relative expression in leaves of *A. thaliana* wild-type Col-0 and mutants *tps03*, *tps10* and *bsmt1* after single *P. xylostella* infestation. Bars represent means ± SE (n = 4 biological replications). Asterisk, $P < 0.02$ (GLM).

Table S1. Volatile organic compounds emitted by *Arabidopsis* wild-type Col-0 after single *P. xylostella* infestation, dual *P. xylostella* and *B. brassicae* infestation and without infestation (Undamaged). Dual infestation was done with either a low (LD) or high (HD) *B. brassicae* density. Plants were sampled at 72 h after insect infestation.

		Treatment (n = 7)			
		Undamaged	*P. xylostella*	Dual LD	Dual HD
ID	Compound	Peak area/g plant fresh weight ± SE			
	Alcohols				
1	1-Penten-3-ol	568 ± 191[a]	1873 ± 334[b]	1567 ± 186[b]	2092 ± 345[b]
14	Methyl-1-octanol	93 ± 25	251 ± 79	242 ± 105	248 ± 126
35	12-Methyl-(*E*,*E*)-2,13-octadecadien-1-ol	122 ± 27	142 ± 35	133 ± 31	148 ± 36
36	2-Methyl-(*Z*,*Z*)-3,13-octadecadien-1-ol	155 ± 35	168 ± 27	163 ± 40	183 ± 46
39	2-Methyl-(*E*,*E*)-3,13-octadecadien-1-ol	86 ± 17	102 ± 25	98 ± 22	105 ± 23
	Terpenoids				
3	β-Citronellene	78 ± 11	100 ± 27	92 ± 29	121 ± 39
4	Camphene	12 ± 1	14 ± 2	17 ± 2	16 ± 3
5	Cyclohexene, 4-ethenyl-1,4-dimethyl-	61 ± 12	79 ± 17	81 ± 17	87 ± 21
6	β-Myrcene	131 ± 14	211 ± 45	171 ± 21	165 ± 28
7	Limonene	591 ± 84	722 ± 135	742 ± 123	755 ± 171
8	1,8-Cineole	10 ± 5	8 ± 1	20 ± 12	10 ± 2
9	Sylvestrene	44 ± 17	69 ± 42	23 ± 5	25 ± 4
10	(*E*)-β-Ocimene	63 ± 11	89 ± 21	72 ± 12	73 ± 15
11	ϒ-Terpinene	245 ± 40	245 ± 49	301 ± 58	287 ± 66
12	Terpinolene	44 ± 7	74 ± 19	61 ± 17	80 ± 34
13	Linalool	59 ± 14	153 ± 48	91 ± 27	92 ± 27
15	Menthone	54 ± 38	33 ± 11	97 ± 74	35 ± 12
16	Isomenthone	13 ± 9 (n=5)	8 ± 2	21 ± 15(n=6)	10 ± 3
17	Menthol	293 ± 177	264 ± 95	527 ± 395	257 ± 128
18	α-Terpineol	60 ± 15	97 ± 25	59 ± 12	63 ± 11

Table S1. (Continued)

