Control of Western flower thrips through jasmonate-triggered plant immunity

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Chapter 1
General introduction

1 Current challenges in agriculture

At present, the agriculture faces different demands. First, it has been predicted that nearly 9.77 billion people will need to be fed by 2050 (UN, 2017). As a consequence, it is estimated that nearly double volume of crop production, compared to 2013, will be required (Ray *et al.*, 2013). And second, there has been an intensification of the global network of ornamental plant species trade that has been accompanied by the increment in their cultivation and the use of pesticides. Increasing the land for the production of horticultural and/or ornamental plants is not the solution, and a great number of researchers have proposed the optimization of plant yield as the most sustainable strategy (Godfray *et al.*, 2010; Foley *et al.*, 2011; Phalan *et al.*, 2011). We need to invest in sustainable agriculture (Garnett *et al.*, 2013), and this might be achieved by optimizing the use of agriculture resources, e.g. water and nutrients (Foley *et al.*, 2011). Also, the generation of high-yield and pest resistant crop varieties through conventional plant breeding or genetic engineering approaches can increase plant yield (Tester & Langridge, 2010). However, most of the cultivated species have been generated by selecting desirable market-related fruit, flower or yield features, while traits conferring resistance to pathogens and herbivores have been lost during the domestication process (Oerke, 2006). As a result, arthropod pests and the diseases they transmit are among the most important factors affecting crop production. Furthermore, these threats are predicted to increase due to current agricultural practices, e.g. monoculture system and global warming (Oerke & Dehne, 2004). To minimize the damaging effects of arthropod pests on horticultural and ornamental crops production, pesticides are used worldwide (Stokstad & Grullón, 2013). However, more than 440 species of insects and mites have been documented to develop pesticide resistances (Roush & Tabashnik, 2012). Moreover, the use of pesticide leads to residue problems in the crops and environment and, therefore, they constitute a threat for untargeted organisms, including humans. European countries have agreed to establish a framework to reduce the adverse effects of pesticides on human health and the environment by promoting the development of Integrated Pest Management (IPM) strategies (directive, 2009). Among these, enhancing host plant resistance by using defense elicitors or the generation of pest resistant cultivars are desirable environmentally-friendly alternatives for pest control.

2 Mechanisms of host plant defense against herbivores

2.1 Constitutive and inducible defenses

To defend themselves against arthropod herbivores, plants have evolved sophisticated defense mechanisms that can be classified into constitutive and inducible. Constitutive defenses are defined as pre-existed morphological or chemical components present in the plant in the absence of herbivory or pathogen infection. Nonetheless, plants may increase their defenses to better protect themselves in response to herbivore or pathogen attacks, i.e. induced defenses (Howe & Jander, 2008). Both constitutive and induced plant defenses can be modulated by the environment as well as by the plant genetics and ontogeny (Karban & Myers, 1989; Franceschi *et al.*, 2005; Köhler *et al.*, 2015). In addition, plants have evolved their immune systems to distinguish their enemies to a certain degree and, thereby, to specifically respond to different types of attacks (Koornneef & Pieterse, 2008). These inducible plant defenses are uniquely initiated after the recognition of molecular patterns associated to herbivory or pathogen attack. These can result from endogenous elicitors derived from injured tissues, the so-called damage-associated molecular patterns (DAMP). Other defense elicitors are components of microbial pathogens (e.g. flagellin, lipopolysaccharides, peptidoglycan, β-glucans and chitin) and they are called pathogen- or

