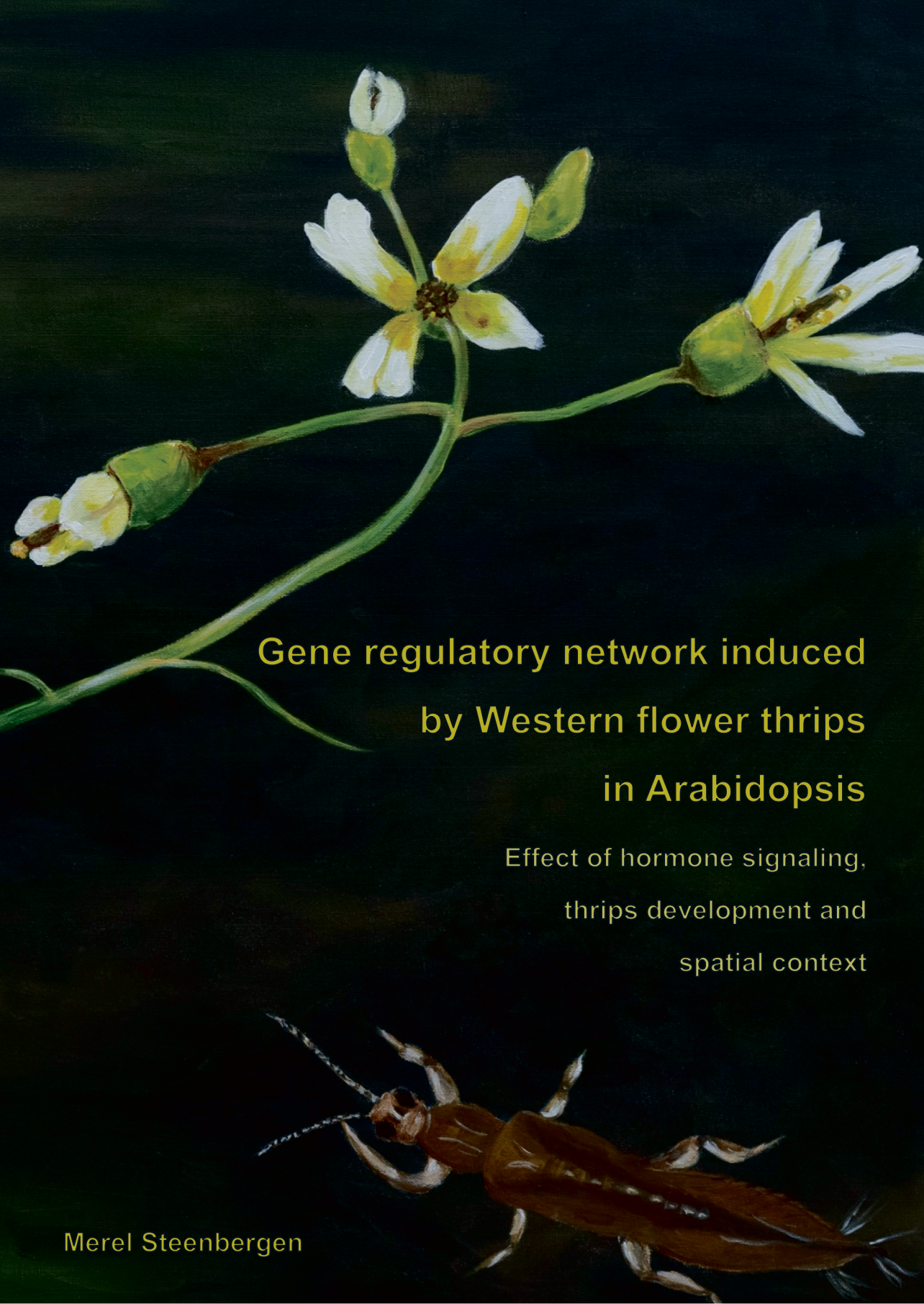

Gene regulatory network induced by Western flower thrips in Arabidopsis

Effect of hormone signaling, thrips development and spatial context

Merel Steenbergen

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Gene regulatory network induced by Western flower thrips in Arabidopsis - Effect of hormone signaling, thrips development and spatial context

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Gene regulatory network induced by Western flower thrips in Arabidopsis

Effect of hormone signaling, thrips development and spatial context

Gen-regulerend netwerk geïnduceerd door de Californische trips in Arabidopsis
Effect van hormoonsignalering, trips ontwikkeling en ruimtelijke context
(met een samenvatting in het Nederlands)

Proefschrift

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door

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geboren op 1 september 1989
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CONTENTS

CHAPTER 1

General introduction: Thrips advisor - Exploiting thrips-induced defenses to combat pests on crops

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Thrips advisor: exploiting thrips-induced defences to combat pests on crops.

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THE PLANT IMMUNE RESPONSE TO HERBIVOROUS INSECTS

Plants and insect herbivores have been interacting with each other for over 400 million years (Labandeira, 2007). Of the estimated 6 million insect species, half are herbivores (Schoonhoven *et al.,* 2005). Additionally, plants have to deal with the different developmental stages within an insect's life cycle, from eggs to larvae, to adults. To maximize reproductive success, plants have evolved a plethora of defense mechanisms that can affect herbivores either directly or indirectly. Direct defenses rely on plant traits that interfere with host-plant selection or herbivore performance (War *et al.,* 2012), while indirect defenses rely on the herbivore's natural enemies (Dicke, 2015). Both direct and indirect defenses can be displayed constitutively, i.e. regardless of herbivore presence, but are more often initiated upon herbivore attack due to the trade-off between plant growth and defence (Züst & Agrawal, 2017).

Herbivore-inducible plant defenses are initiated after perception of the herbivore via the recognition of damage-associated molecular patterns (DAMPs; Duran-Flores & Heil, 2016), herbivory-associated molecular patterns (HAMPs; Mithöfer & Boland, 2008) or egg-associated cues (Hilker and Fatouros, 2016) (Fig. 1). Plants perceive wounding and herbivory through the binding of these cues to pattern recognition receptors. Through yet unknown mechanisms, cellular signaling cascades are activated including the elevation of cytosolic calcium, depolarization of the plasma membrane potential, activation of mitogen-activated protein kinases, and production of reactive oxygen species (Zebelo & Maffei, 2015; Gilroy *et al.,* 2016). These cascades lead to the rapid accumulation of phytohormones such as jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) (Howe & Jander, 2008; Broekgaarden *et al.,* 2015; Nguyen *et al.,* 2016). The phytohormone JA is considered a key player in the defense regulatory network that is effective against herbivores, whereas SA, ET and ABA act as modulators of plant defense by cross-communicating with each other to fine-tune defense responses such that they are effective to the attacker at hand (Reviewed by Nguyen *et al.,* 2016; Erb & Reymond, 2019). The accumulation of JA is not only restricted to the local site of wounding/herbivory but is also extended to distal, yet undamaged parts of the plant via internal and/or external signals, which is advantageous to the plant as it primes those parts to future herbivore attack (Stork *et al.,* 2009; Vos *et al.,* 2013b; Bozorov *et al.,* 2017). Classical grafting experiments on tomato (*Solanum lycopersicum*) and wild tobacco (*Nicotiana attenuata*) revealed that JA is an essential player in long-distance signaling (Li *et al.,* 2002; Bozorov *et al.,* 2017) and the expression of herbivore-induced resistance (Sun *et al.,* 2011).

Some insect herbivore species are able to circumvent the plant's anti-herbivore defenses through the action of specialized defense-suppressing effector molecules present in the insect's saliva (Hogenhout & Bos, 2011; Kant et al., 2015; Giron et al., 2016), or elicitors derived by insect eggs (egg-associated compounds (EACs)).

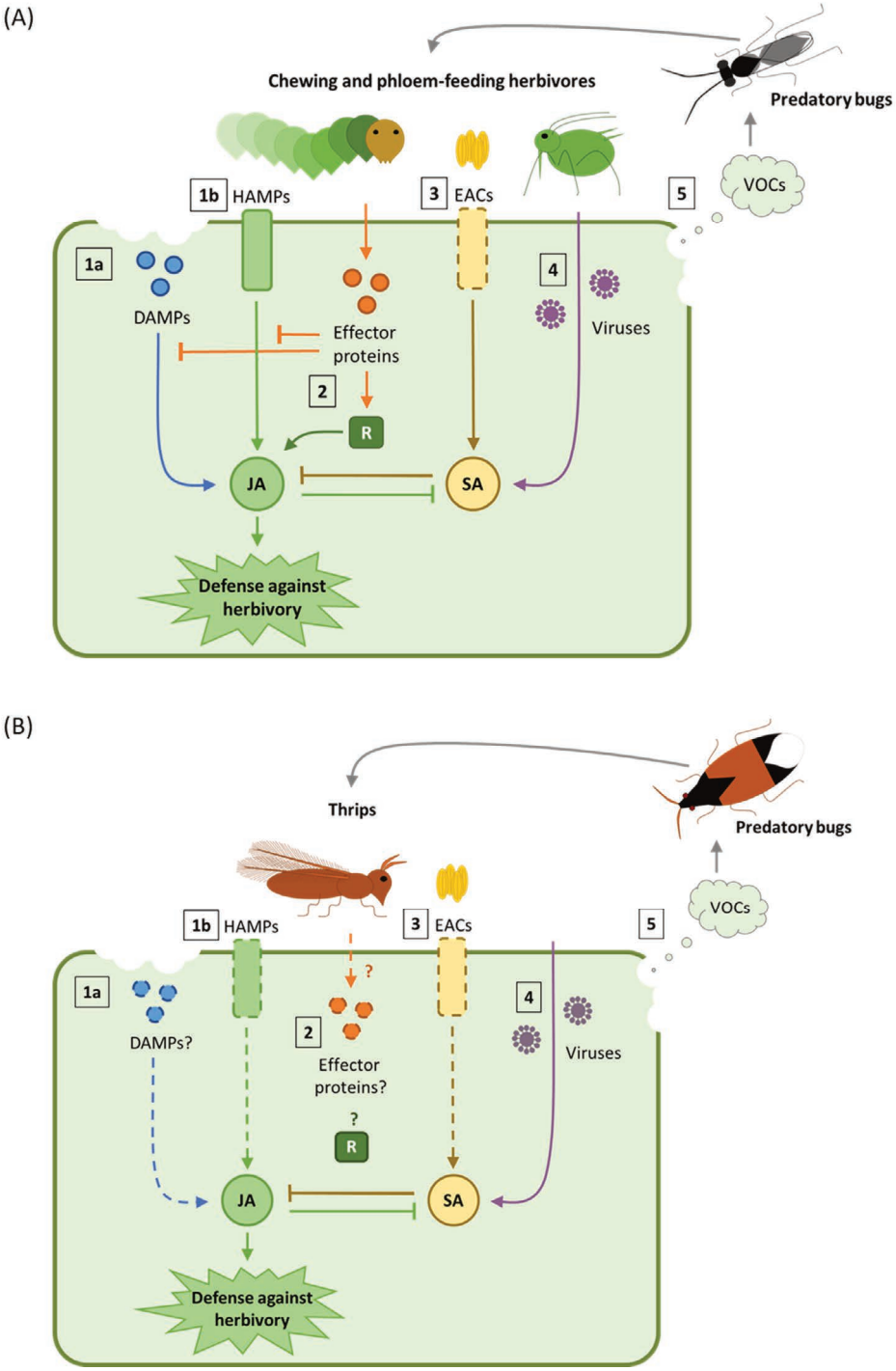

(A)
Chewing and phloem-feeding herbivores
Predatory bugs
1b HAMPs
3 EACs
5
VOCs
1a
DAMPs
Effector proteins
4
Viruses
2
R
JA
SA
Defense against herbivory
(B)
Thrips
Predatory bugs
VOCs
1b HAMPs
3 EACs
5
1a
DAMPs?
?
2
Effector proteins?
4
Viruses
?
R
JA
SA
Defense against herbivory

Figure 1. Molecular components in induced plant responses during feeding by chewing and phloem-feeding herbivores (A) and thrips (B). **(A)** During feeding by chewing and phloem-feeding herbivores, plant defense responses are initiated upon the recognition of damage-associated molecular patterns (DAMPs) (1a) and herbivore-associated molecular patterns (HAMPs) (1b), which activate the biosynthesis of specialized metabolites, such as the phytohormone jasmonic acid (JA), which in turn regulate effective defenses against herbivore attack. Herbivore-derived effector proteins can alter the host's defense response in favor of the insect by suppressing HAMP- or DAMP-induced signaling (2). Plant resistance proteins (R) that recognize pest-derived effector proteins may in turn mount an effective defense response (2). Recognition of insect egg-associated compounds (EACs) triggers salicylic acid (SA) signaling, which through negative crosstalk with the JA response reduces effective plant defenses against the herbivore's offspring (3). Viruses transmitted by insects can interfere with induced anti-herbivory defense responses, possibly also mediated by crosstalk effects of virus-induced SA (4). In response to herbivore feeding, plants emit a specific blend of volatile organic compounds (VOCs) to attract natural enemies of the attacker at hand (5). **(B)** Thrips feeding activates the biosynthesis of JA, resulting in induced plant defenses against thrips. In thrips–plant interactions neither DAMPs (1a) nor HAMPs (1b) have been identified yet. Similarly, thrips effector proteins, their plant targets, or plant R proteins have not been isolated yet (2). In addition, it is unknown whether thrips eggs contain EACs that can be recognized by the host plant or trigger SA signaling (3). Tospoviruses are exclusively transmitted by thrips and can interfere with anti-herbivory plant defenses, possibly through crosstalk of virus-induced SA with thrips-induced JA signaling (4). In response to thrips feeding, plants emit specific VOCs that can attract predatory bugs, such as *Orius laevigatus*, that feed on thrips (5). Uncharacterized and hypothesized receptors and induced response pathways are indicated with dashed lines.

The eggs of some insect species are able to activate the accumulation of SA underneath the egg, thereby increasing the performance of the future progeny (Little *et al.,* 2007; Bruessow *et al.,* 2010). Once the insect larvae emerge from the egg and starts to infest the plant, various morphological and biochemical defenses are induced (War *et al.,* 2018) which can be specific for a single developmental stage of an insect herbivore. In garlic mustard (*Alliaria petiolata*) for example, two compounds were found to involve resistance against two distinct feeding stages of the native American butterfly (*Pieris oleracea napi*). Interestingly, both compounds had no effect on the other stages which demonstrated that the plant had developed specific defense mechanisms against the different developmental stages within the life cycle of the native American butterfly (Renwick *et al.,* 2001). Also differences in the composition of volatile blends, a major form of herbivore-induced indirect defense, was found to be dependent on the developmental stage of the herbivore species. Corn (*Zea mays* L.) plants infested with young *Pseudaletia separata* caterpillars emitted a different volatile blend than corn plants infested with older caterpillars. The volatile blend induced by the young larvae attracted the parasitoid *Cotesia kariyai* while plants infested the older caterpillars did not (Takabayashi *et al.,* 1995). A differential induced plant response upon infestation by a specific developmental stage could be the result of differential gene expression profiles that are characteristic for the physiology of that stage. Transcriptomic analyses on the developmental stages within the life cycle of different herbivore species (Yang *et al.,* 2013a; Vogel *et al.,* 2014; Tian *et al.,* 2015; Chen *et*

al., 2016) revealed unique sets of genes that are exclusively induced during the interaction with a specific developmental stage. For example, a high percentage of effector-encoding genes are active during the first larval stage of the Hessian fly (*Mayetiola destructor*) while older larval stages were more characterized by the activity of genes involved the synthesis of digestive proteases and protective protease-inhibitors (Chen *et al.,* 2016).

As discussed above, there is ample knowledge on the molecular processes that are activated upon infestation with leaf-chewing or phloem-feeding herbivores (Bonaventure, 2012; Hilker & Fatouros, 2016; Züst & Agrawal, 2017; Erb & Reymond, 2019) (Fig. 1). For example, several putative HAMPs (Douglas 2018), egg-associated elicitors (Reymond, 2013), effector proteins and resistance genes have been identified (Hogenhout & Bos, 2011; Douglas, 2018). Additionally, numerous studies demonstrate the specificity of stage-specific gene expression signatures, which may underlie differential plant defense mechanisms against the different developmental stages within an insect herbivore's life cycle (Takabayashi *et al.,* 1995; Renwick *et al.,* 2001). However, knowledge on these molecular mechanisms underlying the interaction between plants and cell-content feeding insects such as thrips is largely lacking. These tiny (1.5-mm or less) insects belong to the order of the Thysanoptera that comprises over 5500 described species. Their fringed wings, i.e., long hairs (cilia) that form a fringe around the wings (Fig. 2A), allow them to remain airborne despite not being strong flyers (Mound, 2005). Several thrips species are pests of commercial crops (Cannon *et al.,* 2007; Diaz-Montano *et al.,* 2011; Mouden *et al.,* 2017) due to causing direct damage to plants through feeding or because they vector economically important plant viruses (Riley *et al.,* 2011). Thrips brings together all the problems of pests in general: they are highly polyphagous, hard to control because of their complex lifestyle and rapid adaptation to pesticides, and they are vectors of destructive viruses.

In this introduction we summarize the current knowledge of thrips-induced plant responses and pinpoint the knowledge gaps that have to be filled for the development of novel thrips control strategies in agroecosystems. First, we will describe the general aspects of the interaction between thrips and plants. Then, we provide an overview of the current fundamental knowledge of thrips-induced plant responses involving phytohormone signaling and specialized metabolites that play a role in direct and indirect defenses followed by suggestions for future research opportunities in plant-thrips interactions to improve sustainable crop production against this pest.

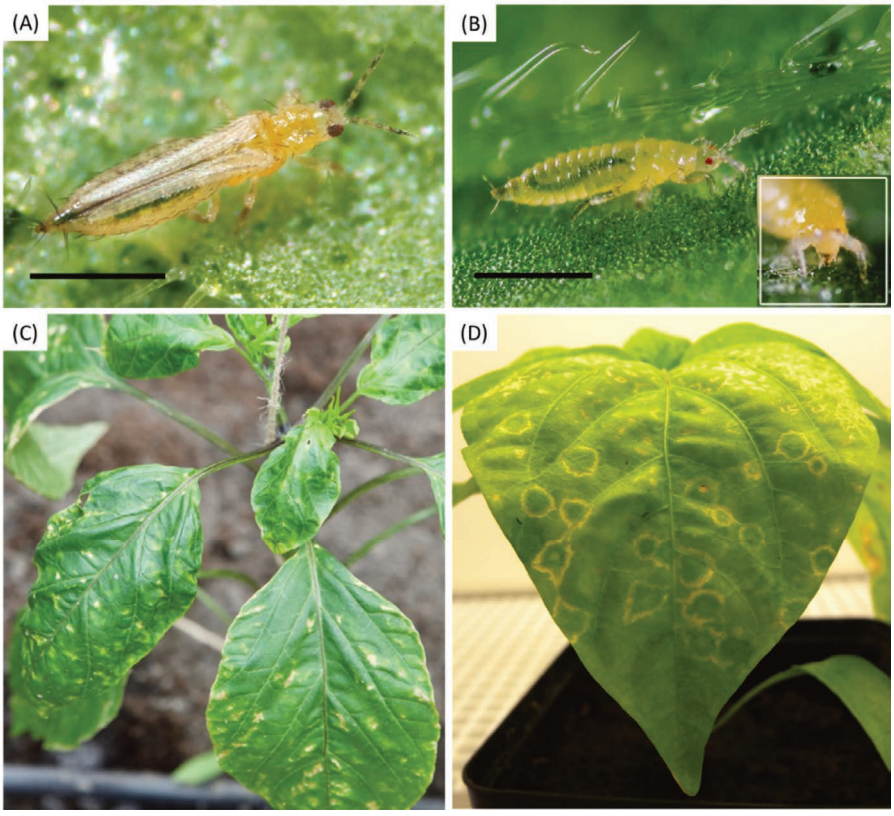


Figure 2. Thrips development and damage symptoms on pepper (Capsicum) plants. (A) Western flower thrips (*F. occidentalis*) adult and **(B)** larva of the same species. Scale bars=0.5 mm. **(C)** Thrips feeding scars, termed 'silver damage'. **(D)** Symptoms of tomato spotted wilt virus (TSWV) infection transmitted by thrips. A, B, and D kindly provided by Jan van Arkel (University of Amsterdam).

THE INTERACTION BETWEEN HERBIVOROUS THRIPS AND PLANTS

Herbivorous thrips feed on leaves, petals, fruits and/or pollen of green plants thereby affecting photosynthesis capacity, plant growth and reproduction (Mound, 2005; Morse & Hoddle, 2006). Female thrips (Fig. 2A) lay their cylindrical or bean-shaped eggs on/into plant tissues that are suitable for their larvae to feed from. Thrips have a haplodiploid sex-determination system, meaning that females and males emerge from fertilized (diploid) and non-fertilized (haploid) eggs respectively. Larvae (Fig. 2B) that hatch from the eggs develop through two stages (L1 and L2) in which they actively feed, and two non-feeding, soil-dwelling stages (prepupa and pupa) in which they prepare for the adult stage. Both adults and larvae insert their stylets into plant tissue and ingest cell content while feeding (Kindt *et al.,* 2003). Probing plant tissue comprises three distinct behavioral phases: (1) piercing, (2) salivation and (3) ingestion of the

cell content (Kindt *et al.,* 2006). The pierced cells collapse or fill up with air, giving the damaged area a silvery appearance (Fig. 2C). The thrips developmental and reproductive rate is highly dependent on environmental conditions, including temperature and the nutritional quality of the host plant. For example, the life cycle from egg to adult of the Western flower thrips (*Frankliniella occidentalis*) can be completed within 45 days at 15°C or within 15 days at 30°C (Cloyd, 2009).

Host plant selection and feeding

Before colonizing a plant, an adult thrips determines host suitability via a sequence of pre-alighting and post-alighting behaviors. Pre-alighting host-recognition behavior, which involves approaching a potential host and landing on it, is mediated by plant volatile organic compounds (VOCs) (Teulon *et al.,* 2002; Koschier *et al.,* 2007; El-Sayed *et al.,* 2009) and/or visual cues such as color (Childers & Brecht, 1996; Teulon *et al.,* 2002), shape (Mainali & Lim, 2011) or size (Papadaki *et al.,* 2008). For example, *F. occidentalis* is attracted to VOCs emitted by cabbage (*Brassica oleracea*), while VOCs from garlic (*Allium sativum*) are repellent to this thrips species (Cao *et al.,* 2014). Additionally, *F. occidentalis* is more attracted to circular shapes than other geometrical forms like rectangular and triangular shapes (Mainali & Lim, 2010). In deciduous forests, color has been demonstrated to be a more dominant factor than odors for the orientation of the introduced basswood thrips (*Thrips calcaratus*), the pear thrips (*Taeniothrips inconsequens*) and the native basswood thrips (*Neohydatothrips tiliae*) (Rieske & Raffa 1995). Flowers can play an important role in pre-alighting host recognition behavior of some thrips species for the reason that flowers emit a mixture of VOCs and display distinct visual cues (Mainali & Lim, 2008; Lim & Mainali, 2009; Boachon *et al.,* 2015).

Final acceptance of a plant is guided by post-alighting behavior to test the plant's suitability for feeding and/or oviposition. The importance of post-alighting testing in thrips host selection has been shown for *F. occidentalis* interacting with the chrysanthemum *Chrysanthemum morifolium* in which thrips were more attracted to linalool-emitting flowers but gradually reversed their preference after landing, probably due to test feeding by thrips (Yang *et al.,* 2013b). Post-alighting host acceptance, but also subsequent feeding and reproduction, is strongly influenced by the plant's nutritional quality and defenses (Leiss *et al.,* 2009b; Baez *et al.,* 2011; Chow *et al.,* 2012; Leiss *et al.,* 2013; Pobozniak & Koschier, 2014; Wang *et al.,* 2014). For example, transgenic potato (*Solanum tuberosum*) plants overexpressing cysteine proteinase inhibitors (PIs), which are known as defensive proteins, deter *F. occidentalis* (Outchkourov *et al.,* 2004). Additionally, leaf age may also correlate with nutritional quality and defenses, which may cause thrips to prefer older leaves and avoid the better-protected young parts of the plant (Joost & Riley, 2008; Scott-Brown *et al.,* 2016; Hunziker *et al.,* 2021). Hybrids of the ragwort species *Senecio jacobaea* and *Senecio aquaticus* showed a negative correlation

between *F. occidentalis* damage and pyrrolizidine alkaloid concentration in the younger leaves (Leiss *et al.,* 2009a). Moreover, UV-B radiation can influence the quality of plant tissue for herbivores either through changes in leaf chemistry and/or reinforcement of the plant cell wall (Lindroth *et al.,* 2000; Stratmann *et al.,* 2000; Rousseaux *et al.,* 2004; Demkura *et al.,* 2010; Kuhlmann & Müller, 2010). For example, solar UV-B radiation strongly reduces damage by the thrips *Caliothrips phaseoli* on soybean (*Glycine max*) leaves (Mazza *et al.,* 1999).

Besides nutritional quality and defense of leaf tissue, pollen can have a significant effect on post-alighting host acceptance as well as on the overall performance of thrips, even allowing the pest to overcome plant resistance (Trichilo & Leigh, 1988). Adding slash pine (*Pinus elliottii*) pollen to leaves of tomato (*Solanum lycopersicum*) or peanut (*Arachis hypogaea*), for example, significantly increased the settling behavior (landing and residence time) of *F. occidentalis* (Riley, 2007).

Virus transmission

In addition to the direct damage caused by feeding, thrips also serve as vectors for plant diseases such as tospoviruses (Riley *et al.,* 2011). Members of the *Tospovirus* genus, among which the Tomato spotted wilt virus (TSWV) (Fig. 2D), can infect thousands of plant species (Whitfield *et al.,* 2005). At present, there are approximately 15 reported thrips species that can serve as vectors for the transmission of 26 different tospovirus species worldwide (Rotenberg *et al.,* 2015). Tospoviruses are generally acquired by immature (L1 and early L2) thrips, when mid gut and salivary glands are still connected, during feeding on a virus-infected plant (Moritz *et al.,* 2004). The efficiency of virus acquisition is highest for L1 larvae and decreases as they develop towards the mature stage (Rotenberg *et al.,* 2015). Once acquired by L1 or early L2 larvae, the virus replicates inside the host vector after a latent period and can then be transmitted into healthy plants by late L2 larvae and adult thrips (Inoue *et al.,* 2002; De Assis Filho *et al.,* 2004). The transmission of tospoviruses can occur as fast as in a single non-ingesting probe in which the virus enters the plant cell along with the secreted thrips saliva (Kindt *et al.,* 2003). During these probes, the plant cell remains largely undamaged which promotes virus transmission (Stafford *et al.,* 2011). However, transmission efficiency is dependent on several factors such as virus isolate, plant variety, thrips species and geographical origin (Van de Watering *et al.,* 1999; Maris *et al.,* 2003; Nagata *et al.,* 2004). Viruses can enhance the transmission efficiency by influencing host plant selection, probing behavior and reproduction of thrips (Maris *et al.,* 2004; Shalileh *et al.,* 2016; Wan *et al.,* 2020). For example, non-viruliferous *F. occidentalis* prefer to feed and oviposit on virus-infected *Capsicum annuum* leaflets over uninfected ones (Ogada *et al.,* 2013) This preference may be mediated by the fact that TSWV-infected plants are a higher quality food source for thrips than healthy plants (Belliure *et al.,* 2005; Blanc & Michalakis, 2016) due to increased availability of

free nutrients (Shrestha *et al.,* 2012; Shalileh *et al.,* 2016) and/or viral-disrupted plant defenses (Abe *et al.,* 2012). Additionally, TSWV-infected *F. occidentalis* males showed a significantly higher number of pre-ingestion/salivation probes on Jimsonweed (*Datura stramonium*) compared to non-viruliferous thrips which may enhance the spread of the virus. Viruliferous females did not significantly change the number of probes, but dispersed more between feeding sites compared to non-viruliferous females, which may also enhance the spread of the virus (Stafford *et al.,* 2011). However, Wan *et al.* (2019) demonstrated that viruliferous females produced substantially more progeny, with significantly more males, suggesting that the shift towards this sex with a greater dispersal and virus transmission capability benefits the virus transmission. Behavioral changes may be related to the fact that TSWV-infected plants are a higher quality food source for thrips than healthy plants (Belliure *et al.,* 2005; Blanc & Michalakis, 2016) due to increased availability of free nutrients (Shrestha *et al.,* 2012; Shalileh *et al.,* 2016) and/or viral-disrupted plant defenses (Abe *et al.,* 2012). Taken from this, a better understanding of the plant virus-insect vector interaction is essential to develop strategies to interfere and control not only the vector thrips, but also virus uptake and transmission.

Economic impact

Many thrips species are pests of commercial food crops and ornamentals worldwide due to the damage caused by feeding and transmission of viral diseases, which reduce yield and market value (Cannon *et al.,* 2007; Diaz-Montano *et al.,* 2011; Stuart *et al.,* 2011). Thrips are such successful pests because of their life-history characteristics (high reproductive capacity, parthenogenetic reproduction, multi-voltinism), hidden life style (pupation in the soil, thigmotactic behavior) and polyphagous nature (broad host plant range) (Morse & Hoddle, 2006; Stuart *et al.,* 2011). *F. occidentalis* is considered economically one of the most important pests worldwide as it feeds on plants of at least 60 families and is the primary vector of plant diseases caused by tospoviruses (Morse & Hoddle, 2006; Mouden *et al.,* 2017). Females can produce 40-100 eggs during their life and *F. occidentalis* generally goes through 2-7 generation cycles per year on field-grown crops, which can increase to 15 generations under greenhouse conditions (Cloyd 2009). Other economically important thrips species include the onion thrips (*Thrips tabaci*), the melon thrips (*Thrips palmi*), the common blossom thrips (*Frankliniella schultzei*) and the avocado thrips (*Scirtothrips perseae*) (Hoddle *et al.,* 2003; Cannon *et al.,* 2007; Diaz-Montano *et al.,* 2011; Seal *et al.,* 2014). Although it is hard to precisely determine the total economic loss caused by thrips, annual crop losses due to fruit scarring and transmission of viral disease are enormous (Hoddle *et al.,* 2003; MacLeod *et al.,* 2004; Riley *et al.,* 2011). Currently, control management of thrips relies mainly on using chemical pesticides. However, due to resistance of thrips to these insecticides, there is an urgent need to intensify the search for plant resistance (Gao *et al.,* 2012).

THRIPS-INDUCED PLANT DEFENSES

Herbivore-induced plant responses are generally organized via a complex network of interacting signaling pathways, orchestrated by several phytohormones, to activate attacker-specific defenses (Erb *et al.,* 2012). Many studies have shown that JA is the most important hormone in the regulation of plant defense against herbivores (Wasternack, 2015) and thrips seems to be no exception to this rule (De Vos *et al.,* 2005; Abe *et al.,* 2008; Escobar-Bravo *et al.,* 2017). The activation of the JA signaling network subsequently leads to the production of various compounds that can serve as direct and/or indirect defense (Howe & Jander, 2008; Okada *et al.,* 2015).

Jasmonic acid signaling

Thrips feeding activities have been shown to activate the biosynthesis of JA (Fig. 1) (Abe *et al.,* 2008, 2009) and the expression of JA-responsive genes (De Vos *et al.,* 2005; Abe *et al.,* 2008, 2009; Selig *et al.,* 2016; Escobar-Bravo *et al.,* 2017). From the total set of genes that are differentially expressed in the thale cress *Arabidopsis thaliana* (hereafter Arabidopsis) during *F. occidentalis* feeding, 69% of the genes were JA-responsive (De Vos *et al.,* 2005). Also in Chinese cabbage (*Brassica rapa subs. pekinensis*) and tomato (*S. lycopersicum)*, JA concentrations increased upon *F. occidentalis i*nfestation, which corresponded with an increased expression of JA-responsive marker genes (Li *et al.,* 2002; Abe *et al.,* 2009; Sarde *et al.,* 2019). The activation of the JA pathway most likely reinforces the plant's resistance to thrips as exogenous application of JA reduces plant susceptibility towards this herbivore (Thaler, 1999; Abe *et al.,* 2009; El-Wakeil *et al.,* 2010), while plants insensitive to JA or deficient in JA accumulation are more susceptible to thrips (Abe *et al.,* 2009). For example, the *S. lycopersicum* mutant *def-1*, which is impaired in JA biosynthesis, was more susceptible to *F. occidentalis* compared to the wild type. However, such differences may not be solely due to differences in induced defenses since the *def-1* mutant also has a lower trichome density than the wild-type (Escobar-Bravo *et al.,* 2017). The availability of mineral nutrients in the soil can also impact plant's degree of JA-depending resistance. Arabidopsis plants grown under potassium-deficient conditions are less susceptible to thrips attack. This effect is most likely mediated by enhanced JA-associated responses, as some responses to potassium deficiency were dependent on COI1, an essential regulator of JA signaling (Armengaud *et al.,* 2010).

The interaction of JA with other phytohormones plays an important role in fine-tuning the magnitude and nature of the final downstream defense response (Pieterse *et al.,* 2012; Schuman & Baldwin, 2016). Research on *A. thaliana* has revealed that the JA pathway consists of two separate branches, which are referred to as the ERF-branch and the MYC-branch (Broekgaarden *et al.,* 2015). ET synergizes the ERF-branch to activate amongst others the

defense-related gene *PDF1.2* while ABA synergizes the MYC-branch to activate amongst others the defense-related gene *VSP2* (Verhage *et al.,* 2011; Vos *et al.,* 2013b). Expression levels of both *PDF1.2* and *VSP2* have been shown to increase in *A. thaliana* upon infestation with *F. occidentalis* (De Vos *et al.,* 2005; Abe *et al.,* 2008; Leon-Reyes *et al.,* 2009), suggesting that both branches are thrips-inducible. However, exogenous applications of ET, which also activates the expression of *PDF1.2*, enhances the susceptibility of *A. thaliana* towards *F. occidentalis* (Abe *et al.,* 2008), suggesting that thrips could potentially interfere with the induced plant defenses. Manipulation of JA-inducible plant defense has been shown for plant interactions with several herbivorous arthropods (Walling, 2008; Alba *et al.,* 2015; Kant *et al.,* 2015) and is most likely due to the presence of effector proteins in the arthropod saliva (Stafford-Banks *et al.,* 2014; Giron *et al.,* 2016; Jonckheere *et al.,* 2016; Villarroel *et al.,* 2016). However, whether thrips secrete effectors in their saliva to modulate JA-mediated defenses and establish a compatible interaction with the host plant is yet unknown (Fig. 1).

The finding that *F. occidentalis* is more attracted to, and performs better on, *C. annuum* plants infected with TSWV compared to non-infected plants (Maris *et al.,* 2004; Ogada *et al.,* 2013; Shalileh *et al.,* 2016) suggests that viruses can also interfere with plant defenses, possibly through interaction of SA with JA signaling (Fig. 1). In *A. thaliana*, TSWV infection led to increased SA concentrations, resulting in reduced *F. occidentalis*-induced JA-mediated defenses (Abe *et al.,* 2012).

Specialized metabolites

Many of the specialized metabolites known to be involved in defense against thrips are constitutively present in the plant (Leiss *et al.,* 2009b; Mellway & Constabel, 2009; Mirnezhad *et al.,* 2010; Barbehenn & Constabel 2011; Nuringtyas *et al.,* 2012; Yang *et al.,* 2012; Wang *et al.,* 2014; Liu *et al.,* 2017), but some have been shown to accumulate in response to thrips infestation. Phenolic compounds, a very common and widespread group of specialized defensive metabolites in plants that confer defense against herbivores, have been found to accumulate in response to thrips infestation (Papadaki *et al.,* 2008; Leiss *et al.,* 2009b; War *et al.,* 2012; Leiss *et al.,* 2013). For example, *F. occidentalis* feeding resulted in enhanced production of phenols (tocopherols) in resistant pepper (*C. annuum*) accessions, but not in susceptible accessions (Maharijaya *et al.,* 2012). A particular class of polyphenolic compounds that act as feeding deterrent to thrips, but also to many other herbivores, are tannins (Whittaker & Kirk, 2004; War *et al.,* 2012). A study on alfalfa *Medicago sativa* lines with different levels of resistance revealed that the increase in tannin content as a response to infestation with the thrips *Odontothrips loti* was faster in resistant than in susceptible lines (Wang *et al.,* 2014).

Some thrips-inducible plant chemicals correlate positively with susceptibility. For example, susceptible pepper (Capsicum spp.) accessions mainly induce the production of

alkanes and fatty acids in response to *F. occidentalis*, whereas resistant accessions did not (Maharijaya *et al.,* 2012). Additionally, California poppy (*Eschscholzia californica*) and greater celandine (*Chelidonium majus*) respond to *F. occidentalis* feeding by enhancing the production of cytotoxic benzophenanthridine alkaloids, but thrips appear to be able to detoxify these compounds (Schütz *et al.,* 2014). These data suggest that thrips may not only withstand plant defenses by manipulating the signaling networks involved (as described above), but also by resisting them directly through metabolic detoxification and/or excretion.

Volatile organic compounds

In response to thrips infestation, plants can change their blend of VOCs to attract natural enemies that predate or parasitize herbivores (Fig. 1), thereby reinforcing the plant's indirect defenses (Janssen *et al.,* 1998; Delphia *et al.,* 2007; Maharijaya *et al.,* 2012). For example, VOCs emitted from eggplant (*Solanum melongena*) infested by *T. palmi* attracted the predatory bug *Orius sauteri* (Mochizuki & Yano, 2007). Chrysanthemum (*C. morifolium*) changes its VOC blend upon infestation by *F. occidentalis* resulting in enhanced attraction of the predatory mite *Neoseiulus* (formerly Amblyseius) *cucumeris* (Manjunatha *et al.,* 1998). In tomato (*S. lycopersicum*), the thrips-induced changes in VOC production/composition are most likely dependent on JA, as wild-type plants significantly increased the overall production of terpenes in response to *F. occidentalis* feeding while the JA-deficient mutant *def-1* did not (Escobar-Bravo *et al.,* 2017). Although not shown for thrips specifically, increased production of terpenes has been associated with the enhanced attraction of predators of herbivorous pests (Dicke *et al.,* 1990; Francis *et al.,* 2004; Köllner *et al.,* 2008). Exogenous application of methyl jasmonate (MeJA) restored the repellence in *def-1* tomato (Escobar-Bravo *et al.,* 2017). Volatiles emitted from cucumber (*Cucumis sativa*) infested by *F. occidentalis* attracted the predatory mite *N. cucumeris* and the generalist predatory bug *Orius laevigatus* (Venzon *et al.,* 1999), the latter being one of the most effective natural enemies of thrips in agricultural production systems (Weintraub *et al.,* 2011; Ardanuy *et al.,* 2016).

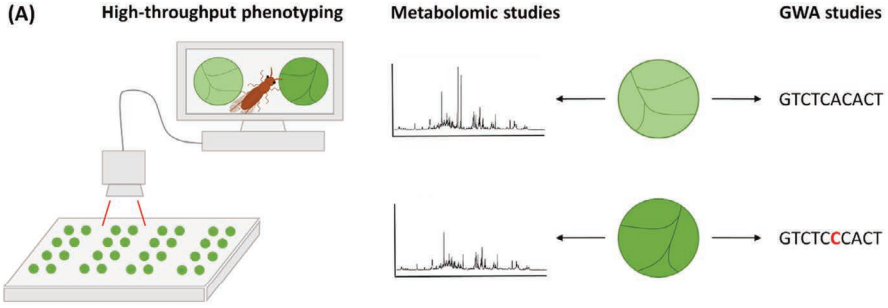
EXPLOITING THRIPS-INDUCED DEFENSES FOR PEST MANAGEMENT

Pest thrips generally have broad host ranges and are responsible for transmission of viruses in many food crops and ornamental plants. Due to the high genomic variation in virus populations, together with high mutation- and recombination rates, monogenic virus resistance can relatively easily be overcome (Drake & Holland, 1999; Harrison, 2002). For example, the widespread introduction of *Capsicum* cultivars carrying the dominant *Tsw*-resistance gene generated a selection pressure that resulted in the rapid emergence of resistance-breaking TSWV isolates (Ferrand *et al.,* 2015; Jiang *et al.,* 2017). Therefore, next to the introduction of

virus-resistance genes, simultaneous breeding for vector resistance is an attractive strategy towards durable virus control in agriculture. Enhancing resistance to pests can decrease transmission of pest-vectored viruses (Maris *et al.,* 2003; Chen *et al.,* 2012; Escobar-Bravo *et al.,* 2016), thereby lowering the selection pressure on the virus and increasing the durability of virus-resistance genes, which could have a large impact on virus outbreaks in monoculture agro-ecosystems. However, there are cases that illustrate that layered resistance is not necessarily straightforward. For example, JA-induced resistance in wild tomato (*S. lycopersicum*) reduced *F. occidentalis* feeding by 75% but this did not lead to a reduction of transmission of TSWV (Thaler *et al.,* 2002). Additionally, effectiveness of (induced) resistance against thrips itself can be highly dependent on environmental conditions (Mazza *et al.,* 1999; Armengaud *et al.,* 2010; Demkura *et al.,* 2010; Kuhlmann & Müller, 2010; Escobar-Bravo *et al.,* 2017). These examples underline the importance of understanding the resistance mechanisms and the virus-thrips-plant interactions in order to aid the development of breeding strategies to control both the pest and the viruses they vector. Below, we describe several opportunities, including future research directions, for meeting the challenge of exploiting knowledge of thrips-induced plant defenses towards more sustainable resistance breeding.

Large scale screening to identify new resistance traits

Since current breeding initiatives are primarily based on identifying resistance sources through large-scale screenings of plant populations, developing reproducible high-throughput screening methods for thrips resistance is of great interest (Kloth *et al.,* 2012; Abd-El-Haliem *et al.,* 2018). At present, host-plant resistance to thrips is mainly determined by monitoring inflicted damage and/or thrips behavior. Thrips damage is usually scored by eye and parameters monitored during behavioral assays, such as survival and reproduction, are usually recorded manually (Abe *et al.,* 2008, 2009; Maharijaya *et al.,* 2011; Leiss *et al.,* 2013), making these assays labor intensive and time-consuming. Since current breeding initiatives are primarily based on identifying resistance sources through large-scale screenings of plant populations, developing more accurate and efficient screening methods is of great interest. Recently, a high-throughput phenotyping platform has been developed that quantifies thrips behavior as a proxy of resistance (Fig. 3A). This method allows screening for host-plant resistance in a multiple, simultaneous two-choice setup with computerized continuous video-tracking by establishing detailed thrips behavioral parameters throughout a period of several hours. With a markedly improved time and resource efficiency, this tool provided comparable resistance scores to manually monitoring inflicted damage on whole plants (Thoen *et al.,* 2016).


(A)
High-throughput phenotyping
Metabolomic studies
GWA studies
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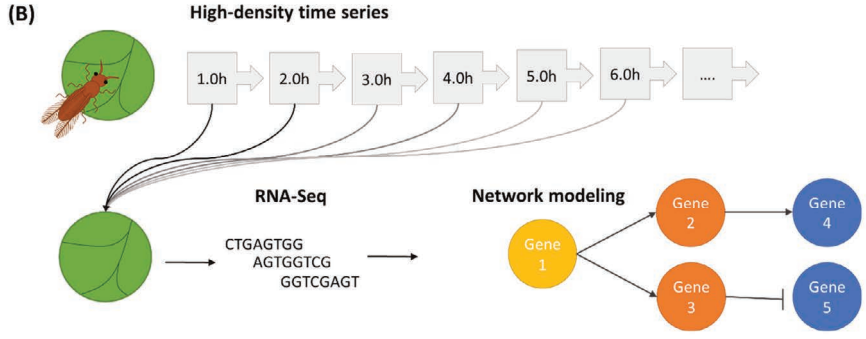
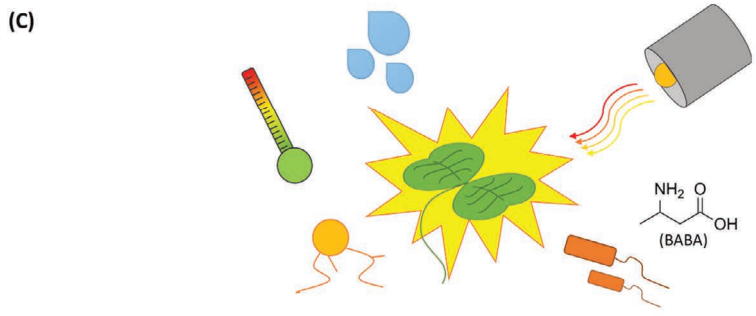

(B)
High-density time series
1.0h
2.0h
3.0h
4.0h
5.0h
6.0h
....
RNA-Seq
CTGAGTGG
AGTGGTCG
GGTCGAGT
Network modeling
Gene 1
Gene 2
Gene 3
Gene 4
Gene 5


(C)
NH2
O
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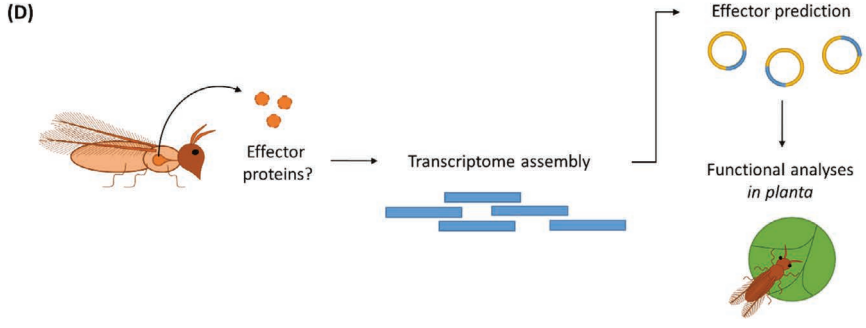
(D)
Effector proteins?
Transcriptome assembly
Effector prediction
Functional analyses
in planta

Figure 3. Strategies to obtain knowledge to enhance plant defense for pest management. (A) Large-scale screenings of plant populations can aid the efficiency of resistance-trait identification. Once different phenotypes have been identified, metabolomic studies along with genome-wide association (GWA) studies can aid the discovery of plant metabolites and genes involved in resistance to thrips. **(B)** High-throughput transcriptome sequencing (by RNA-Seq) from high-density time series will provide insight into the dynamic gene-regulatory network that is induced upon thrips infestation. Such an approach allows the prediction and validation of novel key regulators and their targets for induced defense against thrips. **(C)** Culture parameters such as water availability, light intensity/quality, and temperature can have a great impact on plant defense induction and require optimization for plant growth and defense. Biostimulants, such as chemical substances or microorganisms, are able to enhance crop quality, for example, by improving nutritional status and priming the plant's defense machinery. **(D)** Characterization of proteinaceous effectors in thrips saliva will pinpoint important components of plant resistance to thrips. A bioinformatics pipeline can predict putative effectors from the thrips transcriptome, followed by functional analyses *in planta*.