22	Limonene, 1,2,8,9-diepoxy-	136 ± 34	196 ± 56	203 ± 41	199 ± 51
24	Isodauca-6,9-diene	9 ± 1	11 ± 1	11 ± 1	10 ± 1
25	Longifolene	21 ± 2	25 ± 3	24 ± 3	25 ± 3
26	α-Cedrene	28 ± 2	33 ± 4	30 ± 3	35 ± 5
27	(*E*)-β-Caryophyllene	9 ± 2 (n=5)	7 ± 1 (n=6)	9 ± 1 (n=5)	8 ± 1
28	(*E*,*E*)-α-Farnesene	34 ± 4^{a}	124 ± 33^{b}	94 ± 16^{b}	104 ± 19^{b}
29	(*E*,*E*)-TMTT*	95 ± 31^{a}	791 ± 302^{b}	640 ± 165^{b}	513 ± 158^{b}
	Esters				
19	Methyl salicylate	15 ± 2^{a}	153 ± 73^{b}	81 ± 20^{ab}	67 ± 21^{ab}
20	Linalyl acetate	151 ± 53	202 ± 62	134 ± 48	155 ± 64
21	α-Terpinyl acetate	44 ± 8	58 ± 11	53 ± 13	46 ± 10
23	Neryl acetate	7 ± 1 (n=6)	10 ± 3	8 ± 2	9 ± 3 (n=6)
30	Methyl jasmonate, cis	239 ± 47	193 ± 49	221 ± 66	218 ± 37
41	Nerolidol isobutyrate	187 ± 40	222 ± 57	209 ± 47	235 ± 53
	Others				
2	Allyl isothiocyanate	267 ± 143 (n=5)	347 ± 186 (n=4)	24 ± 11 (n=5)	124 ± 46
34	Farnesyl acetaldehyde	55 ± 12	44 ± 12	68 ± 19	47 ± 13
	Unknown				
31	unknown compound	289 ± 76	358 ± 109	356 ± 96	391 ± 107
32	unknown compound	231 ± 60	288 ± 81	285 ± 74	310 ± 84
33	unknown compound	351 ± 93	433 ± 120	433 ± 117	478 ± 131
37	unknown compound	82 ± 17	98 ± 28	98 ± 22	103 ± 26
38	unknown compound	122 ± 27	137 ± 37	128 ± 30	140 ± 34
40	unknown compound	171 ± 36	198 ± 50	194 ± 42	211 ± 50

Volatile emissions are given as peak area (mean ± SE) per gram plant shoot fresh weight divided by 10^5 with the number of replicates between brackets. If emission of a volatile compound was not found in all the samples of a treatment, volatile emission values are followed by the number of samples in which the compound was detected. Results for the number of samples in which the volatile compound was found were used for calculating the mean volatile emission. Mean values indicated with different letters are significantly different between means (GLM, $P < 0.05$).
* (*E*,*E*)-TMTT, (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

Table S2. Most influential volatile compounds based on their Variable Importance in the Projection (VIP > 1) value between treatments of undamaged plants versus plants infested by *P. xylostella* alone and plants infested by both *P. xylostella* and a low *B. brassicae* density (Dual LD, 5 aphids) versus plants infested by both *P. xylostella* and a high *B. brassicae* density (Dual HD, 25 aphids).

Most influential volatile compounds				
		VIP > 1		
ID	Compound	All treatments	Undamaged vs *P. xylostella*	Dual LD vs Dual HD
	Alcohols			
1	1-Penten-3-ol	2.67	2.31	2.65
14	6-Methyl-1-octanol		1.41	
	Terpenoids			
6	β-Myrcene	1.25	1.22	
9	Sylvestrene			1.42
10	(*E*)-β-Ocimene		1.02	1.51
12	Terpinolene		1.17	1.12
13	Linalool	1.32	1.67	1.88
16	Isomenthone			1.13
18	α-Terpineol			1.77
22	Limonene, 1,2,8,9-diepoxy-	1.09		
24	Isodauca-6,9-diene		1.27	
25	Longifolene	1.54	1.47	1.75
26	α-Cedrene			1.51
28	(*E*,*E*)-α-Farnesene	2.48	2.15	1.99
29	(*E*,*E*)-TMTT*	2.60	2.33	
	Esters			
19	Methyl salicylate	2.33	2.15	
23	Neryl acetate		1.18	1.02
	Others			
2	Allyl isothiocyanate			1.28
34	Farnesyl acetaldehyde			1.42