microbe-associated molecular patterns (PAMPs or MAMPs). Upon herbivory, plants can recognize this type of attack by detecting herbivore-associated molecular patterns (HAMPs). HAMPs are released from the herbivore's oral secretions, saliva, oviposition fluids, digestive wastes, and/or endosymbionts activity (Mithöfer & Boland, 2008; Basu *et al.*, 2017). Some examples are the oral secretion-related protein glucose oxidase, the fatty acid-amino acid conjugates such as volicitin, sulfated fatty acids such as caeliferins, and peptide fragments such as inceptins (Basu *et al.*, 2017). Also, salivary secretions containing ATP hydrolyzing enzymes and ATP synthase (Wu *et al.*, 2012), digestive wastes like the frass of the caterpillar *Spodoptera frugiperda* (Ray *et al.*, 2015), or endosymbionts in *Diabrotica virgifera* (Barr *et al.*, 2010) all have been documented to serve as HAMPs. Once recognized by plants, HAMPs can elicit the expression of defense-related genes, thereby modifying the physical and/or chemical defensive components of the plant.

Induced plant defenses against arthropod herbivores can be divided into direct and indirect defenses. Direct defenses include morphological features such as cuticles waxes, leaf toughness, spines and trichomes (Barton, 2016) and/or production of specialized metabolites and defensive-related proteins that negatively affect herbivore preference (i.e. host plant selection, oviposition, feeding behavior) and/or performance (i.e. growth rate, development, reproductive success) (Howe & Schaller, 2008). Among the above-mentioned mechanisms, the important defensive role of trichomes has been extensively studied for decades. Trichomes are epidermal hairy structures originated from the epidermal cells of plants, which can be divided into non-glandular or glandular types (Werker, 2000). Non-glandular trichomes are unicellular or multicellular hairs, while glandular trichomes are usually multicellular structures provided with specialized glands that can produce and/or secrete diverse chemical substances (Glas *et al.*, 2012). Non-glandular trichomes can provide physical protection against herbivores, while glandular trichome can provide both a physical and chemical barrier in the leaf surface. Glandular trichomes can produce and secrete different allelochemicals that restrain the survival, growth and fecundity of arthropod herbivores. Although trichomes can be present in the plant before herbivory or pathogen infection, their density and chemistry are modulated by abiotic and biotic factors (Peiffer *et al.*, 2009; Escobar-Bravo *et al.*, 2017). Besides trichome induction, many specialized plant chemicals with toxic or repellent properties against herbivores have been described to be induced by herbivory. Some examples include the production of phenolics, terpenoids, alkaloids, cyanogenic glucosides, and glucosinolates (Karban & Myers, 1989; Bennett & Wallsgrove, 1994; Grubb & Abel, 2006). In addition, plants can increase the production of defensive proteins that limit the nutritional value of plant tissues, such as polyphenol oxidases (PPOs) and proteinase inhibitors (PIs) (Chen, 2008; Howe & Schaller, 2008). Finally, herbivory can also induce indirect plant defenses, which consists on the attraction of the herbivore's enemies, often via the release of volatile organic compounds that serve as predatory cues, or by supplying additional food to the predators such as extrafloral nectar (Wu & Baldwin, 2010).

2.2 Hormone-mediated regulation of induced plant defenses

Induced plant defense responses are mainly controlled by the plant hormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Smith *et al.*, 2009; Eyles *et al.*, 2010). In general, chewing-biting (like caterpillars) and cell-content feeding insects (like thrips and spider mites) and necrotrophic pathogens activate the JA signaling pathway (Walling, 2000; Glazebrook, 2005), while the SA pathway is induced by biotrophic pathogens and phloem feeding insects (like aphids and whiteflies) (Glazebrook, 2005). Nonetheless, other hormones like gibberellins, cytokinins, abscisic acid, brassinosteroids and strigolactones participate in the

regulation of these induced plant defenses (Verma *et al.*, 2016). Hence, JA and SA signaling are fine-tuned by other hormones and they also interact through hormonal crosstalk. For instance, antagonistic effects of SA on JA signaling, and vice versa, have been amply described in the literature (Pieterse *et al.*, 2012).