Breeding/engineering for induced defense

Rather than implementing constitutive resistance, breeding (or engineering) crop plants that activate defense only under attack has a cost benefit as resources are reallocated to defense in times of stress only (Pappas *et al.,* 2017). Similarly, inducible volatile (VOC) emission to attract natural enemies upon herbivore damage is preferred to avoid 'false' signals, i.e. natural enemies may become less responsive to constitutively emitted VOCs in the absence of herbivores (Shiojiri *et al.,* 2010). Identification of the plant's key regulators of induced defenses as well as downstream players involved in the gene regulatory network offers ample opportunities for customizing induced defenses against thrips. A robust strategy to reveal thrips-induced plant defenses is to conduct extensive comparative transcriptomics analyses. RNA sequencing (RNA-Seq) is a powerful tool for capturing the spatio-temporal fluctuations of induced plant defenses (Gusberti *et al.,* 2013; Van Verk *et al.,* 2013; Windram *et al.,* 2014; Tzin *et al.,* 2015; Hickman *et al.,* 2017). Conducting computational analysis of RNA-Seq data generated from high-density time series during thrips infestation will elucidate the different phases in time of induced plant defenses in compatible and incompatible interactions (Fig. 3B). Such an approach has been successful in predicting and validating novel key regulators and their direct and indirect targets in plant stress signaling networks underlying resistance or susceptibility to other pests and plant pathogens (Windram *et al.,* 2012; Lewis *et al.,* 2015; Coolen *et al.,* 2016).

Besides insight in timing of defense signaling, such an approach allows quantitative mapping of transcripts directly to metabolic pathways in order to elucidate the expected metabolic changes during plant-thrips interaction. Next, metabolomics can aid the discovery of plant metabolites related to thrips induced resistance (Fig. 3A). In contrast to targeted chemical analyses untargeted metabolomics has revealed some novel compounds involved in constitutive resistance against thrips (Leiss *et al.,* 2009a). Linking metabolomics with

transcriptomics or genome wide association (GWA) studies can lead to the unravelling of candidate genes involved in the production of potentially important defense metabolites (Fig. 3A) (Chen *et al.,* 2014). Functional analysis by over-expression or knock-down of individual candidate genes or complete pathways, allows validation of important signaling networks, genes and defensive compounds. The identification of key players in plant defense and increased knowledge of their function in (crop) plants, will contribute to breeding of thrips-resistant crop varieties as they allow for a targeted search for genetic variation, allelic variants and metabolic diversity in (wild) crop germplasm.

Optimizing culturing conditions

Plants are commonly exposed to changing environmental conditions especially when grown in open-field conditions. In turn, this has a great (temporal) impact on the plant's physiology and chemistry. Plant defense responses to herbivores can be influenced either negatively or positively by environmental (stress) parameters, such as temperature, light intensity/quality and water availability (Fig. 3C) (Gouinguené & Turlings, 2002; Grinnan *et al.,* 2013; Ballaré, 2014; Suzuki *et al.,* 2014; Coolen *et al.,* 2016; Nguyen *et al.,* 2016; Escobar-Bravo *et al.,* 2017). Depending on the external factor, growth-defense trade-offs occur by prioritizing resources in either growth or defense (Vos *et al.,* 2013a). Determining which abiotic conditions influence plant-thrips interactions might help to optimize culture conditions for the reduction of crop damage due to herbivory, while maintaining substantial growth.

The use of UV-B light has been reported to increase constitutive and inducible plant defenses against a wide range of pests (Izaguirre *et al.,* 2007; Mewis *et al.,* 2012; DInh *et al.,* 2013; Zavala *et al.,* 2015), including thrips (Mazza *et al.,* 1999; Demkura *et al.,* 2010; Kuhlmann & Müller, 2010). Reinforcement of UV-B-mediated JA-inducible defenses might be used as a tool to increase crop protection against thrips. However, the (modest) effects of UV-B on plant morphology, growth and yield should be taken into account (Ballaré *et al.,* 2011). Constitutive or primed defenses could stop herbivory at an earlier stage, which minimizes the activation of costly defenses and eventually reduces the energy investment of replacing consumed plant tissue (Frost *et al.,* 2008; Karban, 2011).

Implementing biostimulants

The industry for biostimulants to improve plant productivity is rapidly growing (Yakhin *et al.,* 2017). Treating plants with a biostimulant, which can be a (chemical) substance or a microorganism, enhances crop quality traits such as improved nutritional status, but can also prime a plant to deploy its defense machinery in a faster, stronger and/or more sustained manner while under herbivore attack (Fig. 3C) (Du Jardin, 2015; Martinez-Medina *et al.*, 2016; Muvea *et al.*, 2018; N Pangesti *et al.*, 2013; Pineda *et al.*, 2020). For example, applying the non-protein amino acid DL-β-aminobutyric

acid (BABA) to the soil increases pea aphid (*Acyrthosiphon pisum*) mortality on tic bean (*Vicia faba* var. *minor*), pea (*P. sativum*), broad bean (*V. faba* var. *major*), runner bean (*Phaseolus coccineus*), red clover (*Trifolium pratense*), and alfalfa (*M. sativa*) (Hodge *et al.,* 2005). Colonization of *A. thaliana* roots by the plant growth-promoting rhizobacteria *Pseudomonas simiae* WCS417r (previously known as *P. fluorescens* WCS417r) induces systemic resistance towards the generalist caterpillar *Spodoptera exigua* by activating the JA pathway (Van Oosten *et al.,* 2008). As for resistance towards thrips, onion plants (*Allium cepa*) colonized with the endophytic fungus *Clonostachys rosea* ICIPE 707, *Trichoderma asperellum* M2RT4, *Trichoderma atroviride* ICIPE 710 or *Hypocrea lixii* F3ST1 developed significantly less feeding scars inflicted by *T. tabaci* (Muvea *et al.,* 2014). Another example of the stimulation of JA-mediated defense responses is through endophytic oviposition by *O. laevigatus*, which is known as a thrips predator, but can also significantly reduce *F. occidentalis* feeding damage in tomato (*S. lycopersicum*) by inducing JA-dependent defenses. All in all, the incorporation of biostimulants in agricultural practice may constitute a promising approach for thrips control. Their efficacy could be enhanced if we learn more about their molecular mode of action on the plant and vice versa, how the plant regulates establishment of the beneficial microbes in the rhizosphere.

Capture salivary effectors

The success of thrips on many host plants suggests the existence of a common or general mechanism to perturb plant defenses that facilitates successful feeding and/or protection of the progeny. One such mechanism could be the transfer of salivary effectors. Pathogen and pest effector proteins and small molecules can alter the host's defense responses and/or promote infection (Hogenhout & Bos, 2011) (Fig. 1). Only very recently several proteinaceous effectors that suppress plant defense responses have been identified from aphids (Naessens *et al.,* 2015; Van Bel & Will, 2016; Kettles & Kaloshian, 2016; Thorpe *et al.,* 2016; Mondal, 2017) and spider mites (Villarroel *et al.,* 2016; Schimmel *et al.,* 2017). Based on what we know about the feeding habits of thrips, transferred effectors could either exhibit their action locally at the site of feeding/oviposition or at distance, potentially through hitchhiking on host transport-systems such as the symplast (Khang *et al.,* 2010; Giraldo & Valent, 2013; Zebelo & Maffei, 2015). For larvae that start to feed directly after hatching, manipulating local host defenses could be of crucial importance. So far, no thrips effectors have been identified yet, but a similar approach as for aphids, hessian flies and spider mites, using bioinformatic pipelines (Fig. 3D) (Bos *et al.,* 2010; Zhao *et al.,* 2015; Thorpe *et al.,* 2016; Villarroel *et al.,* 2016) to predict potential effectors from transcriptomes of the insect and the tissue they infest, can be taken to fill this knowledge gap on thrips effectors. The identification of a putative orthologue (>65% identity) of the aphid MP46 effector in the salivary-glands and whole-body transcriptomes of *F. occidentalis* (Stafford-Banks *et al.,* 2014) indicates the potential success of such an approach. Functional analyses of candidate effectors can subsequently be performed using RNAi techniques that silence the expression of salivary effectors. Using this

technique, the salivary effector gene *C002* was silenced in pea aphids (*A. pisum*) resulting in increased aphid mortality (Mutti *et al.,* 2006). Techniques for transferring RNAi into thrips have already been developed (Badillo-Vargas *et al.,* 2015) and can for example be introduced through thrips symbionts (Whitten *et al.,* 2015). Once thrips effectors have been validated, the targets through which such effectors manipulate their host can be identified, which will enable the characterization of the molecular mechanisms involved in the establishment of thrips-host interaction. For non-genetic engineering breeding approaches, natural mutants or alternative alleles of the target plant protein can then be screened for.

OUTLINE OF THE THESIS

Herbivorous thrips are important pests in food and ornamental plant-production worldwide harboring all the problems of pests in general: they are highly polyphagous, become rapidly resistant to pesticides, and are notorious vectors of plant viruses. With the rising demand for more sustainable, safer and healthier food production systems, the development of less invasive and polluting solutions to manage pests is of great importance. Exploiting the natural defense mechanisms of plants against herbivores provides opportunities towards more sustainable resistance breeding. Literature on herbivore-induced plant defenses is currently predominantly focused on leaf-chewing and phloem-feeding herbivores, while far less is known about how plants activate their defenses in response to the cell-content-feeding thrips.

The major aim of this PhD research was to investigate the molecular details by which the model plant Arabidopsis responds to attack by the Western flower thrips (*F. occidentalis*) and unravel the defense responses that play a role in warding off this insect pest. In **Chapter 2**, we first describe the experimental setups that were used in this study. We present methods for thrips rearing and thrips selection, and outline in detail how thrips feeding success and fecundity over a longer period of time is determined. Additionally, we describe the set-up that we used to study the effects of localized thrips infestation on the transcriptome, phytohormone production or resistance against a subsequent attack by thrips or other pests. These assays form the experimental basis for the studies described in **Chapters 3 to 5**. In **Chapter 3** we studied the transcriptional dynamics in single Arabidopsis leaves in response to thrips feeding. Through the combination of high-density time series RNA-sequencing expression data and transcriptional network modeling we were able to capture the structure of the thrips-induced gene regulatory network and the chronology of the associated biological processes that are engaged by Arabidopsis upon attack by thrips. This allowed us to select transcription factors with putative crucial roles in the defense gene regulatory network, which were subsequently biologically validated using a whole-plant thrips performance assay. We confirmed that JA is

the predominant phytohormone modulating the induced defense response against thrips and we identified a number of novel regulators in the thrips-induced plant regulatory network that can be utilized in breeding programs to enhance the development of thrips-resistant crops. In **Chapter 4** we investigated the effect of the developmental stage of thrips on the transcriptomic defense output of Arabidopsis. We show that plants can respond differently to the distinct developmental stages within a thrips life cycle. In response to thrips eggs, plants accumulate SA and enhance the expression of the SA-responsive gene *PR1* at the site of oviposition. In contrast, in response to feeding by the adult female thrips as well as the two larval stages plants mainly enhance the expression of JA-responsive genes. However, the youngest larvae induced a relatively higher expression of the ET-coregulated ERF-branch marker gene *PDF1.2* while the older stages activated higher levels of the ABA-coregulated MYC-branch marker gene *VPS2*. Interestingly, all thrips stages performed significantly worse on the ET-insensitive *ein2-1* mutant, which suggests that the youngest thrips stage might be able to manipulate the plant's defense response through activation of the ERF-branch to antagonize the MYC-branch. This hypothesis correlates with the results in **Chapter 5** in which systemic tissue of a locally thrips-infested plant was shown to express elevated levels of the ERF-branch marker gene *PDF1.2* and the performance of secondary attacking thrips was not affected. In contrast, local infestation by the caterpillar *Mamestra brassicae* systemically induced the MYC-branch marker gene *VSP2* mainly, which was associated with a decrease in thrips performance. Furthermore, prior infestation by thrips shifted the preference of a secondary thrips attack to the youngest leaves. The induction of certain lipoxygenases, which act upstream of the JA pathway (in the younger leaf tissue) might play a role in the differential systemic activation of the JA pathway by the two studied insect herbivores from different feeding guilds. Finally, in **Chapter 6** the findings of the research presented in this thesis are discussed, followed by an outline of suggestions for promising future research in the field of resistance breeding against pest thrips.

CHAPTER 2

Bioassays to evaluate the resistance of whole plants to the herbivorous insect thrips

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ABSTRACT

Thrips are tiny, cell-content feeding insects that are a major pest on crops and ornamentals. Besides causing direct feeding damage, thrips may also cause indirect damage by vectoring tospoviruses. Novel resistance mechanisms to thrips need to be discovered and validated. Induction of jasmonic acid-dependent defenses has been demonstrated to be essential for resistance to thrips, but underlying mechanisms still need to be discovered. For this, it is vital to use robust plant-thrips assays to analyze plant defense responses and thrips performance. In recently developed high-throughput phenotyping platforms, the feeding damage that is visible as silver spots, and the preference of thrips in a two-choice set-up is assessed, using leaf discs. Here, we describe whole-plant-thrips assays that are essential for i) validation of findings obtained by the leaf disc assays, ii) assessment of longer-term effects on thrips feeding success and fecundity, iii) determination of spatial-temporal effects induced by primary thrips infestation on a secondary attack by thrips or other insects or pathogens, and iv) assessment of gene expression and metabolite changes. We present detailed methods and tips and tricks for (a) rearing and selection of thrips at different developmental stages, (b) treatment of the whole plant or an individual leaf with thrips, and (c) determination of feeding damage and visualization of thrips oviposition success in leaves.

INTRODUCTION

Thrips are tiny insects (1.5 mm or less) that are cell-content feeders, greatly affecting commercial food crops and ornamentals worldwide. Besides the direct feeding damage that thrips cause, indirect damage can be inflicted by the tospoviruses that can be transmitted in a non-ingesting probe. The life cycle of thrips is comprised of six stages: the egg stage, two larvae stages (first instar L1 and second instar L2), two pupae stages (prepupa and pupa) and the adult stage (Fig. 1; reviewed by Steenbergen *et al.* (2018)). Female thrips lay their bean-shaped eggs in the epidermal layer of various above-ground plant parts, like leaves, stems and flowers, using a saw-like ovipositor (Tommasini & Maini, 1995; Reitz, 2009). Fertilized (diploid) eggs will develop into female thrips and unfertilized (haploid) eggs will give rise to male thrips. The larvae that hatch from the eggs will go through two larval stages that actively feed from the plant. During the two pupae stages, thrips are non-feeding and move to the soil to prepare for the adult stage. Compared to the larvae, the adults are more mobile because of their fringed wings, allowing them to fly off and look for new suitable host plants (reviewed by Steenbergen *et al.* (2018)). Both larval and adult thrips feed from the plant by piercing with their stylet in epidermal, mesophyll and parenchymal cells and ingesting the contents (Chisholm & Lewis, 1984; Kindt *et al.,* 2003). Emptied cells collapse or fill with air which, after intensive feeding, results in a localized silvery appearance (reviewed by Steenbergen *et al.* (2018)).

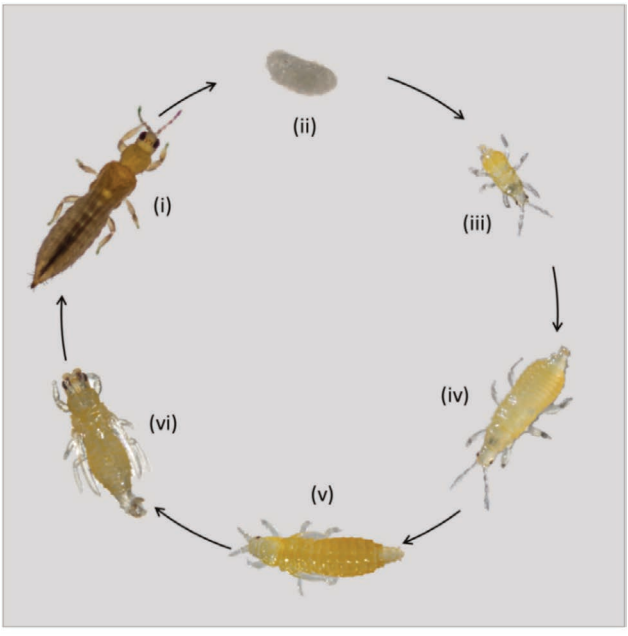

Figure 1: Thrips life cycle. Adult female thrips (i) can lay fertilized or non-fertilized eggs (ii) from which a female or a male thrips larva will emerge, respectively, after 3-4 days (depending on the temperature, which is optimal at 27°C). The first instar larva (L1; iii) will develop into a second instar larva (L2; iv) within two days. The L2 larva will go through a pre-pupa (v) and pupa (vi) phase after 2-3 days, from which an adult thrips emerges after 4-5 days.

Upon probing and ingestion of the plant cells, different defense responses are initiated in the plant. This activation of defenses requires the perception and recognition of specific and general herbivore- and damage-associated molecular patterns (HAMPs and DAMPs). Direct and/or indirect defenses against thrips are coordinated by phytohormones such as jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene (ET). JA has been demonstrated to be essential in the defense against many herbivore species including thrips (reviewed by Steenbergen *et al.* (2018)); e.g., plants insensitive to JA or deficient in JA accumulation are more susceptible to thrips (Abe *et al.,* 2008). Subsequent to triggering of the JA pathway by thrips, different direct and indirect defense mechanisms are activated, like formation of leaf trichomes, and production of phenolic compounds with toxic characteristics and volatile organic compounds that can attract natural predators of thrips. There is a growing body of evidence for the ability of herbivores to manipulate JA-associated defenses through the action of effector proteins in the arthropod saliva that are secreted into the plant cells, but whether thrips harbor these saliva-borne effectors is still unknown (reviewed by Steenbergen *et al.* (2018)). Another form of defense manipulation that has not been described yet for thrips, but that has been demonstrated for chewing herbivores, is that via egg-associated cues that trigger SA signaling, which suppresses anti-herbivore defenses, thereby benefitting the future progeny of thrips (Bruessow *et al.,* 2010).

Thrips are too tiny to measure their weight or length. Therefore, to determine the performance of thrips larvae and adults on different plants, the amount of silver damage inflicted by their feeding is often monitored. Another measure to determine the performance is the reproductive success of thrips on the plants. Moreover, if given a choice between different genotypes or treatments, the preference of thrips for certain plant material, if given a choice, can be assessed. Recently, two high-throughput phenotyping platforms have been developed to quantify host-plant resistance/thrips preference. The first one uses a two-choice setup of half-leaf discs in a 96-well format in which the behavior of the thrips is tracked continuously by a video camera throughout a period of several hours and analyzed with commercial software (Thoen *et al.,* 2016). The second one is based on a no-choice set-up of leaf discs in a Petri dish in which the amount of silver damage inflicted by thrips at one end-point is captured by a photo camera and quantified by freeware (Visschers *et al.,* 2018). These two screening methods will aid to reduce the quantity of material, and the space and time needed to screen for thrips resistance compared to screening of whole plants. Comparisons between leaf disc and/or detached leaf tests with whole-plant tests revealed that the relatively high amount of damage inflicted to the leaf material prior to the start of a leaf disc assay did not influence the thrips resistance scores of a few selected pepper and *Arabidopsis thaliana* (hereafter Arabidopsis) accessions (Maharijaya *et al.,* 2011; Thoen *et al.,* 2016; Visschers *et al.,* 2018).

Nonetheless, good set-ups for whole-plant performance assays are still essential. For example, to study spatial-temporal effects induced by primary thrips infestation on resistance to a secondary attack by thrips or other insects or by pathogens, whole-plant assays are required. Also, it is difficult to maintain leaf discs in good shape, and therefore, in order to study longer-term effects on the thrips performance in terms of feeding and oviposition success, the use of intact plants is essential. Moreover, findings based on leaf disc assays should be validated under greenhouse or field conditions with whole plants. And lastly, for sensitive gene expression or metabolite accumulation assays, it is preferred to introduce as little as possible variation, which demands for less handling and surely not damaging the tissue, before thrips are introduced. Therefore, we describe here several methods for thrips assays on whole plants. We explain how to rear and select different developmental stages of thrips, for which different tools can be hand-made. We present how to treat the whole plant or individual leaves with thrips, for bioassays in which feeding damage or oviposition success can be assessed, or for the study of induced defense responses, locally and systemically.

The use of proper thrips-plant assays, as described here, will help to increase our understanding of the defense mechanisms underlying plant resistance to thrips.

MATERIALS

Equipment used for multiple purposes

1. 1.5-mL Eppendorf tubes.
2. Pipet and tips (200 µL, 1000 µL).
3. Fridge.
4. Vacuum autoclave (344 L) and autoclavable plastic bags (40 × 60 cm).
5. River sand.
6. Potting soil.
7. Modified half-strength Hoagland nutrient solution (Hoagland & Arnon, 1938): 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM KH_2PO_4, 2 mM $MgSO_4$, trace elements, pH 7, 10 mM Fe-ethylenediamine-di[o-hydroxyphenylacetic acid] (Sequestreen).
8. Potting bench.
9. Plant labels.
10. Small trays (150-300 mL; 4 cm high).
11. Big trays (L45 x W30 x H8 cm) with transparent lids.
12. Garden felt.
13. Forceps (curved and straight tip).
14. 15-mL tubes.
15. 1.5-mL safe-lock tubes.

16. Small Petri dishes (Ø 6 cm).
17. Scissors.
18. Cork-borer (Ø 4 mm, Ø 15 mm, Ø 20 mm).
19. Bunsen burner.
20. Matches.
21. Heat-resistant gloves.
22. Nylon mesh (80 micron).
23. Hot glue gun.
24. Liquid nitrogen.

Plant cultivation

Arabidopsis

1. Climate-controlled growth chambers with a 10-h day and 14-h night cycle set at 21°C and 70% relative humidity. Light provided by HPI-T Plus lamps (400W/645 E40) (see Note 1).
2. Seeds of Arabidopsis.
3. 0.1% (w/v) agar.
4. Pots (60 mL, Ø 5.5 cm, height 5 cm) with holes in the bottom for plant cultivation after the seedling stage.

Lettuce

1. A controlled greenhouse compartment with natural light conditions at 18°C and 70% relative humidity.
2. Seeds of *Lactuca sativa* var. *capitate*.
3. Pots (800 mL, L9 x W9 x H10 cm) with holes in the bottom for plant cultivation after the seedling stage.
4. Plastic plant pot saucers (Ø 18 cm).

Instruments for thrips rearing and assays

Containers for synchronized larval rearing and whole plant bioassays

1. 1-L transparent polyethylene terephthalate (PET) jars with screw-cap (Ø 10.4 cm).
2. Butyl septa (Ø 20 mm).
3. Hole saw (Ø 6 cm).

Clip cages for single leaf treatment with thrips

1. Acrylate rings (Ø inside 21 mm, thickness 4 mm, height 5 and 8 mm).
2. Metal hairclips.
3. 4-cm squares of polyether foam (thickness 3 mm).

4. Two-component glue plastic.

Aspirator for collecting thrips of a specific life stage

1. 15-mL plastic, transparent pots with screw-cap (Ø 25 mm).
2. Polytetrafluorethylene tubes (Ø 4 mm, length 30 cm).

Thrips rearing

1. A controlled growth cabinet with a 10-h day and 14-h night cycle at 26°C, 70% relative humidity and a light intensity of 100 µmol m^{-2} sec^{-1}.
2. BugDorms (L60 x D60 x H60 cm) with 150-micron mesh.
3. Common bean pods (Phaseolus vulgaris L.) from the supermarket.
4. Bee pollen.
5. PCR tubes (200 µL) and caps.
6. Paper tissues.
7. Fine paint brush.
8. White 500-mL polypropylene box (L182 x W135 x H35 mm) with transparent lid.

Trypan blue staining

1. Trypan blue solution (lactic acid, glycerol, demi-water and 96% ethanol (1:1:1:3, v:v:v:v) + 0.025% trypan blue (adapted from Koch & Slusarenko (1990).
2. Rack for 15-mL tubes.
3. Floating tube rack for Eppendorf tubes
4. Water bath.
5. Pipette-boy.
6. 25-mL serological pipettes.
7. Empty glass bottle (1L).
8. Lactoglycerol solution (lactic acid, glycerol and demi-water (1:1:1, v:v:v)).
9. Petri dish (Ø 15 cm).
10. Microscope glass slide.
11. 95% ethanol.
12. Stereo zoom optical microscope (10x magnification).

METHODS

Plant Cultivation

Arabidopsis Cultivation

1. Suspend Arabidopsis seeds in 1.0-mL of 0.1% agar in 1.5-mL Eppendorf tubes and store at 4°C for 3 days.
2. Autoclave river sand in double autoclavable bags for 45 min at 120°C.
3. Autoclave a mix of potting soil and river sand (12:5, v:v) in double autoclavable bags twice for 45 min at 120°C with a 24-h interval.
4. Add half-strength Hoagland to sand (150 mL kg^{-1} sand). Fill up the 4 cm high small trays with sand until 1 cm below the edge.
5. Distribute seeds (in 0.1% agar) evenly on sand using a 1000 µL pipet. Place small tray in a big tray (L45 x W30 x H8), add some water to the bottom of the big tray and cover with the transparent lid to ensure 100% relative humidity. Place tray in a growth chamber for 10-14 days.
6. Use a potting bench to mix Hoagland solution (50 mL kg^{-1}) and an equal amount of water with sterile potting soil mixture.
7. Cut garden felt in pieces of L45 x W30 cm (the size of big tray), wet with water, and place in big plant trays (see Note 2).
8. Fill 60-mL pots with soil mix, pots should weigh approximately 70-75 grams, and make a hole of 1.5 cm deep in soil of each pot with the conical end of a 15-mL tube. Place pots in the big trays with felt.
9. To transfer a seedling to planting hole, flood small sand-trays with water and use forceps with a curved tip to loosen up the sand and to gently pull out the seedling.
10. After transfer of seedling into planting hole, close the hole gently by pushing the soil back around the root.
11. Stick a plant label (colored or with text) in each pot to indicate the genotype or treatment. Randomize the different genotypes/treatments in and between trays (40 pots/tray).
12. Cover trays with transparent lids for 2 days to ensure 100% relative humidity. Crack the lids for 1 day before full removal to allow plants to gradually acclimate to a relative humidity of 70% for the following weeks.
13. Every other day, water the plants until the felt is saturated. Once a week, apply 500 mL of Hoagland solution per tray.

Lettuce Cultivation

1. Follow steps 1-6 of 3.1.1 for cultivation of lettuce seedlings.

2. Fill 800 mL pots with sterile potting soil (not a mixture with sand) and place them in a big tray (8 pots/tray; see Note 3). Make a hole of 3 cm deep in the soil of each pot with the conical end of a 15 mL tube.
3. Gently remove a lettuce seedling from sand using your fingers and place it in the planting hole. Close the hole gently by pushing the soil back around the root.
4. From here, follow steps 10, 12 and 13 of 3.1.1 for cultivation of the lettuce plants after transfer of seedlings.

Tools for rearing and collecting of thrips, and for bioassays

Containers for larval rearing and whole plant bioassays

For both larval rearing and whole plant bioassays, modified 1 L transparent PET containers (Ø 10.4 cm) with screw-caps are used (Fig. 2).

1. Using a hot glue gun apply some glue underneath the bottom of a 6-cm Petri dish to attach it on the bottom inside the container.
2. Use a hole saw with a drill to make a hole (Ø 6 cm) in the white screw-cap of container.
3. Cut circles (Ø 8 cm) from the 80-micron mesh and use the glue gun to attach the mesh onto the inside of the screw-cap.
4. Heat the tip of a cork-borer (Ø 20 mm) and create a hole on the side of the container at 2 cm from the bottom. Cover the hole with a 20-mm butyl septum.

Figure 2: Modified PET-container for thrips larval rearing and whole plant bioassays.

Clip cages for single leaf assays on whole plants

For assays in which a localized treatment with a defined number of thrips on one leaf is needed, a clip cage can be used, which allows the treated leaf to remain attached to intact plant (Fig. 3). Clip cage assays are suitable for gene expression or metabolite studies, but also to determine systemic effects of thrips feeding on secondary infestation or infection. By selecting leaves of a similar developmental stage, variation between replicates will be reduced. Clip cages can be ordered online but can also be made in the lab as described here.

1. Hold the two acrylate rings, one of 5 mm height and one of 8 mm height, between the thumb and the index finger.
2. Use a Bunsen burner to heat the tips of metal hair clips. Wear heat-resistant gloves when doing this. When sufficiently heated (the tip will start to glow), each tip of the clip (bottom or top) can be gently pressed into the side of the rings (one tip in the top ring, the other in the bottom one).
3. Cut the 80-micron mesh into squares of 4 x 4 cm and using a glue gun attach a piece of mesh on the outside of the 5 mm ring and a piece on the outside of the 8 mm ring.
4. Cut the polyether foam into squares of 4 x 4 cm and make stacks of 10.
5. Wear heat-resistant gloves when heating the tip of the Ø 15-mm cork-borer in the burner. Press it through the middle of the foam stack to create a hole in each patch.
6. Glue one of the foam patches on the inside of the 5-mm ring and another one on the inside of the 8-mm ring (see Note 4).
7. When the glue is dry, cut the edges of the mesh and the foam.

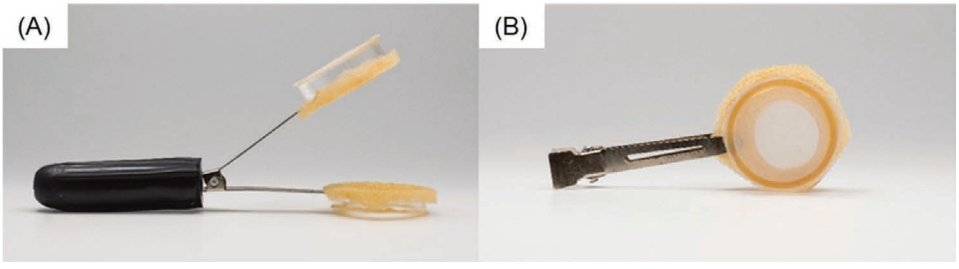

Figure 3: Clip cage. (A) Side view of the clip cage when opened. **(B)** Top/bottom view of the clip cage. Foam is glued on the inside and mesh on the outside of the rings.

Aspirator for adult thrips collection

An aspirator is used to collect the female thrips. Adult females are used for the whole plant bioassays because then both feeding damage as well as oviposition can be assessed as performance parameters. Females are selected from a colony reared in the BugDorm (see section 3.3) and can be recognized by their dark abdomen. Male thrips are smaller and have

a lighter color (Fig. 4). Aspirators can be bought (online) but can also easily be hand-made (Fig. 5), following the protocol below.

1. Heat the tip of a Ø 4-mm cork-borer in a Bunsen burner and pierce 2 holes in the screw-cap of the 15-mL plastic, transparent pots.
2. Cut the silicone tubes in 2 pieces of 30 cm length. Slide tubes through the holes of the screw-cap (until 1 cm below the cap) and glue-shut the opening between the tube and the hole using a glue gun.
3. Cut the 80-micron mesh into small squares of 5 x 5 mm and glue it to the end of one the tubes, facing the inside of the pot.
4. Cut 1 cm off the tip of a 200-µL pipette tip and slide it in the other end of the tube that carries the mesh.

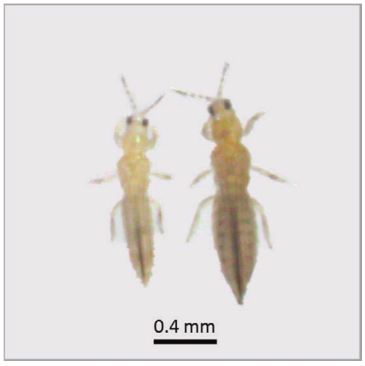

Figure 4: Adult male (left) and female (right) thrips.

Figure 5: Hand-made aspirator to collect thrips.

Thrips rearing and collecting

Adults

When starting a thrips colony, set up 2-3 BugDorms. When you start a colony with 100 adult thrips (mix of males and females), you can have 100-200 adult female thrips after approximately 18 days. To prevent inbreeding, mix the colonies between the BugDorms and import thrips from other thrips sources (e.g. from collaborators) every 6 months.

1. Place the BugDorm in a controlled growth cabinet at 26°C, which is an ideal temperature for thrips.
2. Place 4 plastic plant pot saucers in each BugDorm. Cut pieces of garden felt (Ø 18 cm) to put in the saucers.
3. Place a pot with a 5-week-old lettuce plant on each saucer (see section 3.1.2 for cultivation of lettuce).
4. Add a mix of male and female thrips to each BugDorm.
5. Cover the bottom of a small Petri dish with bee pollen and place the dish in the BugDorm (see Note 5). Refresh the bee pollen weekly.
6. Every other day, water the plants until the felt is saturated. Once a week, apply Hoagland solution (see section 3.1.2).
7. For collection of female thrips, gently shake a thrips-infested lettuce plant from the BugDorm in the white 500-mL box and close it with a transparent lid.
8. Place the pipette tip of the aspirator made in 3.2.3 in your mouth, gently lift the corner of the lid of the box and cover the selected thrips with the end of the other tube to suck it up. Thrips will now be collected in the pot and the mesh at the end of the ‘sucking’ tube will prevent the thrips from moving into that tube.
9. If the desired number of thrips is collected (for example 5 for a leaf- or whole plant-assay, and 100 for synchronized rearing of thrips larvae), then remove the aspirator cap and screw on a normal cap (without holes).

Larvae

After thrips larvae hatch from the eggs, they develop two larval stages. The transition from the first to the second larval stage is difficult to distinguish. First instar larvae (L1) are small and white and transform into yellow and slightly bigger second instar larvae (L2) after 1-2 days, depending on the temperature (Fig. 1). By synchronizing their development, a collection of larvae at a certain developmental stage (L1 or L2) can be obtained.

1. Fold some paper tissues into squares of 10 x 10 cm and place at the bottom of the 1-L container made in section 3.2.1.
2. Rinse the bean pods with water and place 5 beans in each container.

3. Fill a PCR tube with bee pollen and close with its cap. Make a small hole in the bottom of the tube by cutting it with scissors or by piercing it with a needle, through which the thrips can acquire access to the pollen (see Note 5). Place one tube in the container.
4. Collect 100 female thrips as described in 3.3.1 and add them to the container (see Note 6). Allow them to oviposit on the beans for 48 h.
5. After 48 h, repeat step 1 and place the oviposited beans in the female-free container. Add the bee pollen and put the females from step 4 back into the BugDorm.
6. After 4-5 days 100-200 L1 larvae will emerge. They will develop into L2 larvae 2-3 days later.
7. Use a fine paintbrush to collect the L1 or the L2 larva. Moisturizing the brush will improve the sticking of the larva to the brush.
8. Gently place the hairs of the brush on a leaf or in the clip cage and allow the larva to move from the brush. Prevent brushing the larva off as this might damage thrips.

Thrips assays

Feeding damage assays

Measuring thrips feeding damage is a good measure to quantify thrips performance. Thrips silver damage can be estimated in mm^2 by eye or quantified from pictures using software (Visschers *et al.,* 2018). Different thrips developmental stages can be used for this assay, but it should be kept in mind the time between the different stages differs (Fig. 1). When starting with L1 larvae, damage can be recorded for 5 days before they go into the pupae phase. The damage caused by L2 larvae can be recorded for 3 days before the pupae phase starts. The damage inflicted by female adults has to be recorded within 4 days, before their eggs hatch and L1 larvae start to feed from the plant.

Whole-plant damage assay

1. Test at least 10 replicates per treatment and/or genotype. Put 10 mL of water in the Petri dish at the bottom of the hand-made containers (see section 3.2.1). Place an Arabidopsis plant on the Petri dish.
2. Collect thrips at the preferred stage at described in section 3.3.
3. Treat plant with thrips (see Notes 7 and 8).
4. Monitor the amount of feeding damage (see above; Fig. 6A).

Single leaf damage assay

1. Test at least 10 replicates per treatment and/or genotype. Use clip cages (see section 3.2.2).
2. Collect thrips at the preferred stage at described in section 3.3.

3. Transfer thrips to the clip cage (see Note 9).
4. Gently open the clip and place the leaflet in the opening of the clip cage. Gently release grip on the clip so that the leaflet is fixed in the clip (Fig. 6B).
5. Let the clip rest on a neighboring pot to prevent damage on the petiole because of the weight of the clip.
6. Monitor the amount of feeding damage (see above; Fig. 6A; see Note 10).

Oviposition assays

Feeding damage on the plant often correlates with oviposition by the female thrips. As an extra measure of thrips performance, the number of oviposited eggs after feeding by the adult female can be determined. Thrips eggs are kidney shaped and are oviposited in the leaf blades and the petioles (Fig. 7).

1. After damage assessment of a whole-plant assay (see section 3.4.1.1), the rosettes are harvested by cutting the plant at the hypocotyl. Fold the leaves upwards like an umbrella and put the rosette in a 15-mL Falcon tube. When assaying single leaves (using clip cages), cut the treated leaf and place it in a safe-lock Eppendorf tube.
2. Heat up a water bath to 100°C.
3. Add the Trypan blue solution to the leaf tissue (~ 10 mL for a rosette, ~ 0.75 mL for a leaf) and close the tubes with their respective caps.

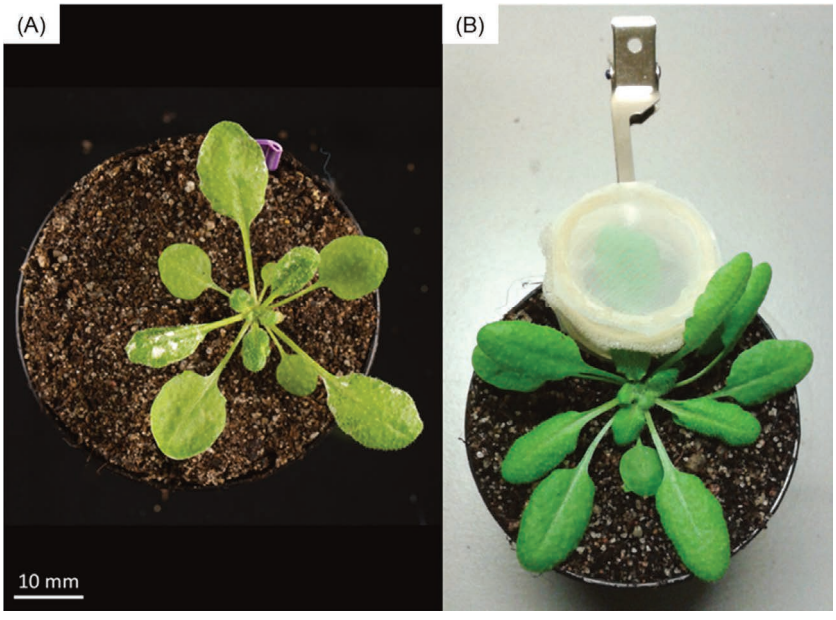

Figure 6: Silver damage on an Arabidopsis plant (A) and application of a clip cage on an Arabidopsis leaf (B).

4. Place the Falcon tubes for 2 min and the Eppendorf tubes for 30 sec in the water bath. Let the solution cool down for 30 min.
5. Pour off the Trypan Blue solution (use forceps to prevent the leaf material from sliding out of the tube) and replace by the lactoglycerol solution until the leaf tissue is fully submerged (~ 7 mL for a rosette, ~ 0.75 mL for a leaf). Samples can be stored at room temperature.
6. Place the rosette in a Ø 15-cm Petri dish containing 95% ethanol. The single leaves are transferred to a microscope glass slide. Use a stereo zoom microscope and count the detected eggs. A magnification of 10x usually suffices, but a higher magnification may be needed if there is a high background staining.

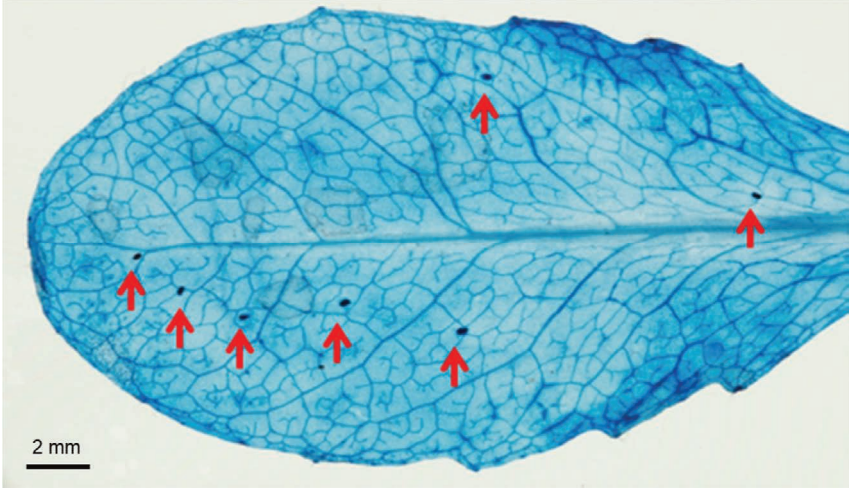

Figure 7: Trypan blue-stained leaf to visualize thrips eggs. Red arrows point to the eggs oviposited in an Arabidopsis leaf.

Assays for thrips-induced changes in gene expression or metabolite accumulation

For measuring the plant's molecular responses to thrips, for example induced changes of gene expression or metabolite accumulation, the use of a single leaf that remains attached to the plant is preferred. The variation between replicates will be reduced by selecting leaves of a similar developmental stage. Moreover, by using a clip cage a defined number of thrips can stay localized on a leaf (Fig. 3).

1. Follow steps 1-5 of 3.4.1.2. Instead of 10 replicates, this type of assays requires a minimum of 5 replicates.
2. Harvest the samples after a preferred period of time (see Note 11) by gently removing the clip and cutting the treated leaf at the petiole. Put the leaf in a safe-lock Eppendorf tube and snap freeze it in liquid nitrogen, after which it can be stored in the -80°C freezer.

NOTES

1. It is important to have separate areas for the cultivation of the plants, the rearing of the thrips, and the execution of the plant-thrips assays. Thrips are difficult to control, since they can easily maneuver though small openings and consequently contaminate other areas. In our case, we performed all of our thrips assays in greenhouse compartments, which are physically separated from our growth chambers (Sylvania Luxline plus lamps F58W/84) where we usually cultivated our Arabidopsis plants until they were moved to the greenhouse at least 24 hours before the execution of the thrips assay. The thrips rearing took place in a separate compartment in the greenhouse. In the beginning, we had also cultivated the Arabidopsis plants in the greenhouse, but the frequency of contamination with thrips became too high.
2. The garden felt will retain the water longer, creating a moist environment for the plants for a longer period of time.
3. No garden felt was used on the bottom of the trays in which lettuce plants were grown. After a few weeks the roots of lettuce start to grow out from under the pots and attach to the felt. Removing the pots from the felt will damage the roots. This root outgrowth does not occur from the pots with Arabidopsis plants.
4. The foam prevents the leaf from damaging. Additionally, the foam closes openings between the two rings at the side where the petiole is placed between the rings. Also, if the cages would be used on leaves of other plant species that might have thicker veins, the foam will close the spaces/openings that are created by the uneven thickness of the leaf.
5. Bee pollen are highly nutritious for thrips and can have a positive effect on e.g. development time, fecundity and longevity.
6. The 15-mL pot of the aspirator containing the 100 female thrips can be opened and placed inside the 1-L rearing container.
7. When a whole plant is treated with adult (female) thrips, the 15-mL pots of the aspirator can be opened and placed (next to the Petri dish) inside the 1-L container.
8. Taken over several days, in our experiments the average temperature inside the 1-L containers was 1.06 and 0.32°C higher than outside the container during the day and night, respectively.
9. To get the female adults in a clip cage, tap against the 15-mL collection pot and hold it upside-down above the open clip cage to release the thrips.
10. Gently remove the clip-cage from the leaf and keep the leaf attached to the plant when assessing the damage. This will prevent wilting of the leaf, which hinders the scoring.

11. We harvested samples for analyses of gene expression and hormone accumulation every other hour until a maximum duration of 2 days (leaves will be too heavily infested after that time).