* (*E*,*E*)-TMTT, (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

Chapter 5

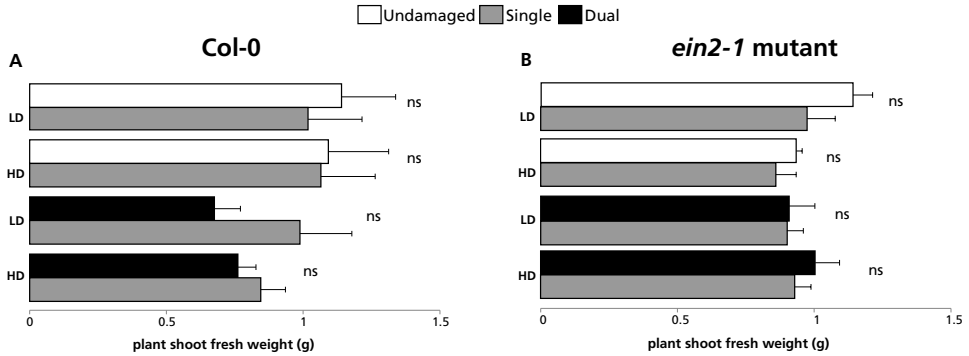

Figure S1. Plant shoot fresh weight of *Arabidopsis* Col-0 wild-type (A) or *ein2-1* mutants (B) of all treatments measured after each pair-wise testing in the olfactometer as indicated in Figure 1 panels A and B. Plants were infested with either a low (LD, 5 aphids) or high density (HD, 25 aphids) of aphids. Bars represent means ± SE (n = 16 – 20 plants). For each treatment combination an independent samples t-test at α = 0.05 was conducted to determine significant differences; ns, not significant.

Table S1. Body mass of *P. xylostella* (mean ± SE) after single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation on *Arabidopsis* wild-type Col-0 and mutants *sid2-1*, *dde2-2*, *myc2*, *ein2-1* and *ora59* at 5 days (or 4 days for *myc2* mutant plants). Plants were infested with either a low (LD) or high (HD) density of *B. brassicae* aphids. Linear Mixed Model table for effect of treatment. Bold numbers indicate significant effects ($P < 0.05$).

								Factor Treatment	
Plant		*P. xylostella* mass (mg)						d.f. = 2	
		Single		Dual LD		Dual HD		F	*P*
Col-0	Wild-type	5.59	± 0.52	5.75	± 0.52	4.33	± 0.48	2.49	0.093
sid2-1	SA induction deficient	4.82	± 0.55	5.80	± 0.52	5.19	± 0.52	0.87	0.423
dde2-2	JA biosynthesis deficient	5.16	± 0.66	4.94	± 0.81	3.92	± 0.68	0.92	0.414
myc2	defective in MYC2	2.90	± 0.36	3.52	± 0.33	3.02	± 0.36	0.91	0.413
ein2-1	defective in ET pathway	5.30[a]	± 0.60	5.53[a]	± 0.60	3.22[b]	± 0.74	3.38	**0.045**
ora59	defective in ET/JA pathway	4.71	± 0.42	3.84	± 0.45	4.30	± 0.57	1.00	0.377

Chapter 6

Because of the large volume of data, supplementary tables for Chapter 6 can be found in electronic form through the following link: https://www.dropbox.com/sh/0agdw9szy1z9voo/AABXBl4Je4lGzj3Mm8RgR_9ja?dl=0

Table S1. Functional clustering analysis (using DAVID Functional Annotation Clustering with enrichment score ≥ 1.3) for pair-wise comparisons of differentially expressed genes in *A. thaliana* at 24 and 48 h after single *P. xylostella* (PT), dual *P. xylostella* and *B. brassicae* at low (LD) or high (HD) aphid density and without infestation (Control; CT). Only GO clusters for biological processes are shown (*: $P < 0.05$ for modified Fisher's exact test, **: $P < 0.05$ with Benjamini-Hochberg adjustment for multiple comparisons)

Table S2. Annotation for differentially expressed genes within each GO cluster for biological processes (using DAVID Functional Annotation Clustering). Only clusters are shown with an enrichment score ≥ 1.3 for pair-wise comparisons as shown in Table S1 for up-regulated (A) and down-regulated (B) genes at 24 and 48 h.