Jasmonic acid signaling

Upon perception of attack by necrotrophic pathogens, herbivory or wounding, early signaling events like ion fluxes and cell membrane depolarization precede biosynthesis and rapid accumulation of JA in plants (Kessler & Baldwin, 2002; Wasternack & Hause, 2013). JA biosynthesis is initiated in the chloroplast, where α-linolenic acid is released from the galactolipids of chloroplast membranes via the action of phospholipases. These enzymatic reactions generate several oxylipins, including the JA precursor 12-oxo-phytodienoic acid (OPDA). OPDA is transported to the peroxisomes and subjected to a series of β-oxidation steps to generate JA (Wu & Baldwin, 2010). JA can be converted into the volatile component methyl jasmonate (MeJA), or conjugated to amino acids, such as isoleucine (Ile), producing the highly bioactive JA-derivative JA-Ile (Fonseca *et al*, 2009). JA-Ile is perceived by the plant in a dose-dependent manner, and it is crucial for the JA-induced molecular responses (Staswick & Tiryaki, 2004; Howe & Jander, 2008). JA-Ile can be perceived by the F-box protein coronatine insensitive1 (COI1) of the E3 ubiquitin-ligase SKP1-Cullin-F-box complex SCF^{COI1} (Sheard *et al.*, 2010). Upon recognition of JA-Ile, COI1 targets the jasmonate ZIM domain (JAZ) transcriptional repressor proteins for degradation via the 26S proteasome. This results in the activation of JA-responsive genes that control, for instance, the synthesis of secondary metabolites (Van Dam *et al.*, 2004), defense-related proteins (Thaler *et al.*, 2001), trichomes (Tian *et al.*, 2014), and volatile organic compounds (Strapasson *et al.*, 2014).

Salicylic acid signaling

SA is rapidly synthesized in plants in response to pathogen infection or attack by phloem feeding insects. It is a phenolic compound that can be synthesized by two different biosynthetic pathways, both requiring chorismate (see also reviewed by Boatwright & Pajerowska-Mukhtar, 2013). The first pathway occurs via the isochorismate synthase, resulting in the production of the SA-precursor isochorismic acid. In the second pathway, chorismic acid is converted into cinnamic acid via phenylalanine. Cinnamic acid is then converted into SA via either benzoic acid or coumaric acid. Activation of the SA-associated defenses is mainly regulated by NONEXPRESSOR OF PR GENES1 protein (NPR1). NPR1 translocates to the nucleus in response to SA accumulation (Ding *et al*., 2018). Then, NPR1 interacts with TGA transcription factors, resulting in the activation of defense-related genes, including for instance the pathogen-related (PR) genes.

2.3 Local and systemic induced plant defenses

Induction of plant defenses can occur locally at the site of attack and systemically in undamaged parts of the plant located at a substantial distance from the challenged area, which is called as a systemic response (Pieterse *et al.*, 2014). The first publication related to induced systemic defense responses against herbivorous arthropods was reported in the 1970s. Local feeding by Colorado potato beetles resulted in a rapid accumulation of PIs in systemic tissues of tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) plants (Green & Ryan, 1972). Since then, lots of experiments have been conducted to uncover the long-distance signal(s) responsible for induced systemic defenses. It has been proposed that the signal propagation through the plant occur through the transport of mobile signals in the phloem

(extracellular pathways) but also trough symplastic (cytoplasmic) pathways. For instance, grafting experiments in tomato have demonstrated that the herbivory-induced JA itself serves as a long distance mobile signal (Sun *et al.*, 2011). In addition, in *Arabidopsis*, it has been shown that local and systemic defense responses are also mediated by reactive oxygen species, electrical signals, and changes in cytosolic Ca^{2+} concentration (see review by Choi *et al.*, 2017).

Although defense-related responses have been reported to occur within minutes in local and systemic tissues, they often vary in their time, organ and magnitude within and among plant species. This variation can be explained by the genetic background, the development plasticity, transmission of long-distance signals, and the vascular architecture of the plant (Van Dam *et al.*, 2001; Arnold & Schultz, 2002; Arimura *et al.*, 2004; Orians, 2005; Howe & Jander, 2008). Importantly, this variation can influence herbivore distribution along the plant canopy, and it can modulate plant-herbivore interactions in specific plant tissues (Lee *et al.*, 2017).