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CHAPTER 3

Architecture of the gene regulatory network induced by Western flower thrips in Arabidopsis

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ABSTRACT

Herbivorous thrips are tiny cell-content-feeding insects that are a major and hard-to-control pest in ornamental and vegetable crops. The western flower thrips (*Frankliniella occidentalis*) feeds on plants of over 60 families and is an important vector of plant pathogenic tospoviruses. Knowledge on plants' natural immune responses to thrips has been scarce but is urgently needed to develop thrips-resistant crops. In this study a high-density RNA-seq time series of thrips-infested *Arabidopsis thaliana* leaves at 12 time points over an 8-h period was performed. Computational analyses revealed that thrips infestation significantly induced transcriptional reprogramming of 2788 genes (65.3% up- and 34.7% downregulated) that could be divided into 20 distinct gene clusters of process-specific co-regulated genes. Already 71% (n = 1981) of the genes became differentially expressed for the first time within 3 h of thrips infestation, which is remarkable considering the small size of the five thrips larva used. The enrichment of bHLH transcription factor (TF) binding motifs in the set of the upregulated genes, but also the specific biological processes associated with those genes, and the overlap of 51% of all thrips-induced differentially expressed genes (DEGs) with MeJA-induced DEGs, suggest that jasmonic acid (JA) is the predominant phytohormone orchestrating the thrips gene regulatory network (GRN). Additionally, smaller sets of genes showed an overrepresentation of WRKY, bZIP, ERF, MYB(-related), NAC, CAMTA, C2H2 and TCP TF binding motifs, which fine-tune the thrips-induced GRN. Using the DREM reconstruction-network tool, a dynamic transcriptional model was generated, including split nodes representing divergence of genes that had been co-regulated up until that point. From each of the 7 co-expressed, upregulated paths several TFs were selected to validate their role in defense regulation to thrips. Compared to wild-type plants, mutants in TFs ERF13, DIV2 and AT5G56840 displayed significantly less thrips feeding damage and oviposition, whereas a mutant in WRKY40 was more susceptible. Collectively, this study revealed a detailed understanding of the complex dynamics and diversity of genes induced upon thrips infestation, which can facilitate further exploration of the thrips-mediated GRN for the identification of novel components that can contribute to the development of more sustainable thrips-resistance breeding.

INTRODUCTION

Plants and insect herbivores have been interacting with each other for over 350 million years. To minimize damage, plants have evolved a plethora of defense mechanisms that can affect herbivore survival and/or performance. These defenses can be either constitutively present, i.e. regardless of herbivore presence, or induced upon herbivore recognition due to feeding (War *et al.,* 2012) or egg deposition (Hilker & Fatouros, 2016). Plants perceive the presence of herbivorous insects through the recognition of damage- and herbivore-associated molecular patterns (DAMPs and HAMPs), which occurs usually by pattern recognition receptors (PRRs) but defense activation by HAMPs can also be independent of PRRs (Diezel *et al.,* 2009; Schäfer *et al.,* 2011). Following recognition, a plant activates general downstream responses such as membrane depolarization, calcium influx, production of reactive oxygen species and activation of mitogen-activated protein kinase (MAPK) signaling cascades that initiate the biosynthesis of phytohormones including jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET) (reviewed by Erb *et al.* (2012), Zebelo & Maffei (2015) and Nguyen *et al.* (2016)). The hormone JA is considered a key player in the defense regulatory network that is effective against different types of herbivores in different plant species. The interplay between hormones fine tunes downstream transcriptional responses, which determines the outcome of the myriad of plant-insect interactions (Pieterse *et al.,* 2012; Caarls *et al.,* 2015).

Most research on plant defenses against insects has been performed using leaf-chewing or phloem-feeding herbivores (Bonaventure, 2012; Hilker & Fatouros, 2016; Züst & Agrawal, 2017). Far less is known about how plants respond and defend themselves to cell-content-feeding herbivores such as thrips. Thrips are tiny insects (1.5-mm or less) belonging to the order of the Thysanoptera which comprises over 5500 described species (Steenbergen *et al.,* 2018). The western flower thrips, *Frankliniella occidentalis*, is considered one of the most economically important pests worldwide as it feeds on plants of at least 60 families and is an important vector of tospoviruses, which cause severe yield losses. The annual crop loss caused by a single tospovirus, such as the Tomato spotted wilt virus, was estimated on $1 billion worldwide (Riley *et al.,* 2011). Thrips undergo six different developmental stages; egg, first instar larva (L1), second instar larva (L2), pre-pupa, pupa and adult. The larval stages and adults feed by piercing the epidermal and mesophyll cells from which they subsequently suck out the cell content (Parker *et al.,* 1995; Tommasini & Maini, 1995). The emptied cells collapse or fill with air, giving the damaged area a silvery appearance (Cloyd, 2009). Thrips infestation also causes stunting of leaves and deformation of flowers and fruits, which affects the yield and market value (Welter *et al.,* 1990; Tommasini & Maini, 1995; Hao *et al.,* 2002). Currently, control management of thrips relies heavily on the use of chemical pesticides. However, because of their small size, rapid reproduction rate and affinity for narrow spaces, the

pesticides must be applied frequently, which led to the development of resistance to major insecticide groups (Morse & Hoddle, 2006; Mouden *et al.,* 2017). By exploiting the plant's natural immune system, alternative and more sustainable plant protection strategies can be developed (Steenbergen *et al.,* 2018).

Plants infested by thrips activate the biosynthesis of specialized metabolites that can serve as direct and/or indirect defenses (Janssen *et al.,* 1998; Leiss *et al.,* 2009; Mellway & Constabel, 2009; Mirnezhad *et al.,* 2010; Barbehenn & Constabel, 2011; Yang *et al.,* 2012; Nuringtyas *et al.,* 2012; Liu *et al.,* 2017). For example, *F. occidentalis* feeding leads to enhanced production of phenolic compounds (tocopherols) in resistant pepper (*Capsicum annuum*) accessions, but not in susceptible accessions (Maharijaya *et al.,* 2012). Additionally, a particular class of polyphenolic compounds (tannins) increased faster in resistant *Medicago sativa* lines upon *Odontothrips loti* infestation than in susceptible lines (Wang *et al.,* 2014). Besides direct defenses, thrips induce indirect defenses in the form of volatile organic compounds (VOCs) (Delphia *et al.,* 2007; Maharijaya *et al.,* 2012) that attract natural enemies of thrips such as the predatory bug *Orius sauteri* (Mochizuki & Yano, 2007), or the predatory mite *Neoseiulus* (formerly *Amblyseius*) *cucumeris* (Manjunatha *et al.,* 1998).

Prior to the activation of direct and indirect defenses against diverse herbivores, various phytohormones are being produced, amongst which JA which has a lead role in the activation of anti-herbivore defenses (War *et al.,* 2012). Thrips infestation also activates JA signaling (Li *et al.,* 2002; Abe *et al.,* 2008, 2009) and induces expression of a number of JA-responsive genes (Li *et al.,* 2002; De Vos *et al.,* 2005; Abe *et al.,* 2008, 2009; Selig *et al.,* 2016; Escobar-Bravo *et al.,* 2017). The JA network is essential for an effective defense response against thrips, as exogenous application of JA enhances the plant's resistance against thrips, and JA-insensitive and/or JA-deficient plants are more susceptible to thrips (Thaler, 1999; Abe *et al.,* 2009; El-Wakeil *et al.,* 2010; Sarde *et al.,* 2019). However, the JA network is also critical for activation of defense responses to other types of insects and even to pathogenic microbes (Pieterse *et al.,* 2012). A microarray analysis of *Arabidopsis thaliana* (Arabidopsis) was used by De Vos *et al.* (2005) to study the overlap and specificity of transcriptome changes that are induced by plant hormones and different pathogens and insects, among which the western flower thrips. This pointed to 69% of the genes being induced by thrips also being JA-responsive. Of these, 41% overlapped with the transcriptional response induced by leaf chewing caterpillars of *Pieris rapae*, while there was an 80% overlap with the response induced by the pathogen *Pseudomonas syringae*. This demonstrates that the induction of JA-responsive genes is central to the transcriptional response induced by different pests and pathogens, but also points to specificity in the responses to different attackers (De Vos *et al.,* 2005).

Measuring global mRNA accumulation forms a robust strategy to uncover the global

picture of the plant response and its underlying regulation mechanisms upon an infliction of a stress like infestation by thrips. The above-mentioned microarray analysis studied the expression of a set of 8,000 genes and tissue was sampled at five time points (3, 12, 24, 48, 72 h). By collecting RNA-seq data of a high-density time series existing of minimally twelve time points in a short time frame, the temporal transcriptional fluctuations can be better captured, which is essential to understand how the dynamic plant response during thrips infestation is regulated. Through extensive comparative transcriptomics analyses the dynamic thrips-induced gene regulatory network (GRN) can be revealed, pointing to different sectors within the network that control specific biological processes, and their key players, like transcription factors (TFs), that orchestrate the defense regulation against thrips. Such systems biology approaches have been successfully applied to studies on plant responses to pathogens, hormones and abiotic stresses (Windram *et al.,* 2012; Bechtold *et al.,* 2015; Lewis *et al.,* 2015; Coolen *et al.,* 2016; Song *et al.,* 2016; Hickman *et al.,* 2017; Hillmer *et al.,* 2017; Zhang *et al.,* 2017; Mine *et al.,* 2018; Arisha *et al.,* 2020; Liu *et al.,* 2020b). The transcriptional response of plants to insects has also been studied, but only at a limited number of time points (usually three time points within an 8-h period) (De Vos *et al.,* 2005; Ehlting *et al.,* 2008; Appel *et al.,* 2014; Martel *et al.,* 2015; Zhou *et al.,* 2015; Davila Olivas *et al.,* 2016; Kroes *et al.,* 2017; Bui *et al.,* 2018; Guo *et al.,* 2019). Here, we performed an in-depth high-density time series RNA-seq study, existing of 12 time points within 8 h of single Arabidopsis leaves infested by L2 larvae of the western flower thrips. Computational analysis of the thrips-induced transcriptional response provided detailed insight in the structure and chronology of the thrips-induced GRN and in the biological processes associated with the different phases of infestation. The dynamic model of the thrips-induced GRN that was generated allowed us to disclose TFs predicted to be involved in regulation of distinct biological processes. A significant role for some of these TFs in thrips defense regulation was confirmed through mutant analyses. This information can greatly facilitate the development of more sustainable thrips-resistance breeding.

RESULTS AND DISCUSSION

Genome-wide dynamic transcriptional changes in single Arabidopsis leaves upon thrips infestation

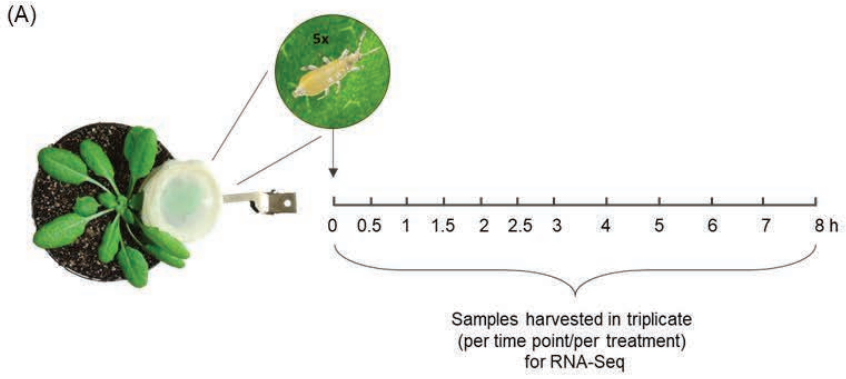
To study the dynamics of thrips-induced transcriptional changes in Arabidopsis over time, we performed a high-resolution time series of the thrips-induced transcriptional response in Arabidopsis leaves using RNA-seq. True leaf number 9 from three independent five-week-old Col-0 plants was treated with either an empty clip cage (Mock) or a clip cage containing five L2 thrips larvae. The plant stayed intact during the assay, meaning that the treated leaf stayed

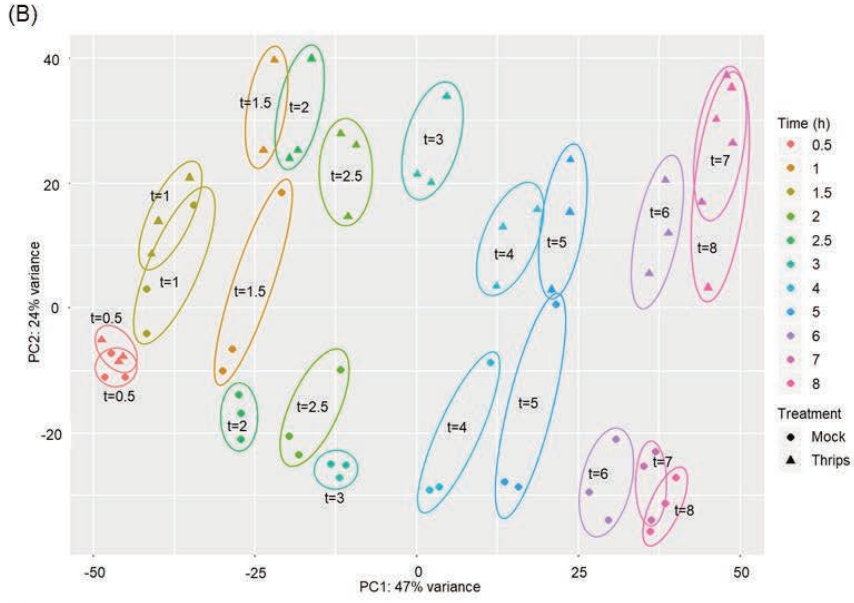
attached to the plant until it was harvested at the indicated time point. We chose L2 larvae because the cell-ingestion rate was highest by this stage compared to the other stages (Kindt *et al.,* 2003), which consequently could result in a more immediate and stronger plant response upon introduction of the thrips on the leaf. We harvested independent samples in triplicate (from three plants; one leaf per plant) at 20 consecutive time points over a 9-h period (0, 0.5, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8 and 9 h; Supplemental Data Set 1). We started with profiling the transcriptional expression of one biological replicate of the whole time series of thrips-infested tissue (Supplemental Fig. 1). Based on showing a major shift in gene expression and/or being part of a logical time range, we selected from these 20 single pilot RNA-seq data time points 12 time points (every 0.5 h, from 0 h until 3 h, followed by every h until 8 h) for performing RNA-seq on the triplicate mock and thrips-infested samples (Fig. 1A). To identify thrips-responsive genes, the RNA-seq data sets were fitted to a generalized linear model that allowed the identification of genes being differentially expressed upon thrips infestation relative to the mock treatment over time. In total 2788 differentially expressed genes (DEGs) were identified (Supplemental Data Set 2).

To visualize expression variation between the replicates, treatments and time points, a principal component analysis (PCA) was conducted on the normalized expression values of the DEGs (Supplemental Data Set 3). The PCA revealed a clear separation of the two components treatment and time, which explained 24% and 47% of the variance, respectively (Fig. 1B). In the first two time points, the thrips and mock samples were still clustered together. From 1.5 h onwards, the different treatments started to separate from each other, indicating the start of transcriptional differentiation as a result of thrips infestation. The number of thrips-responsive DEGs was highest at the 2, 2.5, 3, 6, 7 and 8 h time points (Fig. 1C; Supplemental Data Set 4), which was associated with greater separation between the thrips-infested and mock samples in the PCA (Fig. 1B). The 4 and 5 h time points displayed relatively fewer DEGs, which was correlated with higher variance levels between the biological triplicates at these time points (Fig. 1B and 1C).

Interplay between thrips, MeJA and *P. rapae* signaling pathways

We compared the thrips-induced DEGs set with the DEGs sets derived from high-density time series RNA-seq experiments on exogenous application of methyl JA (MeJA) (Hickman *et al.,* 2017) and feeding by the leaf-chewing caterpillar *Pieris rapae* (Coolen *et al.,* 2016). We found that half (51.1%) of the thrips-responsive DEGs (n = 1424) were also differentially expressed by MeJA treatment (Fig. 2A; Supplemental Data Set 5). From these, 893 DEGs were upregulated and 335 DEGs were downregulated by both thrips and MeJA (Fig. 2B and C), suggesting a role for JA especially in the upregulation of thrips-responsive genes.


(A)
5x
0 0.5 1 1.5 2 2.5 3 4 5 6 7 8 h
Samples harvested in triplicate
(per time point/per treatment)
for RNA-Seq


(B)
PC2: 24% variance
PC1: 47% variance
Time (h)
0.5
1
1.5
2
2.5
3
4
5
6
7
8
Treatment
Mock
Thrips

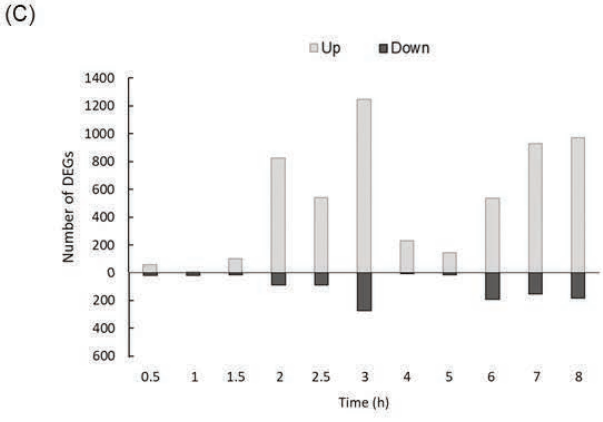

(C)
Up
Down
Number of DEGs
Time (h)

Figure 1: Experimental set-up and dynamics of transcriptional changes of the high-density time series experiment performed on Arabidopsis upon thrips infestation over time. (A) Experimental set-up of the high-density time series experiment. Leaf number 9 of 5-week-old wild-type Arabidopsis (Col-0) plants was treated with either five L2 thrips larvae that were contained by a clip cage or just received an empty clip cage (Mock). At every indicated time point, three biological replicates of the mock and thrips-infested leaves were harvested and snap frozen. Whole-genome transcriptional expression was profiled using RNA-seq technology. **(B)** Principal component analysis (PCA) of the 2788 DEGs during an 8-h thrips or mock treatment. The different colors and symbol shapes indicate the different time points and treatments, respectively. The biological replicates per time point/treatment are encircled. **(C)** The total number of thrips responsive genes per time point showing significant up- or downregulation. A pairwise comparison was performed between mock- and thrips-treated samples at singular time points using the set of 2788 DEGs during thrips infestation over time, as identified by GLM analysis. Light and dark gray indicate upregulation and downregulation of expression, respectively.

Furthermore, 59.8% of the thrips-responsive DEGs (n = 1666) were also differentially induced by *P. rapae* (Fig. 2A), of which 1186 DEGs were upregulated and 230 DEGs were downregulated by both insect herbivores; 62% of the overlapping insect-induced DEGs were also induced by MeJA treatment in the same direction (Fig. 2B and C). The shared insect-induced DEG sets contain genes that are essential for the biosynthesis of JA (e.g. *LOX2* and *AOCs*) and the regulation of the JA response (e.g. *JAZs* and *MYC2*) (Supplemental Data Set 5; Wasternack & Hause, 2013), which confirms the central role of JA in defense responses to insects. This large overlap that we detect between the thrips-, MeJA- and *P. rapae*-responsive DEGs, indicates a major role of JA in the regulation of plant responses to insects of different feeding guilds. Moreover, it also validates the biological relevance of studies using exogenous MeJA as a proxy for the JA pathway component in insect-induced responses.

Amongst the thrips-induced DEGs set, 28.2% (n = 513) and 55.2% (n = 534) of the up- and downregulated DEGs, respectively, were exclusively responsive to thrips and not to MeJA treatment or *Pieris rapae* infestation (Fig. 2B and C). Interestingly, in the specific, upregulated DEGs set, genes involved in the defense response to fungi and bacteria and SA-associated processes were found, amongst which *WRKY60* (Xu *et al.,* 2006), *AZI1* (Jung *et al.,* 2009) and *PBS3* (Nobuta *et al.,* 2007). In the specific, downregulated set, genes involved in abiotic (photoperiodic) stimulus response were found to be overrepresented amongst which the TFs *HY5* and *PRR5* (Burko *et al.,* 2020; Li *et al.,* 2020). The exclusive thrips-responsive DEGs might have specific functionality in plant resistance to cell-content-feeding insects like thrips.

Clustering of DEG expression profiles and characterization of biological processes

To visualize the overall spatiotemporal regulation of genes and to separate the thrips-responsive DEGs into tightly regulated groups of co-expressed genes with similar expression profiles, we used the time-series clustering algorithm SplineCluster (Heard *et al.,* 2006).

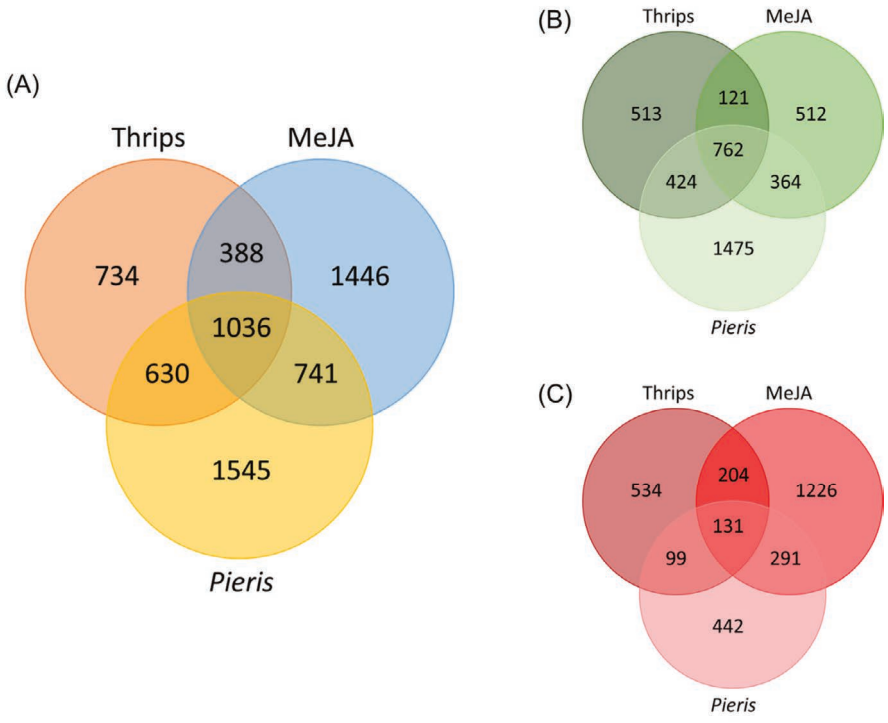


Figure 2: Venn diagrams showing the number of DEGs and their overlap between time series experiments with thrips, MeJA and *Pieris rapae.* (A) The total number of DEGs of all three treatments, **(B)** the upregulated DEGs and **(C)** the downregulated DEGs in comparison to their respective mock treatments (Supplemental Data Set 5).

This led to grouping of the 2788 DEGs into 20 clusters (c1 to c20) of genes that share similar expression profiles (Fig. 3). Based on hierarchical clustering of the clusters (Fig. 3A), clusters 1 to 12 (c1-c12) were considered as the clusters containing genes that were upregulated in response to thrips feeding, while clusters 13 to 20 (c13-c20) were considered as the clusters containing the genes that were downregulated by thrips feeding (Supplemental Data set 6). Many clusters showed a sustained induced expression pattern (Fig. 3B), while a transient change in expression was displayed by only a few clusters. This is different from the dominating transient expression behavior of clusters identified in our previously analyzed high-resolution time series of plants that had received a one-time treatment with exogenous MeJA, in which case a temporary JA signal was perceived (Hickman *et al.,* 2017) instead of ongoing signals such as during infestation by the thrips in this study.

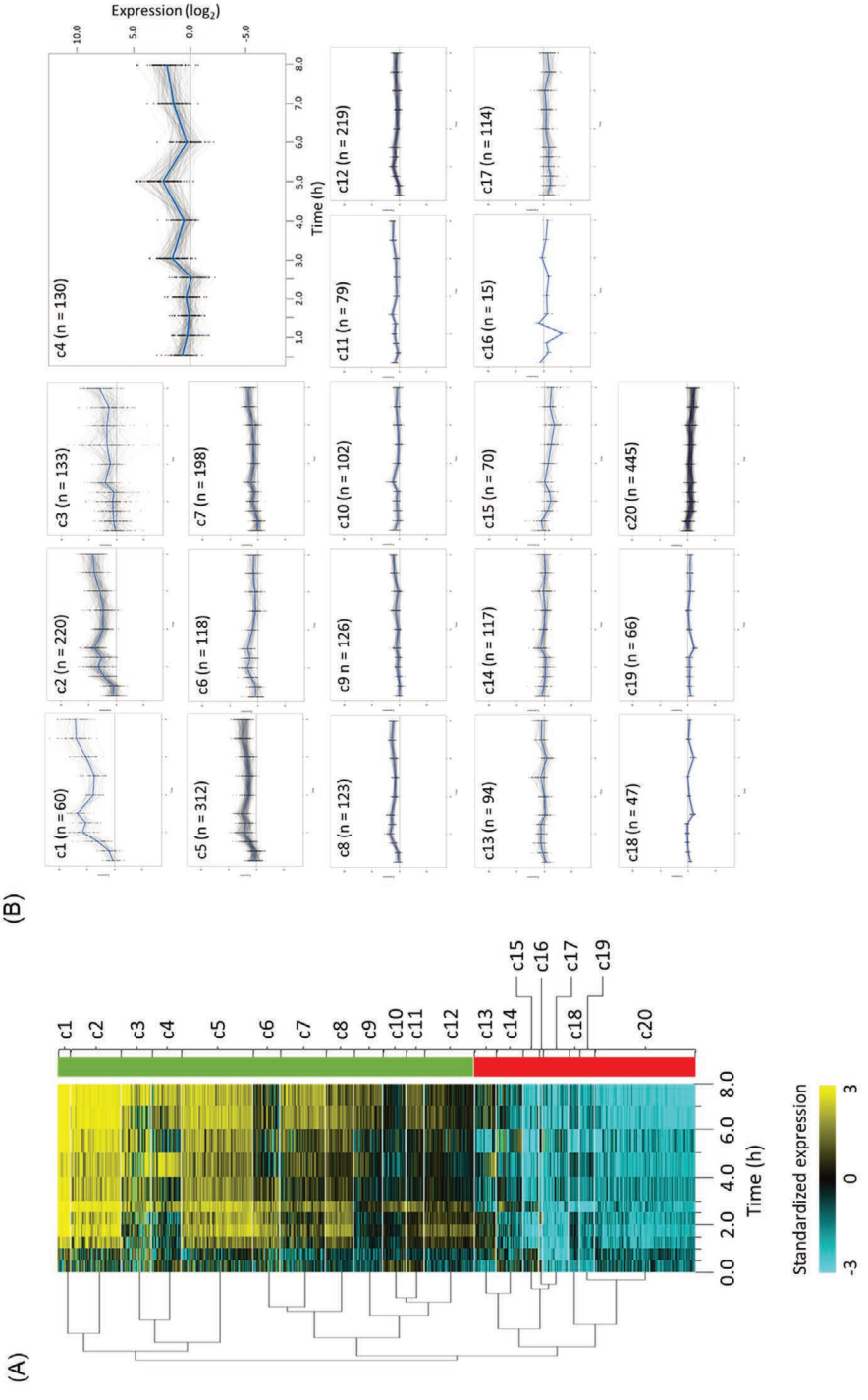

(A)
c1
c2
c3
c4
c5
c6
c7
c8
c9
c10
c11
c12
c13
c14
c15
c16
c17
c18
c19
c20
0.0
2.0
4.0
6.0
8.0
Time (h)
Standardized expression
-3
0
3
(B)
Expression (log2)
10.0
5.0
0.0
-5.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Time (h)
c1 (n = 60)
c2 (n = 220)
c3 (n = 133)
c4 (n = 130)
c5 (n = 312)
c6 (n = 118)
c7 (n = 198)
c8 (n = 123)
c9 n = 126)
c10 (n = 102)
c11 (n = 79)
c12 (n = 219)
c13 (n = 94)
c14 (n = 117)
c15 (n = 70)
c16 (n = 15)
c17 (n = 114)
c18 (n = 47)
c19 (n = 66)
c20 (n = 445)

Figure 3: Expression profiles in clusters of thrips-induced co-expressed DEGs. **(A)** SplineCluster analysis partitioned the set of 2788 genes showing differential expression in response to thrips into twenty distinct co-expressed gene clusters (c1–c20). Each row of the heatmap represents an individual gene and indicates level of expression, with yellow and blue indicating increased and decreased expression (standardized on a per gene basis across mock and thrips treatments), respectively. The green and red bar indicate the annotation of clusters as upregulated (c1-c12) or downregulated (c13-c20), respectively. **(B)** The individual gene expression profiles (log_2-fold change [thrips/mock]), y-axes) of clusters c1-c20 are shown as grey lines with the mean expression level in blue. The x-axes indicate the time in h past introduction of thrips on the leaf. Both axes' scales are the same over all the graphs; cluster c4 is enlarged as an example, showing the labeling of the axes in detail. See Table 1 for associations of the cluster's GO terms and examples of genes (Supplemental Data Set 6).

Next, we investigated in which biological processes the co-expressed DEGs in each cluster of the thrips experiment were involved. Therefore, overrepresentation of the DEGs for functional categories was analyzed for Gene Ontology (GO) term enrichment (Maere *et al.,* 2005) (Supplemental Data set 6). Not surprisingly, multiple upregulated clusters were overrepresented for functional terms associated with JA signaling, e.g., 'response to jasmonic acid', 'response to wounding' and rather general terms such as 'response to stress', 'response to stimulus' and 'response to other organism' (Table 1). Yet, it is evident from their partitioning in different clusters that the DEGs are at least partly differently regulated over time, leading to distinct expression behaviors in the different clusters. In addition, some functional categories were linked solely to a single cluster. For instance, from the clusters representing upregulated genes, cluster c8 showed specific enrichment in genes involved in 'tryptophan metabolic process' and 'indole-containing compound metabolic process' (Table 1). Tryptophan is a precursor of indole-containing secondary metabolites, such as glucosinolates that are involved in plant defense against herbivores (Radwanski & Last, 1995), although to the best of our knowledge they have not yet been reported to contribute to resistance to thrips. Apart from clusters associated to JA signaling, there are a few clusters pointing to a possible involvement of SA signaling in the thrips-induced GRN. For example, c4 shows enrichment of genes that are associated with 'response to bacteria' such as *PBS3* (Table 1), which encodes for an enzyme that is involved in disease-resistance signaling and is required for the accumulation of SA (Nobuta *et al.,* 2007). Amongst the downregulated clusters, a GO term associated to auxin ('response to auxin') is specifically enriched in c20 (Table 1). This likely reflects the trade-off of plants to switch their resources from growth to defense (Züst & Agrawal, 2017). A selection of (well-studied) genes and the most important GO terms that belong to the different clusters are listed in Table 1. The diverse and specific functional enrichment of the distinct gene clusters shows that our data set captured enough information to identify different regulatory processes within the thrips-induced GRN that are associated with distinct biological functions.

Table 1: Functional enrichment associated with genes in the 20 co-expressed gene clusters showing responsiveness to thrips. The top-most significant GO terms within each cluster, maximized to five and having a *P*-value <0.01. GO terms were assigned by BiNGO and further summarized using REVIGO. Cluster 1-12 contain upregulated clusters/processes, cluster 13-20 downregulated clusters/processes. Asterisks behind specific GO terms indicate GO terms that are linked solely to a single cluster within the thrips data set. The final column displays examples of genes that are associated with the GO term and present within the respective cluster. A dash indicates that there is no significant enrichment of GO terms, and thus no examples are genes are depicted either. Full results used to derive this table are available in Supplemental Data Set 6.

Cluster number	GO term	*P*-value	Example genes
1	Response to jasmonic acid	9.96E-06	*VSP1, MYB15*
	Response to wounding	1.43E-04	*JAZ10, TPS03*
	Response to organic substance	2.82E-04	*ERF11, ERF13*
	Response to stress	1.97E-03	*WRKY62, RAP2.6*
2	Response to stimulus	1.00.E-10	*GRX480, ORA47*
	Response to stress	1.90E-09	*ERF1, OPR3*
	Response to wounding	3.24E-08	*VSP2, THI2.1*
	Response to chemical	4.36E-07	*STZ, WRKY40*
	Defense response	5.27E-07	*WRKY48, LOX3*
3	Response to stimulus	3.90E-09	*ORA59, PDF1.2*
	Response to stress	9.40E-09	*PR4, HSFA2*
	Response to biotic stimulus	1.77E-08	*DGL, CYP81D11*
	Response to other organism	7.15E-07	*PYK10, AIG1*
	Defense response	1.44E-05	*AZI1, PDF1.4*
4	Multi-organism process	4.12E-06	*NPF5.2, DMR6*
	Response to other organism	2.66E-05	*WRKY60, PR5*
	Response to biotic stimulus	4.74E-05	*HSP90.1, MES9*
	Response to bacterium	1.70E-03	*PAD4, PBS3*
5	Response to stimulus	1.00E-300	*MYC2, LOX2*
	Response to other organism	1.00E-300	*EDS5, bHLH017*
	Response to wounding	1.00E-300	*JMT, AOS*
	Response to jasmonic acid	1.00E-300	*JAZ1, WRKY18*
	Response to stress	1.00E-300	*ERF2, MYB108*
6	-	-	-
7	Response to stimulus	2.00E-03	*HSFB2A, MYB34*
8	Tryptophan metabolic process*	3.00E-10	*CYP83B1, TSB2*
	Indolalkylamine metabolic process	3.00E-10	*ASB1, ASA1*
	Indole-containing compound biosynthetic process	5.00E-10	*CYP79B2, IGPS*
	Indole-containing compound metabolic process*	1.30E-09	*TSA1, WRKY33*
	Carboxylic acid biosynthetic process	4.10E-09	*ACX1, CYT1*
9	Response to endoplasmic reticulum stress*	2.69E-03	*PDIL1-2, UNE5*
10	-	-	-
11	Response to chitin	5.26E-06	*WRKY22, ANAC062*
	Response to carbohydrate	3.53E-04	*ATL2, PUB24*

12	Sulfur compound metabolic process	7.42E-04	*APK, APS3*
	Response to stimulus	1.36E-03	*ABI1, HB-7*
	Response to chemical	3.67E-03	*MYB77, RGL1*
13	Response to heat	1.00E-300	*MBF1C, HSP101*
	Response to temperature stimulus*	1.81E-07	*MBF1C, EGY3*
	Protein folding*	3.25E-07	*CPN60BETA2, BIP1*
	Response to abiotic stimulus	1.63E-05	*ABCI21, JAR1*
	Response to hydrogen peroxide	5.08E-05	*BAG6, HSP70*
14	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	Translation	1.51E-08	*PRL24A*
	Cellular macromolecule biosynthetic process	1.44E-06	*RPS6*
	Macromolecule biosynthetic process	1.59E-06	*RPL19B*
	Gene expression	3.65E-06	-
	Cellular protein metabolic process	6.63E-05	*CPN60A, BOB1*
19	-	-	-
20	Response to hormone	2.62E-08	*BRS1, TAA1*
	Response to auxin*	2.43E-07	*IAA16, SAUR6*
	Response to endogenous stimulus	3.88E-07	*CCA1, MYB43*
	Response to organic substance	8.58E-07	*TAR2, HAT2*
	Response to chemical	3.61E-03	*ATHB13, ARL*

Enrichment of TF DNA binding motifs in thrips-responsive DEGs

Transcription factors are key regulators of dynamic transcriptional changes, such as occur during thrips infestation. The overrepresentation of DNA motifs for TF binding in the promotor sequence of co-expressed thrips-responsive DEGs can aid in the understanding and reconstruction of the thrips-induced GRN. We screened for overrepresentation of these motifs in each of the up- and downregulated clusters of co-expressed genes (Supplemental Data Set 7). The motifs corresponding to the DNA binding sites of bHLH and bZIP TFs were found to be overrepresented in the upregulated genes within clusters c1, c2, c5, c6, c7, c8, c9 and c12 (Fig. 4). Members of these two TF families are known key regulators of JA-associated processes (Goossens *et al.,* 2016; Hickman *et al.,* 2017). Within the bHLH TF family, the subclass bHLH IIIe contains the well-known bHLH TF MYC2 (Goossens *et al.,* 2016), which is represented as a DEG in cluster c5 (Table 1). The MYC2 TF directly targets a large group of genes, among which hundreds of genes that encode TFs themselves, thereby shaping a large part of the JA-responsive GRN (Hickman *et al.,* 2017; Zander *et al.,* 2020). Additionally, MYC2 is an important player in the regulation of defenses against thrips (Thoen, 2019; Wu *et al.,* 2019). The overrepresentation of bHLH binding sites in many of the thrips-upregulated clusters corroborates the key role of MYC2 and other bHLH TFs such as bHLH017 (Table 1) in the

thrips GRN.

It has been shown that members of the bZIP TF family are essential for the activation of JA- and ET-dependent defense mechanisms but are also required for the synthesis of SA. Most bZIP TFs bind to an ACGT core sequence within an A-box (TACGTA), C-box (GACGTC), or G-box (CACGTG) (Jakoby *et al.,* 2002). Cluster c1 shows an overrepresentation of bZIP binding motifs containing a G-box sequence (Fig. 4), which can also be bound by bHLH TFs. Other clusters that show a significant overrepresentation of the G-box are clusters c2, c5, c6, c7, c8, c9, c10 and c12. In contrast, the C-box was significantly overrepresented in clusters c4 and c7 (Fig. 4). There are only four bZIPs reported to bind to the C-box (bZIP9, bZIP10, bZIP25 and bZIP63) (Dröge-Laser *et al.,* 2018), but none of them were DEGs in our thrips data set. Moreover, neither one of their interactors was transcriptionally induced in our thrips data set (The Biological General Repository for Interaction Data sets (https://thebiogrid.org) (Oughtred *et al.,* 2019)). This suggests that either these C-box containing genes are regulated by yet unknown TFs or that the bZIPs are post-transcriptionally modified by thrips infestation. Protein phosphorylation and protein interaction have been demonstrated to control bZIP activity (Dröge-Laser *et al.,* 2018). In the case of bZIP10 this leads to stimulation of pathogen-induced hypersensitive response, basal defense responses and reactive oxygen-induced cell death (Kaminaka *et al.,* 2006), which is in line with the enriched biological functions of genes belonging to cluster c4 of our thrips-induced network.

WRKY binding sites are overrepresented in clusters c4, c5, c10 and c11 (Fig. 4). WRKYs are known to be involved in stress-related abiotic and biotic responses, among which SA- and ABA-associated processes (Pandey & Somssich, 2009). In accordance with the W-box enrichment, several WRKY genes are present in clusters c4, c5 and c11 (Table 1). Amongst these WRKYs are *WRKY60* and *WRKY18* that are known to be induced in response to SA (Xu *et al.,* 2006) and ABA (Chen *et al.,* 2010). Another member is *WRKY22*, which is induced by SA and upon pathogen recognition (Dong *et al.,* 2003) but also during feeding by the aphid *Myzus persicae* (Kloth *et al.,* 2016), which is a sucking insect herbivore. WRKY22 was hypothesized to modulate the interplay between the SA and JA pathways, making Arabidopsis more susceptible to *M. persicae* (Kloth *et al.,* 2016).

In the set of downregulated genes, enrichment of specific TF binding sites was less prevalent (Fig. 4). There was a slight overrepresentation of bHLH and bZIP binding sites in c13, c14 and c20. MYB-related TF motifs were specifically overrepresented in cluster c14. MYB-related TFs are reported as regulators of the circadian clock and anthocyanin biosynthesis (Nguyen & Lee, 2016). No significant overrepresentation of a biological process was detected in this cluster by our GO term analysis (Table 1). However, *PRR5* is present in c14, which acts as a transcriptional repressor of the circadian clock-associated MYB TF CCA1 (c20) (Nakamichi *et al.,* 2012) (Supplemental Data Set 6).

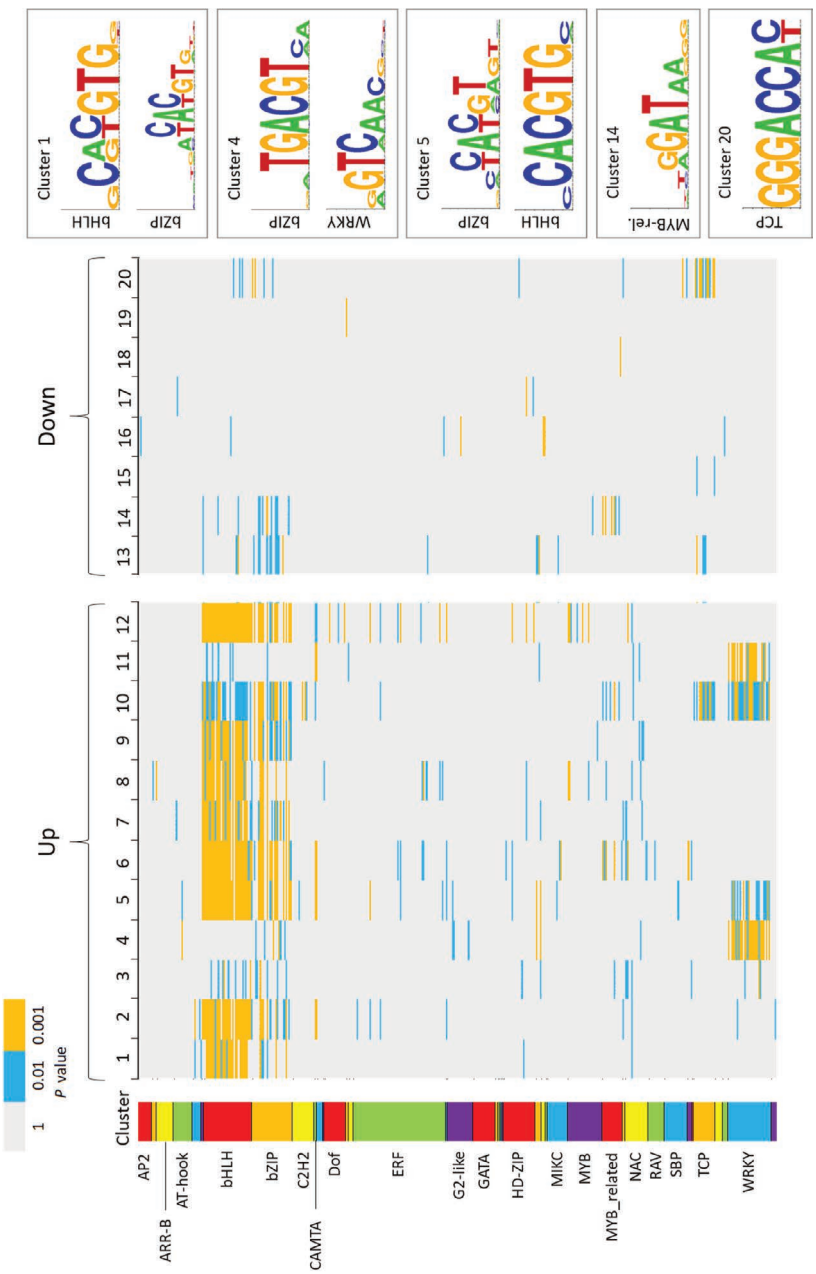


Figure 4: Motif enrichment in clusters of thrips-induced co-expressed DEGs. Overrepresentation of 441 known TF DNA-binding motifs within each distinct thrips-responsive cluster. Rows indicate motifs, yellow and blue indicate overrepresentation of motifs with $P < 0.001$ or $P < 0.01$, respectively. On the right, sequence logos of selected forward motifs that are significantly overrepresented in some of the clusters are depicted. Full results used to derive this figure are available in Supplemental Data Set 7.

Moreover, the AthaMap web tool (Hehl & Bülow, 2014) revealed that the upstream region of *PRR5* itself can be bound by 7 TFs of the MYB TF family, amongst which are *CCA1* and *MYB59*, which are present in our data set (in c20 and c9, respectively). TCP binding motifs were specifically overrepresented in cluster c20 which could link to the modulation of sets of target genes involved in plant growth and development (Li *et al.,* 2015). In line with this, auxin-associated genes, encoding for SAUR-like auxin-responsive proteins (Danisman *et al.,* 2013), IAA16 (Rinaldi *et al.,* 2012) and HAT2 (Challa *et al.,* 2019), are downregulated in cluster c20 (Table 1). Collectively, the downregulation of genes associated with growth and development, which requires a functional circadian system (Inoue *et al.,* 2018), accompanied the upregulation of genes associated with defense, reflecting the trade-off between growth and defense (Huot *et al.,* 2014).