Table S3. Annotation for genes up- or down-regulated based on $\log_2$-fold change ratios (measured relative to non-infested control samples) at 24 (A), 48 (B) h and of interaction significant genes within each cluster in response to single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation at 24 and 48 h (C). Plants were infested with either a low (LD) or high (HD) density of *B. brassicae* aphids.

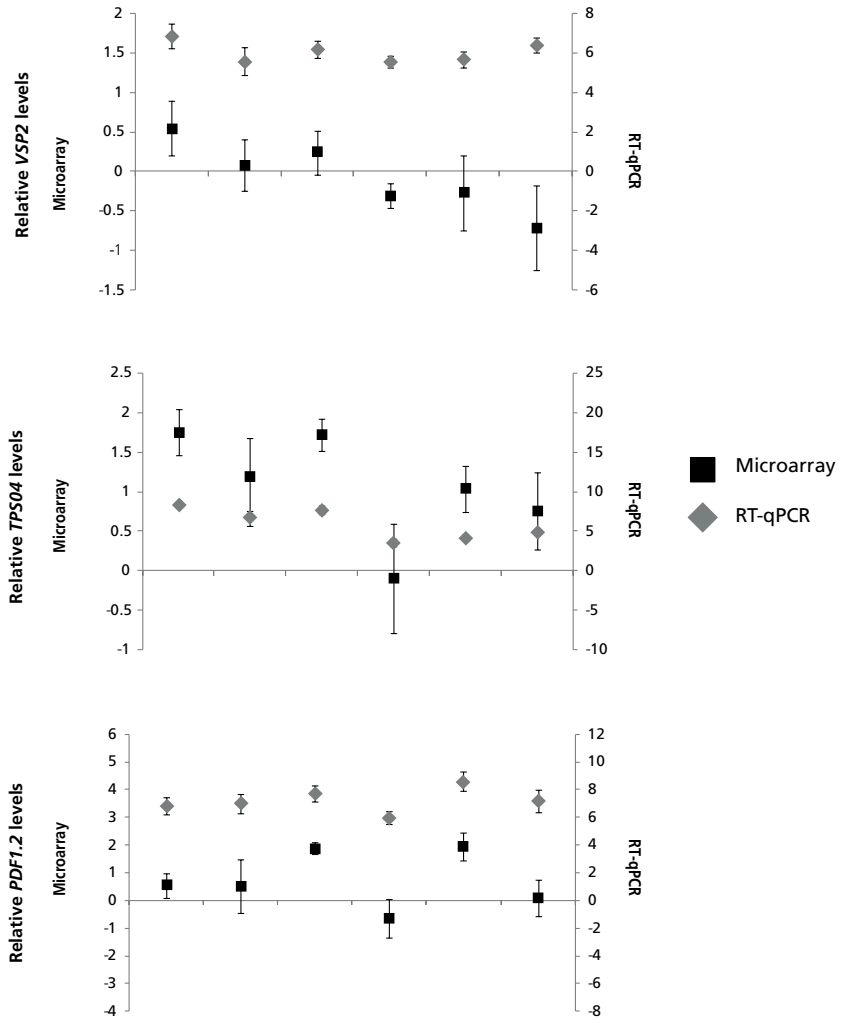

Figure S1. Validation of microarray expression data by quantitative real-time PCR for *VSP2*, *TPS04* and *PDF1.2* gene expression. Gene expression was measured in leaves of *A. thaliana* sampled for the microarray analysis after single *P. xylostella* and dual *P. xylostella* and *B. brassicae* infestation (Dual). The black squares show the microarray data and the grey diamonds show the RT-qPCR data. Plants were infested with either a low (LD, 5 aphids per plant) or high (HD, 25 aphids per plant) density of *B. brassicae* aphids. Gene expression was measured relative to non-infested control samples. Symbols represent means ± SE (n = 4 biological replicates).