2.4 Within-plant variation of constitutive and inducible defenses

Within an individual plant, leaves of different development stage might differ in their degree of constitutive defenses, and they might respond differently to biotic stresses as well (Takabayashi *et al.*, 1994; Constabel *et al.*, 2000; Bezemer *et al.*, 2004; Steimetz *et al.*, 2012). For instance, young maize leaves have been reported to induce higher levels of 1,4-benzoxazin-3-one derivatives than older leaves (Köhler *et al.*, 2015). In another example, wounding or exogenous MeJA treatments triggered a much stronger expression of PPO in young poplar (*Populus trichocarpa* × *Populus deltoids*) leaves than in older leaves (Constabel *et al.*, 2000). According to the optimal defense theory, this phenomenon can be explained by the higher contribution of young leaves to plant fitness (Harper, 1989; Iwasa *et al.*, 1996; Van Dam *et al.*, 1996). Importantly, this asymmetric distribution of plant defenses along the plant canopy can shape the foraging behavior of arthropod herbivores (Köhler *et al.*, 2015). For instance, many generalist herbivores display preferential feeding for basal and less protected parts of their host plant (Meyer & Montgomery, 1987; Bodnaryk, 1991; Leiss *et al.*, 2009b). Exploring the differences in constitutive and inducible chemical defenses within the plant canopy would help to identify resistant factors and develop plant protection strategies.

2.5 Activation of JA signaling by the *Pseudomonas syringae*-derived phytotoxin coronatine

JA-associated plant defense responses can be artificially activated by natural and synthetic elicitors. For instance, exogenous application of systemin, JA, MeJA, oligogalacturonides, and chitosan all have been documented to induce JA signaling pathway, and to enhance plant resistance to herbivorous arthropods in different plant species (Doares *et al.*, 1995; Bergey *et al.*, 1996; Wu *et al.*, 2008). Another extensively studied example of natural defense elicitors of JA signaling is the phytotoxin coronatine (COR). COR is a polyketide produced by various *Pseudomonas syringae* pathovars, including pv. *atropurpurea, glycinea, maculicola, morsprunorum* and *tomato* (Zhao *et al.*, 2001). COR is composed of two moieties, the polyketide coronafacic acid and coronamic acid (Ichihara *et al.*, 1977; Slawiak & Lojkowska, 2009). Both the structure and function of COR mimic the bioactive molecule JA-Ile. COR binds with high affinity to COI1 and activates the JA signaling pathway (Geng *et al.*, 2014). Yet, this phytotoxin is ca. 1000-fold more active than JA-Ile in activating downstream JA signaling pathway *in vitro* (Katsir *et al.*, 2008). Among the biological activities, COR induces chlorosis, hypertrophy and ET release (Kenyon & Turner, 1990b; Kenyon & Turner, 1990a).

In *Arabidopsis*, *P. syringae* pv. *tomato* infection results in a significant increase in COR levels during the first 24 h, followed by large increases after 48 h (Schmelz *et al.*, 2003). Due to the antagonistic interactions between JA and SA signaling pathways (Takahashi *et al.*, 2004), COR-mediated activation of JA signaling suppresses the SA-dependent defenses responses in the plant (Zhao *et al.*, 2003; Block *et al.*, 2005; Brooks *et al.*, 2005; Uppalapati *et al.*, 2007). Suppression of SA defenses increase the plant susceptibility to *P. syringae*. Hence, in coronatine-insensitive *Arabidopsis* mutants, *P. syringae* elicits both elevated levels of SA and expression of defensive PR proteins, which suppress bacterial growth (Kloek *et al.*, 2001). Notably, activation of JA signaling by COR-producing *P. syringae* strains can alter plant resistance to arthropod herbivores that are susceptible to these defenses (Stout *et al.*, 1999; Cui *et al.*, 2005). This hormonal crosstalk employed by *P. syringae* might set the basis to investigate whether COR and/or other *P. syringae*-derived defense elicitors could be exploited in agricultural systems to increase plant resistance to insect pests.