Chronology of thrips-induced gene expression and biological processes

To gain insight into the chronology and temporal regulation of the dynamic thrips-induced responses, we determined the timepoint at which each gene became differentially expressed for the first time. A pairwise comparison analysis was conducted on singular time points to determine which genes were differentially expressed at that given time point, of which a portion was differentially expressed for the first time (ftode = first time of differential expression; Supplemental Data Set 4). Genes were assigned as up- or downregulated genes according to their clustering (c1-c12 containing upregulated DEGs, c13-c20 containing downregulated DEGs). Within each set of genes that became up- or downregulated for the first time per time point, the number of TF genes was determined. Within 3 h of thrips infestation, most of the total number of thrips-induced DEGs were induced for the first time (>80% of the upregulated DEGs and >55% of the downregulated DEGs). The least transcriptional changes were detected at the 0.5, 1, 4 and 5 h time points, but the latter two time points also contained most biological variation in their respective mock samples, which reduces the statistical power in comparisons (Fig. 1B). The highest number of genes that became differentially expressed for the first time was at 2 h for the upregulated genes and at 3 h for the downregulated genes (Table 2). Within the first 0.5 h, eight TF genes were differentially expressed, and these were assigned as DEGs at multiple time points, suggesting that they have a key role in determining the architecture of the thrips GRN. For example, *ERF11* and *ANAC090* were already induced at 0.5 h and were DEGs at ≥ 4 time points. Of the 207 TF genes induced in our time series, 46 were significantly induced at more than 5 of the studied time points, among which *MYC2*, *ORA47*, *ERF1*, and *WRKY40*, suggesting that they play prominent roles in shaping the GRN. On the other hand, 59 TFs were significantly differentially expressed at only one of the assayed time points. This suggests that these genes have a temporary effect on the thrips-induced GRN. An example of such a transient gene is WRKY22, which, as described previously,

belongs to the WRKY DNA-binding motif-enriched cluster c11 and is reported to be a potential target in Arabidopsis for *M. persicae* to promote host plant susceptibility (Kloth *et al.,* 2016).

Table 2: Number of DEGs up- or downregulated for the first time during thrips infestation. For each time point the total number of DEGs is presented as well as the number of DEGs that are differentially expressed for the first time (ftode = first time of differential expression) at that given time point. The number of TFs within the ftode gene set is specifically indicated (TFs ftode) per time point. Full results used to derive this table are available in Supplemental Data Set 4.

Up				Down			
Time point (h)	Total	ftode	TFs ftode	Time point (h)	Total	ftode	TFs ftode
0.5	50	50	8	0.5	26	26	4
1	4	3	1	1	20	18	4
1.5	101	97	15	1.5	19	14	0
2	814	742	86	2	99	94	16
2.5	540	69	11	2.5	88	72	14
3	1221	513	63	3	303	292	33
4	233	18	0	4	8	6	1
5	136	13	1	5	28	23	2
6	526	54	4	6	203	172	18
7	920	128	9	7	159	116	6
8	967	133	8	8	188	135	8
Total	5512	1820	206	**Total**	1141	968	106

To investigate the chronology of biological processes that were altered during thrips infestation, we screened different time frames of ftode genes for overrepresentation of functional categories. Generally, different processes were induced in distinct time frames (Fig. 5A; Supplemental Data Sets 8-10). Within the first 3 h, the upregulated genes that became differentially expressed over time were associated with processes such as ‘innate immune response’, ’response to wounding’, ‘response to jasmonic acid’ and ‘response to chitin’. From 3 h onward, genes associated with ’response to oxidative stress’ and ‘phenylpropanoid biosynthetic process’ became activated (Fig. 5A), and furthermore, new genes belonging to the aforementioned categories became differentially expressed after 3-8 h as well. Within the downregulated set of genes, fewer GO terms became enriched over time (Fig. 5A); these were mainly involved in growth and development (‘response to auxin’ and ’shoot system development’ (Mller & Leyser, 2011)) or associated with the synthesis of metabolites such as ‘glycosides’, which have been demonstrated to be related to thrips resistance in *Capsicum* spp. (Macel *et al.,* 2019; Maharijaya *et al.,* 2019).

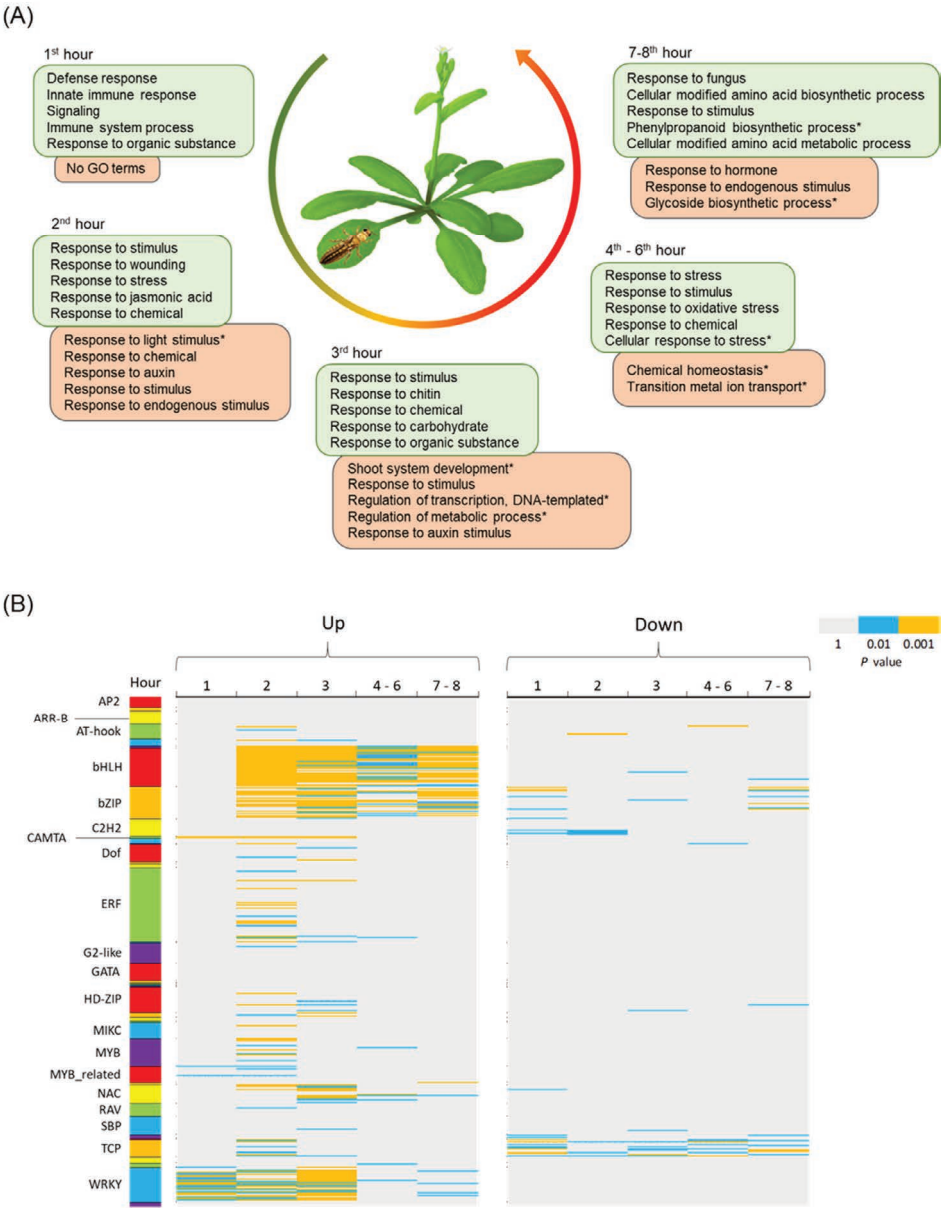


Figure 5: Chronology of thrips-induced biological processes and motif enrichment in genes that become differentially expressed for the first time within a certain time frame. (A) Overview of the biological processes of DEGs that were induced for the first time at different time frames, with green and red indicating the top-most significant GO terms within each time frame, maximized to five, of upregulated and downregulated processes ($P < 0.01$, and the GO term should contain at least five DEGs). DEGs were grouped in time frames of 1 h for the first 3 hours and subsequently in time frames of multiple hours based on the number of DEGs that became differentially expressed for the first time. These groups of DEGs were subsequently analyzed for GO term overrepresentation.

Asterisks behind specific GO terms indicate GO terms that are linked solely to a single time frame within the thrips data set. **(B)** Enrichment of 441 known TF DNA-binding motifs in up- and downregulated DEGs that were differentially expressed for the first time per time frame in response to thrips infestation. Rows indicate motifs, with yellow and blue indicating a minimum *P*-value of 0.001 and 0.01, respectively. Full results used to derive this figure are available in Supplemental Data Sets 8-11.

Overrepresentation of TF binding sites during temporal gene expression

Next, the enrichment of DNA binding motifs of ftode genes within each time frame was analyzed to associate timing of potential TF activity with temporal induction of specific DEGs and biological processes (Fig. 5B; Supplemental Data Sets 11). Genes containing WRKY TF binding motifs were upregulated within the 1st hour of thrips feeding. Since WRKY genes themselves were not among the DEGs in the 1st hour, the presence of the WRKY binding motifs may be related to posttranslational regulation of WRKY proteins. The *WRKY18* and *WRKY40* genes were induced in the 2nd hour; both genes are also induced by *Spodoptera littoralis* infestation and play a role in resistance against this herbivore (Schweizer *et al.,* 2013). In addition, the CAMTA motif showed a similar enrichment pattern over time as the WRKY motif: they were present in genes within the first 3 h of thrips infestation. This finding confirms the link between CAMTAs and WRKYs during biotic stress responses (Galon *et al.,* 2008). Additionally, a recent paper by Bjornson *et al.* (2021) corroborated the role of post transcriptionally regulated CAMTAs in early general stress responses. The six known CAMTA TFs in Arabidopsis regulate the expression of genes that impart abiotic stress tolerance and SA-mediated immunity (Kim *et al.,* 2019). This may point to a role for CAMTAs in regulating responses to thrips infestation, possibly by suppression of SA signaling to aid enhancement of effective JA signaling. Enriched binding sites for a large variety of TFs was observed in the 2nd hour of thrips infestation (bHLH, bZIP, ERF, MYB and TCP TFs) (Fig. 5B), which is in line with the large number of genes and GO terms within this time frame (Table 2 and Supplemental Data Set 4, 8 and 9). Starting from the 3rd hour, mainly bHLH TF binding sites remained being enriched whereas this was not the case for the other previously engaged TFs, supporting the role of bHLH TFs as key activators over a broad time range of our experiment. Finally, within the 3rd hour there was also enrichment in NAC TF binding sites (Fig. 5B). Genes within the NAC TF family can be involved in both stress responses and development (Chen *et al.,* 2008). An example of a NAC TF within the set of upregulated genes 3h after thrips infestation is *ANAC062*, which is a member of all five top-most significant GO terms during the 3rd hour of thrips infestation (Fig. 5A; Supplemental Data Set 8). *ANAC062*, also known as *NTL6*, was found to be induced in response to cold stress, ABA (Seo & Park, 2010) and chitin (Libault *et al.,* 2007).

In the downregulated DEGs, a relatively small overrepresentation of bZIP binding sites was detected within the 1st h, and at the last time frame (7-8 h). Clearly, TCP binding sites were

enriched over the whole time course. An overrepresentation of genes with a C2H2 binding site were found to be enriched in the 2nd h and 4-6 h. C2H2 TFs play an important role in the regulation of abiotic stress responses in plants (Han *et al.,* 2020). These findings point to important roles for members of multiple TF families, i.e., bHLH, bZIP, ERF, MYB, NAC, WRKY, CAMTA, C2H2 and TCP, as key regulators of temporal gene expression leading to activation or repression of specific biological processes in time as part of the response to thrips feeding.

Gene regulatory network modelling

The analyses above revealed a broad array of different regulatory motifs that underlie thrips-mediated transcriptional reprogramming that is associated with specific biological processes. Here, we extend these analyses to improve our prediction of key TFs that drive temporal expression of specific co-expressed genes. We used DREM (Dynamic Regulatory Events Miner) (Schulz *et al.,* 2012) to infer a dynamic thrips GRN model. DREM defines paths of co-expressed genes and identifies bifurcation points from which groups of co-expressed genes begin to diverge. Using our time series expression data as input, DREM defined a GRN model with 8 unique paths to which genes with similar expression patterns were assigned (Fig. 6A). A total of 9 bifurcation nodes were identified in the network, of which 7 appeared within the first 3 h, which makes sense since by then most genes had become differentially expressed (Fig. 1C; Table 2). These bifurcation events point to complexity of the network that is controlled by multiple TFs at certain time points.

First, we wanted to evaluate to what extent the genes within the co-expressed clusters that we previously identified with SplineCluster (Fig. 3B) were present in the 8 co-expressed DREM paths (Fig. 6B, Supplemental Fig. 2, Supplemental Data Set 12). Each path contained genes of multiple clusters, but 15 out of the 20 clusters were assigned primarily to one path only (containing ≥ 60% of their genes). For example, path 1 was mainly represented by genes from clusters c1 (95.0%) and c2 (67.3%), but this path also contained genes from c3 (6.2%) and c5 (25.7%). Meanwhile, c3 was more represented in path 2 (42.6%) and 6 (33.3%). The downregulated genes of clusters c15-c20 were all represented by path 8, which contains a temporary split between 1 h and 3 h, after which the two sub-paths merge again. The overrepresentation of multiple SplineCluster clusters within a single DREM path suggests a tight transcriptional coregulation of these clusters and may therefore improve the prediction of responsible TFs. Likewise, splitting up of clusters over different paths disentangles specific regulation patterns.

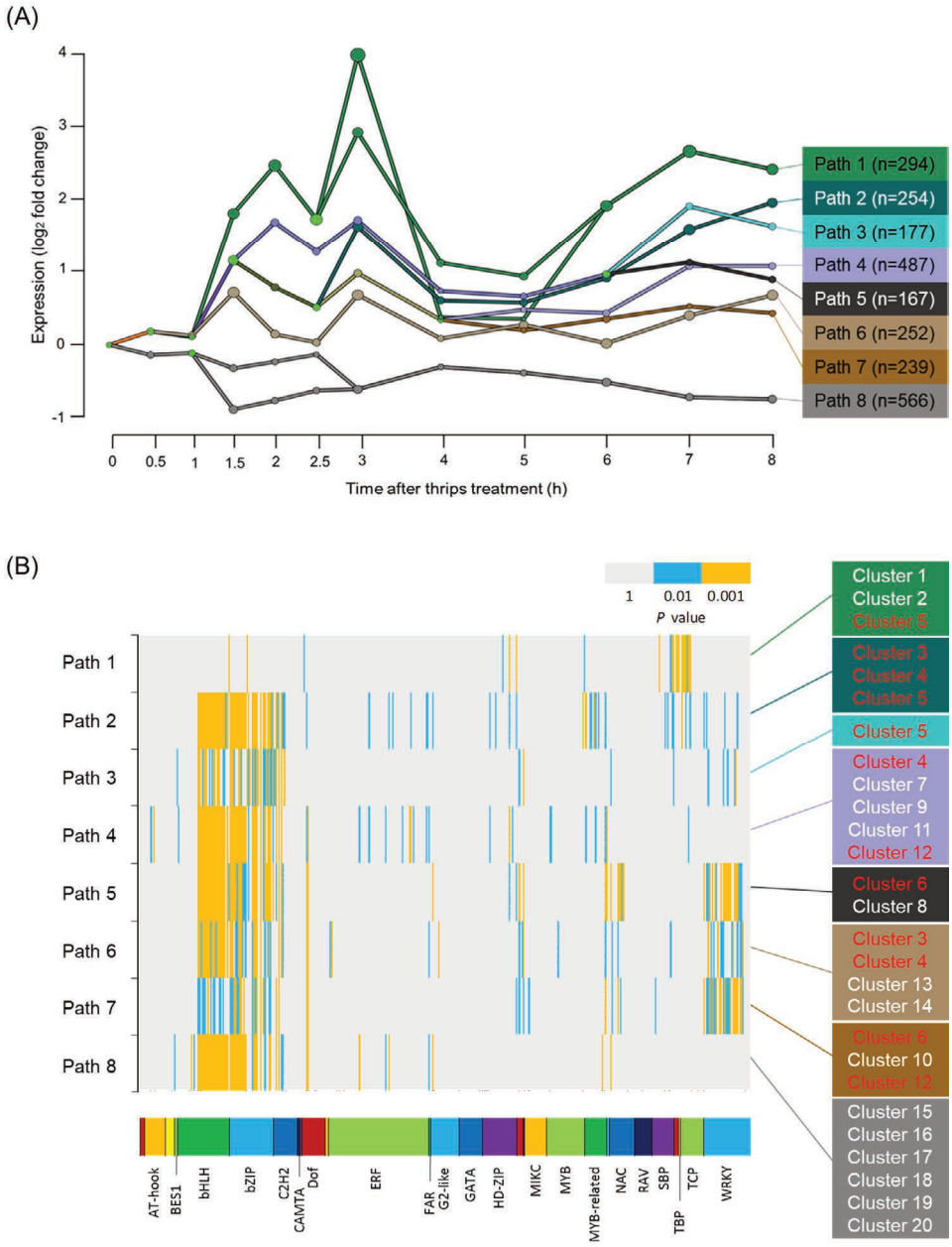


Figure 6: DREM-based GRN model and motif enrichment of thrips-induced DEGs in co-expression paths. **(A)** GRN model constructed by DREM 2.0. The x-axis represents a scaled timeline of the experiment. The y-axis represents the level of gene expression (log_2-fold change [thrips/mock]) normalized to the expression at the 0 h time point. Each line represents a path of co-expressed genes. Split nodes (light green) represent points at which paths diverge. **(Continues on next page)**

Figure 6 (continued). (B) Overrepresentation of 441 known TF DNA-binding motifs in genes present within each DREM path. Rows indicate the different DREM paths. Columns represent the TF DNA-binding motifs, as indicated by the bar under the rows. Yellow and blue indicate an overrepresentation with a minimum *P*-value of 0.001 and 0.01, respectively. The boxes on the right represent the overrepresented clusters as designated by SplineCluster (Fig. 3A). White-lettered-clusters indicate clusters of which ≥60% of the genes within that cluster are present in the indicated path and thus overrepresented in a single path; red-lettered-clusters indicate clusters of which 25%-60% of the genes within that cluster are present in the indicated path and are thus overrepresented in multiple clusters. Full results used to derive this figure are available in Supplemental Data Sets 12 and 13.

Next, we analyzed each DREM path for overrepresented binding sites for TF families and determined the biological coherence associated to these paths (Fig. 6B; Table 3; Supplemental Data set 12 and 13). As expected, most paths (path 1-7) showed an overrepresentation of genes with bHLH and bZIP TF binding sites (Fig. 6B). Especially DREM path 1 harbored the typical herbivory-related processes such as 'response to wounding' and 'response to jasmonic acid' and contained the JA master regulator TF MYC2, which contributed to the top 5 of significant GO terms (Table 3). Paths 2 and 4 showed overrepresentation of motifs that correspond to WRKY TF binding sites. DEGs within these paths mainly originated from clusters c4 and c11 (e.g., c11 members *WRKY22* (Table 1 and 3) and *WRKY25* (Table 3)). Additionally, path 4 showed some enrichment for binding sites for NAC TFs, which was more evident than in the single clusters c7, c9 and c11 from which genes were combined in path 4. Fig. 5B indicates that NAC TF binding sites started being overrepresented in the 3rd hour after thrips infestation, suggesting that NAC family members may be causally related to the splitting of path 4 (e.g., by the activation of path members *ANAC062* and *ANAC068*; Table 3) from path 2 at 2.5 h (Fig. 5B). Path 8, which mainly contained genes that were downregulated in response to thrips, specifically showed an overrepresentation of genes containing binding sites for TCP TFs. No known TCP TFs were present in this path and neither in any other path as they were not differentially expressed in our experiment, but TCPs have been suggested to be targets for posttranscriptional downregulation (Palatnik *et al.,* 2003). In summary, compared to SplineCluster, the DREM model provided more insight in the coherence of co-expressed clusters, but also in the divergence of paths that had been co-regulated until a certain time point.

Table 3: Functional enrichment associated with TF genes in the 8 DREM paths showing responsiveness to thrips. The top-most significant GO terms within each cluster, maximized to five and having *P*-values <0.01, assigned by BiNGO in Cytoscape and further summarized using REVIGO. Asterisks behind specific GO terms indicate unique GO terms within the data set. The last column displays examples of TFs that are associated with the indicated GO term within the respective DREM path, with a maximum of two example TFs. Green-colored TFs indicate selected TFs for thrips performance assays that we got homozygous mutants for orange-colored TFs indicate selected TFs that we did not succeed to get homozygous mutants for. A TF is assigned to only a single GO term and not to multiple ones within a path, unless it is the only TF or one of the only two TFs that are subjected to that term; asterisks indicate that the TF is linked to all GO terms listed per path. A dash indicates that no TFs are associated with the GO term. Full results used to derive this table are available in Supplemental Data Set 12.

Path number	GO term	*P*-value	Example TF genes
1	Response to wounding	5.53E-39	*MYC2*, WRKY40*
	Response to stimulus	1.99E-34	*ERF11, ERF13*
	Response to stress	1.02E-32	*ANAC019, ANAC072*
	Response to jasmonic acid	1.27E-25	*ANAC055, MYB47*
	Response to chemical	7.91E-24	*MYB15, STZ–*
2	Response to stimulus	1.07E-18	*CEJ1*, WRKY53**
	Response to stress	5.72E-16	*MYB2, WRKY75*
	Response to biotic stimulus	1.57E-13	*CEJ1, WRKY53*
	Response to chemical	1.53E-12	*MYB113, ERF105*
	Response to other organism	2.59E-12	*CEJ1, WRKY53*
3	Tryptophan metabolic process*	5.74E-13	*MYB34**
	Indole-containing compound metabolic process	1.55E-10	*MYB34*
	Response to stress	8.35E-10	*ERF5, HSFB1*
	Response to other organism	9.60E-10	*ERF104, MYB34*
	Response to biotic stimulus	1.88E-09	*ERF104, MYB34*
4	Response to chitin	2.51E-13	*bZIP60*, ANAC062**
	Response to stimulus	5.73E-13	*SCL-3, WRKY25*
	Response to chemical	5.50E-11	*ANAC036, ANAC068*
	Response to carbohydrate	3.11E-10	*MYB59, WRKY22*
	Response to biotic stimulus	1.18E-08	*ANAC091, WRKY8*
5	Carboxylic acid biosynthetic process	2.05E-06	-
	Response to jasmonic acid	2.71E-05	*ERF4, MYB73*
	Response to endogenous stimulus	4.03E-04	*BT4, ERF98*
	Cellular modified amino acid metabolic process	4.31E-04	*MYB75**
	Aromatic compound biosynthetic process	6.20E-04	*WRKY33, MYB75*
6	Response to stress	6.73E-15	*HSFA2*, DREB1A**
	Response to temperature stimulus	2.28E-14	*MBF1C*, DREB1C*
	Response to stimulus	5.18E-14	*WRKY60, ORA59*
	Response to abiotic stimulus	5.03E-12	*GATA12, HSFA7A*
	Response to chemical	6.46E-10	*FUS3, MBF1C*

(Continues on next page)

Table 3. (continued)

7	Response to chemical	9.58E-09	*ATL6, WRKY15*
	Response to stimulus	3.07E-07	*NPR3, PIL1*
	Response to endogenous stimulus	2.20E-06	*BIM1, ERF10*
	Oligopeptide transport	2.23E-05	-
	Cellular response to endogenous stimulus	5.84E-05	*HB-7, RGL1*
8	Response to auxin*	1.09E-06	*HAT2*, IAA4**
	Response to endogenous stimulus	1.35E-05	*CCA1*, HY5*
	Response to organic substance	5.61E-05	*MYB43, ARR7*

Thrips performance assay for biological validation of candidate regulatory genes

Each DREM path contained thrips-responsive co-expressed genes that share a high similarity in associated biological processes that are potentially regulated by a select group of TFs. Therefore, we hypothesized that by knocking out TF genes that are part of a DREM path, the regulation of that path and its associated biological responses may be perturbed, causing the plants to become more susceptible or more resistant to thrips. Potential TF candidates were selected for a thrips bioassay with mutants when the average number of normalized sequencing reads of the thrips-infested samples was ≥100 over the whole time course. Moreover, to increase our chance of selecting a TF that is specifically regulating defenses against thrips, the TF had to be more than 2-fold higher induced in the thrips experiment than in a previously reported *P. rapae* experiment (a chewing herbivore; (Coolen *et al.,* 2016) (Table 4; Supplemental Data Set 14). Using the abovementioned criteria, we isolated mutants of a total of 18 candidate TFs, representing every upregulated DREM path. Most of the selected TFs belong to path 1, which is related to their high gene expression levels and their ftode < 3 h of the start of thrips infestation (Table 4).

All mutants were subjected to a no-choice, whole plant performance assay with female thrips. Two different parameters were assessed: feeding damage and oviposition. The oviposition was only determined when a significant difference in feeding damage was observed. The JA-insensitive mutant *coi1-34* and the MYC triple mutant *myc2,3,4*, which are enhanced susceptible to thrips (**Chapter 4**; Abe *et al.,* 2009; Wu *et al.,* 2019) were used as positive controls. Indeed, *coi1-34* and *myc2,3,4* showed significantly more feeding damage and a higher number of oviposited eggs compared to the Col-0 wild-type (Fig. 7). One of the selected mutant lines, *wrky40*, also showed significantly more feeding damage (Fig. 7A) and a higher number of oviposited eggs (Fig. 7B) in comparison to Col-0. WRKY40 was assigned to DREM path 1 and is associated with GO terms such as ‘response to wounding’ and ‘response to stress’ (Table 3; Suppl Data Set 15). The *WRKY40* gene is also induced by the green leaf volatile E-2-hexenal that is produced upon wounding, herbivory or pathogen infection (Mirabella *et al.,* 2015). Moreover, induction of *WRKY40* by the chewing caterpillar *Spodoptera*

littoralis is dependent on a functional JA pathway (Schweizer *et al.,* 2013) although our previous transcriptome study revealed that *WRKY40* is not significantly induced by exogenous MeJA treatment (Hickman *et al.,* 2017). Conversely, WKRY40 itself is a positive regulator of JA signaling (Pandey *et al.,* 2010). Similar to our thrips findings, a *wrky40* mutant is enhanced susceptible to *S. littoralis* (Schweizer *et al.,* 2013).

Table 4: Selection criteria of candidate TF genes for which mutants were tested in thrips bioassays to validate the thrips GRN. Membership to a certain path (derived from DREM) and cluster (derived from SplineCluster) is indicated. First time of differential expression (ftode) is depicted. Genes were considered as potential candidates when they had an average normalized count number of ≥ 100 over the entire time course in the thrips-induced samples and were present in an upregulated cluster (c1-c12). Additionally, genes were selected if they had a more than 2-fold higher induction level (log_2-fold change) in the thrips data set than in the *Pieris rapae* data set (Coolen *et al.,* 2016) (on average over the 3 h and 6 h timepoints, which reflect the only overlapping time points in the thrips and *P. rapae* data sets). A cross-mark in this column indicates that the thrips-induced gene is not present in the *P. rapae* DEGs set. Full results used to derive this table are available in Supplemental Data Set 14.

Path	Cluster	ftode	Locus name	Gene name	TF family	Average (normalized) counts in thrips samples	Log_2 fold change [thrips/*Pieris*]
1	1	0.5 h	AT1G28370	ERF11	AP2/ERF	472.7	4.0
1	1	2 h	AT2G44840	ERF13	AP2/ERF	508.8	4.2
1	1	1.5 h	AT3G23250	MYB15	R2R3-MYB	656.7	5.1
1	1	3 h	AT2G39030	NATA1	-	9439.5	4.6
1	2	2 h	AT3G15500	ANAC055	NAC	109.2	3.0
1	2	2 h	AT1G27730	STZ	C2H2-type	1185.8	4.6
1	2	1.5 h	AT1G80840	WRKY40	WRKY	1825.1	3.8
1	5	2 h	AT4G27410	ANAC072	NAC	3221.7	1.4
2	5	2.5 h	AT4G23810	WRKY53	WRKY	683.8	2.5
2	6	2 h	AT5G56840	-	MYB-like	209.7	1.4
3	5	2 h	AT3G19580	ZF2	C2H2	685.0	1.1
4	9	3 h	AT1G50420	SCL-3	GRAS	642.8	X
4	11	3 h	AT3G02150	PTF1	TCP	710.9	X
5	6	2 h	AT1G76590	PLATZ2	PLATZ	811.0	1.4
5	8	2 h	AT2G27230	LHW	bHLH	3449.4	X
6	3	1 h	AT2G26150	HSFA2	HSF	2310.9	X
6	4	3 h	AT2G25000	WRKY60	WRKY	177.1	X
7	10	3 h	AT5G04760	DIV2	MYB	507.7	X

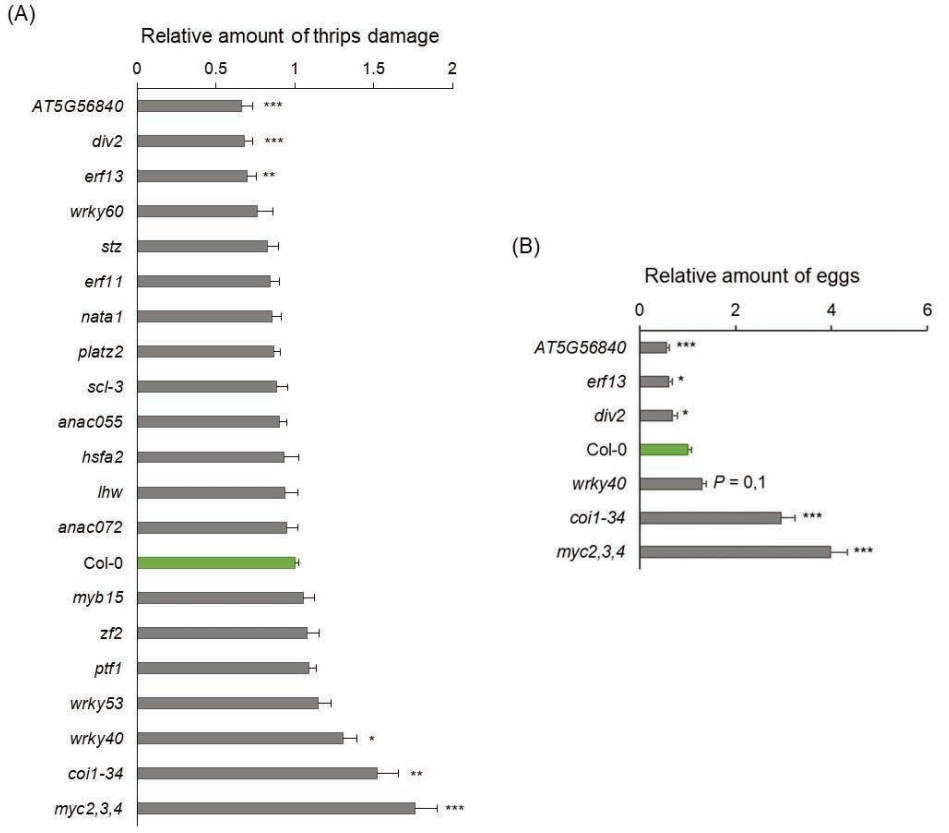


Figure 7: Performance of thrips on different Arabidopsis genotypes. The amount of thrips feeding damage **(A)** and oviposited eggs **(B)** on different mutant lines relative to the wild-type Col-0 (set to 1; bar in green). The data originate from 16 independently performed experiments in which every mutant line was tested twice (n = 17-25) and Col-0 was included in every experiment (n = 147). The number of eggs were only counted in the genotypes (in grey) that showed a significantly altered feeding damage. Asterisks indicate statistically significant differences between the mutant and Col-0 (Student's t-test, $*$ = $P < 0.05$, $**$ = $P < 0.01$, $***$ = $P < 0.001$). Error bars represent SE, n = 17-25 plants.

The mutants in ERF13, DIV2, and AT5G56840 showed significantly fewer feeding scars compared to the wild-type Col-0 and harbored less thrips eggs compared to Col-0 (Fig. 7B). This suggests that these TFs act as negative regulators of plant defense against thrips, and that thrips potentially manipulate the plant to activate these genes for their own benefit. ERF13 (path 1) is a member of the B-3 sub-family of AP2-ERF TFs (reclassified to group IX in Nakano *et al.*, 2006) and can be transcriptionally triggered in response to different biotic and abiotic conditions (McGrath *et al.,* 2005; Winter *et al.,* 2007; Lee *et al.,* 2010a; Hasegawa *et al.,* 2011). Schweizer *et al.* (2013) reported that an *erf13* mutant is more susceptible to the caterpillar *S.*

littoralis. In their qPCR experiments no effects on *PDF1.2* and *VSP2* expression levels were detected in this mutant after insect feeding. However, their gene chip analyses did reveal overall enhanced induction levels of *S. littoralis*-induced genes in the *erf13* mutant (Schweizer *et al.,* 2013). Accordingly, MeJA treatment activated an increased level of *PDF1.2* expression in the *erf13* mutant compared to Col-0 (Caarls *et al.,* 2017). This suggests that ERF13 negatively regulates JA responses, which may explain the enhanced resistance of *erf13* to thrips (Fig. 7). The fact that *erf13* mutants are on the other hand more susceptible to the caterpillar *S. littoralis* (Schweizer *et al.,* 2013) may point to differential effects of shared plant responses to thrips versus caterpillars. DIV2 (path 7) is an R-R-type MYB transcription factor that is involved in salt stress and ABA signaling (Fang *et al.,* 2018). Loss-of-function *div2* mutants show improved salt tolerance, increased levels of ABA under control conditions, and higher ABA sensitivity during seed germination (Fang *et al.,* 2018). Higher ABA levels, such as found in *div2*, might promote the biosynthesis of JA (Adie *et al.,* 2007), explaining the increased tolerance of *div2* to thrips. Not much is known about AT5G56840 (path 6), apart from the fact that it is a member of the MYB-like TF family, hardly responsive to ABA, ET, JA or SA, or to salt stress, and that it is mainly expressed in the petioles of mature leaves (Yanhui *et al.,* 2006; Klepikova *et al.,* 2016). AT5G56840 is orthologous to a MYB TF in tea plants (*Camellia sinensis*), which negatively regulates the biosynthesis of the polyphenol epigallocatechin-3-gallate (Zheng *et al.,* 2019). Perhaps, AT5G56840 has a similar negative role in the production of a polyphenol that bears a defensive activity against thrips in Arabidopsis.

We used the DAP-seq database of O'Malley *et al.* (2016) about *in vitro*-verified TF binding sites in genomic DNA to learn more about the molecular effects of the above-described TFs for which we validated a role in defense against thrips. Unfortunately, DIV2 is not included in this database. DAP-seq of WRKY40 identified 860 target genes, which are also present in the thrips data set, which is more than expected based on the number of targets in the whole genome (30.8% of the thrips DEGs are targets versus 22.3% of all genes) (Supplemental Data set 15). The genome wide target genes of WRKY40 are mainly related to 'phosphate-containing compound metabolic process'. The target genes of WRKY40 in the thrips DEGs set are mainly present in clusters c4, c5, c10 and c11 (>35% of the DEGs in these clusters can be targeted by WRKY40, Supplemental Data Set 15), where they are associated with processes such as 'defense response to fungus', 'multi-organism process', and 'response to chemical'. The target genes of WRKY40 are mainly represented in paths 2, 3, 4 and 7, which correlates with the enrichment of WRKY binding motifs (Fig. 6B). Paths 2, 4 and 7 are tightly connected, e.g., paths 4 and 7 originate from the same path, which diverged after 4.0 h of thrips infestation, and this joint path had diverged from path 2 after 2.5 h of thrips infestation. Since *WRKY40* was already differentially expressed for the first time at 1.5 h after thrips infestation and stayed differentially expressed at 6 other time points thereafter, it can be hypothesized that WRKY40

regulates the early divergence starting at 1.0 h of these paths from paths 1 and 6.

ERF13 has 844 potential target genes in the thrips data set, which is in line with the expected number of targets based on the whole genome data (~30%) (Supplemental Data Set 16). The genome-wide targets of ERF13 are related to development-associated processes. The target genes of ERF13 in the thrips DEGs set are mainly overrepresented in clusters c11, c12 and c13 (>35% of the DEGs in these clusters can be targeted by ERF13), where they are associated with processes such as 'response to chitin' and 'response to wounding'. While ERF13 itself is present in cluster c1 and path 1, its potential target genes are mainly located in path 4 and 7.

DAP-seq of AT5G56840 identified 791 target genes that are present within our thrips data set (Supplemental Data Set 17). This number is in line with the expectations for this TF that binds genome-wide to ~25% of all genes according to the DAP-seq data. The processes that are enriched amongst the genome-wide target genes of AT5G56840 are related to 'response to (endogenous) stimulus'; amongst the thrips-induced target genes of AT5G56840 that are mainly assigned to clusters c1, c6, c10 and c15 (>35% of the DEGs in these clusters can be targeted by AT5G56840), more specific processes related to 'response to chitin', 'secondary metabolic process', and 'multi-organism process' become apparent. AT5G56840 itself is present in cluster c6 and path 2, and most of its potential target genes can be found in path 7. Since AT5G56840 became differentially expressed for the first time after 2 h of thrips infestation it can be suggested that this TF was a driver of the split at 2.5 h, when the ancestor of path 7 diverged from path 2.

Taken together, the thrips-induced GRN was biologically validated using mutants of a selection of candidate TFs belonging to different TF families. Four TFs, WRKY40, ERF13, DIV2 and AT5G56840, turned out to be either positive or negative regulators of defense against thrips, of which most targets were assigned to DREM path 7. The potential target genes of WRKY40, ERF13, and AT5G56840 can be involved in different relevant biological processes, but whether these processes are affected by the mutation in the TF, explaining their altered resistance to thrips, needs to be further investigated.

CONCLUSION AND PERSPECTIVE

Extensive computational analyses of RNA-seq data derived from a high-resolution time series is an effective strategy to reveal the dynamics of plant transcriptional reprogramming, and the underlying gene regulatory interactions. This strategy has been reported for plants that are exposed to different conditions such as biotic and abiotic stress (Bechtold *et al.,* 2015; Coolen *et al.,* 2016; Davila Olivas *et al.,* 2016; Song *et al.,* 2016; Hickman *et al.,* 2017; Hillmer *et al.,* 2017; Zhang *et al.,* 2017; Mine *et al.,* 2018; Arisha *et al.,* 2020). While GRNs have been

described for plant responses to pathogens (Windram *et al.,* 2012; Lewis *et al.,* 2015; Liu *et al.,* 2020b), the level of detail obtained for GRNs that are induced by insects is generally smaller, as fewer time points were analyzed (Ehlting *et al.,* 2008; Appel *et al.,* 2014; Martel *et al.,* 2015; Zhou *et al.,* 2015; Davila Olivas *et al.,* 2016; Kroes *et al.,* 2017; Bui *et al.,* 2018; Guo *et al.,* 2019). Moreover, only few studies have reported on transcriptomic changes that are induced upon attack by cell content feeding insects, such as thrips (De Vos *et al.,* 2005). Here, we describe the execution and analysis of a high-resolution time-course of RNA-seq-based gene expression data that are obtained from Arabidopsis leaves upon infestation by L2 larvae of the western flower thrips. This provided an excessive level of insight into the structure and chronology of the thrips-induced GRN, which allowed us to predict which TF families and individual TF members regulated genes associated with certain biological processes. We validated four potential TFs to play a significant role in the plant's induced defense response during thrips infestation.

Temporal dynamics of the thrips-induced GRN and role of TF families

Our temporal transcriptome data provided insights into the timing of thrips-mediated transcriptional responses, the biological processes targeted, patterns of coregulation of gene expression and novel network regulators. We observed that Arabidopsis responded strikingly rapidly to thrips feeding, as demonstrated by the segregation of the transcriptome profiles of the mock- and thrips-treated plants at 1.5 h after the start of the experiment (Fig. 1B). Additionally, of the total number of 2788 DEGs within the 8.0-h time course, 71.3% (52.9% up- and 18.5% downregulated) of the DEGs (n = 1990) became differentially expressed for the first time within 3 h of introduction of thrips on the leaves (Table 2). This is especially remarkable considering the tiny size of the thrips and the little damage that the five larvae had caused on the leaf. The DEGs transcriptionally activated within the first 3 h of thrips infestation were found to be involved in rather general processes such as 'response to wounding' and 'response to jasmonic acid', which were mainly transcriptionally controlled by members of the bHLH TF family, of which many known JA pathway regulators, e.g., JAZs and MYC2 (Fig. 5A; Supplemental Data Set 10). There was also a substantial set of DEGs that became upregulated in the 3rd hour, which contained a WRKY-binding motif (Fig. 5A; Supplemental Data set 8). Although a few WRKY-binding motif enriched clusters (c4 and c11) pointed to a possible involvement of SA signaling in the thrips-induced GRN related to the overrepresentation of biological processes such as 'response to bacterium' (c4), hardly any SA accumulates in response to thrips feeding and thrips are not affected by exogenous SA application (**Chapter 4**). Alternatively, WRKY TFs can be associated with the stimulation of JA signaling (Brotman *et al.,* 2013; Chen *et al.,* 2021) and several WRKY TFs with an anti-herbivore effect have been described (Skibbe *et al.,* 2008; Schweizer *et al.,* 2013; Hu *et al.,*

2015). Also in our study, the activation of *WRKY40* has been demonstrated to be essential for the plant's defense against thrips, as the *wrky40* mutant showed more thrips damage (Fig. 7A).

Additionally, smaller sets of DEGs within the first 3 h of thrips infestation showed an overrepresentation of e.g., ERF- and MYB-binding motifs fine-tuning the activation or repression of specific biological processes in time as part of the response to thrips feeding. (Fig. 5B). The ERF TF family contains many members, which regulate a variety of developmental and physiological processes in plants (Nakano *et al.,* 2006). The *ERF13* gene is activated by MeJA treatment and herbivory (Supplemental Data Set 5A; Schweizer *et al.,* 2013; Coolen *et al.,* 2016; Caarls *et al.,* 2017; Hickman *et al.,* 2017)) but acts as a negative regulator of thrips defense as the *erf13* mutant showed significantly less thrips feeding damage and a lower number of oviposited eggs (Fig 7). The MYB TF family is also a large family containing members that play important roles in different biological processes (Cao *et al.,* 2020). In response to thrips, the *DIV2* gene encoding an R-R type MYB TF was significantly induced. DIV2 acts as a negative regulator of thrips-induced defense since the *div2* mutant showed significantly less thrips feeding scars and oviposited eggs. Although it can be speculated that DIV2's negative action on defense occurs through suppression of ABA signaling (Fang *et al.,* 2018; Jeong *et al.,* 2018), no ABA-associated processes were overrepresented in the down-regulated genes in response to thrips.

The DEGs transcriptionally downregulated within the first 3 h of thrips infestation were mainly overrepresented with TCP binding motifs. Genes controlled by members of the TCP TF family are generally known to be involved in processes associated to plant growth and development (Li, 2015). This was in line with the overrepresentation of the biological processes 'response to auxin' and 'shoot system development' (Fig. 5A; Supplemental Data set 9).

From 3-h onward, fewer GO terms were enriched in the set of downregulated DEGs, although the enrichment of TCP binding motif in these DEGs remained. In the set of upregulated DEGs, the significant enrichment for bHLH binding sites also persisted over time. This confirms again the importance of bHLH TF for the regulation of defense against thrips in Arabidopsis, together with the association of the biological process 'response to wounding' and 'phenylpropanoid biosynthetic process' (Fig. 5A; Supplemental Data Set 8). The phenylpropanoid pathway is responsible for the synthesis of a diverse group of plant secondary metabolites such as phenolic acids, flavonoids, lignans, and stilbenes (Dong & Lin, 2021; Singh *et al.,* 2021). Multiple studies have shown that the phenylpropanoid pathway can be activated in response to wounding and herbivory (Bernards & Båstrup-Spohr, 2008; Singh *et al.,* 2021). Moreover, phenylpropanoid-derived metabolites can facilitate plant defenses against different insect herbivores (Felton *et al.,* 1999; Johnson & Dowd, 2004; Kaur *et al.,* 2010; Porth *et al.,* 2011; An *et al.,* 2019; Dixit *et al.,* 2020; He *et al.,* 2020). Also against thrips, the phenolic acid chlorogenic acid in chrysanthemum (Dendranthema grandiflora) deters thrips

infestation (Leiss *et al.,* 2009b). Additionally, the orthologous gene of one of our selected candidate TFs, the MYB-like AT5G56840, was found to negatively regulate the biosynthesis of the polyphenol epigallocatechin-3-gallate TF in tea (*C. sinensis*) (Zheng *et al.,* 2019), which is in agreement with the enhanced plant tolerance to thrips when AT5G56840 is knocked out (Fig. 7).

The DREM network-reconstruction tool provided more insight in the coherence of co-expressed clusters identified with SplineCluster. The overrepresentation of specific clusters within a single DREM path, enriched for distinct biological processes, implied a tight transcriptional coregulation of these clusters, directed by specific TFs. Matching with the SplineCluster analysis, most DREM paths were overrepresented with genes containing a bHLH or WRKY TF binding site (Fig. 6B). Also in terms of the biological processes that were enriched in these paths largely overlapped with the processes found by SplineCluster, but were more specific per path. Our model contained a series of split nodes, of which most occurred within the first 3 h of thrips infestation, that are regulated by a variety of TF families. From our selection, TFs WRKY40, ERF13, DIV2 and AT5G56840 were validated as positive or negative regulators of significant processes in resistance to thrips. The target genes of these TFs were commonly present in DREM path 7 and were generally found to be associated with 'response to chitin'. Chitin is an essential component in the exoskeleton of all insect herbivores (Doucet & Retnakaran, 2012), including that of thrips (Kubota, 1989; Seal, 2011; Doucet & Retnakaran, 2012; Mouden *et al.,* 2017).