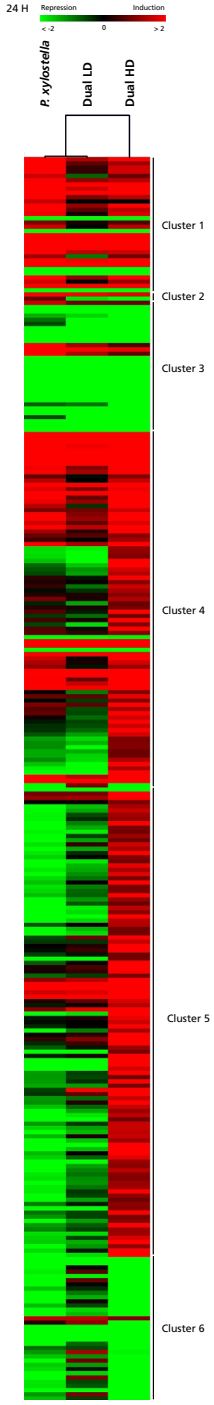

Figure S2. Cluster analysis and heat map showing average $\log_2$-fold change ratios (measured relative to non-infested control samples) of genes expressed in *A. thaliana* at 24 h after single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (LD) or high (HD) density of *B. brassicae* aphids. Hierarchical clustering (HCL) was performed with Spearman correlation using average link method. Red indicates up-regulated genes, while green shows down-regulated genes. Black represents no change in expression. Each row in the columns corresponds to a single gene, and genes belonging to different clusters are indicated by letters.

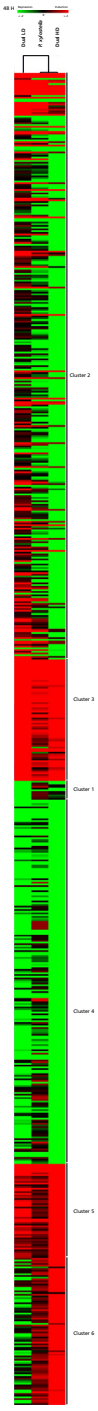

Figure S3. Cluster analysis and heat map showing average $\log_2$-fold change ratios (measured relative to non-infested control samples) of genes expressed in *A. thaliana* at 48 h after single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (LD) or high (HD) density of *B. brassicae* aphids. Hierarchical clustering (HCL) was performed with Spearman correlation using average link method. Red indicates up-regulated genes, while green shows down-regulated genes. Black represents no change in expression. Each row in the columns corresponds to a single gene, and genes belonging to different clusters are indicated by letters.

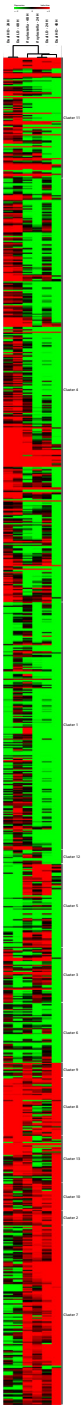

Figure S4. Cluster analysis and heat map showing average $\log_2$-fold change ratios (measured relative to non-infested control samples) of genes expressed in *A. thaliana* during 24 and 48 h of single *P. xylostella* or dual *P. xylostella* and *B. brassicae* infestation. Plants were infested with either a low (Dual LD) or high (Dual HD) density of *B. brassicae* aphids. Shown are expression ratios for interaction significant genes (two-way ANOVA, $P < 0.05$). Hierarchical clustering (HCL) was performed with Spearman correlation using average link method. Red indicates up-regulated genes, while green shows down-regulated genes. Black represents no change in expression. Each row in the columns corresponds to a single gene, and genes belonging to different clusters are indicated by letters.

The research described in this thesis was financially supported by
The Netherlands Organization for Scientific Research (NWO/ TOP GO grant 854.10.010 to M.D.)

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Printed by GVO drukkers & vormgevers B.V.