3 The experimental system

In this thesis I have explored how variations in constitutive and JA-associated inducible defenses correlate with the plant susceptibility to Western flower thrips *Frankliniella occidentalis* in cultivated tomato (*S. lycopersicum*) and chrysanthemum (*Chrysanthemum* × *morifolium* Ramat), two economically important plant species for which Western flower thrips represent one of the most damaging insect pests affecting their production worldwide. In addition, I have investigated whether the exogenous application of *P. syringae*-derived defense elicitors, i.e. COR, might elicit the positive effects of JA on plant defenses against this insect pest.

3.1 The Western flower thrips

Economic impact and biology

Western flower thrips (WFT), *F. occidentalis* (Pergande) (Thysanoptera: Thripidae), was first described in 1895 from specimens collected in California, USA. It has become a global agriculture and horticulture pest since 1970s, when insecticide resistant strain(s) emerged due to intensive pesticide use in Western North American greenhouses (Immaraju *et al.*, 1992; Kirk & Terry, 2003). Since then, WFT has spread to the East North America, and then to Europe and the rest of the world, this being mainly boosted by the global horticulture and floricultural trade (Kirk & Terry, 2003; Wu *et al.*, 2017). In the Netherlands, WFT was first recorded in 1983, on a glasshouse of African violets, and it has become the most common thrips species in Dutch greenhouses (Vierbergen, 2001; Messelink, 2014). It has been estimated to cause annual loses of 55 million euros only in vegetable and ornamental crops cultured in Dutch greenhouses (see also MacDonald *et al.*, 2002).

Several features make WFT a serious agricultural pest. First, it is a highly polyphagous insect that feeds on more than 250 plant species from nearly 60 different families, including fruiting and leafy vegetables, horticultural plants and fruit trees (Lewis, 1997). Second, because of its small size (less than 2.0 mm length) and cryptic habit, it is often unnoticed in the crops until serious levels of infestation take place. Furthermore, typical hiding and feeding behavior in tiny crevices of flowers or leaves makes this pest difficult to control by pesticides (Jensen, 2000). Third, it has a short developmental time and a high reproductive potential. The life cycle of WFT from egg to adult takes from 14 to 21 days to be completed at a moderate temperature (20-25°C) (**Fig. 1**); although it can be shortened to less than 10 days at 30°C (Reitz, 2008). Depending on the host plant species, WFT may produce up to 300 eggs per female, leading to more than 200 offspring per female and up to

five generations per year under field conditions (Robb, 1989; Lewis, 1997; McDonald *et al.*, 1998). And fourth, WFT easily develops insecticide resistance due to the short generation time, high fecundity and its haplodiploid sex-determination system (Jensen, 2000).