Resemblance between the thrips-, MeJA- and *P. rapae*-induced signaling pathways

SplineCluster analysis identified multiple groups of thrips-responsive DEGs with distinct transcriptional signatures that are enriched in different biological processes. Many of the clusters containing upregulated DEGs were involved in JA signaling and showed overrepresentation of biological processes associated to 'response to jasmonic acid' and 'response to wounding' (Table 3). The set of upregulated DEGs in response to thrips showed a large overlap (41.9%) with the DEGs upregulated in response to MeJA and *P. rapae* (Fig. 2; Supplemental Data Set 5; Coolen *et al.,* 2016; Hickman *et al.,* 2017), which confirms the key role of JA in the regulation of plant responses to insects of different feeding guilds and the relevance of using exogenous MeJA as a proxy for the JA pathway component in insect-induced responses. For example, enhanced levels of *ERF13* and *AT5G56840* were observed in response to MeJA and *P. rapae* infestation (Supplemental Data set 5), although the role of ERF13 differs per plant-insect interaction, e.g. *erf13* mutant plants are more susceptible to the caterpillar *S. littoralis* (Schweizer *et al.,* 2013) while being more resistant to thrips (Fig. 7). Furthermore, JA-independent and thrips-specific responses are clearly also activated. For instance, the induction of MeJA-nonresponsive *WRKY40* is observed in response to *P. rapae*

and thrips (Supplemental Data set 5), and a mutant in *wrky40* shows enhanced susceptibility to both thrips and the caterpillar *S. littoralis* (Schweizer *et al.,* 2013), suggesting that this TF plays a role in insect-general defense responses. Finally, DIV2 was found to be specifically induced by thrips only. Taken from this, it should be considered that although the majority of the transcriptomic changes are JA-responsive, other subsets are specifically induced depending on the attacker at hand.

The set of downregulated DEGs in response to thrips showed a smaller overlap with the set of DEGs downregulated in response to MeJA and *P. rapae* (13.5%) (Fig. 2; Supplemental Data Set 5; Coolen *et al.,* 2016; Hickman *et al.,* 2017). Nevertheless, the main downregulated biological process shared between these data sets are associated with 'response to auxin stimulus', demonstrating again that during these interactions, plants prioritize the activation of defenses over plant-development associated processes (Denancé *et al.,* 2013).

In conclusion, this study provides detailed insight in the transcriptional dynamics of the thrips-mediated GRN in single Arabidopsis leaves and serves as a valuable recourse for functional studies on previously unknown signaling components in plant-thrips interactions. JA-associated processes were predominantly upregulated in response to thrips. Through the combination of RNA-seq expression data and transcriptional network modeling we identified several TFs that play a significant role in the resistance against thrips. Although the exact regulatory interactions with downstream targets of the thrips-induced TFs need to be confirmed, this data set facilitates further exploration of the thrips-mediated GRN for the identification of novel components that can contribute to the development of more sustainable thrips-resistance breeding.

MATERIAL AND METHODS

Plant material and cultivation

The wild-type and mutant *Arabidopsis thaliana* (Arabidopsis) plants used in this study were in the Columbia ecotype (Col-0) background. The following T-DNA insertion mutants were obtained from the Nottingham Arabidopsis stock center: *at5g56840* (SALK_002490C), *platz2* (SALK_016183C), *anac072* (AT4G27410; SALK_063576C), *div2* (AT5G04760; SALK_000139C), *hsfa2* (AT2G26150; SALK_008978C), *lhw* (AT2G27230; SAIL_588_F06), *myb15* (AT3G23250; SALK_151976C), a*nac055* (AT3G15500; SALK_011069C), *nata1* (AT2G39030; SALK_044395), *scl-3* (AT1G50420; SALK_023428C), *stz* (AT1G27730; SALK_004580), *wrky53* (AT4G23810; SALK_034157C), *wrky60* (AT2G25000; SALK_120706C) and *zf2* (AT3G19580; SALK_132562C). Mutations were identified through PCR genotyping by using primer pairs flanking the T-DNA insertion site for the mutant

(Supplemental Table 1). Double homozygous lines were made by crossing. The mutant line *ptf1* (AT3G02150) was kindly provided by Richard Immink (Wageningen University, the Netherlands). The mutant line *wrky40* was kindly provided by Robert Schuurink (University of Amsterdam, the Netherlands). Seeds of *coi1-34* were kindly provided by Prof. E.E. Farmer (University of Lausanne, Switzerland), *myc2/3/4* (AT1G32640/AT5G46760/AT4G17880; Fernández-Calvo *et al.,* 2011) by Roberto Solano. The mutant lines *erf11* (AT1G28370; SALK_166053) and *erf13* (AT2G44840; GK_121A12) were previously isolated in our lab (Caarls *et al.,* 2017).

Seeds were stratified for 48 h in 0.1% agarose at 4°C. Subsequently, seeds were sown on river sand that was supplemented with 250 mL of modified half strength Hoagland solution containing 10 µM sequestreen (CIBA-Geigy, Basel, Switzerland) per kg of sand (Steenbergen *et al.,* 2020). The tray with sown sand was covered with a lid to ensure 100% relative humidity. From then on plant cultivation took place in a controlled greenhouse compartment with a 10-h day and 14-h night cycle at 21°C and 70% relative humidity (outside the closed tray). Two-week-old seedlings were transplanted to 60-mL pots containing a river sand:soil mixture (5:12, v:v) that had been autoclaved twice for 45 min with a 24-h interval. The autoclaved soil was supplemented with 50 mL/kg modified half strength Hoagland solution. After transplanting, plants were kept under 100% relative humidity by placing the lid on the tray for two days, after which the lids were cracked so that the plants could gradually acclimate to the relative humidity of 70% in the greenhouse for the remainder of the experiment. Plants were watered every other day and received 10 ml of modified half-strength Hoagland solution once a week. For all the experiments, 5-week-old, non-flowering (vegetative stage) plants were used.

Thrips rearing

Western flower thrips (*Frankliniella occidentalis* (Pergande)), originally collected from pepper (*Capsicum annuum*) leaves (Enza Zaden, Netherlands), were reared in a climate chamber (10-h day, 14-h night, 26°C) on common beans (*Phaseolus vulgaris*) and lettuce (*Lactuca sativa* var. *capitata*) in bugdorms (https://shop.bugdorm.com/) (Steenbergen *et al.,* 2020).

Experimental set-up for RNA-seq experiment

Leaf number 9 (counted from oldest to youngest leaf) of 5-week-old Arabidopsis Col-0 plants received an empty clip cage (Mock) or a clip cage containing five L2 larvae (Fig. 1). The clip cages were made as described by Steenbergen *et al.* (2020). At the indicated time point, leaf number 9 was harvested and snap frozen from three individual mock- and thrips-treated plants. The 19 time points following treatments were: 0.5, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8 and 9 h. The biological replicates were treated and harvested according to a time schedule during the day (Supplemental Data Set 1). The experiment started at 8:45,

which was 15 minutes after the lights were turned on in the greenhouse compartment and was arranged such that the early time points (0.5 — 3.5 h) all had received the treatments within the 1st hour and were harvested in chronological order.

RNA-sequencing

Deep-frozen Arabidopsis leaves were homogenized for 2 x 45 seconds using a mixer mill (Retsch, Haan, Germany) set to 30-Hz. Total RNA was extracted using the Rneasy Mini Kit (Qiagen) including a DnaseI treatment step. Quality of RNA was checked by determining the RNA Integrity Number (RIN) using an Agilent 2100 Bioanalyzer and RNA 6000 Nano Chips (Agilent, Santa Clara, United States). Samples required a minimum of 200 ng of high-quality RNA (RNA Integrity Number (RIN) ≥ 8). KeyGene N.V. prepared libraries using Illumina TruSeq Stranded mRNA Library Preparation Kit. Samples were barcoded and randomized/pooled. Quality control (QC) checks, qPCRs and MiSeq Nano runs were performed on randomized sets of samples. Subsequently, randomized sets of samples were sequenced on the HiSeq2500 platform using V4 chemistry and single-end (2 x 125 base pairs) sequencing with a final output of ~40 million reads per sample. Each mix of samples were sequenced on different flow cells to account for technical variation.

Basecalling was performed as described by Hickman *et al.* (2017) using the Casava v1.8.2. pipeline with default settings apart from the argument '—use-bases-mask y50,y6n', to provide an additional FASTQ file containing the barcodes for each read in each sample. Sample demultiplexing was performed by uniquely assigning each barcode to a sample reference, which only allowed a maximum of two mismatches and barcode nucleotides with a quality score of 28 or more.

RNA-seq data analyses

Read alignment and counting was performed as described by Van Verk *et al.* (2013). Reads were aligned to the Arabidopsis genome (TAIR v10) using TopHat v2.0.4 (Trapnell *et al.,* 2009) with the parameters set on: 'transcriptome-mismatches 3', 'N 3', 'bowtie1', 'no-novel-juncs', 'genome-read-mismatches 3', 'p 6', 'read-mismatches 3', 'G', 'min-intron-length 40', 'max-intron-length 2000'. HTSeq-count v0.5.3p9 (Anders *et al.,* 2015) was used to count the number of reads aligning to each gene based on TAIR v10 annotation with the settings: '-stranded no', '-i gene_id'.

All statistics associated with testing for differential gene expression were performed with R v3.3.2 (http://www.r-project.org). The count data were normalized for differences in sequencing depth between samples using the median-count ratio method available in the DESeq2 v1.14 R Bioconductor package v3.4 (Anders & Huber, 2010).

Next, a generalized linear model (GLM) with a log link function and a negative binomial distribution was fit for each gene as described by Hickman *et al.* (2017) to identify genes that were differentially expressed in thrips versus mock samples. Genes were considered as differentially expressed when their expression level was supported by at least 20 counts, a two-fold change (log_2-fold change > 1.0 or < -1.0 [thrips/mock]) during at least one time point, and an adjusted *P*-value (corrected for multiple testing with the Benjamin-Hochberg procedure) ≤ 0.01. Based on these selection criteria, we identified a total of 2788 differentially expressed genes (DEGs) between mock and thrips-infested samples over the time course.

To visualize the transcriptional relatedness and variation between samples, we performed a principal component analysis (PCA) using the *ggplot2* package v2.2.1 in R (Love *et al.,* 2015). The PCA plot that was generated with a former GLM output (3044 DEGs), revealed that one of the samples was an extreme outlier (thrips 1.5 h replica 2, Supplemental Fig. 3). Therefore, this sample was excluded from the data set after which the GLM was executed again, revealing the 2788 DEGs we continued our analyses with.

Using the DESeq2 R Bioconductor package (Anders & Huber, 2010), a pairwise comparison was performed for each time point, using the Pearson correlation measure, to determine the log_2-fold change and *P*-value of each of the 2788 GLM-identified DEGs at the given time point. As a default, DESeq2 (< v1.16.0) shrinks the fold changes to generate more accurate log_2 fold changes estimates in case of low counts or high dispersion values for a gene. Using the output or the pairwise comparison, the first time point of differential expression (ftode) for each of the 2788 DEGs was determined by the following criteria: the first time point at which a gene was significantly differently expressed ($P \leq 0.01$) at a more than two-fold level (log_2-fold change > 1.0 or < -1.0 [thrips/mock]). There were 352 DEGs that had passed the GLM test but did not match the criteria of the pairwise comparison. To assign them to a ftode, these 352 DEGs were manually assigned to a time point where the gene had the lowest *P*-value.

Clustering of gene expression profiles

Temporal expression profiles of the DEGs were clustered using SplineCluster (Heard *et al.,* 2006). Clustering was performed using the log_2-fold changes of each time point (thrips-treated versus mock), with a prior precision value of 10^{-4}, the default normalization procedure and cluster reallocation step (Heard, 2011). All other optional parameters remained as default.

Analyses of TF families, promoter motifs, and TF-target genes

To determine which TF families are overrepresented within a set of DEGs, TF family annotations were retrieved from the TF database PlantTFDB v3.0 (Jin *et al.,* 2014) and tested for enrichment using a cumulative hypergeometric distribution as described by Hickman *et al.* (2017).

For the analysis of known motifs, characterized DNA-binding specificities for 580 Arabidopsis TFs from studies using protein-binding microarrays (PBMs) were utilized to identify DNA motifs that are overrepresented in each of the up- and downregulated clusters (Franco-Zorrilla *et al.,* 2014; Weirauch *et al.,* 2014). The presence or absence of a given motif within a promoter (500 bp upstream of the transcription start site) was determined using FIMO (Grant *et al.,* 2011). A promoter was considered to contain a motif if it had at least one match with a *P*-value ≤ 0.0001.

We used the TF-gene interaction (DAP-seq) data of O'Malley *et al.* (2016) to determine the targets of the most significant thrips-induced TFs; WRKY40, ERF13, AT5G56840 and DIV2. DAP-seq peaks with a FriP (fraction of reads in peaks) score ≥5% were retrieved from the Plant Cistrome Database (O'Malley *et al.,* 2016).

Gene ontology analysis

Gene ontology (GO) enrichment analysis on gene clusters was performed using BiNGO in Cytoscape v3.7.0 (Shannon *et al.,* 2003; Maere *et al.,* 2005). Overrepresentation for the GO categories 'Biological Process' were identified with use of the 'GO_Full' database and by computing a *P*-value using the hypergeometric test and Benjamini & Hochberg False Discovery Rate (FDR) for multiple testing correction ($P \leq 0.05$). GO terms were summarized by removing redundant GO terms using REVIGO (size of the resulting list set to 'small (0.5)'. Other settings were kept as default, apart from the species used to find the GO terms was set to '*Arabidopsis thaliana*') (Supek *et al.,* 2011).

DREM analysis

The software DREM (Dynamic Regulatory Events Miner) 2.0 was used (Schulz *et al.,* 2012). A DREM model was generated, based solely on the time series expression data, provided as $\log_2$-fold changes of thrips/mock treatment per time point ($\log_2$-fold change > 1.0 or < -1.0 [thrips/mock]), as was generated by the DESeq2 pairwise comparison (described in 'RNA-seq data analyses'). Most settings were kept as default, apart from the settings for the search options 'Allow path merges' that was turned on and 'Use regulator-gene interaction data to build model' that was deselected, and in model selection options the 'Penalized Likelihood Node Penalty' was set to 25.

Thrips performance assays

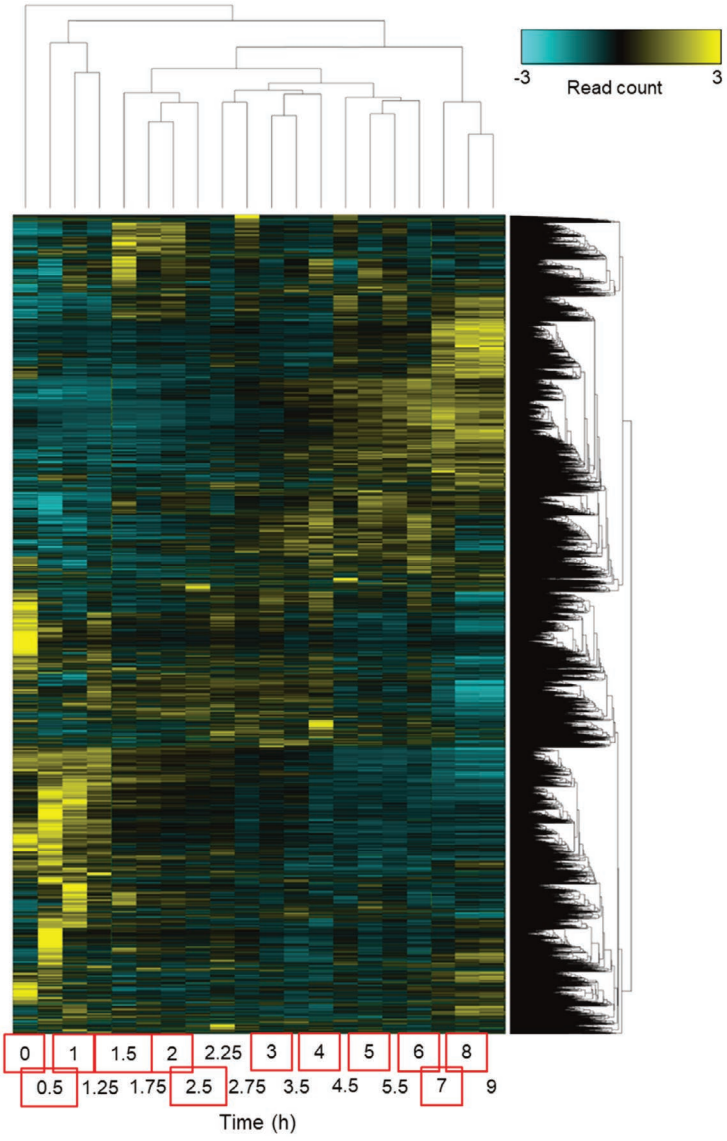
For the whole-plant performance assay, a 5-week-old plant in a 60-mL pot was placed in a 1-L PET jar with nylon mesh, as described by Steenbergen *et al.* (2020) **(Chapter 3).** Five adult female thrips were introduced in each jar, and after four days the feeding damage was estimated in mm^2 by counting the damaged spots on the leaves of each plant. As an extra

measure of thrips performance, the number of oviposited eggs was examined by staining with Trypan blue (lactic acid, glycerol and demi-water (1:1:1, v:v:v) plus 0.025% trypan blue, and an equal volume of 96% ethanol (1:1, v:v) (adapted from Koch & Slusarenko, (1990)), as described by Steenbergen *et al.* (2020). Samples were boiled for 2-3 min, the trypan blue solution was removed, after which a lactoglycerol solution (lactic acid, glycerol and demi-water (1:1:1, v:v:v) was applied for de-staining. Eggs were counted using a binocular.

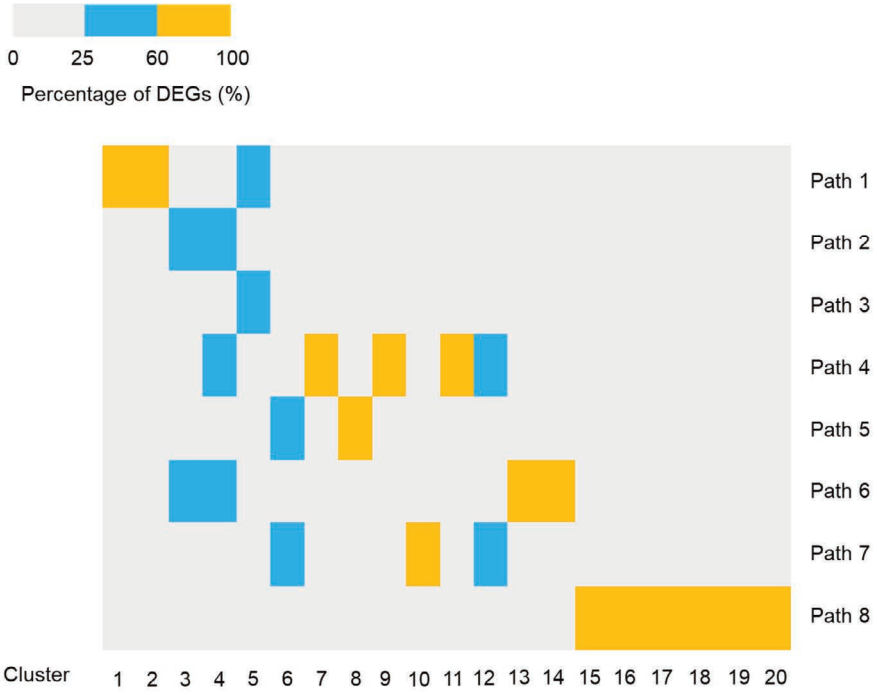
ACKNOWLEDGEMENTS

The authors want to thank colleagues from the Plant-Microbe Interactions lab (Utrecht University, the Netherlands) and the Entomology lab (Wageningen University, the Netherlands) for their help during the RNA-seq experiment and KeyGene N.V. (Wageningen, the Netherlands) for RNA sequencing. This work is part of the research STW perspective program Green Defenses Against Pests and supported by the Netherlands Organization for Scientific Research (NWO, grant no. 13555) in collaboration with Bejo Zaden (Warmenhuizen, the Netherlands), East-West International BV (Bangkok, Thailand), Enza Zaden (Enkhuizen, the Netherlands), Keygene N.V. (Wageningen, the Netherlands), Rijk Zwaan (De Lier, the Netherlands) and Syngenta (Basel, Switzerland). Several authors have additionally been supported by VENI grant no. 13087 (CB), ERC advanced grant no. 269072 (CMJP) and VIDI grant no. 11281 (SCMvW).

SUPPLEMENTAL INFORMATION



Supplemental Figure 1: Expression profiles of Arabidopsis genes over time after thrips infestation. Each row represents an individual gene, with the total of 33540 genes within each column representing a time point after which thrips was introduced on the leaf. Per time point, yellow indicates a relatively high expression of the gene at that time point whereas blue indicates a relatively low expression of the gene at that time point (standardized on the number of counts across all genes per time point). The relationship between the time points is shown by the dendrogram on top of the heatmap. Time points outlined in red were chosen for RNA-seq of the two remaining thrips and the three mock replicates.



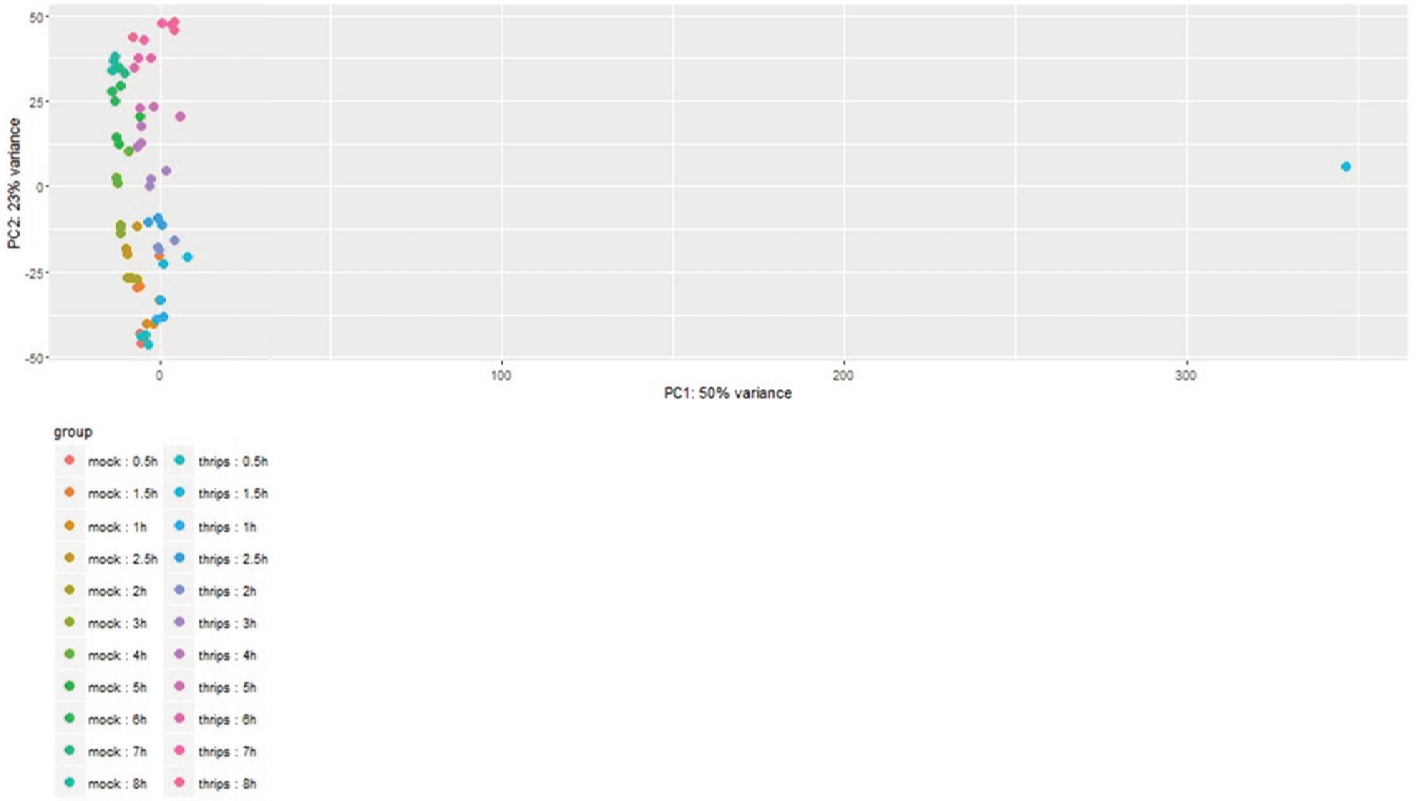
Supplemental Figure 2: Enrichment of SplineCluster-clusters per DREM path induced by thrips infestation. The percentage of DEGs within each cluster that follows the expression pattern of a certain path. Yellow and blue represents an overrepresentation with a minimum percentage of 25% and 60%, respectively, of the DEGs within a certain cluster that is present in a path. Full results used to derive this figure are available in Supplemental Data Set 12.

Supplemental Table 1: List of primers used for genotyping T-DNA mutants.

Primers used for genotyping T-DNA mutants				
Gene	**Locus name**	**SALK/SAIL ID**		**Sequence ID**
-	AT5G50915	SALK_141414C	LP	GCCTTCCCTGTTACCTATTCG
			RP	TCTAACATAAATTACCCGCCG
-	AT5G56840	SALK_002490C	LP	ACTTGCTCTTCATTCCGAATG
			RP	AACCAGCAAAGGAAGAGGAAG
ANAC072	AT4G27410	SALK_063576C	LP	AGTGATCGAGTGCTTCAGGAC
			RP	ACTCGTGCATAATCCAGTTGG
DIV2	AT5G04760	SALK_000139C	LP	TGACGATAAAGGTCGTGTGC
			RP	CTTGCCTTATGACCGGATTC
HSFA2	AT2G26150	SALK_008978C	LP	AAGGTTCCGAACCAAGAAAAC
			RP	TCCTTCCACGTTACTTCAAGC
LHW	AT2G27230	SAIL_588_F06	LP	CGTTTTGTCTTTGTTTAGCCC
			RP	TGAAACTCCCCAACTGTTGTC
MYB15	AT3G23250	SALK_151976C	LP	GAAGATGGGGTTGAAGAGAGG
			RP	CTAAGAGATCTTGTTCCCCGC
NAC055	AT3G15500	SALK_011069C	LP	GAGCGATAGCGAGCAAAGTC
			RP	TCAAGACCTAATCGGGTTGC
NATA1	AT2G39030	SALK_044395	LP	ATTTGGACGTCGTTGACCTC
			RP	TTTTTAACCGGACAGCGTTC
PLATZ2	AT1G76590	SALK_016183C	LP	AGCATTTCAAATGCCGAAAG
			RP	GTTTCTGCTCTCTCGGTTGC
SCL-3	AT1G50420	SALK_023428C	LP	AGAAAAATGGTTGCGTCTGC
			RP	TATGCGATGTTGCAGGCTAG
STZ	AT1G27730	SALK_004580	LP	GAGGGAATTTTCAAGGAAACG
			RP	CTAGTAGCGTGTCCAACTCCG
WRKY53	AT4G23810	SALK_034157C	LP	TATCAATGGATTTGATGCGG
			RP	CGGGGAAAGTTGTGTCAATC
WRKY60	AT2G25000	SALK_120706C	LP	CAGGGCATAGTCAATGGGTC
			RP	TTGTTCTCCGAATTAACCCG
ZF2	AT3G19580	SALK_132562C	LP	TTGTTGCTAACAAGCATGTGC
			RP	GCCAGAATCAAAGAACCTTCC

SALK T-DNA insert:
LBb1.3 ATTTTGCCGATTTCGGAAC

SAIL T-DNA insert:
LB1_SAIL GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
LB2_SAIL GCTTCCTATTATATCTTCCCAAATTACCAATACA
LB3_SAIL TAGCATCTGAATTTCATAACCAATCTCGATACAC



Supplemental Figure 3: Principal component analysis (PCA) of the initial GLM output (3044 DEGs) during an 8-h thrips and mock treatment. The different colors indicate the different time points and treatments. Included is the outlier of the 1.5 h thrips-infested sample.

Supplemental Data Set 1. Experiment set-up RNA-seq time series experiment.

Supplemental Data Set 2. Main expression (average shrunken log2 fold change) and *P*-values of DEGs [thrips/Mock] per time point.

Supplemental Data Set 3. Normalized expression values (read counts) of all genes and biological replicates over an 8-h period after thrips and mock treatments.

Supplemental Data Set 4. Time point(s) at which differentially expressed genes (DEGs) became differentially expressed for the first time (ftode) or again (ade).

Supplemental Data Set 5. Overview of DEGs overlapping and not overlapping in response to thrips, MeJA and *Pieris rapae*.

Supplemental Data Set 6. Distribution of 2788 DEGs in 20 co-expressed clusters identified by Splinecluster and their associated GO terms.

Supplemental Data Set 7. Enrichment of known TF DNA binding motifs in each of the 20 co-expressed clusters.

Supplemental Data Set 8. GO-terms overrepresented in upregulated genes that became differentially expressed for the first time per time frame.

Supplemental Data Set 9. GO-terms overrepresented in downregulated genes that became differentially expressed for the first time per time frame.

Supplemental Data Set 10. Top 5 GO-terms overrepresented in up- and downregulated gene sets per time frame.

Supplemental Data Set 11. Enrichment of known TF DNA binding motifs of genes in each of the up- and downregulated time frames.

Supplemental Data Set 12. Enrichment of genes and GO terms per DREM path.

Supplemental Data Set 13. Enrichment of known TF DNA binding motifs of genes within each DREM path.

Supplemental Data Set 14. Overview of TFs in the upregulated DREM paths 1-7 fitting the mutant selection criteria based on minimum average read count > 100 and 2-fold change [thrips/Pieris].

Supplemental Data Set 15. Targets of WRKY40.

Supplemental Data Set 16. Targets of ERF13.

Supplemental Data Set 17. Targets of AT5G56840.

CHAPTER 4

Differential phytohormonal signaling induced by the different developmental stages of the Western flower thrips

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ABSTRACT

The Western flower thrips, *Frankliniella occidentalis*, is one of the most important pest insects worldwide. This cell-content feeding insect goes through six developmental stages including egg, two larval stages and adult. Most studies on thrips-induced plant responses only focus on the regulation of defense upon feeding by the adult stage. Almost nothing is known about the plant's response towards the other developmental stages of the thrips life cycle. In this study we analyzed the function of the plant defense-related hormones salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET) in *Arabidopsis thaliana* to infestation by the individual thrips developmental stages. We found that plants enhanced the expression of the SA-responsive marker gene *PR1* at the site of oviposition, while all feeding stages activated JA-associated marker genes. The ABA-coregulated MYC-branch of the JA pathway, represented by the marker gene *VSP2*, was induced to the highest extent by adult females, followed by L2 larvae, while L1 larvae were the lowest inducers. Interestingly, L1 larval feeding highly activated the ET-coregulated ERF-branch of the JA pathway, which is represented by the marker gene *PDF1.2*. Nevertheless, enhanced ET levels were detected irrespective of the developmental stage. However, ABA and JA(-Ile) levels were relatively higher induced by adults and L2 larvae than by L1 larvae. The role of these phytohormones in defense against the different feeding thrips stages was further investigated using performance assays with wild-type plants that were exogenously treated with these phytohormones, and with mutant plants that were affected in hormone sensitivity or production. Overall, ABA and SA perturbations had no effect. All thrips stages fed less on plants treated with methyl jasmonate (MeJA), which correlated with enhanced feeding damage inflicted on the JA-insensitive mutant plants *coi1-34* and *myc2,3,4*, confirming the essential role of JA in defense against thrips. Unexpectedly, exogenous application of 1-aminocyclopropane-carboxylic acid (ACC), a precursor of ET, reduced the feeding performance of all thrips stages, but the ET insensitive mutant *ein2-1* also showed a lower feeding performance by most of the thrips stages. Possibly, L1 larvae repress defenses by shifting the JA pathway towards the ERF-branch away from the MYC-branch. This hypothesis is supported by our finding that in experiments with sequential feeding by different stages of thrips, L1 larvae as second feeders turned out to perform equally well as on non-pre-fed plants, while the other stages performed worse.

INTRODUCTION

Plants have to cope with a wide variety of insect species. Of the estimated 6 million insect species, half are herbivores (Schoonhoven *et al.,* 2005). For plants to be able to defend themselves against herbivorous insects, they have evolved sophisticated defense mechanisms that can be either constitutively present or induced upon herbivore attack. The plant's immune system can be activated upon recognition of cell damage (damage-associated molecular patterns (DAMPs)) and herbivore-associated molecular patterns (HAMPs) that can be present in the herbivore's saliva or eggs (Mithöfer & Boland, 2008; Duran-Flores & Heil, 2016; Hilker & Fatouros, 2016). Subsequent to the perception of these cues, a signaling cascade is triggered resulting in the accumulation of phytohormones including salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET), which further activate downstream transcriptional responses. SA and JA are considered core players in the plant's immune response. The SA pathway mediates defenses against (hemi)biotrophic pathogens, while the JA pathway is predominantly initiated by and effective against herbivores and necrotrophic pathogens (Pieterse *et al.,* 2012). The JA signaling pathway is comprised of two branches. In the so-called MYC-branch, ABA plays a synergistic role with JA; it is primarily activated by and effective against insect herbivores. In the so-called ERF-branch, ET plays a synergistic role with JA and is primarily activated by and effective against necrotrophic pathogens. Different hormone signaling pathways can exhibit negative crosstalk, which allows fine-tuning of defense responses to the attacker at hand and suppression of unnecessary and ineffective defenses, which can be considered as a cost-saving strategy (Vos *et al.,* 2013a, 2015) .

Besides dealing with different insect herbivore species, plants also have to deal with the different developmental stages within an insect's life cycle, from egg to larvae, to adult. Egg deposition can be considered as the first step of infestation. Plants can prepare themselves for future larval attack by triggering defenses that directly kill the eggs, for example, by the formation of neoplasms or necrotic tissue, or indirectly via the action of volatiles that attract egg parasitoids (Hilker & Fatouros, 2016). Insect oviposition can also prime the plant's defenses by activating the biosynthesis of JA that will target the future feeding larvae (Kim *et al.,* 2012). However, some insect herbivore species have evolved a defensive strategy to circumvent the anti-herbivore defenses of the plant during the egg stage by inducing SA accumulation at the site of oviposition, which suppresses the JA pathway and thereby increases the performance of the future progeny (Bruessow *et al.,* 2010).

Once insect larvae successfully emerge from the eggs and start to eat and develop on the plant, plants can respond with various morphological and biochemical defenses (War *et al.,* 2018), which can be exclusively effective against a single developmental stage of an insect

herbivore (Zhang *et al.,* 2018; Liu *et al.,* 2020a). In garlic mustard (*Alliaria petiolate*) for example, two distinct glycosides were found to protect the plant against either one of the two different feeding stages of the native American butterfly (*Pieris oleracea napi*), with one compound blocking the feeding of the first instars, and the other deterring later instars (Renwick *et al.,* 2001). The fact that plants produce distinctive defense compounds against different larval stages of an insect herbivore suggests that the different developmental stages within an insect herbivore's life cycle have developed different mechanisms to overcome its host plants defenses. Several transcriptomic studies on different insect developmental stages have demonstrated that the gene expression profiles between life stage transitions were dramatically different (Yang *et al.,* 2013a; Vogel *et al.,* 2014; Tian *et al.,* 2015; Chen *et al.,* 2016). For example, in wheat (*Triticum aestivum* L.) feeding by the first larval stage of the Hessian fly (*Mayetiola destructor*) induced a high percentage of effector-encoding genes, while later larval stages upregulated genes involved in synthesis of digestive proteases and protective protease-inhibitors (Chen *et al.,* 2016). These findings prove that there are stage-specific gene expression signatures which may underlie differential plant defense mechanisms against the different developmental stages within an insect herbivore's life cycle. Despite this knowledge, most resistance studies in plant-insect interactions focus on the plant's responsiveness and performance to a single life stage without considering the responsiveness to the other stages within the insect life cycle.

An example of an insect herbivore of which the plant's responsiveness to the full life cycle has not been studied yet is the Western flower thrips (*Frankliniella occidentalis*). These tiny cell content-feeding insects are major pests in food and ornamental plant-production areas worldwide. Thrips undergo six different developmental stages; egg, first instar larva (L1), second instar larva (L2), pre-pupa, pupa and adult. Female thrips lay their bean-shaped eggs into the plant tissues that are suitable for the larvae to feed from. During the two larval stages, thrips are actively feeding. When preparing for the adult stage, thrips go through two pupal stages which are soil-dwelling and non-feeding (Steenbergen *et al.,* 2018). Most studies on the plant's responsiveness to thrips rely on the use of adult thrips or a mix of different ages of thrips. Thrips feeding has been shown to activate the biosynthesis of JA and the expression of JA-responsive genes (De Vos *et al.,* 2005; Abe *et al.,* 2008, 2009; Selig *et al.,* 2016; Escobar-Bravo *et al.,* 2017). Activation of the JA pathway, specifically the MYC-branch of the JA pathway, enhances the plant's tolerance against adult thrips (Abe *et al.,* 2008, 2009; Wu *et al.,* 2019). This has been demonstrated using the *Arabidopsis thaliana* (hereafter Arabidopsis) *coi1-1* mutant and the tomato *def1* mutant, which are impaired in JA signal transduction, and the Arabidopsis *myc2* mutant that is a knockout of the JA-regulated MYC2 TF, which all suffered more feeding damage and increased oviposition (Abe *et al.,* 2009; Escobar-Bravo *et al.,* 2017; Wu *et al.,* 2019). An exogenous JA treatment enhanced the plant's tolerance against thrips

(Abe *et al.,* 2008, 2009; Escobar-Bravo *et al.,* 2017; Sarde *et al.,* 2019). Additionally, exogenous application of 1-aminocyclopropane-carboxylic acid (ACC), a precursor of ET, on Arabidopsis leaf discs resulted in increased feeding damage by adult female thrips, suggesting that activating the ERF-branch is in the advantage of thrips. However, the ET-insensitive mutant *ein2-1* suffered similar levels of feeding damage as wild-type plants, and the authors proposed that only JA functions in the wound response against thrips (Abe *et al.,* 2008). Whether this hormonal response is similar when plants are fed on by L1 larvae and L2 larvae has never been investigated in detail.

In this study we analyzed the response of Arabidopsis to different thrips developmental stages, i.e., eggs, L1 larvae, L2 larvae and adults. For this we analyzed the transcriptional dynamics of selected defense marker genes, together with the induced levels of defense hormones responsible for the induction of those genes, upon feeding by the different thrips developmental stages. Furthermore, the function of these defense hormones in plant tolerance against all feeding thrips stages was studied further by exogenously treating wild-type plants with SA, methyl jasmonate (MeJA), ABA and ACC, and by using hormone-related mutant plants. Our results show that thrips eggs locally induced a SA response, while all the different feeding stages mainly induced a JA response. However, the ABA-coregulated MYC-branch marker gene *VSP2* was induced to the highest extent by adult females and to the lowest extend by L1 larvae. Oppositely, L1 larval feeding highly activated the ET-coregulated ERF-branch marker gene *PDF1.2*, while adult feeding did not. We found that modulating the ET response, by applying its precursor ACC in high levels or by genetically blocking downstream ET-mediated responses, negatively affected the performance of thrips. Performance assays in which plants were pre-infested by different thrips stages revealed that sequential feeding by a different thrips stage significantly reduced the performance of L2 larvae and adult female thrips. In contrast, L1 larvae performed equally well on pre-infested plants as on non-pre-infested plants. We thus hypothesize that the L1-preferentially induced ERF-branch plays a role in the repression of anti-thrips defenses.

RESULTS

Molecular response to thrips eggs and feeding adult female thrips

To monitor whether molecular changes take place in Arabidopsis after oviposition and feeding by female thrips, we used transgenic lines containing the promoters of the defense-related genes *PR1* and *LOX2*, and the GCC-box motif that can be bound by ERF transcription factors, coupled to the beta-glucuronidase (GUS) reporter gene. Previous studies demonstrated that plants accumulate SA and thereby activate expression of SA-responsive genes such as *PR1* at sites where eggs of different herbivore species have been oviposited (Little *et al.,* 2007;

Bruessow *et al.,* 2010; Caarls *et al.,* 2021). To our knowledge, this has never been demonstrated for thrips eggs. *LOX2* is an enzyme involved in JA biosynthesis and serves as a marker for activation of the JA pathway by insects (Koornneef *et al.,* 2008; Chauvin *et al.,* 2013; Zhai *et al.,* 2013; Chauvin *et al.,* 2016). The GCC-box can be bound by ERF transcription factors that activate the ERF-branch of the JA pathway leading to expression of e.g. the *PDF1.2* gene (Pré *et al.,* 2008; Zarei *et al.,* 2011). A strong *PR1*::*GUS* activity was shown around the thrips eggs (Fig. 1A), while *LOX2*::*GUS* and *GCC*::*GUS* were not active (Fig. 1B and C). In control *PR1*::*GUS* plants there was some background GUS activity (Fig. 1D), but not in the *LOX2*::*GUS* and *GCC*::*GUS* control plants (Fig. 1E and F). Upon female thrips infestation, no *PR1*::*GUS* activity was observed (Fig. 1G). *LOX2*::*GUS* was activated (Fig. 1H) and a slight activation of *GCC*::*GUS* around the main veins was observed after thrips feeding (Fig. 1I). Zooming in on the feeding sites (Fig. 1J-L), it was clear that the cells surrounding the emptied thrips feeding sites solely activated *LOX2*::*GUS* and not *PR1*::*GUS* or *GCC*::*GUS*. These results confirm that thrips eggs locally stimulate the SA response pathway, and that adult thrips feeding activates the JA response pathway, with a stronger activation of the MYC-branch than the ERF-branch.

Temporal transcriptional changes in response to infestation by thrips larvae and adults

Adult female thrips feeding has been demonstrated to activate the biosynthesis of JA (De Vos *et al.,* 2005; Abe *et al.,* 2008, 2009) and the expression of JA-responsive genes (De Vos *et al.,* 2005; Abe *et al.,* 2008, 2009; Selig *et al.,* 2016; Escobar-Bravo *et al.,* 2017). Whether this also applies to feeding by L1 and L2 larvae is unknown. Additionally, whether the thrips-induced JA response is coregulated by ABA or ET has also not extensively been studied yet. To analyze the transcriptional dynamics of genes regulated by defense hormones upon feeding by the different thrips developmental stages, we performed a time series experiment with plants infested with either L1 or L2 larvae, or female adults. For this, leaf number 9 received a clip cage containing five thrips and this treated leaf was harvested for gene expression analysis (Van de Wetering *et al.,* 1998). We monitored the expression of the hormone-mediated defense marker genes *PR1* (SA), *VSP2* (MYC-branch of JA signaling) and *PDF1.2* (ERF-branch of JA signaling) after 2, 4, 6, 8 and 28 h of infestation. All thrips stages significantly enhanced the expression of *VSP2* compared to non-infested plants, that had received an empty clip cage (Fig. 2A). Adult thrips feeding resulted in higher transcript levels of *VSP2* compared to the larval stages. Interestingly, the transcript levels of *PDF1.2* were only significantly increased after 28 h of L1 larvae feeding. Feeding by L2 larvae and female adults did not significantly increase the transcript levels of *PDF1.2* at any of the time points. Expression of the SA-responsive gene *PR1* could not be detected after feeding by any of the three stages (data not shown).

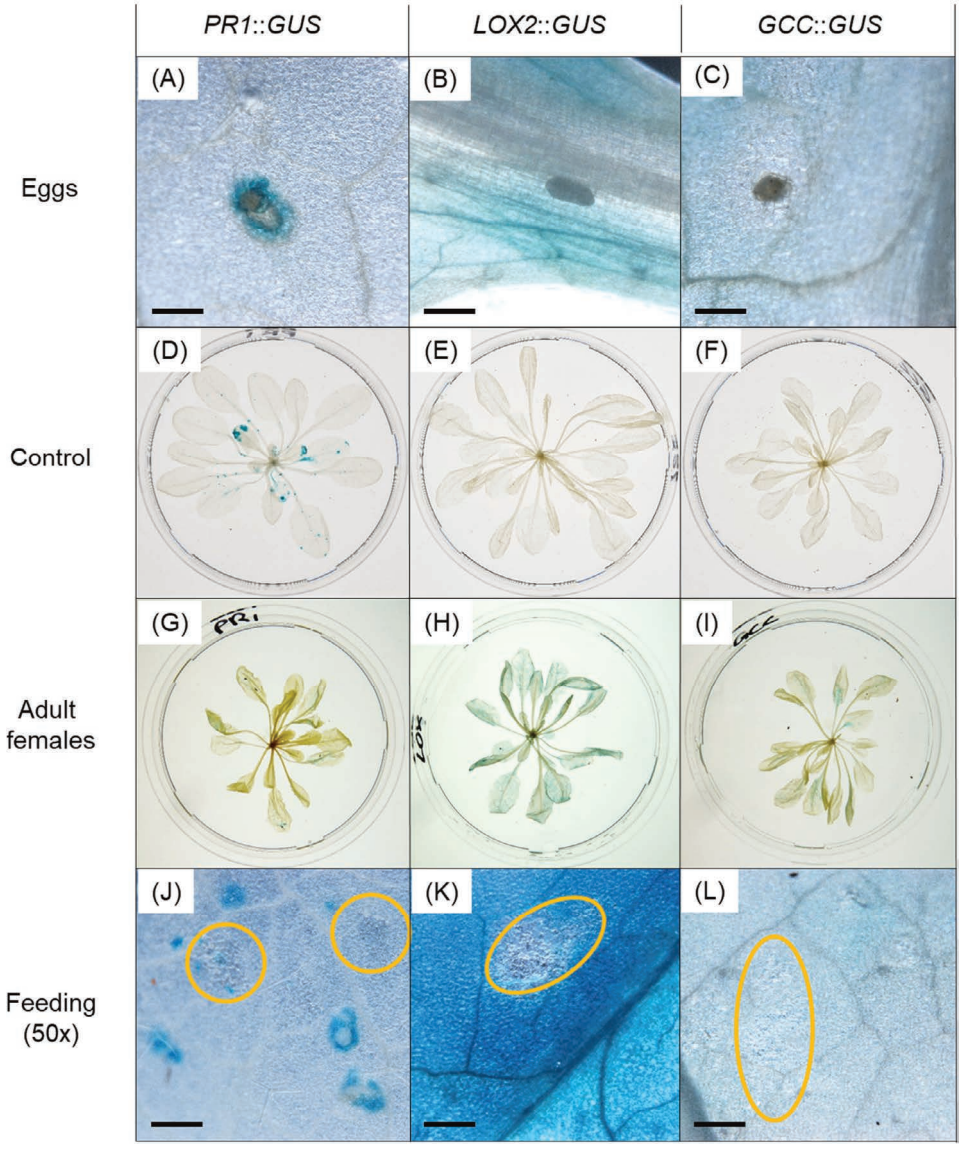

Figure 1: Activity of the promoters of the defense-related genes *PR1* and *LOX2,* and the GCC-motif that can be bound by ERF transcription factors, upon egg deposition and feeding by female adult thrips. Five-week-old plants were treated with 5 adult female thrips for 4 days after which the whole plant was harvested for GUS staining. **(A-C)** Close-up of GUS activity in response to thrips eggs (bar = 125 µm). **(D-F)** Control plants, which did not receive female thrips. **(G-I)** GUS activity in response to 4 days of female thrips infestation**. (J-L)** Close-up of the emptied cells as a result of 4 days of female thrips infestation (encircled in yellow; bar = 200 µm).

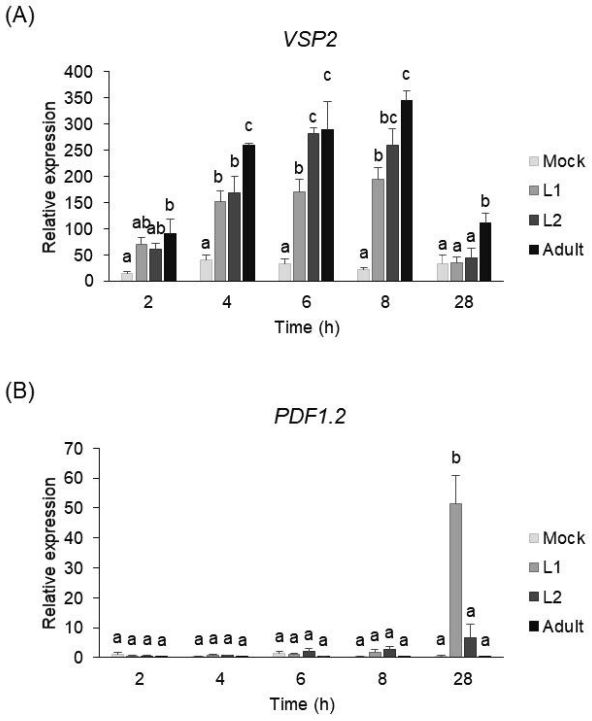

Figure 2: Differential JA response in wild-type Arabidopsis (Col-0) plants in response to feeding by thrips of different developmental stages. RT-qPCR analysis of **(A)** *VSP2* and **(B)** *PDF1.2* gene expression levels in leaf number 9 of 5-week-old Arabidopsis plants after 2, 4, 6, 8 and 28 h of feeding by 5 thrips (L1 larvae, L2 larvae or female adults) in a clip cage. Control plants (Mock) were treated with an empty clip cage. Different letters indicate statistically significant differences between the treatments within the indicated time point (ANOVA, Tukey post-hoc test; $P < 0.05$). Error bars represent SE, n = 3-5 plants.

These results confirm that all feeding stages activate the JA pathway and not the SA pathway and show that the plant responds differently to L1 larval infestation compared to L2 larval and female adult infestation, namely with higher ERF-branch activation.

Hormone accumulation over time during feeding by thrips larvae and adults

Together with JA, signaling by the phytohormones ABA and ET promotes the expression of *VSP2* and *PDF1.2*, respectively, during feeding by the insect *Pieris rapae* (Vos *et al.,* 2013b, 2019). To determine whether defense-related hormone accumulation changed during infestation by thrips, we measured the levels of SA, JA-Ile (biologically most active form of JA), ABA, JA, and ET in leaf number 9 of Arabidopsis in response to L1 larvae, L2 larvae and female adults. Unexpectedly, L2 larval infestation led to a significant increase in SA content in the plant after 8 and 28 h compared to the mock treatment (Fig. 3A). The SA levels in tissue fed on by L1 stage and adult thrips did not significantly differ from the mock condition at these time points,

although a slight trend to enhanced SA levels was detected. JA-Ile levels were significantly higher after 8 and 28 h of feeding by L2 larvae and adult female thrips (Fig. 3B), while the induction of JA-Ile by L1 larvae infestation was significant only at the 8-h time point and a trend was visible at 28 h. Similar findings were observed for the JA levels (Fig. 3C). The ABA concentrations were significantly higher after 8 and 28 h of feeding by L2 larvae and female adults (Fig. 3D). The L1 larvae did enhance the level of ABA after 8 h, but there was no longer a significant difference with the mock treatment at the 28-h time point. The concentration of ET was significantly enhanced by L2 larval and adult female thrips infestation at both time points (Fig. 3E).

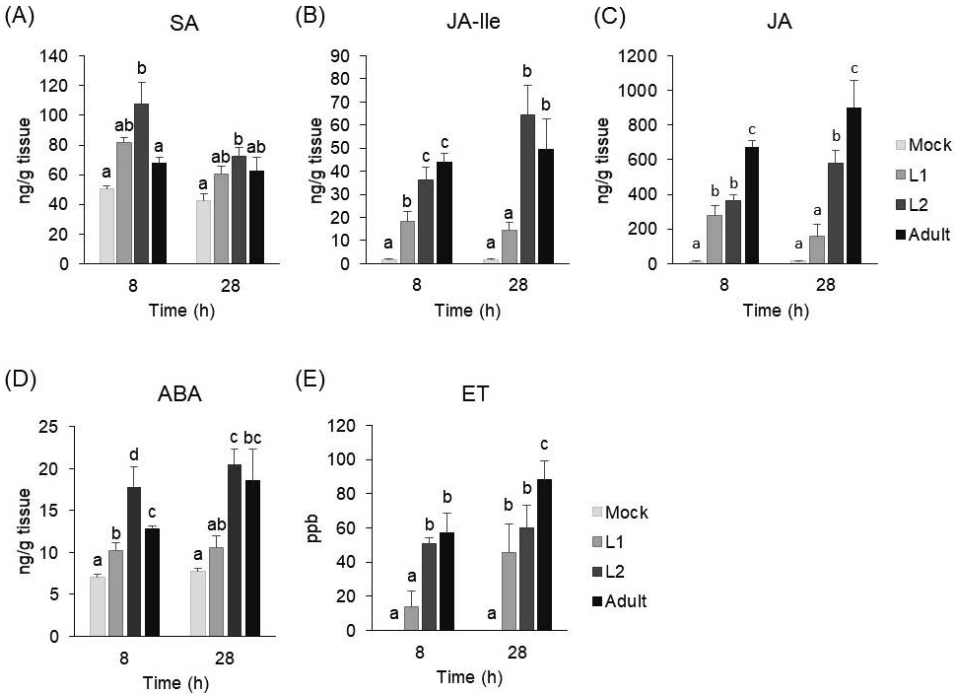

Figure 3: Production of SA, JA-Ile, JA, ABA, and ET in wild-type Arabidopsis (Col-0) plants during feeding by different thrips developmental stages. Leaf number 9 of 5-week-old Arabidopsis (Col-0) plants was assayed at 8 and 28 h after 5 thrips (L1 larvae, L2 larvae or female adults) were placed in a clip cage on leaf 9 **(A-D)** or in a 10-cc vial containing leaf 9 **(E)**. Control plants (Mock) did not receive any thrips. **(A-D)** Absolute values (ng/g fresh weight (FW)) of SA, JA-Ile, JA and ABA levels measured by Triple Quad LC/MS/MS (5 pooled leaves per sample). **(E)** ET levels were measured by a gas chromatograph. Different letters indicate statistically significant differences between the treatments within the indicated time point (ANOVA, Tukey post-hoc test; $P < 0.05$). Error bars represent SE, $n = 5$.

The L1 larvae significantly induced ET at 28 h, but there was already a clear trend at 8 h. In conclusion, only L2 larvae feeding enhanced the accumulation of SA. L2 larvae and female

adult thrips significantly enhanced the levels of JA-Ile, JA, ABA and ET at both 8 and 28 h. Feeding by L1 larvae generally also induced these hormones but to a lower extent and at one of the measured time points not reaching a significantly higher level than the mock treatment. This different effect on hormone accumulation between the developmental stages of thrips may be related to the lower amount of feeding damage inflicted by L1 larvae compared to the older stages (Supp. Fig. 1).

Effect of phytohormones on the performance of thrips larvae and adults

It has previously been demonstrated that activation of the JA pathway reinforces Arabidopsis resistance to adult female thrips (Abe *et al.,* 2009). On the other hand, exogenous application of ACC, the precursor of ET, has been shown to profit the performance of female thrips on Arabidopsis leaf discs. Here, we exogenously applied SA, MeJA, ABA and ACC to wild-type Arabidopsis 24 h prior to introduction of thrips at different developmental stages (L1, L2 and adult). Furthermore, the function of the corresponding hormones in tolerance to thrips was studied using hormone-deficient or -nonresponsive mutants. We monitored the amount of feeding damage inflicted by all thrips stages. When the plants were treated with adult females also oviposition was determined.

Pre-treatment with SA or ABA had no significant effect on the performance of all the thrips developmental stages. Plants treated with MeJA or ACC showed a significant reduction of feeding damage inflicted by L1 and L2 larval thrips (Fig. 4A and B). The MeJA- and ACC-induced decrease in damage by adult thrips was not statistically significant, but a trend was visible (P = 0.15 and P = 0.35, respectively; Fig. 4C). The number of eggs laid by the adult females in all the different hormone-treated plants was not statistically different from that in the mock-treated plants (Fig. 4D).

In line with the results of the hormone pre-treated plants, the absence of SA in the deficient mutant *sid2-1* or the absence of ABA in the deficient mutant *aba2-1* had no significant effect on the feeding performance by the different thrips stages, with an exception for *aba2-1* that showed significantly less feeding damage by L2 larvae (Fig. 5). The JA-insensitive mutants *coi1-34* and *myc2,3,4* exhibited significantly more thrips feeding damage inflicted by all thrips developmental stages (Fig. 5), which correlates with the reduced damage on the MeJA pre-treated plants (Fig. 4). Additionally, adult female thrips laid significantly more eggs on these JA-insensitive mutants (Fig. 5D). These results demonstrate that JA plays a major role in the plant's induced defense against all feeding thrips stages.

The *ora59* mutant, which is defective in the ERF transcription factor ORA59 that is induced by ET and JA and regulates *PDF1.2* expression, showed the same damage severity caused by all three thrips stages as the wild-type plants. Apparently, neither the expected enhanced MYC-branch activity nor the reduced ERF-branch in the *ora59* mutant conferred an

altered level of protection against thrips. Interestingly, the ET-insensitive mutant *ein2-1* showed significantly fewer feeding scars caused by L1 larvae and female adults (Fig. 5A and C) and a significantly lower number of eggs oviposited by the female adults (Fig. 5D).

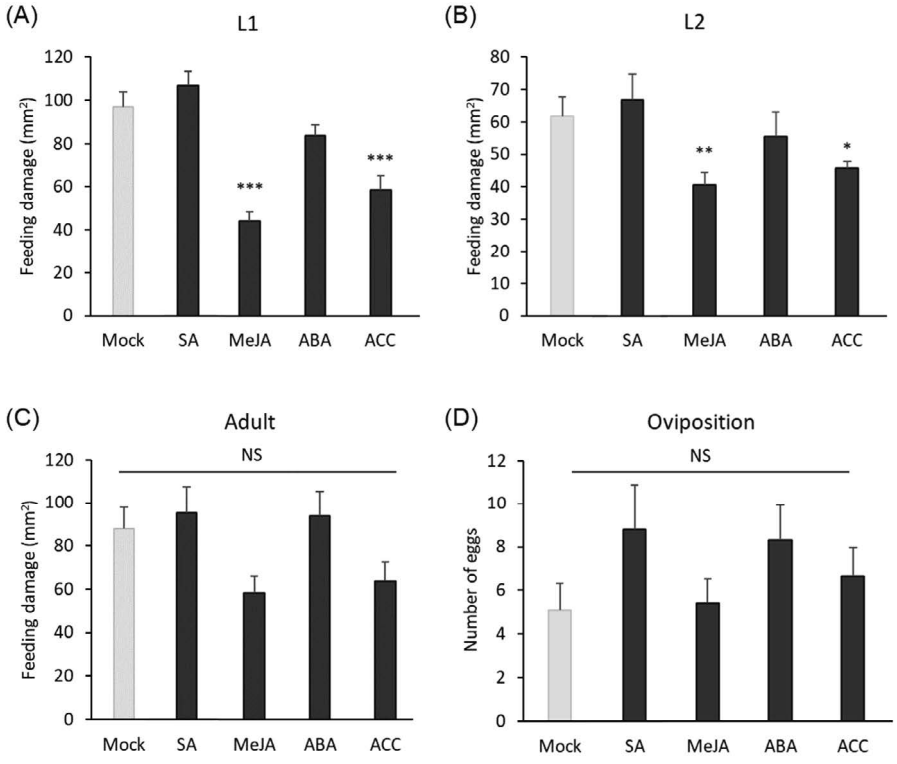

Figure 4: Effect of exogenous application of different phytohormones on the performance of the different thrips developmental stages. The rosettes of 5-week-old wild-type Arabidopsis (Col-0) plants were dipped in a mock solution or a solution containing 1 mM SA, 100 µM MeJA, 100 µM ABA or 1 mM ACC 24 h prior to the introduction of thrips. Whole plants received five thrips of different developmental stage: **(A)** L1 larvae, **(B)** L2 larvae or **(C)** adult females for 5, 3 or 4 days, respectively, after which feeding damage (in mm^2) was assessed. The plants that were treated with adult females were harvested for Trypan blue staining to visualize the eggs **(D)**. Asterisks indicate a statistically significant difference between the indicated treatment and the mock treatment (ANOVA, Dunnett post-hoc test; $*$ = $P < 0.05$, $**$ = $P < 0.01$, $***$ = $P < 0.001$, NS = not significant). Error bars represent SE, n = 7-10 plants.

This result indicates that severe modulation of the ET response, such as by applying its precursor ACC in high levels or by genetically blocking downstream ET-mediated responses, has a negative effect on the performance of L1 larvae and adult female thrips. Nevertheless, the L2 larval stage was insensitive to the perturbation caused by the *ein2-1* mutation. Taken together, these results indicate that SA and ABA have no or minor effects on thrips feeding. In

contrast, JA, potentially with ET, plays a significant role against the three different feeding stages of thrips, where L1 larvae and adult females display a higher sensitivity to JA and ET signaling than L2 larvae.

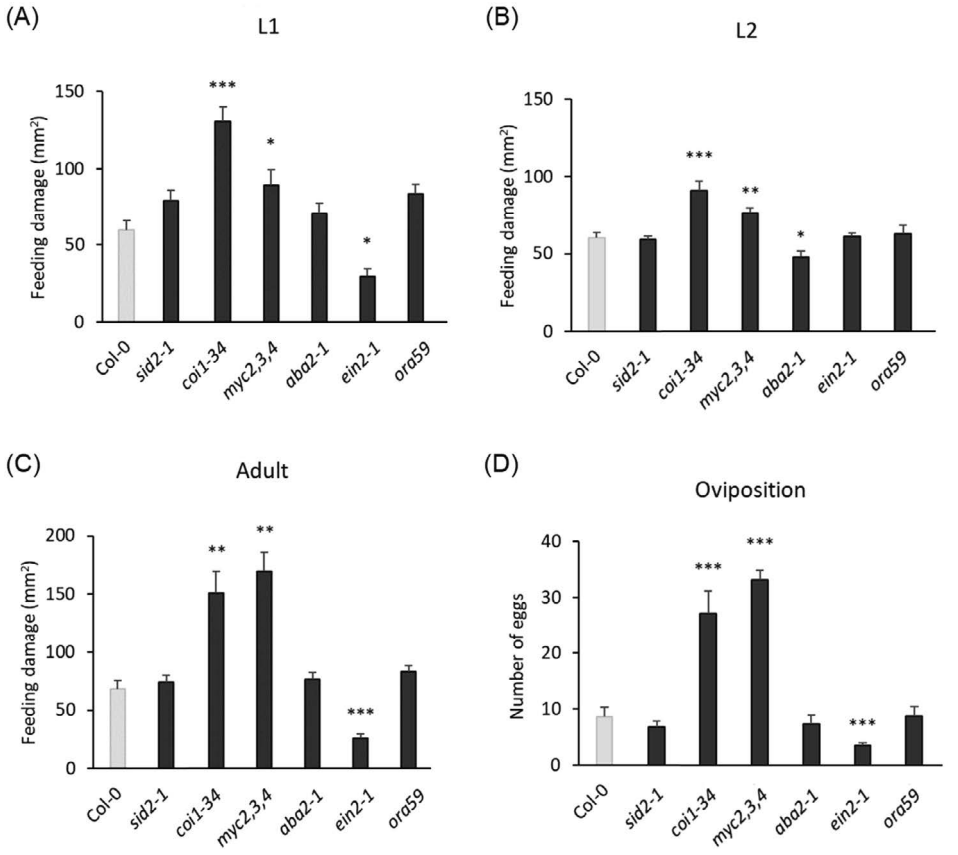

Figure 5: Effect of hormone-deficient or -insensitive plants on the performance of different thrips life stages. Five-week-old wild-type Arabidopsis (Col-0) plants and mutant plants *sid2-1, coi1-34, myc2,3,4, aba2-1, ein2-1* and *ora59* were treated with 5 thrips of different a developmental stage: **(A)** L1 larvae, **(B)** L2 larvae or **(C)** adult females for 5, 3 or 4 days, respectively, after which the area of feeding (mm^2) was assessed. The plants that were treated with adult females were harvested for Trypan blue staining to visualize the eggs **(D)**. Asterisks indicate a statistically significant difference between the indicated treatment and the mock-treated plants (ANOVA, Dunnett post-hoc test; $* = P < 0.05$, $** = P < 0.01$, $*** = P < 0.001$). Error bars represent SE, n = 8-10 plants.

Effect of pre-infestation with different thrips feeding stages on performance of the other stages

Our previous results suggest that JA and ET signaling define the plant's tolerance to thrips (Fig. 3-5). We also showed that L2 larvae and female adults mainly activated the JA/ABA signaling pathway marker gene *VSP2*, while L1 larvae induced *VSP2* to a lower extent and induced the

JA/ET signaling marker gene *PDF1.2* to a much higher extent than the L2 and adult stages. We hypothesized that the differential activation of the MYC- and ERF-branches of the JA pathway during feeding by the different thrips feeding stages is related to defense activation by the plant versus defense repression by the thrips. To investigate this, we used whole-plant thrips performance assays to test the effects of pre-infestation on subsequent thrips performance at different developmental stages. To correct for the amount of feeding damage that was caused by the 24-h pre-infestation it was subtracted from the damage that was measured after the subsequent thrips infestation for 3 days.

Standing out from the results depicted in Figure 6 is the performance of L1 larvae, which was not affected by a 24-h pre-infestation with L2 larvae or female adults (Fig. 6A). In contrast, L2 larval and adult female performance were significantly reduced after a 24-h pre-infestation with the other thrips stages (L1 and adult or L2, respectively; Fig. 6B-D). Thus, all three thrips feeding stages can activate plant defenses, which are effective to a subsequent infestation by L2 larvae or female adults but are not effective against L1 larvae. This may be explained by a capacity of L1 larvae to suppress the defenses activated by L2 larvae and adult thrips. Possibly, plant defense activation is correlated with JA signaling and *VSP2* induction, while defense repression is correlated with ET signaling and *PDF1.2* induction.

DISCUSSION

Plants have to deal with not only a large variety of insect herbivores, but also with the different developmental stages that an insect herbivore goes through, from eggs to larvae to adults. Transcriptomic studies on different stages of several pest insects demonstrated significant changes in gene expression between the successive developmental stages (Yang *et al.,* 2013a; Vogel *et al.,* 2014; Tian *et al.,* 2015; Chen *et al.,* 2016). These differential gene expression profiles characterize specific functions for each developmental stage of the pest insect. This could result in a differential counter-defense response by the plant on which the pest insect is developing. This has been shown in garlic mustard (*A. 101etiolate*) where two distinct compounds were found to be involved in the resistance against either early or late instars of the native American butterfly (*P. oleracea napi*) (Renwick *et al.,* 2001). Nothing is known about the plant responses against the different stages of the cell-content feeding insect thrips, which goes through six developmental stages. Most studies on thrips resistance have been conducted with adult thrips and mainly focus on the role of JA as the key player in the defense against thrips (Abe *et al.,* 2008, 2009; Selig *et al.,* 2016; Escobar-Bravo *et al.,* 2017). Here we studied the phytohormonal response and associated gene expression in response to the different thrips developmental stages, Additionally, the function of these defense hormone signaling pathways in tolerance to the feeding thrips stages was studied.

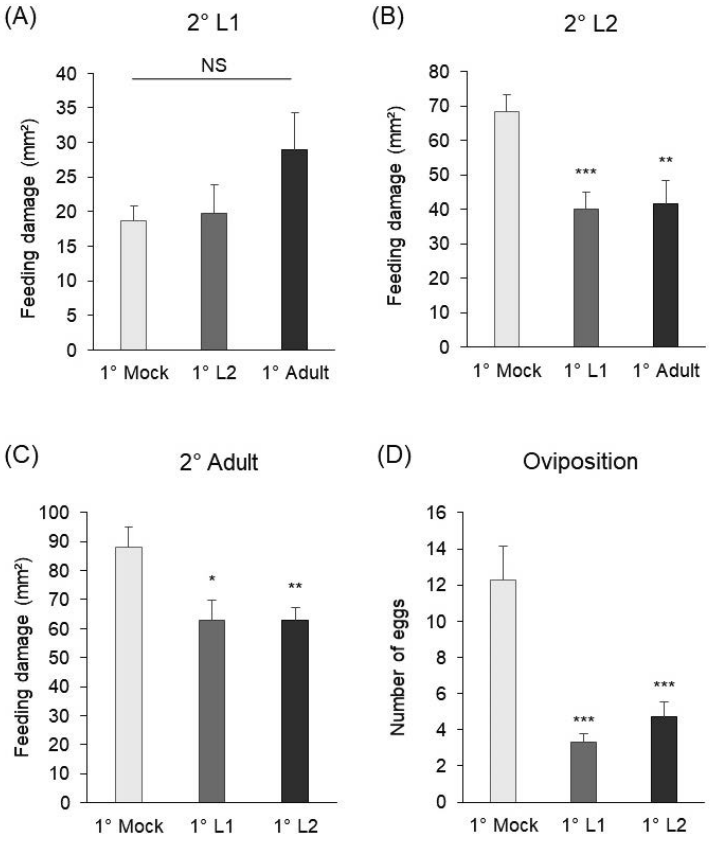

Figure 6: Effect of pre-infestation with different thrips feeding stages on performance of the other stages. Whole 5-week-old wild-type Arabidopsis plants were pre-infested with 5 thrips, either L1 larvae, L2 larvae or adult females (indicated by 1° on the x-axis). Control plants (Mock) were not pre-infested with thrips but were like the thrips-treated plants placed in an enclosed jar. After 24 h, the 1° thrips were removed and the inflicted damage was assessed (mm^2). After that, plants received five thrips were at a different development stage (indicated by 2° in the figure) than when used for the pre-infestation. After 3 days, feeding damage (mm^2) was assessed of **(A)** L1 feeding on plants that had been pre-infested by L2 larvae or adult female thrips, **(B)** L2 feeding on plants that had been pre-infested by L1 larvae or adult female thrips and **(C)** adult female feeding that had been pre-infested by L1 or L2 larvae. The plants that received female adults as second interactor were harvested for Trypan blue staining to visualize the eggs **(D)**. Asterisks indicate a statistically significant difference between the indicated treatments (ANOVA, Dunnett post-hoc test; NS = not significant, $*$ = $P < 0.05$, $**$ = $P < 0.01$, $***$ = $P <$ 0,001). Error bars represent SE, n = 10 plants.

Thrips eggs locally activate the SA-responsive marker gene *PR1*

Previous studies have shown that the egg extracts of different insect herbivores triggers the accumulation of SA which benefits the future progeny by suppressing anti-herbivore defenses (Little *et al.,* 2007; Bruessow *et al.,* 2010; Stahl *et al.,* 2020). Here we demonstrated that thrips eggs also induce local SA responses, as evidenced by the activation of the SA-responsive

gene *PR1* (Fig. 1). Whether thrips egg-induced SA pathway activation would benefit the performance of the future thrips progeny, which is sensitive to JA-dependent defenses (Fig. 4 and 5), would be interesting to investigate. Our study showed that exogenous application of SA did not alter performance of thrips at different feeding stages (Fig. 4). Other egg studies tested extracts of *P. brassicae* eggs (Bruessow *et al.,* 2010), but the collection of thrips eggs that are laid in the epidermal layer of the leaf are hardly visible by eye and is much more challenging. Additionally, compared to ovipositing butterflies, female thrips feed from the plant before oviposition which will activate a herbivore-induced response. Therefore, to solely study the egg-induced response, it is essential to avoid any response induced by feeding female thrips. A possibility to collect thrips eggs would be by letting female thrips lay eggs through parafilm (Murai & Loomans, 2001), but a remaining challenge is that a large number of female thrips is required to get enough eggs for extraction.

Differences in transcriptional changes induced by different thrips stages are not caused by differential hormone accumulation

Several studies have monitored the expression of SA- and JA-responsive genes in Arabidopsis during thrips feeding. Abe *et al.* (2008, 2009) showed that feeding by adult thrips resulted in the activation of both SA- and JA-associated marker genes *PR1, VSP2* and *PDF1.2* (Abe *et al.,* 2008, 2009). In our study, we found that feeding by adult female thrips increased the SA concentration in the infested leaf (Fig. 3A) but the SA response marker gene *PR1* was not activated by any of the thrips feedings stages (data not shown). Moreover, in our experiments the adult thrips significantly enhanced the expression of *VSP2* (Fig. 2A), but not *PDF1.2* (Fig. 2B). Feeding by L2 larvae resulted in comparable expression levels as found for adult thrips feeding. Feeding by L1 larvae also resulted in an enhanced expression of *VSP2* (Fig. 2A), but interestingly, only L1 larval feeding resulted in a significant activation of *PDF1.2* (Fig. 2B). This *PDF1.2* induction was also reported by De Vos *et al.* (2005) who monitored the expression of different phytohormone-associated marker genes during thrips larval feeding. Although the authors did not specify which developmental stage was used, RNA blot and microarray analyses revealed that in their experiments thrips larvae activated *PDF1.2,* while *VPS2* was not induced at the time points tested (De Vos *et al.,* 2005).

Quantity, composition and timing of the different hormones determine the activation of a specific signaling pathway. Within the JA signaling pathway, the relative contribution of ABA and ET signaling determine the outcome of the crosstalk between the MYC- and ERF-branch (Vos *et al.,* 2013b, 2019). Because L1 larvae induced lower levels of *VSP2* compared to the other stages, but higher levels of *PDF1.2*, it was hypothesized that L1 larval feeding stimulates higher levels of ET, and L2 larvae and adult female thrips higher levels of ABA. However, we found that all feeding stages significantly enhanced the accumulation of almost all the

measured hormones (Fig. 3), with the overall trend of highest levels of JA(-Ile), ABA and ET upon adult female thrips feeding, followed by L2 larval feeding. The hormone levels induced by L1 larvae were always lower compared to the levels induced by the other stages. This could be explained by the lower ingestion rate of L1 larvae. A study by Kindt *et al.* (2003) demonstrated that the ingestion rate of cell contents was the lowest for L1 larvae (Kindt *et al.,* 2003). We also found this correlation, with L1 larvae inflicting less damage compared to L2 larvae and adults (Supp. Fig. 1). Nevertheless, increasing the number of L1 larvae on a leaf, which expectedly results in enhanced feeding damage, led to enhanced induction levels of both *VSP2* and *PDF1.2* (Supp. Fig. 2), indicating that the amount of damage cannot explain the differences in induction levels of *VSP2* versus *PDF1.2* by the different thrips developmental stages. Overall, there was no causal correlation between the differential induction of the tested defense marker genes *PR1, VSP2* and *PDF1.2* with the different defense hormone accumulation levels, as induced by the three thrips feeding stages.

Effect of phytohormones on the performance of thrips larvae and adults

The differential activation of *VSP2* and *PDF1.2* may be caused by either the plant actively triggering different defense mechanisms against the different thrips stages, or by the thrips stages having different abilities to manipulate the plant's defenses by actively inducing a certain pathway and thereby suppressing the other. In order to relate the performance of all the feeding thrips stages to the relative hormone and gene expression levels, we monitored the amount of damage inflicted by each thrips developmental stage on plants pre-treated with SA, MeJA, ABA and ACC as well as on hormone-deficient mutants *sid2-1* (SA-deficient),*coi1-34* and *myc2,3,4* (JA insensitive), *aba2-1* (ABA-deficient), *ein2-1* (ET insensitive) and *ora59* (JA/ET insensitive). In line with previous findings for adult thrips performance, plants that were treated with MeJA suffered significantly less damage inflicted by L1 and L2 larvae (Fig. 4). This correlated with the finding that all three thrips stages inflicted significantly more feeding damage on the *coi1-34* and *myc2,3,4* mutant plants, which are insensitive to JA (Fig. 5). In contrast to what was reported by Abe *et al.* (2009), our study shows that exogenous application of ACC had a negative effect on thrips performance (Fig. 4). This difference may be related to the leaf-disc set-up used by Abe *et al.* (2009) that somehow might have enhanced the repressive effect of ET on defense against thrips. Interestingly, the ET-insensitive mutant *ein2-1* was more resistant to L1 larvae and female adults (Fig. 5). This could be the result of an enhanced production of JA in the *ein2-1* mutant, which was demonstrated for *ein2-1* mutants that were subjected to the chewing caterpillar *Pieris rapae* or the necrotrophic fungus *Alternaria brassicola* (Penninckx *et al.,* 1998; Vos *et al.,* 2019). Vos *et al.* (2019) also showed that *ein2-1* plants were more resistant to *P. rapae* caterpillars, which was correlated with an increased activation of *VSP2*.

L1 larvae potentially manipulate defenses induced by L2 larvae and adult thrips

Because of the differential activation of the MYC- and ERF-branch of the JA pathway during feeding by the different thrips developmental stages, we hypothesized that activation of one of the branches stimulates plant defense against thrips, while the other is related to defense suppression/enhanced plant susceptibility. In this study we found that a 24-h pre-infestation with one of the feeding thrips stages significantly reduced the performance of L2 larvae and female adults (Fig. 6B-D), while the performance of L1 larvae was not affected by a pre-infestation by one of the other thrips stages (Fig. 6A). Compared to the Mock (no pre-infestation), there was even a slight but non-significant increase in damage inflicted by the L1 larvae after a 24-h pre-infestation with female adults. It could thus be suggested that L1 larvae have the capacity to circumvent the defenses activated by L2 larvae and adult thrips. A study by Abe *et al.* (2013) revealed that when adult leafminers (*Liriomyza trifolii*) were allowed to feed on Arabidopsis for 7 days, the first 3 days both *VSP2* and *PDF1.2* levels were induced. However, after 3 days, when the eggs oviposited by the adults hatched, larvae started to feed from the plant as well, and at this stage *VSP2* levels decreased and *PDF1.2* increased. The auteurs proposed that this differential gene induction was related to differences in feeding style between adults and larvae. However, it could also be possible that the young larvae harbor effector proteins that suppress MYC-branch activation through enhanced activity of the ERF-branch. Effectors have successfully been identified for several insect herbivore species (Bos *et al.,* 2010; Zhao *et al.,* 2015; Thorpe *et al.,* 2016; Villarroel *et al.,* 2016). The L1 larvae of the Hessian fly (*M. destructor*) are the most active in secreting effector proteins for defense manipulation (Chen *et al.,* 2016).

Collectively, this study demonstrated that thrips eggs locally accumulated SA, while all the feeding thrips stages, though to a different extend, triggered the accumulation of JA and induced associated marker gene expression. Although all feeding stages enhanced the expression of the MYC-branch marker gene *VSP2*, L1 larvae exclusively activated the ERF-branch marker gene *PDF1.2.* The results from the different thrips feeding performance assays using plants pre-treated with hormones, pre-infested by thrips or mutated in hormone pathways imply that the plant response to different developmental stages of thrips is complex and likely not fully explainable by the canonical hormone-regulated defense responses. However, we found indications for plant defense activation via JA signaling associated with *VSP2* induction. Moreover, defense repression may occur via ET signaling associated with *PDF1.2* induction, which is most apparent during L1 larval infestation. Full transcriptome profiling of individual thrips developmental stages and of the infested leaf tissue might reveal stage-specific gene expression signatures and aid the search for resistance mechanisms against thrips.

MATERIALS AND METHODS

Plant material and cultivation

The wild-type and mutant *Arabidopsis thaliana* (Arabidopsis) plants used in this study were in the Columbia ecotype (Col-0) background. Seeds of *coi1-34* were kindly provided by Prof. E.E. Farmer (University of Lausanne) and *myc2,3,4* (AT1G32640/AT5G46760/AT4G17880; Fernández-Calvo *et al.,* 2011) by Prof. R. Solano. Seeds of Arabidopsis accession Col-0, mutants *sid2-1* (Nawrath & Métraux, 1999), *coi1-34, myc2,3,4, aba2-1* (Koornneef *et al.,* 1982)*, ein2-1* (Alonso *et al.,* 1999) and *ora59* (Zander *et al.,* 2014) (Nawrath & Métraux, 1999) and transgenic lines *PR1::GUS* (Koornneef *et al.,* 2008)*, LOX2::GUS* (Jensen *et al.,* 2002) and *GCC::GUS* (Pré *et al.,* 2008; Zarei *et al.,* 2011) were stratified for 48 h in 0.1% agarose at 4°C. Subsequently, seeds were sown on river sand that was supplemented with 250 mL of modified half strength Hoagland solution containing 10 uM sequestreen (CIBA-Geigy, Basel, Switzerland) per kg of sand (Steenbergen *et al.,* 2020). The tray with sown sand was covered with a lid to ensure 100% relative humidity. From then on, plant cultivation took place in a controlled greenhouse compartment with a 10-h day and 14-h night cycle at 21°C and 70% relative humidity (outside the closed tray). Two-week-old seedlings were transferred to 60-mL pots containing a river sand:soil mixture (5:12, v:v) that had been autoclaved twice for 45 min with a 24-h interval. The autoclaved soil was supplemented with 50 mL kg^{-1} modified half-strength Hoagland solution. After transplanting, plants were kept under a RH of 100% for 2 days, after which the lids were cracked so the plants could gradually acclimate to the relative humidity of 70% in the greenhouse for the remainder of the experiment. Plants were watered every other day and received 10 mL of modified half-strength Hoagland solution once a week.

Thrips rearing

Western flower thrips (*Frankliniella occidentalis* (Pergande)), originally collected from pepper (*Capsicum annuum*) leaves (Enza Zaden, Netherlands), were reared in a climate chamber (10-h day, 14-h night, 26°C) on common beans (*Phaseolus vulgaris*) and lettuce (*Lactuca sativa* var. *capitata*) in bugdorms (https://shop.bugdorm.com/) (Steenbergen *et al.,* 2020).

GUS assay

GUS staining was performed on 5-week-old plants after being exposed to female adult thrips for 4 days. Whole plants were submerged in a GUS staining solution (1mM X-Gluc, 100 mM NaPi buffer, pH 7.0, 10 mM EDTA and 0.1% (v:v) Triton X-100), vacuum infiltrated and incubated overnight at 37°C, and chlorophyll was further destained with 96% ethanol (Spoel *et al.,* 2003). Images were taken with a Zeiss Fluorescent microscope fitted with a camera.

Thrips assays

Gene expression and phytohormone analyses

Leaf number 9 was selected of 5-week-old Arabidopsis plants. For ET measurements the leaf was cut and placed in a vial (see "Phytohormone analysis – ET measurements"); for all other assays the plants stayed intact and the leaf received a clip cage. The vial or clip cage received no thrips (mock) or 5 individuals of L1 larvae, L2 larvae or female adults were added. L1 and L2 larvae were collected with a fine paintbrush, adult female thrips were collected using an aspirator and transferred into a clip cage. Clip cages were hand made in the lab (mesh from Kabel Zaandam B.V., pore size 80-micron; plastic rings (Ø 21 mm, height 5 and 8 mm) from Kunststofshop.nl; metal clips from Kapperskorting.nl; polyether foam (3 mm thick) in between plastic rings from Crica B.V.). An individual leaf corresponded to a single replicate and 5 biological replicates per treatment were harvested after the time points indicated in the legends.

Whole-plant performance assay

Five-week-old plants were placed individually in PET jars (Nipak B.V., 1-L straight cylinder jar, HxLxW: 139x104x104 mm) (Steenbergen *et al.,* 2020; **Chapter 2**). Each plant received a pre-treatment with 5 thrips, which were either L1 larvae, L2 larvae or adult females. Control plants (Mock) were not pre-treated with thrips. Feeding damage was estimated in mm^2 by assessing the damaged spots on the entire plant. Plants that were subsequently infested by female adults were collected in 15-mL falcon tubes for oviposition count (see Material and Methods, 'Trypan Blue Staining').

Trypan blue staining

To visualize thrips eggs, plants were stained with a Trypan Blue mix (adapted from Koch & Slusarenko, 1990): lactic acid, glycerol and demi-water (1:1:1, v:v:v) plus 0.025% Trypan blue. An equal amount of this total volume was supplied with an equal volume of 96% ethanol. The Trypan blue mix was added into the tubes until the whole plant was covered. Tubes were boiled in a water bath for 2-3 min and subsequently cooled down for 30 min. The Trypan blue solution was removed after which a lactoglycerol solution (lactic acid, glycerol and demi-water (1:1:1, v:v:v) was applied for destaining. Eggs were visualized under a binocular where they can be recognized as dark blue, kidney-shaped structures.

RNA extraction and RT-qPCR

Total RNA was isolated as described by Oñate-Sánchez & Vicente-Carbajosa (2008). RNA was pre-treated with DNAse I (Fermentas) to remove genomic DNA. DNA-free RNA was converted into cDNA using RevertAid H minus Reverse Transcriptase (Fermentas) and an oligo-dT primer. PCR reactions were performed in optical 384-well plates (Applied Biosystems).

To monitor the synthesis of double stranded DNA, SYBR® Green was detected with the ViiATM 7 Real-Time PCR system. A standard thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. After cycle 40, the amplicon dissociation curves were recorded by heating from 60°C to 95°C with a ramp speed of 1.0°C min^{-1}. Transcript levels were calculated relative to the reference gene At1g13320 (Czechowski *et al.,* 2005) using the $2^{-\Delta\Delta CT}$ method as described by Schmittgen & Livak, (2008).

The AGI numbers for the genes studied by RT-qPCR are At2g14610 (*PR1*), At5g24770 (*VSP2*), and At5g44420 (*PDF1.2*).

Phytohormone analysis

SA, JA, JA-Ile and ABA,

For the quantification of ABA, JA, JA-Ile, OPDA and SA approximately 100 mg of leaf tissue, existing of a pool of 5 number-9-leaves (see 'Thrips assays – Gene expression and phytohormone analyses') was collected in 2-mL safe-lock tubes containing 2 glass beads (3-mm Ø). Five replicates were sampled. A maximum of 25 mg of frozen leaf material was used for sample extraction; 1 mL of 10% cold MeOH:H_2O (v:v) containing stable-isotope-labelled internal standards D_6-SA, D_5-JA and D_6-ABA was added after which samples were further processes and analyzed by UHPLC-MS/MS as described by (Floková *et al.,* 2014).

ET measurements

For the analysis of ET production (parts per billion) leaf number 9 of 5-week-old Arabidopsis was placed in a sealed 10-cc glass vial. Five replicates were sampled. Air was drawn off each vial using a 1-mL syringe and manually injected into a gas chromatograph (Syntech Spectras GC955; Groningen, The Netherlands) equipped with a Haye Sep 80/100 column and photo-ionization detector (oven T = 105°C; minimum detectable concentration, 0.01 µL L^{-1}).

Chemical treatments

The leaves of 5-week-old Col-0 plants were dipped in a solution containing either 1 mM SA (Malinkrodt Baker, Deventer, the Netherlands), 100 µM MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands), 100 µM ABA (Sigma, Steinheim, Germany) or 1.0 mM ACC and 0.015% (v:v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands). MeJA and ABA solutions were diluted from a 1000-fold concentrated stock in 96% ethanol (final concentration 0.1% (v:v) 96% ethanol). As a control, the mock solution contained 0.015% (v:v) Silwet L77 and 0.1% (v:v) 96% ethanol.

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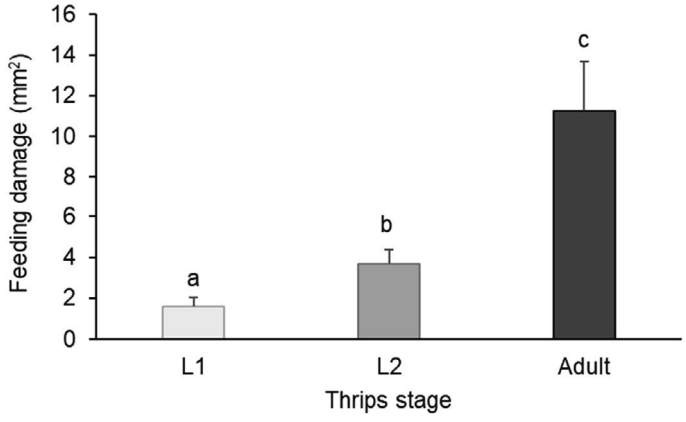
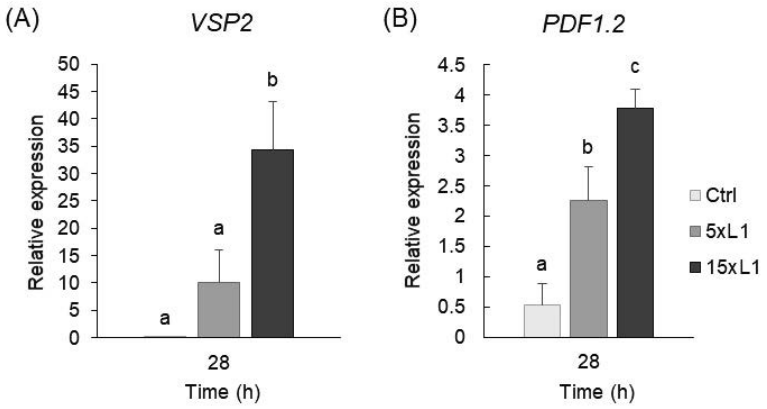
SUPPLEMENTAL INFORMATION



Supplemental Figure 1: Average feeding damage inflicted by an equal number of L1, L2 and adult thrips within 48 h. Five thrips of L1 larvae, L2 larvae or adult females were allowed to feed on a whole plant for 48 h after which the inflicted damage area was monitored. Different letters indicate statistically significant differences between the treatments within the indicated time point (ANOVA, Tukey post-hoc test; $P < 0.05$). Error bars represent SE, n = 9-10 plants.



Supplemental Figure 2: Differential JA response in wild-type Arabidopsis (Col-0) plants in response to feeding by different densities of L1 larvae. RT-qPCR analysis of **(A)** *VSP2* and **(B)** *PDF1.2* gene expression levels in leaf number 9 of 5-week-old Arabidopsis plants after 28 h of feeding by 5 or 15 thrips at L1 larval stage in a clip cage. Control plants (Mock) were treated with an empty clip cage. Different letters indicate statistically significant differences between the treatments (ANOVA, Tukey post-hoc test; $P < 0.05$). Error bars represent SE, n = 3-5 plants.

CHAPTER 5

Differential activation of JA defense responses in local and systemic Arabidopsis leaves by insects from different feeding guilds

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ABSTRACT

Feeding by tissue-chewing or cell-content feeding herbivores results in local accumulation of the hormone jasmonic acid (JA), a major player in the regulation of anti-herbivore plant defenses. In preparation for future herbivore attack, undamaged leaves of plants that are fed on a single leaf by tissue-chewing insects also activate the JA pathway. It is unknown whether such long-distance activation of JA signaling is also initiated by cell-content feeding herbivores, such as thrips, and whether this leads to protection against a subsequent thrips attack. To test this, single mature *Arabidopsis thaliana* leaves were treated with either the cell-content feeding western flower thrips (*Frankliniella occidentalis*) or the tissue-chewing caterpillar *Mamestra brassicae* (Mamestra), after which we determined the systemic expression of different JA-responsive marker genes and the level of protection against subsequent thrips infestation. We demonstrate that herbivory by either of the insects locally enhanced the expression levels of *PDF1.2* and *VSP2*, which are markers for the activation of the ERF- and MYC-branch of the JA pathway, respectively. Systemically, only thrips and not Mamestra feeding induced *PDF1.2*, which correlated with enhanced preference of subsequently attacking thrips for the youngest systemic leaf tissue. Additionally, *LOX2*, *LOX3* and *LOX4* were only systemically induced by thrips and not by Mamestra. In comparison to thrips, Mamestra induced a relatively higher systemic expression of *VSP2* in the mature and older systemic leaf tissue, which was associated with enhanced deterrence of thrips. Taken together, this study demonstrates that herbivory by insects from different feeding guilds differentially triggers genes related to the JA pathway and has different consequences for subsequent thrips feeding behavior on local and systemic leaves of different ages.