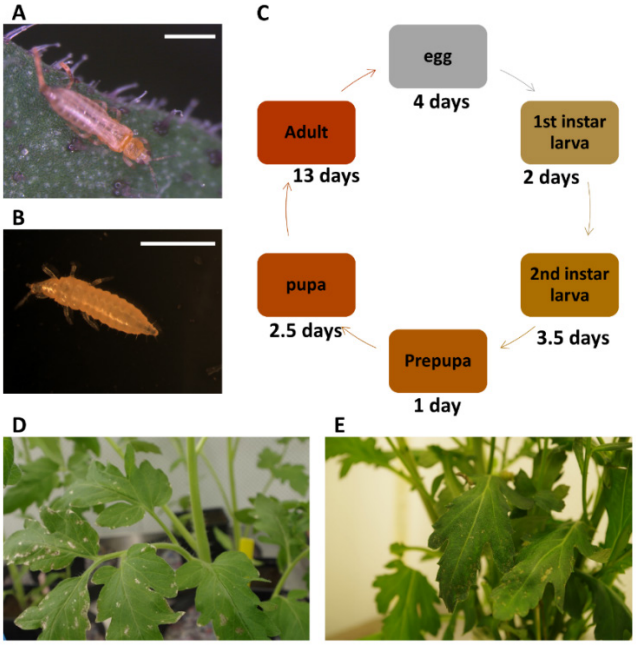

Fig. 1 Development and feeding damage by Western flower thrips. Photographs of (**A**) adult and (**B**) first instar larva of Western flower thrips (*F. occidentalis*). (**C**) life cycle of *F. occidentalis* at 25ºC. Photographs of the typical feeding damage, also termed as "silver damage" on leaves of (**D**) tomato and (**E**) chrysanthemum plants. Scale bars = 0.5 mm. The pictures in A and B were kindly provided by María José Rodríguez.

WFT can cause direct damage by feeding on different parts of the plant (Tommasini & Maini, 1995). WFT penetrates the epidermal and sub-epidermal plant cells with stylet-like mouthparts, sucking out the entire cell sap (Jensen, 2000; Maris *et al.*, 2004). Empty cells are then filled with air resulting in silvery or necrotic patches on leaves, flowers and fruits, which is so-called 'silver damage' (Jensen, 2000). In addition, WFT feeding on developing tissues leads to distortion of flowers and leaves, affecting the photosynthetic ability and fertility of crops and, ultimately, decreasing the crop yield (de Jager *et al.*, 1995; Shipp *et al.*, 2000). Indirect damage by WFT results from the transmission of tospoviruses, of which the *tomato spotted wilt virus* (TSWV) is especially important. It has been estimated that TSWV alone causes an annual economic loss of $19 million in The Netherlands (Rugman-Jones *et al.*, 2010). Until now, more than 1000 plant species belonging to 84 families have been documented to be TSWV hosts, which makes TSWV as one of the most widespread and host-ranged viruses (Parrella *et al.*, 2003). TSWV is transmitted by several species of thrips, of which WFT is one of the most important vectors (Ullman *et al.*, 1989). TSWV is only acquired by first- and second-instar WFT larvae and transmitted by second larval instars or adults (Ullman *et al.*, 1993; Wijkamp & Peters, 1993). As WFT can feed on an ample array of plant species, viruliferous individuals can efficiently transmit the virus across neighboring species.

WFT control in agriculture systems

Current control of WFT mainly relies on the use of insecticides and biological control. Most chemicals, however, have a short-term effectiveness in part due to the cryptic life style of WFT, and a frequent spraying of 3-5 days interval is generally required (Brødsgaard, 1994; Daughtrey *et al.*, 1997; Berndt *et al.*, 2004). General biological control agents of WFT include predatory mites (Gerson & Weintraub, 2007), bugs (Blaeser *et al.*, 2004) and entomopathogenic fungi (Wang & Zheng, 2012). However, biological control is often inefficient because of the limited feeding habit of the predator. For instance, both *Neoseiulus barkeri* Hughes and *Amblyseius cucumeris* Oudenmans primarily prey on WFT first instar larva only (Van der Hoeven & Van Rijn, 1990). Combining two biocontrol control agents, however, seems to not solve this situation and negative interactions might happen between or among biological control agents when simultaneously preying on WFT (Wu *et al.*, 2016). Furthermore, some predators can feed on the plant as well when WFT populations are not very high, thus causing damage in the plant (Briese, 2005).