INTRODUCTION

To protect themselves from insect herbivore attack, plants possess different direct and indirect defense mechanisms (War *et al.,* 2012; Dicke, 2015). Such defenses can be expressed constitutively, but to prevent unnecessary expression of costly defenses, many defenses are induced only upon an actual attempt of the herbivore to infest the plant (Züst & Agrawal, 2017). Induction of anti-herbivore defenses is initiated by perception and recognition of diverse cues, such as herbivore-associated molecular patterns (HAMPs; Mithöfer & Boland, (2008)) and endogenous damage-associated molecular patterns (DAMPs; (Duran-Flores & Heil, 2016). Subsequent to herbivore recognition, cellular signaling cascades are triggered including the elevation of cytosolic calcium, depolarization of the plasma membrane potential, activation of mitogen-activated protein kinases, and production of reactive oxygen species (Zebelo & Maffei, 2015; Gilroy *et al.,* 2016). These cascades lead to rapid accumulation of phytohormones such as jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET) (Howe & Jander, 2008; Broekgaarden *et al.,* 2015; Nguyen *et al.,* 2016), which regulate distinct defense signaling pathways in the plant. Cross-communication between these hormone-inducible pathways allows fine-tuning of the defense response directed to the attacker at hand (Pieterse *et al.,* 2012). JA is considered as a core player in plant defense against herbivorous insects and necrotrophic pathogens. Two different branches of the JA pathway are distinguished: the MYC-branch, which is primarily activated by and effective against herbivores, and the ERF-branch, which is primarily activated by and effective against necrotrophic pathogens. In *Arabidopsis thaliana* (hereafter Arabidopsis) the MYC-branch is controlled by the bHLH-type transcription factors (TFs) MYC2, MYC3, MYC4 and requires activation of JA and ABA signaling (Anderson *et al.,* 2004; Dombrecht *et al.,* 2007; Fernández-Calvo *et al.,* 2011; Niu *et al.,* 2011). The ERF-branch is regulated by the ERF-type TFs ERF1 and ORA59 and requires JA and ET signaling (Penninckx *et al.,* 1998; Lorenzo *et al.,* 2003; Anderson *et al.,* 2004; Pré *et al.,* 2008). Activation of the MYC- and ERF-branch leads to expression of the marker genes *VSP2* and *PDF1.2*, respectively (Verhage *et al.,* 2011; Vos *et al.,* 2013b).

Mechanical wounding as well as feeding by tissue-chewing or phloem feeding insects has been demonstrated to induce accumulation of JA and to activate JA-responsive genes, not only at the local site of wounding/feeding, but also in distal parts of the plant (Farmer *et al.,* 2014; Salvador-Recatalà *et al.,* 2014). The accumulation of JA in distal, undamaged parts of the plant can be initiated by either airborne or vasculature-transported signals (Frost *et al.,* 2008; Ruan *et al.,* 2019). Internal signals travel through the vascular system depending on the interleaf connectivity (parastichies), which is developmentally determined. Arabidopsis, like most other flowering plants and ferns, has a spiral phyllotactic leaf development, causing leaves to share a vascular connection with every third (n±3), fifth (n±5) and eighth leaf (n±8)

(Dengler, 2006; Mousavi *et al.,* 2013). Local wounding of a leaf was demonstrated to result in accumulation of JA and the highly bio-active JA conjugate JA-Ile in an unwounded, vascular connected leaf, but not in a non-connected leaf (Glauser *et al.,* 2009; Chauvin *et al.,* 2013). A similar pattern was shown for the expression of *JAZ10*, an important regulator of the JA response and serving as a marker gene for the JA pathway (Mousavi *et al.,* 2013; Heyer *et al.,* 2018), and for *VSP2*, serving as a marker for the MYC-branch of the JA pathway (Chauvin *et al.,* 2016). Also in other plant species it has been demonstrated that the systemic accumulation of JA and activation of JA-responsive defenses upon mechanical wounding, either or not with the application of herbivore oral secretions, is constrained to vascular connectivity (Orians *et al.,* 2000; Schittko & Baldwin, 2003; Orians, 2005; Frost *et al.,* 2007; Hettenhausen *et al.,* 2014). The production of reactive oxygen species, the elevation of cytosolic calcium, and depolarization of the plasma membrane potential are considered as key regulators of local and systemic signaling responses (Choi *et al.,* 2017). Changes in the cytosolic calcium concentration are dependent on glutamate receptor-like (GLR) ion channels (Toyota *et al.,* 2018), which have been demonstrated to mediate systemic defenses associated with accumulation of JA-Ile and activation of *JAZ10* (Mousavi *et al.,* 2013; Choi *et al.,* 2017; Toyota *et al.,* 2018). The exact mechanism by which calcium mediates the wound-induced increase of JA-Ile has yet to be identified. It has been suggested that upon wounding, calcium-dependent phosphorylation of the JAV1-JAZ8-WRKY51 complex leads to its disintegration, thereby relieving the suppression of JA biosynthesis (Yan *et al.,* 2018). Calcium-dependent control of LOX mRNA accumulation and LOX activity could also influence JA biosynthesis (Bonaventure *et al.,* 2007).

The activation of JA signaling in distal, yet undamaged parts of the plant is advantageous because it prepares the tissue for subsequent herbivore attack (Stork *et al.,* 2009; Vos *et al.,* 2013b; Bozorov *et al.,* 2017). Vos *et al.* (2013b) demonstrated that local infestation of Arabidopsis by the tissue chewing caterpillar *Pieris rapae* primes the systemic leaves for enhanced ABA-dependent activation of *VSP2*, which is associated with enhanced resistance to subsequent *P. rapae* attack. Little is known about the systemic responses induced upon feeding by insects that do not damage the vasculature, such as thrips (Kindt *et al.,* 2003). These tiny insects (1.5-mm or less) are major pests in many vegetable and ornamental crops worldwide. Both adults and larvae of thrips feed on the leaves, stems, petals and/or fruits by piercing their stylets into the epidermal cells and ingesting their contents. Pierced cells collapse or fill with air, which gives the damaged area a silvery appearance (Steenbergen *et al.,* 2018). Thrips infestation has been demonstrated to lead to local JA accumulation in Arabidopsis (Abe *et al.,* 2008, 2009) and induction of JA-responsive genes in different thrips-host plant systems (De Vos *et al.,* 2005; Abe *et al.,* 2008, 2009; Selig *et al.,* 2016; Escobar-Bravo *et al.,* 2017). Activation of the JA pathway is very important for plant defense to thrips infestation, as revealed

by reduced feeding damage upon exogenous application of JA (Thaler, 1999; Abe *et al.,* 2009; El-Wakeil *et al.,* 2010) and oppositely, increased feeding damage on JA-deficient or -non-responsive plants (Abe *et al.,* 2009; Escobar-Bravo *et al.,* 2017; Chen *et al.,* 2018; Sarde *et al.,* 2018).

In this study we investigated the ability of the cell-content feeding western flower thrips (*Frankliniella occidentalis*) to activate JA-associated defenses in systemic, undamaged leaves of Arabidopsis. We demonstrated that compared to the tissue-chewing caterpillars of *Mamestra brassicae* (Mamestra), local thrips infestation systemically activated relatively higher expression levels of ERF-branch genes and lower levels of MYC-branch genes. Prior infestation by thrips shifted the preference of a subsequent thrips attack to the youngest leaves, whereas prior infestation by Mamestra led to increased deterrence of thrips from the primary-attacked mature leaves. These findings point to the possibility that thrips can modulate the plant's defenses in the younger tissue such that it benefits the performance of thrips.

RESULTS

Effect of local thrips or Mamestra feeding on systemic JA responses in vascular connected and non-connected leaves

We investigated the activation of JA-responsive defenses in vascular connected and non-connected leaves in response to the cell-content feeding herbivore thrips in comparison to the tissue chewing herbivore Mamestra. Therefore, we treated (mature) leaf number 9 (leaf n) with either an empty clip cage (Mock) or a clip cage containing 5 individuals of thrips (L2 larvae) or an L1 Mamestra caterpillar. After 24 h, the expression of different JA-responsive genes was monitored in the locally treated leaf n, a vascular connected leaf (number 12, referred to as n+3) and a non-connected leaf (number 8, referred to as n-1) (Fig. 1A). *JAZ10* was chosen as a marker for the activation of the JA pathway. The expression of the key TF genes *MYC2* and *ERF1*, as well as their respective target marker genes *VSP2* and *PDF1.2*, was assessed to study the activation of the MYC- and ERF-branch of the JA pathway, respectively.

The *JAZ10* gene was strongly induced locally by both herbivores but not systemically (Fig. 1B). The MYC- and ERF-related genes nearly all showed a local induction and some a systemic induction, depending on the attacking herbivore. Mamestra feeding resulted in a significant increase in *MYC2* transcript levels in the local leaf only. This correlated with an enhanced expression level of its target gene *VSP2*, which was also apparent in both the vascular connected and non-connected leaves, albeit to a lesser extent (Fig. 1C and D). Thrips infestation, on the other hand, did not induce *MYC2* expression and the *VSP2* transcript level was enhanced in the local leaf only. The *ERF1* gene was induced by both thrips and Mamestra in the locally infested leaf and this was associated with an enhanced expression level of its

target gene *PDF1.2* in the local leaf. However, only thrips and not Mamestra significantly enhanced *PDF1.2* expression in the vascular connected and non-connected leaves.

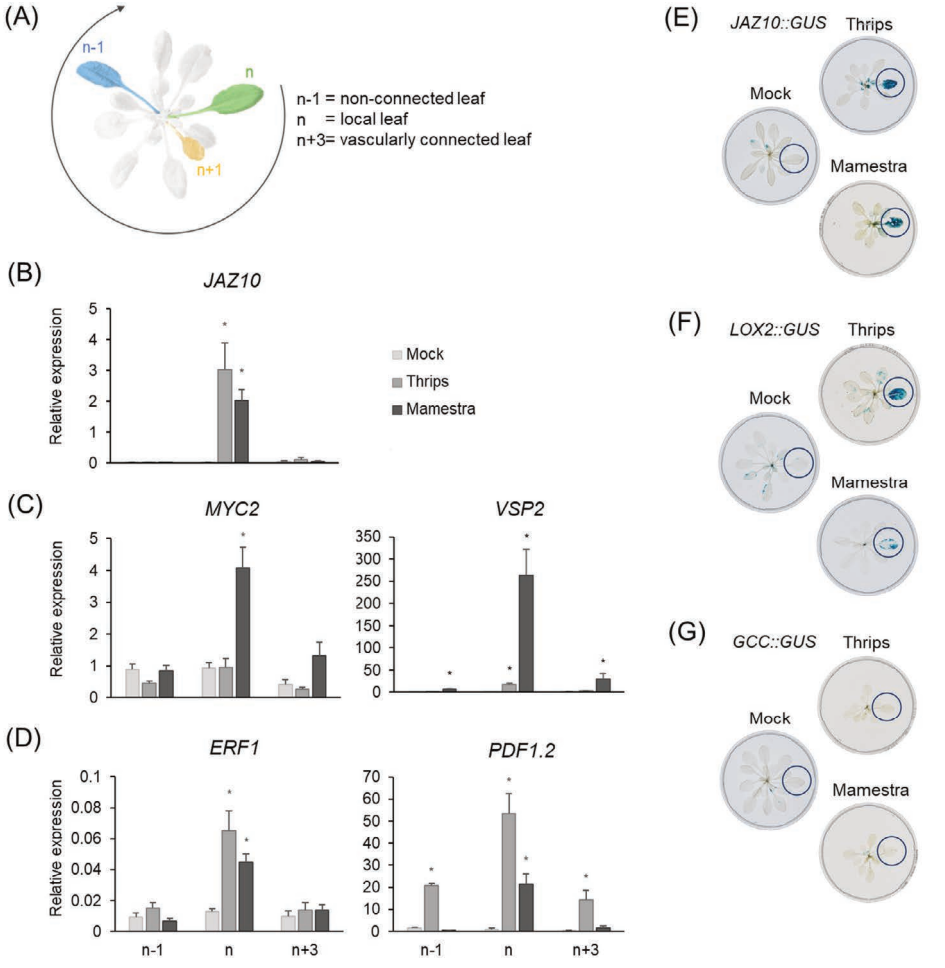

Figure 1: Effect of local thrips or Mamestra infestation on the expression of JA-responsive marker genes in vascular connected and non-connected leaves. (A) Illustration of Arabidopsis rosette growth spiral depicting the locally infested leaf (number 9, referred to as n, in green), the vascular connected leaf (number 12, referred to as n+3, in yellow) and the non-connected leaf (number 8, referred to as n-1, in blue) used in Fig. 1B-D. The arrow indicates the direction of the leaf development from old to young. Leaf number 9 (n) was treated with either an empty clip cage (Mock) or a clip cage containing 5 individuals of thrips (L2 larvae) or a single L1 Mamestra caterpillar for 24 h. The distal leaves (n-1 and n+3 in 1B) were not treated. **(B-D)** Quantification of the expression of JA-responsive marker genes *JAZ10, MYC2*, *VSP2*, *ERF1* and *PDF1.2*, relative to the reference gene At1g13320, after a 24-h local treatment of leaf n. Asterisks indicate statistically significant differences between the indicated herbivore treatment and the mock treatment (Student's *t*-test; $P < 0.05$). Error bars represent SE, n = 3-5 plants. **(E-G)** Representative Arabidopsis plants, stained to determine the GUS activity of *JAZ10::GUS*, *LOX2::GUS* and *GCC::GUS* lines after a 24-h local treatment (encircled leaf in 1E-G represents leaf n as displayed in 1A).

We also assayed GUS reporter lines to monitor the activity and localization of JA-associated gene expression after 24 h of local thrips or Mamestra infestation. We used the transgenic lines *JAZ10::GUS* (reporting JA pathway activity according to Acosta *et al.* (2013)), *LOX2::GUS* (reporting MYC-branch activity according to Jensen *et al.* (2002)), and *GCC::GUS* (reporting ERF-branch activity by activation of the GCC-box motif by ERF TFs according to Zarei *et al.* (2011)). Strong *JAZ10::GUS* and *LOX2::GUS* activity was observed in the leaves that were locally treated with thrips or Mamestra, whereas *GCC::GUS* activity was not induced by neither of the two herbivores. *JAZ10::GUS* activity was not systemically enhanced by Mamestra, while some patchy expression in mock- and thrips-treated plants was detected (Fig. 1E-G). The *LOX2::GUS* line showed some activity in the leaves adjacent to the local Mamestra-infested leaf (correlating with the systemic activation of the MYC-branch marker gene *VSP2*; Fig. 1C and F) and also the youngest leaves of the thrips-infested *LOX2::GUS* plants displayed some elevated GUS activity.

Collectively, these results suggest that the two studied insects from different feeding guilds both activate JA-responsive genes. Although this response is partly overlapping, thrips feeding shows a relatively higher induction of the ERF-branch, while the MYC-branch was relatively higher activated by Mamestra which was even more evident in the systemic leaves. The expression patterns of the studied JA pathway genes did not solely follow the vascular connections between the leaves of the insect-attacked plants.

Effect of local thrips or Mamestra feeding on subsequent thrips performance

Next, we investigated whether the differential induction of the ERF- and MYC-branch by local thrips or Mamestra infestation could lead to a distinct impact on subsequent thrips infestation on the individual leaves of a whole plant (Fig. 2A). Pre-treatment of leaf n with thrips for 24 h did not significantly enhance the amount of damage inflicted by a 4-day subsequent thrips attack over the whole plant (Fig. 2B). Local pre-treatment with Mamestra resulted in an overall significant reduction of damage inflicted by a subsequent thrips attack. Investigation of the oviposition success, which was also assessed 4 days after application of female thrips on the plant, revealed no significant differences in the total number of eggs deposited after the different pre-treatments, although a lower number was found after both the herbivore pre-treatments compared to the control (Fig. 2C).

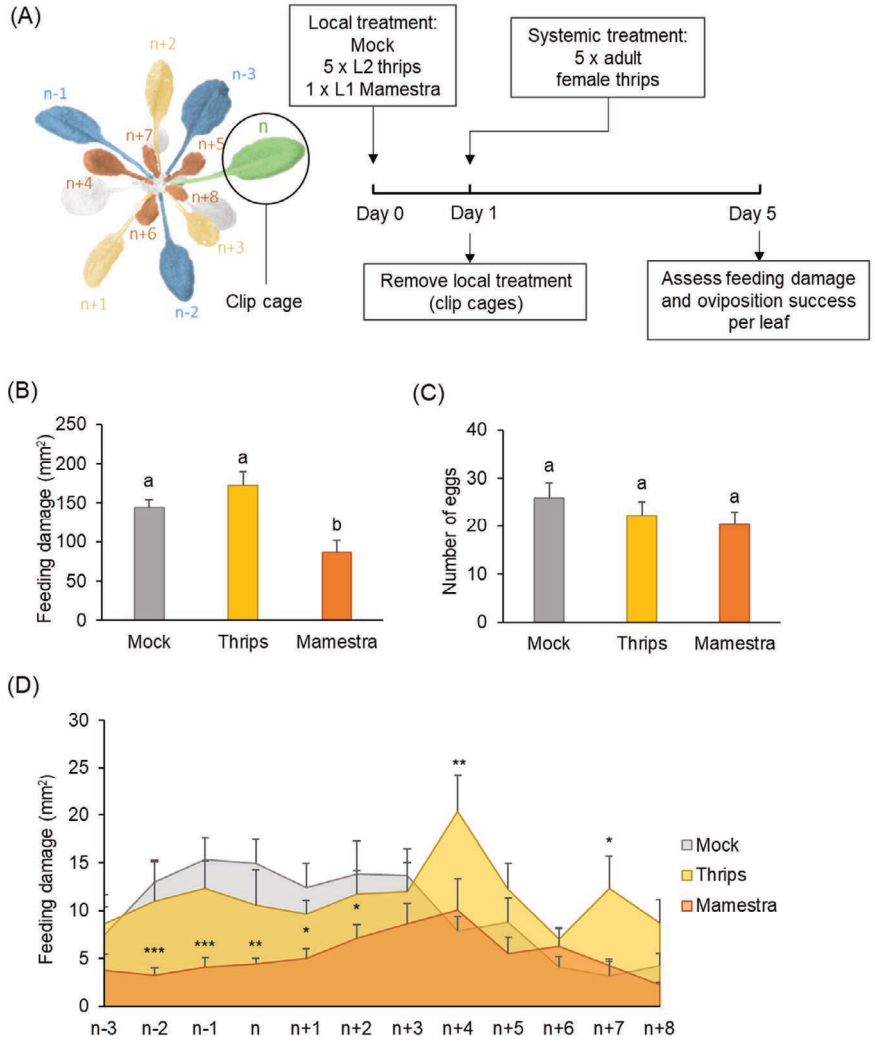

Figure 2: Effect of local thrips or Mamestra infestation on thrips performance on systemic leaves. (A) Experimental timeline of thrips performance assay upon local pre-treatments with thrips or Mamestra. Leaf number 9, (leaf n), of 5-week-old wild-type Arabidopsis (Col-0) plants was treated with either an empty clip cage (Mock), a clip cage containing 5 individuals of thrips (L2 larvae), or a clip cage with a single L1 Mamestra caterpillar for 24 h. Then, the cages and the insects were removed after which 5 adult female thrips were allowed to feed from the whole plant for 4 days. Thrips feeding damage and oviposition on leaves n-3 > n+8 was scored individually. See Figure 3A for further details on leaf age. **(B)** Average thrips feeding damage (mm^2 leaf tissue) on the sum of leaves (n-3 > n+8) per plant inflicted by adult female thrips. **(C)** Average number of eggs oviposited by adult female thrips on the sum of leaves (n-3 > n+8) per plant. **(D)** Average thrips feeding damage (mm^2 leaf tissue) on individual leaves, inflicted by adult female thrips. Different letters indicate statistically significant differences in thrips feeding damage as a result of the different local pre-treatments (ANOVA, Duncan post-hoc test; $P < 0.05$). Asterisks indicate statistically significant differences between the herbivore pre-treatments and the mock pre-treatment on the indicated leaves (Student's *t*-test; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.01$). Error bars represent SE, $n = 10$ plants.

When finely monitoring the individual leaves of all plants, we observed that in the mock-treated plants thrips preferred to feed from leaves that were mature at the time of damage assessment in comparison to youngest, not yet fully developed leaves (Fig. 2D). Local pre-infestation of mature leaf n by thrips resulted in a shift in preference, namely the young leaves n+4 and n+7 were significantly more infested, while all the mature, older leaves were slightly, but not significantly, less preferred. In contrast, after a local pre-treatment with Mamestra, thrips inflicted significantly less damage on the fully developed leaves n-2 till n+2, while the younger leaves were damaged to the same extent as in the mock-treated plants. Taken together, in our set-up local prior infestation by thrips or Mamestra differentially influenced the preference of a subsequent thrips attack for leaves of different ages, which could not be linked to the vascular connections between leaves.

Effect of local thrips or Mamestra feeding on systemic JA responses in leaves of different ages

As shown in Figure 1B-D, infestation by either thrips or Mamestra activated JA-responsive genes. In the two systemic leaves assayed, the ERF-branch was upregulated specifically by thrips while the MYC-branch was upregulated specifically by Mamestra. Here, we aimed to study JA-responsive gene expression in more leaves of different ages to determine whether the observed preference of thrips for youngest leaves versus the deterrence of mature leaves in Mamestra pre-infested plants (Fig. 2D), correlates with a differential expression of the ERF- and MYC-branches in these leaves, respectively. To investigate this, we monitored JA-responsive marker gene expression at 24 h after introduction of the insects on leaf n (leaf 9) in the following 4 different leaf-age categories: 3 mature leaves that were older than the treated leaf (n-3 > n-1), the treated leaf itself (n), the next 3 younger leaves that were (nearly) fully grown (n+1 > n+3), and the following 5 youngest leaves (n+4 > n+8) (Fig. 3A).

Like shown in Figure 1B, the transcript levels of *JAZ10* were significantly enhanced in the local leaf that was treated with thrips or Mamestra (Fig. 3B). Only after local thrips pre-infestation, a small but significant increase of *JAZ10* expression was detected in the younger leaf category (n+1 > n+3), which is in line with our GUS staining data (Fig. 1E), but this did not correlate with a differential thrips feeding performance on this leaf category (Fig. 2D). Similar to what can be observed in Figure 1C, the transcript levels of *MYC2* and its target gene *VSP2* were significantly enhanced in the leaf locally infested by Mamestra, while thrips only locally induced *VSP2* (albeit to a lower level than Mamestra) and not *MYC2* (Fig. 3C). Unexpectedly, only thrips and not Mamestra enhanced the expression of *MYC2* systemically, which was apparent in the older (n-3 > n-1) and younger (n+1 > n+3) leaf categories. The expression of *VSP2* was activated by both insects in all systemic samples, although the transcript levels were relatively higher induced by Mamestra than by thrips. The overall relatively higher activation of

the MYC-branch response genes after Mamestra infestation may thus be partly involved in the observed enhanced protection against thrips in the mature leaf tissue.

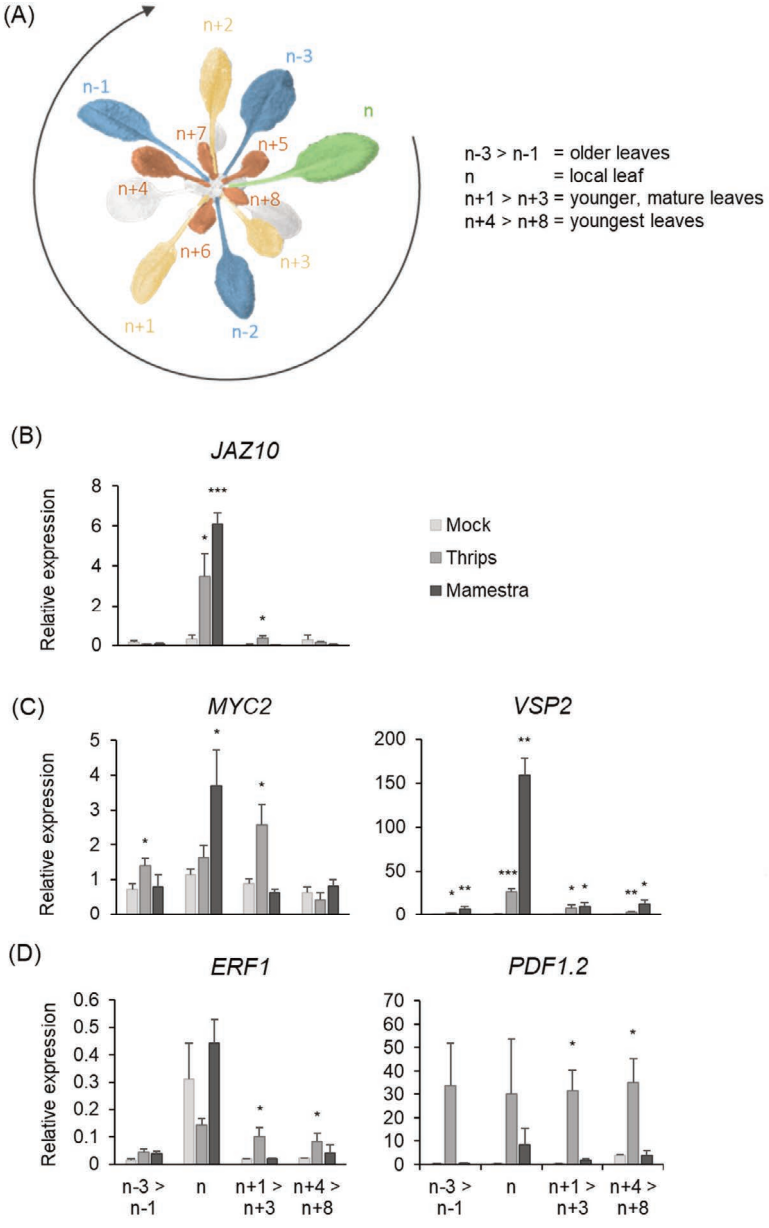

Figure 3: Effect of local thrips or Mamestra on the expression of JA-responsive marker genes in 4 leaf-age categories. (A) Illustration of Arabidopsis rosette growth spiral depicting the locally infested leaf (number 9, referred to as n, in green), the 3 older leaves (n-3 > n-1, in blue), the 3 younger, mature leaves (n+1 > n+3, in orange) and the 5 youngest leaves (n+4 > n+8, in red) used in Fig. 3B-D and Fig. 4. The arrow indicates the direction of the leaf

development from old to young. **(B-D)** Quantification of the expression of JA-responsive marker genes *JAZ10, MYC2*, *VSP2*, *ERF1* and *PDF1.2*, relative to the reference gene At1g13320. Leaf number 9 (n) was treated with either an empty clip cage (Mock) or a clip cage containing 5 individuals of thrips (L2 larvae) or a single L1 Mamestra caterpillar for 24 h, while the distal leaf-age categories (n-3 > n-1, n+1 > n+3 and n+4 > n+8) were not treated. Asterisks indicate statistically significant differences between the indicated herbivore treatment and the mock treatment (Student's *t*-test; $P < 0.05$). Error bars represent SE, $n = 3$-5 plants.

In locally infested leaves, the *ERF1* and *PDF1.2* genes were not statistically significantly induced by either thrips or Mamestra (Fig. 3D). However, for *PDF1.2* a clear trend to enhanced local expression, especially after thrips infestation, was visible (Fig. 3D). Systemically, thrips pre-infestation significantly enhanced the expression of both *ERF1* and *PDF1.2* in the younger leaf categories (n+1 > n+3 and n+4 > n+8), whereas in line with Figure 1D no effects by Mamestra pre-infestation were detected systemically (Fig. 3D). The observed enhanced activity of the ERF-branch after thrips infestation in the systemic tissue, may thus be partly controlling the observed enhanced preference of thrips for the youngest leaf tissue (as activation of the MYC-branch would inhibit their performance). However, there was no strict correlation between the expression of the studied JA-responsive marker genes in the different leaf age categories and the level of protection against thrips feeding after the local pre-infestations by thrips or Mamestra.

Lipoxygenases are systemically induced in response to local thrips infestation

The Arabidopsis genome contains four 13-lipoxygenase genes (encoding *LOX2*, *LOX3*, *LOX4* and *LOX6*) that all contribute in a coordinated fashion to the wound-stimulated biosynthesis of JA and resistance against insects (Chauvin *et al.,* 2013, 2016). LOX2 is responsible for the bulk of the JA biosynthesis, while LOX6 is specifically involved in the initiation of early JA biosynthesis and JA-mediated responses, e.g. induction of *LOX3*, *LOX4*, *JAZ10* and *VSP2* gene expression in wounded, but especially in unwounded leaves (Chauvin *et al.,* 2016). We showed above that both thrips and Mamestra activate the MYC- and ERF-branch, but the MYC-branch seems more highly activated by Mamestra, while the ERF-branch is higher expressed upon thrips infestation. This differential activation is apparent in local but especially in systemic tissue. Here, we studied whether the activation of the different JA branches is associated with activation of the *LOX2*, *LOX3*, *LOX4* or *LOX6* genes upon local thrips or Mamestra infestation. In general, we found that both insects locally induced *LOX2*, *LOX3* and *LOX4*, the induction level by Mamestra being relatively higher than by thrips (Fig. 4). Systemically, thrips had a larger effect on these three LOX genes than Mamestra, as demonstrated by significantly enhanced expression levels of *LOX2*, *LOX3* and *LOX4* in both the younger (n+1 > n+3) and the youngest (n+4 > n+8) leaf-age categories of thrips-infested plants, while in Mamestra-infested plants only *LOX2* expression was significantly enhanced in

the youngest (n+4 > n+8) and the oldest (n-3 > n-1) leaf category (Fig. 4). The *LOX2* expression pattern was mostly in line with the observed *LOX2::GUS* activity in whole rosettes (Fig. 1F). The expression level of *LOX6* was not significantly changed in most of the leaf categories by any of the two insects, although a general trend to a reduced *LOX6* expression upon insect feeding was observed, which was statistically significant in the younger leaf category (n+1 > n+3) after Mamestra pre-infestation. Thus, *LOX2*, *LOX3* and *LOX4* were upregulated by thrips and Mamestra, while *LOX6* was slightly downregulated. In young, systemic leaf tissue, especially thrips induced the *LOX2*, *LOX3* and *LOX4* genes, which correlated with the activation of the ERF-branch in this tissue.

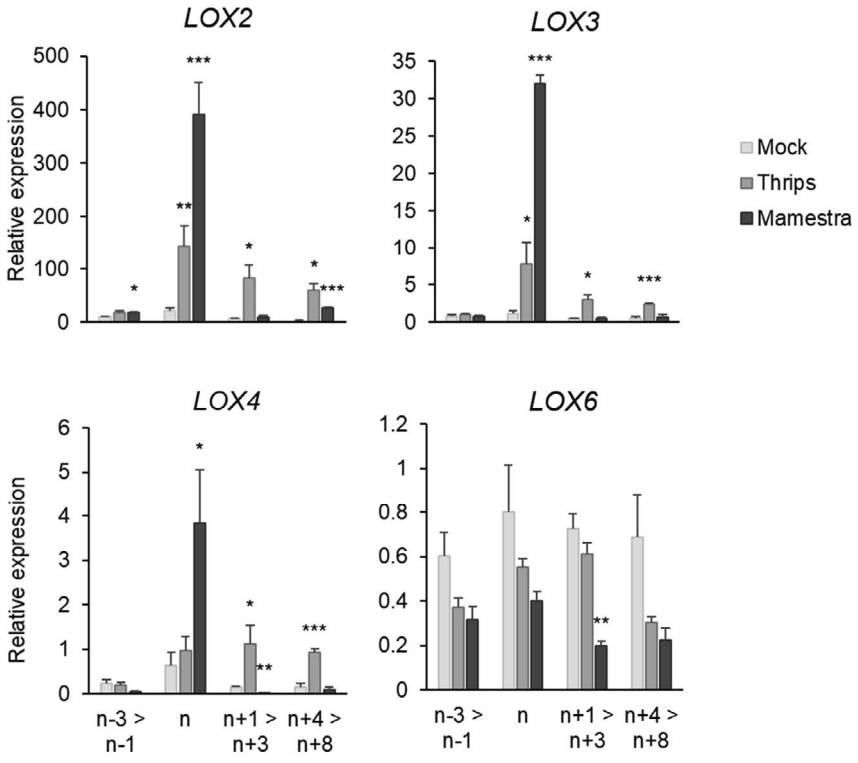

Figure 4: Expression of *LOX2*, *LOX3*, *LOX4* and *LOX6* in 4 leaf-age categories after local thrips or Mamestra infestation. See legend of Figure 3 for further details.

DISCUSSION

Mechanical wounding and feeding by chewing herbivores activate signaling cascades in the plant that lead to the activation of the JA pathway, which prepares the plant to defend itself against a subsequent attack not only locally, but also systemically in distal parts of the plant (Li *et al.,* 2002; Howe & Jander, 2008; Vos *et al.,* 2013b; Zebelo & Maffei, 2015). Here, using

Arabidopsis, we demonstrate that local infestation by either the tissue chewing caterpillar Mamestra or the cell-content feeding insect thrips, led to systemic activation of the MYC-branch. In contrast, the ERF-branch was exclusively systemically activated by thrips and not by Mamestra. Subsequent thrips feeding performance on the systemic leaves of local pre-infested plants also differed, where thrips preferred the youngest leaves on thrips-pre-infested plants, while thrips was deterred from mature leaves on Mamestra pre-infested plants.

Herbivore-induced defenses did not follow vascular connections

Studies on systemic signaling in response to mechanical wounding or chewing caterpillars have demonstrated a rapid elevation in expression levels of the JA-responsive genes *LOX2*, *LOX3*, *LOX4*, *LOX6*, *JAZ10* and *VSP2* in the locally treated leaf, referred to as leaf n, and the leaves directly connected to leaf n; n±3, n+5 and n+8 (Chauvin *et al.,* 2013; Mousavi *et al.,* 2013; Salvador-Recatalà *et al.,* 2014; Chauvin *et al.,* 2016; Heyer *et al.,* 2018). In our study, both thrips and Mamestra infestation enhanced the activity of *JAZ10* in the local leaf but not in the systemic leaves (Fig. 1B, D and Figure 3B). Nevertheless, the observed systemic induction of *VSP2*, *PDF1.2* and *LOX2*, *LOX3*, and *LOX4* (Fig. 1C and D, Fig. 3C and D, Fig. 4) suggests that the JA pathway was systemically activated by the insects. In contrast to our gene expression study at 24 h after introduction of the insects on the plants, most studies on the systemic *JAZ10* activation monitored the expression of this gene within 1 h after wounding (Glauser *et al.,* 2009; Chauvin *et al.,* 2013; Gasperini *et al.,* 2015; Chauvin *et al.,* 2016; Heyer *et al.,* 2018). Nonetheless, Hoo *et al.* (2008) showed that local wounding and herbivory by *Spodoptera exigua* induced the expression of *JAZ10* in systemic leaves after 24 h, which suggests that the activation of this gene is not only restricted to an early phase within the JA response. Still, it would be interesting to investigate why in our experimental set-up there was no substantial systemic induction of *JAZ10* or *LOX6*.

The systemic expression of various JA-responsive genes in locally thrips- or Mamestra-infested plants was independent of the vascular connections between the local and systemic leaves (Fig. 1B-D, Fig. 3B-D, Fig. 4). A potential explanation for the expression of these genes in leaves that do not share a vascular connection with the locally infested leaf, could be that the JA pathway was activated via airborne signals (Orians, 2005; Frost *et al.,* 2008). In a hybrid poplar (*Populus deltoides* x *nigra*), feeding by gypsy moth larvae (*Lymantria dispar* L.) releases volatiles that prime the adjacent leaves with little or no vascular connectivity to the infested leaf (Frost *et al.,* 2007). Volatile-mediated induction of JA-mediated resistance can occur within a couple of hours (Engelberth *et al.,* 2004; Ton *et al.,* 2007). JA can be methylated into methyl-jasmonate (MeJA) which mediates the induction of long-distance resistance within a plant, both internally as well as externally (Farmer & Ryan, 1990; Heil & Ton, 2008). Thus, airborne signaling could have overcome the vascular constraints on internal systemic signaling and led

to activation of JA-associated gene expression in both the vascular connected and non-connected leaves in our experimental system. Earlier sampling may reveal whether there can be an initial restriction of internal systemic signaling to the vascular connected leaves and whether the pattern of induction by the piercing sucking thrips in the systemic leaves overlaps or is different compared to the chewing Mamestra.

Differential herbivore-induced JA-associated defenses and subsequent thrips preference in a leaf-age dependent manner

Thrips and Mamestra infestation could both activate the expression of *PDF1.2* and *VPS2* in a locally infested leaf. We did observe that there were subtle differences in the gene expression levels induced by the different herbivores in the locally infested leaves, which was most apparent for *ERF1* in Figure 1D versus Figure 3D. This could be due to variation in the amount of damage as well as in the amount of saliva secreted during feeding by insects in different experiments (Peiffer & Felton, 2009). Nevertheless, while *VSP2* was systemically induced by both insects throughout all investigated leaves, *PDF1.2* was exclusively systemically induced by thrips and not by Mamestra (Fig. 1C and D, Fig. 3C and D). This suggests that thrips infestation triggers partly different signals than Mamestra does, which may be related to differences in feeding behavior or recognition of different insect-specific compounds by the plant.

When the performance of subsequently released thrips was monitored on the whole plant, differential effects by thrips or Mamestra pre-infestation became apparent. We demonstrated that overall, thrips performed equally well on thrips-pre-infested plants and on mock-treated plants (Fig. 2B and C). A study by Pappas *et al.* (2018) with thrips (*F. occidentalis*) on cucumber (*Cucumis sativus* L.) came to the same conclusion. However, a more detailed look on individual leaves in our study indicated that on thrips pre-treated Arabidopsis the preference was shifted to the youngest leaves. Especially leaves n+4 and n+7 incurred significantly more thrips damage, while on mock-pre-treated Arabidopsis plants thrips preferred to feed from mature leaves (n-2 till n+3) (Fig. 2D). In literature both similar and contrasting results for thrips feeding (and oviposition) preference on old or young leaves have been documented for different plant species (De Kogel *et al.,* 2002; Joost & Riley, 2008; Leiss *et al.,* 2009a; Van Haperen *et al.,* 2019).

In our study, the shift in preference of thrips for the youngest leaf-category in thrips-pre-infested plants correlated with a significantly lower level of *MYC2* transcript compared to the other leaf-age categories (Supplemental Fig. 1). This could potentially be the result of intra-pathway antagonism between the ERF- and MYC-branch (Lorenzo *et al.,* 2004; Dombrecht *et al.,* 2007; Fernández-Calvo *et al.,* 2011; Verhage *et al.,* 2011). A recent study by Wu *et al.* (2019), as well as results from **Chapter 3** and **Chapter 4**, showed that *myc* mutant plants are

enhanced susceptible to thrips. Thus, modulation of MYC-branch activity by thrips may be a strategy to increase thrips performance. This would be analogous to the activation of the ERF-branch by oral secretions of *Pieris rapae* caterpillars, which rewires the wound-induced MYC-branch to the *P. rapae*-preferred ERF-branch of the JA pathway (Verhage *et al.,* 2011).

Plants locally pre-treated with Mamestra were found to be overall more resistant to thrips, and this was apparent in all the mature leaves (older than leaf n+3) but not in the youngest leaves (Fig. 2D). This may be explained by a generally higher induction of the MYC-branch and a lower induction of the ERF-branch in comparison to plants induced by thrips (Fig. 1C and D, Fig. 3C and D). A number of previous studies also demonstrated a negative effect on thrips performance when plants were pre-infested by other herbivore species (Agrawal *et al.,* 1999; Pappas *et al.,* 2018). However, the underlying mechanisms of this subsequent decrease in thrips feeding performance still requires further investigation. Taken together, these data suggest that thrips are negatively affected by a local pre-infestation by Mamestra, and that thrips pre-infestation leads to improved thrips feeding performance in very young tissue, possibly by suppression of defenses through activation of ERF-MYC antagonism.

Interestingly, the exclusive systemic ERF-branch activation by thrips correlated with the expression of *LOX2*, *LOX3* and *LOX4* in the young leaf-age categories (Fig. 3D, Fig. 4). In contrast to the enhanced feeding performance of thrips in this LOX-expressing young systemic tissue, tissue-chewing caterpillars of *Spodoptera littoralis* preferred to consume younger leaves of plants that are mutated in these four LOXs (Chauvin *et al.,* 2013). Together, these data suggest that LOXs may be differentially involved in the JA pathway, depending on the attacker at hand, whereby induction of *LOX2*, *LOX3* and *LOX4* in young leaf tissue may specifically be part of the thrips-induced ERF-branch activation and/or making the young tissue preferable for thrips.

MATERIALS AND METHODS

Plant material and cultivation

Seeds of *Arabidopsis thaliana* (Arabidopsis) accession Col-0 and transgenic lines *JAZ10::GUS* (Acosta *et al.,* 2013), *LOX2::GUS* (Jensen *et al.,* 2002) and *GCC::GUS* (Zarei *et al.,* 2011) were stratified for 48 h in 0.01% agarose at 4°C. Subsequently, seeds were sown on river sand that was supplemental with modified 250 mL kg^{-1} half strength Hoagland solution containing 10 µM sequestreen (CIBA-Geigy, Basel, Switzerland) (Steenbergen *et al.,* 2020) and placed in a tray that was covered with a lid to ensure 100% relative humidity. Seeds were allowed to germinate and the seedlings were cultivated for 2 weeks in a controlled greenhouse compartment with a 10-h day and 14-h night cycle at 21°C and 70% relative humidity. Two-week-old seedlings were transferred to 60-mL pots containing a river sand:soil mixture (5:12,

v:v) that had been autoclaved twice for 45 min with a 24-h interval. The autoclaved soil was supplemented with 50 mL kg^{-1} half strength Hoagland solution. After transplanting, plants were kept under 100% relative humidity for 2 days, after which they were gradually acclimatized to a relative humidity of 70% for the following weeks. Plants were watered every other day and received 10 mL of modified half-strength Hoagland solution (Hoagland & Arnon, 1938) containing 10 uM sequestreen (CIBA-Geigy, Basel, Switzerland) once a week. For all the experiments, 5-week-old, non-flowering (vegetative stage), plants were used.

Insect rearing

Western flower thrips (*Frankliniella occidentalis* (Pergande)), originally collected from pepper (*Capsicum annuum*) leaves (Enza Zaden, Netherlands), were reared in a climate chamber (10-h day, 14-h night, 26°C) on common beans (*Phaseolus vulgaris*) and lettuce (*Lactuca sativa* var. *capitata*) in bugdorms (https://shop.bugdorm.com/) (Steenbergen *et al.*, 2020).
Mamestra brassicae eggs were obtained from the laboratory of Entomology at Wageningen University & Research where they were reared as described previously (Pangesti *et al.,* 2015).

Herbivore infestation for gene expression analyses

Leaf number 9 (n) of 5-week-old wild-type Arabidopsis Col-0 plants was treated with either an empty clip cage (Mock), a clip cage containing 5 individuals of L2 thrips larvae or a single L1 Mamestra caterpillar (Fig. 1). After 24 h, the locally infested leaf, and systemic leaves of 3-5 biologically independent replicas were harvested for gene expression analyses. For more details on the methods, see Steenbergen *et al.* (2020).

RNA extraction and RT-qPCR

Total RNA was isolated as described by Oñate-Sánchez & Vicente-Carbajosa (2008). RNA was treated with DNAse I (Fermentas) to remove genomic DNA. DNA-free RNA was converted into cDNA using RevertAid H minus Reverse Transcriptase (Fermentas) and an oligo-dT primer. PCR reactions were performed in optical 384-well plates (Applied Biosystems). To monitor the synthesis of double stranded DNA, SYBR® Green was detected with the ViiATM 7 Real-Time PCR system. A standard thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. After cycle 40, the amplicon dissociation curves were recorded by heating from 60°C to 95°C with a ramp speed of 1.0°C min^{-1}. Relative quantification of gene transcript levels was performed using the comparative method as described by Schmittgen and Livak (2001). Expression values were normalized using the reference gene *PP2AA3* (At1g13320) (Czechowski *et al.,* 2005) The AGI numbers for the genes studied are AT5G13220 (*JAZ10*), At1g32640 (*MYC2*), At5g24770 (*VSP2*), (At3g23240 (*ERF1*), At5g44420 (*PDF1.2*), At3g45140 (*LOX2*), At1g17420 (*LOX3*), At1g72520 (*LOX4*) and

At1g67560 (*LOX6*). All primers used for qRT-PCR are listed in Supplemental Table 1.