WFT-plant interactions

WFT feeding induces JA signaling pathway and this response is required to increase plant resistance against this insect (De Vos *et al.*, 2005; Abe *et al.*, 2008; Abe *et al.*, 2009; Kawazu *et al.*, 2012). Accordingly, exogenous application of JAs has been found to increase plant resistance to WFT in cotton (*Aphis gossypii*) (Omer *et al.*, 2001), Arabidopsis (Abe *et al.*, 2008), Chinese cabbage (*Brassica rapa*) (Abe *et al.*, 2009) and tomato (*S. lycopersicum*) (Thaler *et al.*, 2001; Escobar-Bravo *et al.*, 2017). Morphological, chemical and enzymatic-related defenses induced through the activation of JA-dependent defenses (Traw & Bergelson, 2003; Boughton *et al.*, 2005; Tian *et al.*, 2012; Chu *et al.*, 2017; Escobar-Bravo *et al.*, 2017) probably accounts for the enhanced resistance to this insect pest.

Host plant resistance to WFT

Host plant resistance to WFT can be mediated by the constitutive expression of morphological and chemical plant defensive traits, but also by the induction of these or other defenses. For instance, foliar wax content has been found to be negatively correlated with WFT feeding in Gladiolus spp. (Zeier & Wright, 1995). In addition, constitutive levels of certain primary and secondary metabolites, as well as defense enzymes, have been associated with plant resistance to WFT (Mouden *et al.*, 2017). Low concentration of certain aromatic amino acids has been observed to correlate with a reduced WFT feeding damage in lettuce (*Lactuca sativa*), tomato (*S. lycopersicum*), sweet pepper (*Capsicum annuum*) and cucumber (*Cucumis sativus*) (Mollema & Cole, 1996). Variations in constitutive levels of secondary metabolites such as isobutylamide, chlorogenic and feruloyl quinic acid in chrysanthemum (Tsao *et al.*, 2005; Leiss *et al.*, 2009b), jacobine and jaconine in *Senecio (Leiss et al., 2009a)*, trichome-derived acyl sugars in tomato (Mirnezhad *et al.*, 2010), pyrethrins in *Tanacetum cinerariifolium* (Yang *et al.*, 2012), and luteolin and β-alanine in *Daucus carota* L. (Leiss *et al.*, 2013) all have been found to correlate with WFT resistance. Furthermore, genetic engineering for the expression of cysteine proteases inhibitors has been demonstrated to reduce WFT offspring and survival in transgenic potato (*S. tuberosum*) plants (Outchkourov *et al.*, 2004). Notably, induction of certain chemical and morphological defenses has been demonstrated to correlate with WFT resistance or susceptibility as well. For instance, light intensity-mediated reinforcement of type-VI trichome associated chemical defenses has been shown to increase WFT resistance in tomato (*S. lycopersicum*) (Escobar-Bravo *et al.*, 2018). In pepper, Maharijaya *et al*. (2012) showed that while susceptible pepper (Capsicum spp.) accessions induced the production of alkanes and fatty acids in response to WFT infestation, resistant accessions did not.

3.2 Tomato and chrysanthemum

Tomato

Cultivated tomato (*S. lycopersicum* L.) is one of the main consumed vegetable in the world, with an estimated global production of around 177 million tons per year (FAOSTAT, 2016). China, India, EU, USA, Turkey, Egypt, Iran, Brazil, Mexico and Russia produced more than 81% of the total global tomato fruit yield in 2016. In the Netherlands, tomato production was 900 thousand tons in 2016, making tomato production come fifth after potatoes, sugar beet, onions, and wheat (FAOSTAT, 2016). Tomato fruit is a rich source of vitamins A and C, potassium, folic acid and carotenoids, which are positively associated with human health (Giovannucci, 1999; Perveen *et al.*, 2015). Furthermore, carotenoids cannot be synthesized in human tissues, being exclusively obtained from our diet. Tomato fruit also contains other antioxidant compounds, which include flavonoids and phenolic acids (Wardale, 1973). Flavonoids and polyphenols have shown many beneficial properties for human health including anti-cancer, anti-inflammatory, immunomodulatory, and anti-thrombotic activities (Lee & Zhu, 2005; García-Lafuente *et al.*, 2009). Altogether, these features make tomatoes an important nutrient source for the human diet.