Thrips performance after local pre-treatment with thrips or Mamestra caterpillars

Leaf number 9, referred to as leaf n, of 10 independent 5-week-old Arabidopsis plants was treated with a clip cage containing either 5 individuals of L2 thrips larvae or a single L1 Mamestra caterpillar (Fig. 2A). The different larvae were placed in a clip cage using a fine paintbrush. Control plants received an empty clip cage (Mock). After 24 h, the cages and insects were removed from the local leaf after which 5 adult female thrips were allowed to feed from the whole plant for 4 days. Feeding damage was estimated in mm^2 by assessing the damaged spots on individual leaves. The feeding damage inflicted on the local leaf by the pre-treatment with the thrips was scored visually and subtracted from the damage inflicted by the challenging thrips that was monitored at the end of the experiment. After 4 days, feeding damage by the adult thrips was determined on leaves n-3 till n+8 after which the leaves were collected in a 15-mL falcon tube for oviposition count (see Material and Methods - Trypan blue staining). For more details on the methods applied here, see Steenbergen *et al.* (2020).

Trypan blue staining

To visualize thrips eggs, leaves of the 10 independent replicas from the performance assay were stained with a Trypan blue mix (adapted from Koch & Slusarenko (1990): lactic acid, glycerol and demi-water (1:1:1, v:v:v) plus 0.025% Trypan blue). This mix was supplied with an equal volume of 96% ethanol in 50-mL tubes until the whole rosette was covered. Tubes were placed in a container with boiling water for 2-3 min and subsequently cooled down for 30 min. Then, the staining solution was removed after which a lactoglycerol solution (lactic acid, glycerol and demi-water (1:1:1, v:v:v) was applied for de-staining. Eggs were visualized under a binocular where they can be recognized as dark blue, kidney-shaped structures (Steenbergen *et al.,* 2020).

GUS assay

Leaf number 9 of 5 independent 5-week-old plants was infested with 5 individuals of L2 thrips larvae that were enclosed in a clip cage. After 24 h, *LOX2::GUS* and *GCC::GUS* plants were submerged in a GUS staining solution (1 mM X-Gluc, 100 mM NaPi buffer, pH 7.0, 10 mM EDTA and 0.1% (v:v) Tritin X-100), vacuum infiltrated and incubated overnight at 37°C after which the GUS staining solution was replaced by 70% ethanol (Spoel *et al.,* 2003). For *JAZ10::GUS* plants a different protocol was used. Because *JAZ10::GUS* plant are highly sensitive to wounding and activate high levels of the reporter gene upon harvesting of the rosette, the tissue was first treated with 90% acetone at room temperature for at least 30 min to prevent endogenous GUS activities which might create background staining. After the

acetone fixation, plants were washed with a 50 mM phosphate buffer and stained with a GUS solution containing 50 mM phosphate buffer, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.05% Triton X-100 and 1.2 mM X-Gluc. After vacuum infiltration, plants were incubated overnight at 37°C after which the staining solution was replaced by 96% ethanol.

Statistical analyses

All the statistical analyses were performed using SPSS. For the gene expression experiments, within each leaf category, the relative expression levels of the genes of interest from each herbivore treatment was compared to the relative expression level of the corresponding gene in the mock treatment using a Student's *t*-test. The effect of the different local herbivore pre-treatments on the systemic performance of thrips was compared with the systemic performance of thrips on mock treated plants using a one-way ANOVA and a Duncan post-hoc test.

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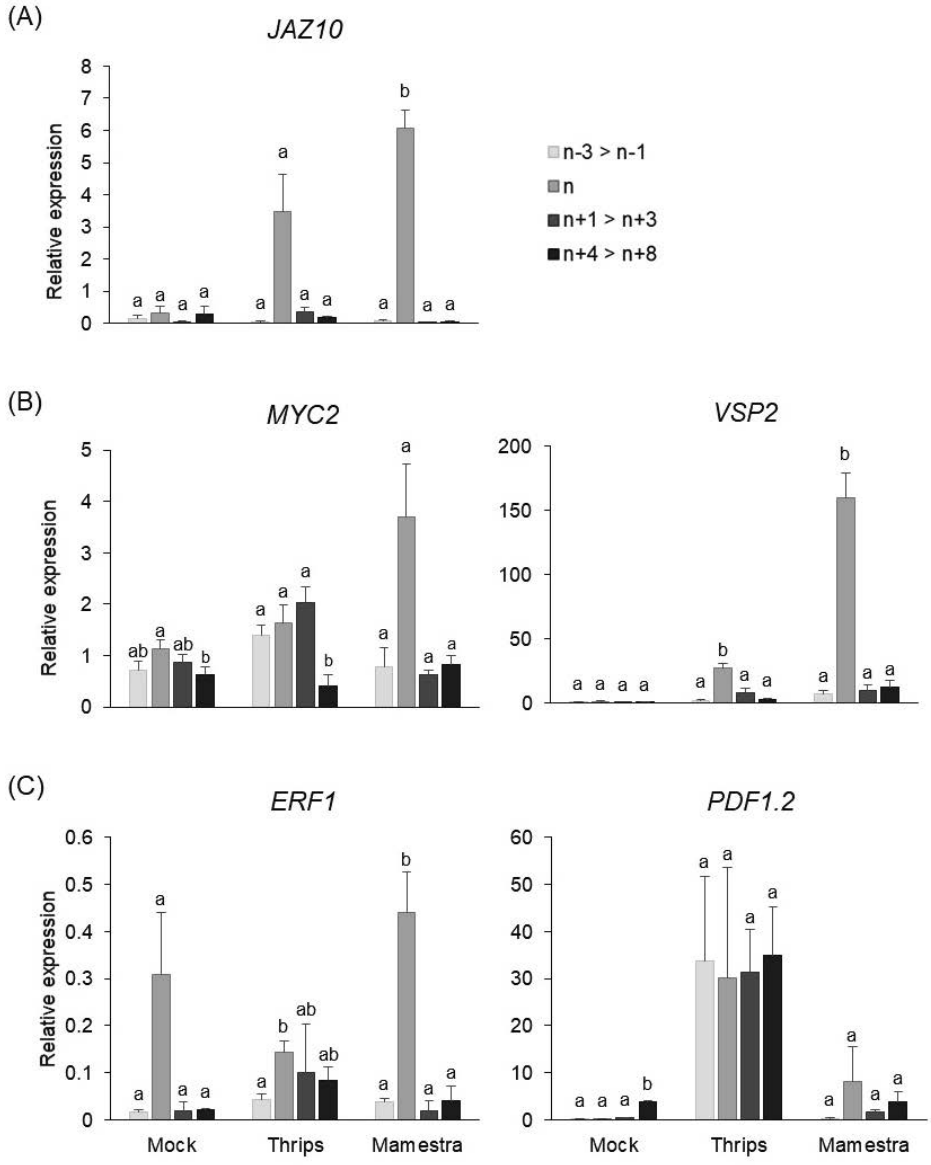
SUPPLEMENTAL INFORMATION



Supplemental Figure 1: Expression of JA-responsive marker genes after local thrips or Mamestra infestation in 4 leaf-age categories. See legend of Figure 3 for further details. Different letters indicate statistically significant differences between the different leaf-age categories of the mock-, thrips- or Mamestra-treated plants (ANOVA, Duncan post-hoc test; $P < 0.05$). Error bars represent SE, n = 3-5 plants.

CHAPTER 6

General discussion

Herbivorous thrips are considered as one of the most economically important pests worldwide. They cause direct damage by feeding on plants of at least 60 families including many vegetable and ornamental crops, and indirect damage by transferring devastating plant viruses. Their high and parthenogenetic reproductive capacity and hidden lifestyle (pupation in the soil, thigmotactic behavior) make thrips hard to control. Current pest management control relies mainly on the use of chemical pesticides, to which thrips can develop resistance. Exploiting the natural defense mechanisms of plants against thrips can provide the opportunity towards more sustainable resistance breeding. Knowledge on the molecular mechanisms underlying induced defense responses upon thrips feeding has been scarce. RNA sequencing (RNA-seq) is a powerful tool for studying transcriptional changes during plant defense activation upon thrips infestation (Steenbergen *et al.,* 2018; **Chapter 1**). In this thesis, we performed RNA-seq on a high-density time series, to study the temporal fluctuations of thrips-induced defenses in single Arabidopsis leaves (**Chapter 3**). To achieve this, we used an experimental set-up that we describe in **Chapter 2**. In the RNA-seq study we confirmed that jasmonic acid (JA) is the predominant phytohormone modulating the induced defense response against thrips and we discovered several novel regulators to play a key role in the thrips-induced plant regulatory network (**Chapter 3**). However, depending on the thrips developmental stage, the predominant JA-induced response is modulated differently (**Chapter 4**). For example, L1 larvae induced a relatively higher expression level of the ethylene (ET)-coregulated ERF-branch marker gene *PDF1.2,* which correlated with defense repression, while the older developmental stages activated higher levels of the abscisic acid (ABA)-coregulated MYC-branch marker gene *VPS2,* which was found to be associated with enhanced plant resistance. In the final chapter, we found that thrips are able to modulate the plant's defenses in the systemic, yet undamaged parts of the plant (**Chapter 5**). In the current chapter I summarize the most important findings reported in this thesis, deliberate on the role of novel transcriptional regulators within the thrips-induced JA pathway and finally discuss potential future research opportunities to continue investigations on the molecular mechanisms underlying the induced defense response upon thrips feeding.

JA as main regulatory hormone of thrips-induced defenses

Plants have evolved sophisticated defense mechanisms that can be induced upon herbivore attack. Upon the recognition of cell damage via damage-associated molecular patterns (DAMPs) (Duran-Flores & Heil, 2016), or herbivores via herbivore-associated molecular patterns (HAMPs) in the herbivore's saliva (Mithöfer & Boland, 2008) or insect eggs via egg-associated molecular patterns (EAMPs) (Reymond, 2013; Hilker & Fatouros, 2016), signaling cascades are triggered resulting in the accumulation of phytohormones, including salicylic acid (SA), JA, ABA and ET (Erb *et al.,* 2012; Reymond, 2021). The final hormone composition and

responsiveness to them are dependent on the infestation strategy of the attacker at hand and the environmental conditions of the plant, which determines the final immune response (Aerts *et al.,* 2021). In plant-herbivore interactions, JA is considered as the core player in the plant's immune response against herbivores, while SA, ABA and ET act as modulators of plant resistance to herbivores (Erb & Reymond, 2019). Although several studies have shown the importance of JA in plant-thrips interactions (De Vos *et al.,* 2005; Abe *et al.,* 2008, 2009; Selig *et al.,* 2016; Escobar-Bravo *et al.,* 2017), knowledge on the role of the other phytohormones and the induced molecular mechanisms underlying the thrips-induced response was lacking. In **Chapter 3-5** we confirm that JA is indeed essential for the induced defense against all feeding thrips stages. In **Chapter 4** we show that all feeding thrips stages (1) trigger the accumulation of JA(-Ile) in the plant, though to a different extend, (2) perform significantly worse on plants that received an exogenous MeJA treatment, and (3) perform significantly better on JA-insensitive mutants *coi1-34* and *myc2,3,4*. In **Chapter 3** we demonstrate that thrips L2 larval infestation significantly enhanced a considerable number of genes that were correlated with JA-associated processes, such as 'response to jasmonic acid', 'response to wounding' and 'phenylpropanoid biosynthetic process'. Additionally, most genes that were transcriptionally activated by thrips contain a TF binding site that can be bound by a member of the bHLH TF family such as MYC2, a well-known master regulator of anti-herbivore responses (Kazan & Manners, 2013). Moreover, a substantial set of the upregulated genes shows overlap with the gene set induced by exogenous MeJA application (48.5%, Hickman *et al.,* 2017) and *Pieris rapae* infestation (65.1%, Coolen *et al.,* 2016), which also verifies the importance of JA against thrips. However, a substantial set of genes is not overlapping with the above-mentioned gene expression data sets indicating that specific responses towards thrips infestation are induced.

Role of SA, ABA and ET in modulating the thrips-induced defense response

Activation of the SA pathway antagonizes the JA pathway and can therefore negatively affect the herbivore-induced, JA-dependent defenses (Pieterse *et al.,* 2012). In plant-insect interactions, the SA pathway can be induced by e.g. endosymbionts present in the oral secretion of an insect herbivore (Chung *et al.,* 2013) or insect eggs (Reymond, 2013; Hilker & Fatouros, 2016). Western flower thrips were found to be associated with Enterobacteriaceae such as *Erwinia* and *Pantoea* (De Vries *et al.,* 2001; Chanbusarakum & Ullman 2008; Facey *et al.,* 2015), but whether these endosymbionts are able to manipulate anti-thrips plant responses requires further investigation. Instead, Tospoviruses, which are exclusively vectored by thrips (Rotenberg *et al.,* 2015), are known to activate the SA pathway, thereby antagonizing the JA pathway in the plant, which benefits the performance of thrips (Abe *et al.,* 2012). In our research, experiments were performed with virus-free western flower thrips. In **Chapter 3** we

did not observe SA-associated processes to be overrepresented upon thrips infestation by L2 larvae, suggesting that potential thrips-associated endosymbionts did not modulate the thrips-induced defense response. Also, in **Chapter 4**, no induction of the SA-associated marker gene *PR1* could be detected, despite the observation of slightly elevated SA levels after L2 larval infestation. In the same chapter, we demonstrated that *PR1* was strongly activated in leaf tissue containing thrips eggs. This has been confirmed for several insect herbivore species (Hilker & Fatouros, 2016; Reymond, 2021), but has not previously been shown for thrips. For caterpillars, the egg-induced accumulation of SA negatively interferes with the JA pathway, making plants more susceptible to future larvae (Bruessow *et al.,* 2010). Whether this is true for the plant-thrips interaction should be examined further by assessing for example the hatching success and larval survival on hormone-deficient or -hypersensitive mutant plants. Furthermore, research on the chemical nature and perception (receptors) of thrips eggs, as well as the induced signaling events and transcriptional changes in the plant are needed to gain more knowledge on this interaction.

The phytohormones ABA and ET interact with the JA pathway as co-regulators for the activation of the MYC- and ERF-branch, respectively. The MYC-branch is primarily activated by and effective against insect herbivores, whereas the ERF-branch mainly activated by and effective against necrotrophic pathogens (Pieterse *et al.,* 2012). These two distinct branches antagonize each other such that activation of the ERF-branch decreases the expression of the MYC-branch marker gene *VSP2*, whilst activation of the MYC-branch reduces the expression of the ERF-branch marker gene *PDF1.2* (Vos *et al.,* 2013b, 2019). A study by Abe *et al.* (2008) demonstrated that adult female thrips induced the expression of both *VSP2* and *PDF1.2*, while we found that adult female thrips mainly activated the MYC-branch marker gene *VSP2* (**Chapter 4**). L1 and L2 larval infestation also induced the expression of *VSP2* in Arabidopsis, while L1 larval infestation additionally strongly activated the ERF-branch marker gene *PDF1.2* (**Chapter 4** and Fig. 1). The induction of *PDF1.2* upon thrips larval infestation has also been demonstrated by De Vos *et al.* (2005), although it is not clear which larval stage was used in that study. Our studies on the role of ABA and ET in tolerance against thrips feeding (**Chapter 4**) revealed that all thrips stages significantly enhanced ABA and ET levels in Arabidopsis, but performance studies on hormone mutant plants or plants pre-treated with hormones shows that thrips feeding was mainly affected by perturbations in ET signaling and not ABA signaling (**Chapter 4**). The significantly lower amount of thrips feeding damage on the *ein2-1* mutant for example could be explained by the fact that ET-insensitive mutants are still able to enhance the expression of *VSP2* upon thrips feeding, potentially to an even higher extent, while the expression of *PDF1.2* and other ET/JA-inducible genes in these mutants was suppressed (Abe *et al.,* 2008; Vos *et al.,* 2019). The induction of *VSP2* seems to be related with defense against thrips (Miyaji *et al.,* 2014), which we also deliberated in **Chapter 5**. In this chapter we

demonstrated that the caterpillar *Mamestra brassicae* induced higher levels of *VSP2* in the locally infested leaves as well as in the non-infested systemic leaves than L2 larvae did, which resulted in enhanced systemic resistance to adult female thrips (**Chapter 5**). The induction of *PDF1.2* by L1 larvae (**Chapter 4**) could in this case be suggested to be induced to manipulate the plant by circumventing the activation of anti-herbivore defenses associated with the activation of *VSP2* (Fig. 1). A way for insect herbivores to manipulate the host plant is via the action of effector proteins in the insect's saliva (Hogenhout & Bos, 2011; Kant *et al.,* 2015; Giron *et al.,* 2016). No thrips effectors have been identified yet, but there are genomic approaches available that have been successful in identifying effector proteins in aphids, Hessian flies and spider mites (Bos *et al.,* 2010; Zhao *et al.,* 2015; Thorpe *et al.,* 2016; Villarroel *et al.,* 2016; Abd-El-Haliem *et al.,* 2018). A study on Hessian flies demonstrated that each developmental stage expressed unique sets of genes, with first instar larvae expressing a high percentage of effector-encoding genes while subsequent/older stages expressed genes that encode for enzymes that detoxify plant-based compounds (Chen *et al.,* 2016). This corresponds to a transcriptomic study on the salivary glands of adult thrips, which also contained genes encoding for proteins involved in the detoxification and inhibition of plant defenses (Stafford-Banks *et al.,* 2014).

Novel regulators of defense against thrips

While the importance of the MYC-branch within the JA pathway and ET signaling for plant tolerance against thrips is now demonstrated, hardly anything is known about other molecular mechanisms underlying the thrips-induced defense response in plants. Using a high-throughput time series set-up, we were able to build a thrips-induced gene regulatory network (GRN) that allowed us to pinpoint novel regulators of induced responses (**Chapter 3**). The most significant candidates that we revealed, WRKY40, ERF13, DIV2 and AT5G56840, have distinct roles in the thrips-mediated GRN, but are all thought to be closely connected within the JA pathway (Fig. 1). The *wrky40* mutant was significantly more susceptible to thrips, indicating it is a positive regulator of the thrips-induced defense response. Schweizer *et al.* (2013) demonstrated that *wrky40* mutant plants showed a significantly reduced expression of *VSP2*, which we hypothesize to be essential for enhanced plant tolerance against thrips. *WRKY40* can be induced by MeJA (Wang *et al.,* 2008), but not exclusively (Schweizer *et al.,* 2013). The expression of *WRKY40* can also be triggered by ABA and vice versa, but only in the presence of WRKY18 and WRKY60 (Chen *et al.,* 2010), which are both induced by thrips (**Chapter 3**). Interestingly, one of our other regulator candidates, DIV2, can potentially be regulated by WRKY40 (**Chapter 3**). A few studies demonstrated that *div2* mutant plants harbor higher levels of ABA than wild-type plants (Fang *et al.,* 2018; Jeong *et al.,* 2018), which suggests that DIV2 is a negative regulator of ABA. Therefore, it can be suggested that WRKY40 acts as a negative

regulator of DIV2 (Fig. 1), which would explain the increased tolerance of *div2* mutant plants to thrips (**Chapter 3**). One of our other candidate transcriptional regulators, AT5G56840, was found to potentially regulate the expression of DIV2 (**Chapter 3**). Not much is known about AT5G56840, only that it negatively regulates the biosynthesis of the polyphenol epigallocatechin-3-gallate in tea (*Camellia sinensis*) (Zheng *et al.,* 2019). This polyphenol was found to promote the biosynthesis of JA in Arabidopsis (Hong *et al.,* 2015) and ABA in tomato (*Solanum lycopersicum*) (Ahammed *et al.,* 2020). Therefore, it can be suggested that this TF acts as a negative regulator of the JA/ABA pathway, which would corroborate with the enhanced tolerance to thrips in mutants knocked out in AT5G56840, potentially via the regulation of DIV2 (Fig. 1). The role of our final candidate transcriptional regulator, ERF13, in the thrips-induced GRN was more difficult to define. The expression of *ERF13* is not induced by exogenous ET or ABA application (Oñate-Sánchez & Singh, 2002; Lee *et al.,* 2010b), but it is in response to exogenous MeJA and SA application (Oñate-Sánchez & Singh, 2002; Caarls *et al.,* 2017). Upon caterpillar attack, the expression of *VSP2* and *PDF1.2* were not significantly affected in the *erf13* mutant compared to wild-type plants (Schweizer *et al.,* 2013). However, treating *erf13* mutant plants with MeJA resulted in higher expression levels of *PDF1.2* than *VSP2*, suggesting that ERF13 is a negative regulator of the ERF-branch marker gene *PDF1.2* (Caarls *et al.,* 2017; Fig. 1). This would correlate with our finding that *erf13* mutant plants showed enhanced thrips tolerance compared to wild-type plants (**Chapter 3**). Taken from this, there is still a lot unknown regarding the exact downstream targets and function of the identified regulator candidates in the thrips GRN. Even though some of the identified regulators were found to be important in other plant-insect interactions as well, they must have specific downstream targets that are exclusively effective against thrips. For example, the expression of *ERF13* is strongly dependent on a functional MYC-branch, but the *erf13* mutant was found to be more susceptible to herbivory by *S. littoralis* (Schweizer *et al.,* 2013), while it was more resistant to thrips (**Chapter 3**). Therefore, studying the transcriptional reprogramming of genes in these mutants during thrips infestation could elucidate which gene sets and thus biological processes are affected in these mutants and thus may play a critical role in thrips defense.

Next steps in thrips control management

There is a considerable amount of literature on the induced defenses activated in response to chewing and phloem-feeding insects, while most of the identified cues in these plant-insect interactions have yet to be identified for cell-content feeding insects such as thrips. This thesis has contributed to understanding the molecular mechanisms and hormonal modulation induced in Arabidopsis during its interaction with thrips. However, this work has only added a few pieces to a huge puzzle by providing valuable insights into the thrips-induced transcriptional changes, patterns of coregulation and associated biological processes targeted.

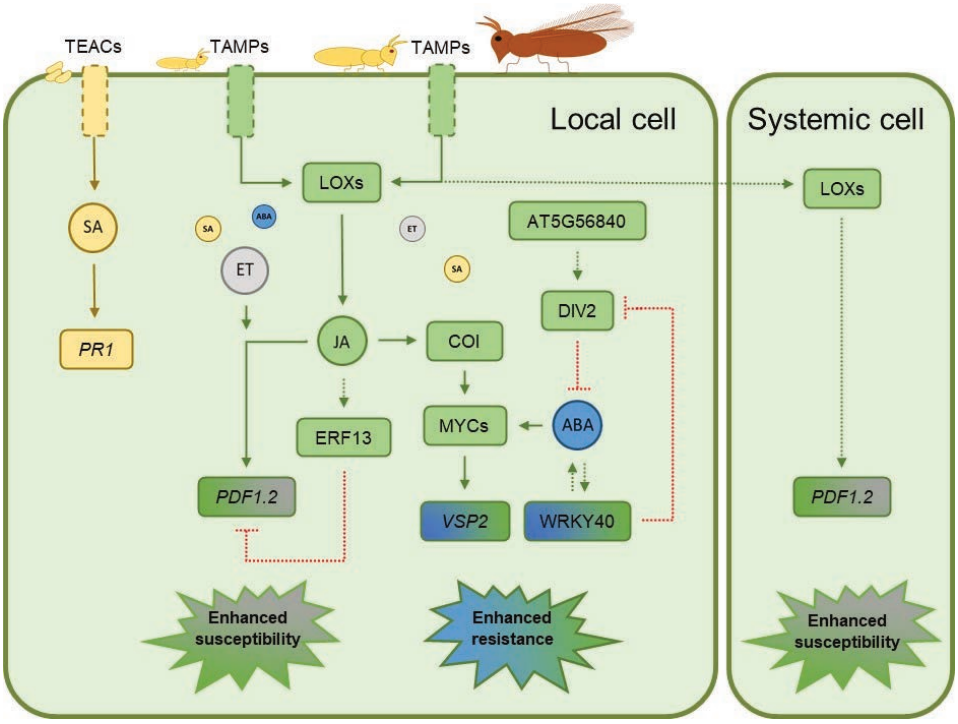


Figure 1. Molecular components during plant-thrips interactions. Thrips egg-associated compounds (TEACs) of thrips eggs are recognized by the plant by yet unknown receptors and trigger the local accumulation salicylic acid (SA) followed by the enhanced expression of the marker gene *PR1*. When L1 larvae emerge from the eggs and start to feed from the plant, thrips-associated molecular patterns (TAMPs) are recognized by yet unknown receptors of the plant and activate the biosynthesis of jasmonic acid (JA). Although all feeding thrips stages enhance the accumulation of the phytohormones JA, ethylene (ET) and abscisic acid (ABA), yet to different extends, L1 larval feeding mainly triggers the ERF-branch of the JA pathway accompanied with the activation of the marker gene *PDF1.2*, resulting in enhanced susceptibility to thrips. Feeding by L2 larvae and adult female thrips on the other mainly activates the MYC-branch of the JA pathway which is accompanied with the activation of the marker gene *VSP2* and enhanced resistance to thrips. Local L2 larval feeding also enhances the expression of *PDF1.2* in undamaged, systemic leaf tissue, resulting in enhanced susceptibility to thrips. The RNA-seq time series experiment performed in this thesis revealed several candidate regulators, WRKY40, ERF13, DIV2 and AT5G56840, to have distinct roles in the thrips-mediated GRN but are thought to be closely connected to the JA pathway. Dashed lines represent hypothesized receptors and hypothesized connections between molecular components. Blocked lines indicate negatively regulated responses and green arrows positively regulated responses.

Additionally, the differential hormonal modulation in Arabidopsis when oviposited by thrips eggs or infested by L1 or L2 larvae or thrips adults, stresses the importance of studying the gene expression signatures/GRN specifically induced per thrips developmental stage. Therefore, we analyzed the transcriptional changes of the 4 significant candidate genes identified in **Chapter 3** over time in response to all feeding thrips stages and observed that all feeding stages are

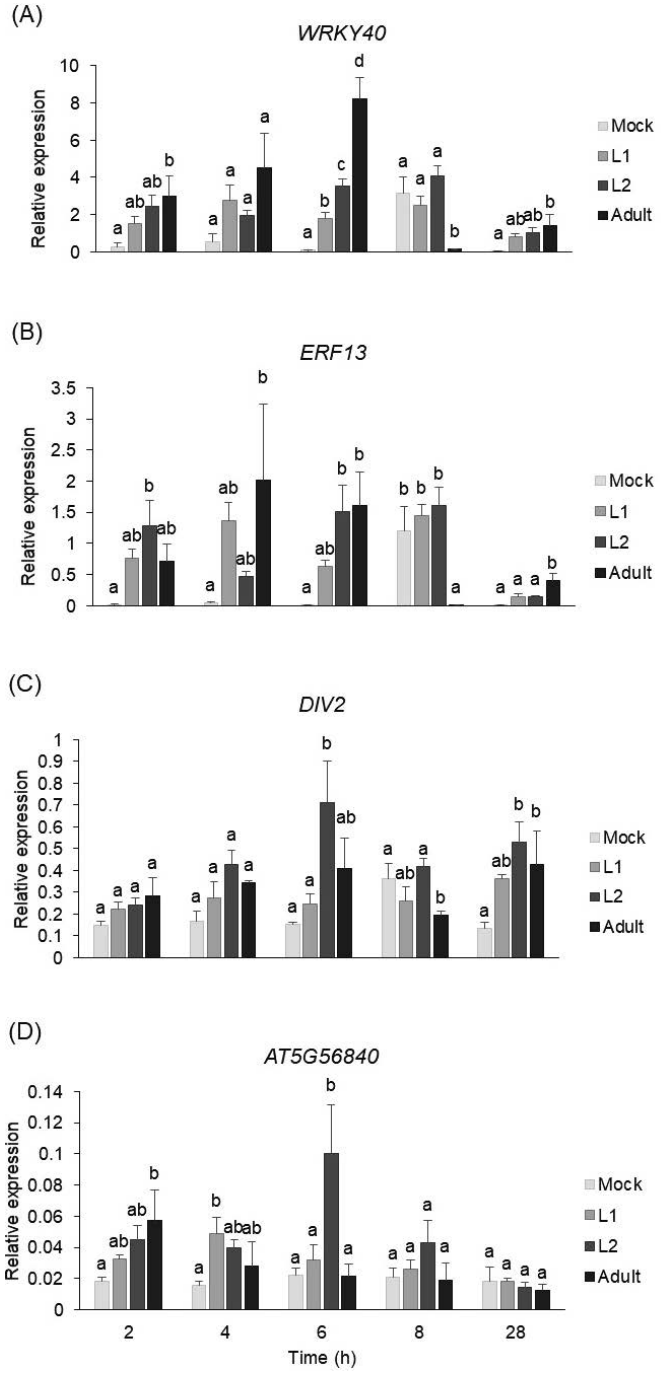
able to induce these candidate genes (Supplemental Fig. 1), but there was no clear trend/correspondence between the expression patterns and the performance of each thrips developmental stage (**Chapter 4**). Only a few studies demonstrated that the different developmental stages within an insect's life cycle differentially affect the plant defense regulatory network (Gheysen & Mitchum, 2019; Liu *et al.,* 2020a). Furthermore, plants also go through a natural life cycle and the induced defense response may vary throughout the plant's life cycle, but also between different tissues, organs, and leaves (old versus young leaves) (Brütting *et al.,* 2017; Schäfer *et al.,* 2017; Aničić *et al.,* 2018; Ochoa-López *et al.,* 2020). Therefore, a better understanding on the life cycle dependent variations in the induced defense mechanisms and functionality of key genes in plant-insect interactions is essential for successful resistance breeding (Kant & Schuurink, 2021).

Apart from studying the transcriptional changes induced upon thrips infestation, other technologies that provide information on for example the translatome, proteome and metabolome (Lee & Bailey-Serres, 2019; Zander *et al.,* 2020; Allwood *et al.,* 2021) should also be considered as, for example, changes in the transcriptome do not always correlate with changes in the proteome (Sharma *et al.,* 2017; Li *et al.,* 2018). Additionally, the use of these techniques should not only be restricted to locally infested leaves. Also in the systemic undamaged leaves of the plant transcriptional reprogramming occurs, but this response may be insufficient in activating downstream target genes without a secondary herbivore attack (Vos *et al.,* 2013b).

Another level of complexity in plant-thrips interactions is understanding the crosstalk between SA and JA in virus-thrips interaction. A recent study on the modulation of transcriptional changes during tomato spotted wilt (TSWV) virus – thrips interaction revealed that although TSWV infection mainly induced SA-related genes in tomato, only 29% of the JA-related responses were suppressed upon viral infection (Nachappa *et al.,* 2020). However, in interactions like these, timing, magnitude, and order of elicitation will determine the interplay between SA and JA pathways and the modulation of SA-JA crosstalk (Thaler *et al.,* 2012), which can also differ depending on leaf age (Berens *et al.,* 2019).

Collectively, there are still a lot of undiscovered areas in the plant-thrips interactions that require further investigation. Transcriptomic studies provide a valuable source of information on the dynamics and architecture of the thrips-induced plant responses which allows the discovery of key regulators. However, there are additional approaches that should be considered: (1) translatomics, proteomics and metabolomics, (2) the life cycle dependent variation in plant–herbivore interactions, and (3) identifying thrips egg-associated molecular patterns, thrips salivary proteins and their host targets. Knowledge on this will aid the sustainable development of thrips-resistance crops.

SUPPLEMENTAL INFORMATION



Supplemental Figure 1: Differential response of candidate transcription factors (Chapter 3**) in wild-type Arabidopsis (Col-0) plants in response to feeding by thrips of different developmental stages.**

RT-qPCR analysis of **(A)** *WRKY40,* **(B)** *ERF13,* **(C)** *DIV2* and **(D)** *AT5G56840* gene expression levels in leaf number 9 of 5-week-old Arabidopsis plants after 2, 4, 6, 8 and 28 h of feeding by 5 thrips (L1 larvae, L2 larvae or female adults) in a clip cage. Control plants (Mock) were treated with an empty clip cage. Different letters indicate statistically significant differences between the treatments within the indicated time point (ANOVA, Tukey post-hoc test; $P < 0.05$). Error bars represent SE, n = 3-5 plants.

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SAMENVATTING

Trips komt op veel verschillende plantensoorten voor en is wereldwijd een zeer gevreesde plaag. Trips beschadigt het gewas door plantencellen aan te prikken en leeg te zuigen waarna de cellen inklappen. Dit resulteert in een waas van zilvergrijze vlekken en misvorming van de aangetaste bladeren, bloemen of vruchten met daarbij zwarte stippen van uitwerpselen. Daarnaast kan trips virussen (tospovirussen) overbrengen van plant naar plant wat nog meer economische schade kan toebrengen aan verschillende gewassen. Om trips te bestrijden wordt vooral chemische bestrijding toegepast. Echter, trips is in staat om hieraan te ontsnappen of er resistentie tegen te ontwikkelen. Dit komt voornamelijk door hun kleine omvang, verborgen levenswijze (in bv bloemknoppen) en hoge reproductiesnelheid. Met de toenemende behoefte aan meer groene alternatieven om schadelijke insecten zoals trips aan te pakken, is het essentieel om meer inzicht te krijgen in de natuurlijke weerbaarheid van planten tijdens tripsvraat. De huidige kennis en aanknopingspunten/mogelijkheden met betrekking tot het aanvullen van de ontbrekende fundamentele kennis die noodzakelijk zijn voor het bevorderen van de natuurlijke weerbaarheid van planten tegen trips zijn samengevat in **Hoofdstuk 1**. Om de weerbaarheid van de plant, in zowel enkele bladeren als de gehele plant, tegen trips te toetsen, hebben we een aantal niet-destructieve biotoetsen ontworpen (**Hoofdstuk 2**).

Het natuurlijke afweersysteem van planten wordt geactiveerd wanneer de plant specifieke signalen, toegebracht door zijn aanvaller, opmerkt. Dit zijn signalen die voortkomen uit bijvoorbeeld vraatschade van het blad, componenten uit het speeksel van een etend insect, of componenten van eitjes die in/op het blad worden gelegd. Na herkenning van deze signalen, activeert de plant een afweerreactie die afgestemd is op de aanvaller, zoals trips, beschreven in dit proefschrift. Plantenhormonen spelen een belangrijke rol in het aansturen van cellulaire signaleringsroutes, die leiden naar afweerreacties, en andere biologische processen. Het plantenhormoon jasmonzuur (JA) is essentieel voor het activeren van de afweer tegen insecten zoals trips, maar ook tegen necrotrofe pathogenen (ziekteverwekkers die zich voeden met vernield plantenmateriaal). Samen met JA, zorgt de aanwezigheid van de plantenhormonen ethyleen (ET) en abscisinezuur (ABA) voor de activatie van verschillende takken binnen de JA signaleringsroute: de ERF-tak en de MYC-tak, respectievelijk. Binnen deze signaleringsroutes worden complexe signaleringsnetwerken geactiveerd of onderdrukt via de regulatie van genexpressie door transcriptiefactor eiwitten. Dit resulteert in de productie van specifieke fysische en/of chemische afweerstoffen die afgestemd zijn tegen de aanwezige aanvaller.

Aangaande de signaleringsroutes die geactiveerd worden door de plant als reactie op tripsvraat is er niet veel bekend. Om hier meer inzicht in te krijgen is in dit proefschrift op gedetailleerd niveau onderzocht hoe de modelplant *Arabidopsis thaliana* (zandraket, hierna Arabidopsis) reageert op vraat door de Californische trips (*Frankliniella occidentalis*). Zodoende hebben we in **Hoofdstuk 3** de verandering van alle ~27.000 genen van Arabidopsis bladeren geanalyseerd op 12 tijdstippen binnen een tijdspanne van 8 uur tripsvraat. Met behulp van bioinformatische analysemethoden hebben we 2788 genen geïdentificeerd die significant werden geactiveerd of onderdrukt ten gevolge van tripsvraat. Het merendeel van de genen die geactiveerd worden zijn, zoals verwacht, geassocieerd met biologische processen die aangestuurd worden binnen de JA signaleringsroute. Vergeleken soortgelijke gen-reguleringsnetwerk studies waar de dynamiek van Arabidopsis genen gedurende JA toediening of vraat door rupsen van het kleine koolwitje (*Pieris rapae*) is bestudeerd, bleek dat een groot deel van de door tripsvraat-geactiveerde genen overeenkwam met deze twee studies (48.5% en 65.1%, respectievelijk). Echter, een substantieel deel van de geïdentificeerde genen wordt specifiek geïnduceerd door trips. Door middel van de reconstructie van het tripsvraat-aangedreven gen-reguleringsnetwerk waren we in staat om transcriptiefactor eiwitten te selecteren die mogelijk verantwoordelijk zouden kunnen zijn voor de regulatie van genen die een belangrijke rol spelen in de afweerreactie van planten tegen trips. Een aantal van deze kandidaat transcriptiefactoren hebben we nader onderzocht middels biotoetsen met mutanten waarvan er vier, WRKY40, ERF13, DIV2 en AT5G56840, grote veranderingen in de afweer tegen trips bleken te hebben. Van deze vier transcriptiefactoren bleek WRKY40 een positieve regulator van de afweer tegen trips te zijn, terwijl de transcriptiefactoren ERF13, DIV2 en AT5G56840 negatieve regulatoren van de afweer tegen trips bleken te zijn.

In **Hoofdstuk 4** onderzochten we of er verschillen zijn in de hormonale regulatie van afweer in Arabidopsis gedurende de interactie met verschillende levensstadia van trips. De levenscyclus van trips bestaat uit 6 stadia: het eistadium, twee larve stadia, een voorpop, pop, en het volwassen stadium. Wanneer trips eitjes in het plantenweefsel legt, accumuleert de plant het plantenhormoon salicylzuur (SA), gepaard gaande met expressie van het merker gen *PR1*. Het hormoon SA is essentieel voor het activeren van de afweer tegen biotrofe pathogenen (ziekteverwekkers die zich voeden met levend plantenmateriaal) en is in staat om de JA signaleringsroute te onderdrukken. In het geval van tripseieren kan dat een voordeel zijn voor de toekomstige uitkomende larven aangezien JA tripsvraat onderdrukt. Gedurende tripsvraat (door de twee larve stadia en het volwassen stadium) detecteerden we geen expressie van het *PR1* gen meer. Daarentegen activeren de verschillende voedende trips stadia verschillende takken binnen de JA signaleringsroute: het tweede larve stadium en de volwassen vrouwtjes activeren vooral de MYC-tak van de JA signaleringsroute met het merker

gen *VSP2*, terwijl het eerste larve stadium sterk de ERF-tak met het merker gen *PDF1.2* activeert. Activatie van de ERF-tak lijkt de plantenafweer tegen trips te onderdrukken. Dit werd duidelijk na biotoetsen met een mutant die geen functionele ERF-tak meer had. Zowel de twee larve stadia als de volwassen vrouwtjes richtten minder vraatschade aan op deze mutant wat suggereert dat een effectieve activering van de ERF-tak de MYC-tak onderdrukt.

Deze hypothese correleert met resultaten uit **Hoofdstuk 5** waarin we onderzochten of lokale tripsvraat de JA signaleringsroute kan activeren in naastgelegen, onbeschadigde Arabidopsis bladeren. Lokale tripsvraat bleek zowel de MYC-tak merker *VSP2* als de ERF-tak merker *PDF1.2* te activeren. In de naastgelegen, onbeschadigde bladeren werd vooral *PDF1.2* geactiveerd. Een herbivoor met een andere voedingswijze, rupsen van de kooluil (*Mamestra brassicae*), activeerde in lokaal aangevreten bladeren hogere levels van *VSP2* dan trips, en in de naastgelegen, onaangetaste bladeren werd ook *VSP2* waargenomen. Deze activatie resulteerde in significant minder tripsvraat op de onbeschadigde (vooral oudere) bladeren wanneer trips op een plant werd geïntroduceerd die daarvoor aangevreten was door rupsen van de kooluil. Planten die eerder op een enkel blad door trips waren aangevreten, lieten meer tripsvraat zien op de onbeschadigde (vooral jongere) bladeren wanneer deze voor de tweede keer in contact werden gebracht met trips. Alles bij elkaar laat dit zien dat de activatie van het merker gen *PDF1.2* bij tripsvraat gepaard gaat met onderdrukking van een trips-effectieve afweerreactie, terwijl de activatie van het merker gen *VSP2* bij vraat door rupsen van de kooluil resulteert in verhoogde afweer tegen trips. Mogelijk speelt de activatie van bepaalde lipoxygenase (LOX) enzymen, die betrokken zijn bij de synthese van JA, een rol bij deze verschillende reacties. In onbeschadigde bladeren van trips-aangevreten planten hebben we namelijk waargenomen dat de lipoxygenase genen *LOX2*, *LOX3* en *LOX4* werden geactiveerd, terwijl dit niet het geval was in onbeschadigde bladeren van planten die door rupsen van de kooluil waren aangevreten.

Het werk wat in dit proefschrift beschreven staat biedt nieuwe inzichten in hoe planten hun afweerreactie reguleren gedurende tripsvraat. Door middel van de reconstructie van de het gen-reguleringsnetwerk waren we in staat om nieuwe transcriptiefactor eiwitten te identificeren die een significante rol spelen in de plantenafweer tegen trips. De afweerreactie die geactiveerd wordt door de plant is afhankelijk van het trips-ontwikkelingsstadium, en van de locatie van de vraatschade (lokaal aangevreten versus naastgelegen, onbeschadigd jonger of ouder blad). Deze verschillende effecten dienen nader onderzocht te worden in verschillende plant-insecten interactie studies. Deze nieuwe resultaten omtrent plant-trips interacties bieden een goede basis voor vervolgonderzoek die kunnen bijdragen aan de ontwikkeling van gewassen die beter bestand zijn tegen trips.

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Finally, an era has come to an end. This may sound a bit double, because I had an amazing time in the Plant-Microbe Interactions (PMI) group, but finally finishing my PhD thesis has been such a relief… My first encounter with the PMI group was in the beginning of 2010, when I had no other option than to follow several plant biology-related courses. I was never interested in plant biology, until I had no other option than to follow the Bachelor courses 'Plant-microbe interactions' and 'Plant, adaptation and defense'. This was the year I met some of the core members of the PMI group such as Dr. Peter Bakker, Prof. Dr. Ir. Corné Pieterse and Prof. Dr. Saskia van Wees. Under the supervision of Saskia and one of her, at that time, PhD students Adriaan Verhage, we (Judith, Pascal and I) performed a mini research project in the PMI group. Saskia was so enthusiastic about me (she has a good eye for people with weird and fun personalities), she encouraged me to start a Master's in Plant Biology and to perform my major research in the PMI group. So, in 2011, I started my Master's in Plant Biology and started my major research project in the PMI group. After my minor research project at the University of Lausanne in the group of Philippe Reymond, Saskia wanted to write a research proposal with me. Although this proposal did not get granted, shortly after I received my Master's degree, Saskia and Corné offered me a PhD position with the topic to study the transcriptional dynamics of *Arabidopsis thaliana* during thrips infestation. Now that my PhD track is officially finished, it is time to thank some people for their supervision, help, friendship, emotional support, etcetera... Get the tissues out, let's go...

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you are, keep up the nice work!

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CURRICULUM VITAE

Merel was born on the 1st of September 1989, in Gouda, the Netherlands. In 2006, Merel finished her secondary education and obtained her VWO diploma at the Emmauscollege, Rotterdam, the Netherlands. Afterwards, she started her Biology study at the Utrecht University, the Netherlands, and obtained her Bachelor of Science in February 2011. After a 6-month gap period, Merel started with the Master Environmental Biology at the Utrecht University. During her Master studies, two research internships were carried out. The first, a 9-month internship, was at the Plant- microbe Interactions group of the Utrecht University under the supervision of Dr. Irene A. Vroegop-Vos and Prof. Dr. Saskia C.M. Van Wees. During this internship she studied how environmental factors and disease pressure influenced growth and seed production of Arabidopsis plants when infected with the biotrophic pathogen *Hyaloperonospora arabidopsidis*. For her second internship, Merel moved to Lausanne, Switzerland, for 7 months to work in the Department of Plant Molecular Biology of the University of Lausanne under the supervision of Dr. André Schmiesing and Prof. Dr. Philippe Reymond. During this research internship, she studied the molecular responses induced in Arabidopsis plants in response to *Pieris brassicae* eggs. After this internship, in April 2013, she enrolled in the 'Experimental Plant Sciences (EPS) MSc talent program', in which she wrote a PhD proposal together with Prof. Dr. Saskia C.M. Van Wees. Unfortunately, this proposal was not granted. Merel obtained her Master of Science in December 2013. In January 2014 she was offered to work as a PhD student in a recently granted research program, the 'Green defense Against Pests (GAP)' program which was part of the Technology Foundation TTW (formerly STW) and partly financed by the Netherlands Organization for Scientific Research (NWO) and private agricultural companies Bejo Zaden, East-West Seed, Enza Zaden, KeyGene, Rijk Zwaan, and Syngenta Seeds. Before this project could start, Merel first worked in the Plant-microbe Interactions groups as a research assistant for 6 months. In August 2014 her PhD project started under the supervision of Prof. Dr. Saskia C.M. Van Wees, Prof. Dr. Ir. Corné M.J. Pieterse and Dr. Colette Broekgaarden. During her PhD, Merel studied the transcriptional dynamics of the gene regulatory network induced by thrips in Arabidopsis. This thesis is the result of her PhD research. After her PhD, Merel started working as a researcher at Chrysal International BV (May 2019). At Chrysal, Merel is involved in different research projects, focused on the improvement of the pre- and post-harvest quality of cut flowers and potted plants.

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