Cultivated tomato (*S. lycopersicum* L.) belongs to the Solanaceae family. This family originated in South America and contains many of the most important cultivated plants such as potato, tomato, pepper, eggplant, petunia and tobacco. Tomato breeding for fruit yield, taste and nutritional quality have generated more than 7500 cultivated varieties (Bai & Lindhout, 2007; Korir *et al.*, 2014). Yet, important agricultural traits such as resistance to biotic and abiotic stresses were gradually lost during tomato domestication. As a consequence, most cultivated tomatoes are highly susceptible to a wide array of diseases and arthropod pests, including WFT (Kennedy & Barbour, 1992; Bai & Lindhout, 2007).

One of the main and most important components of tomato defenses against herbivorous arthropods is the leaf trichomes (Kang *et al.*, 2010a; Kang *et al.*, 2010b). Cultivated tomatoes possess non-glandular (type III, V and VIII) and glandular (type I, VI and VII) trichomes types (Glas *et al.*, 2012). Non-glandular trichomes can physically hinder the movement, feeding and oviposition of arthropod herbivores. Type VI glandular trichome, which is the most abundant glandular-type in the leaf surface, can also affect host plant selection and herbivore growth, survival and fecundity (Duffey, 1986). Type-VI glandular trichomes produce and secrete a wide variety of specialized metabolites including terpenoids, phenolics and acyl sugars (Kang *et al.*, 2014). Despite their constitutive expression in the plant, glandular trichome density and chemistry can be induced by the application of JA (Degenhardt *et al.*, 2010; Cevallos-Cevallos *et al.*, 2012; Dobritzsch *et al.*, 2015) or its volatile methyl jasmonate (MeJA) (Boughton *et al.*, 2005; Tian *et al.*, 2012), which can increase tomato resistance to herbivorous arthopods (Escobar-Bravo *et al*., 2018).

Chrysanthemum

Chrysanthemum [*Chrysanthemum* × *morifolium* Ramat. (Asteraceae)], bred as early as ca. 1000 BC in China and Japan, is one of the economically most important greenhouse ornamentals worldwide (Fletcher, 1992). It is the second most important cutting flowers just after roses in the Netherlands. The Netherlands is also the largest exporting country of cut-chrysanthemum to intra-EU, amount annually to €232 million (Hanks, 2015). The number of chrysanthemum varieties is extremely large, with about 15000 and 6000 listed in Japan and in the National Chrysanthemum Society in Britain, respectively (Teixeira da Silva *et al.*,

2013). Chrysanthemum is primarily propagated asexually by cultivating asexual vegetative stem cuttings (Teynor *et al.*, 1989).

Modern garden chrysanthemums are most likely derived from interspecific hybrids between *Chrysanthemum indicum* and *C. vestitum* native in Eastern Asia being the center of genetic resources of this genus (Zhao *et al.*, 2009). Due to the dense screening and selection of chrysanthemum varieties varying in flower color, size and shape, commercial varieties lacks resistance traits to biotic or abiotic stresses (Teixeira da Silva *et al.*, 2013). Hence, most commercial chrysanthemum cultivars are susceptible to many arthropod pests including the leaf miner *Liriomyza trifolii* (van Dijk *et al.*, 1992), the cotton aphid *Aphis gossypii* (Guldemond *et al.*, 1994) and WFT (*F. occidentalis*) (Leiss *et al.*, 2009b). Yet, there are still variations in the levels of pest resistance. Such variations have been associated to differences in trichome density and antioxidant leaf properties in some cultivars (Leiss *et al.*, 2009b; Deng *et al.*, 2010; He *et al.*, 2011). Thus, determining constitutive and inducible defense traits against arthropod pests in chrysanthemum might be used for the generation of resistant varieties by plant breeding strategies.

4 Outline of this thesis

In **chapter 2** I investigated how JA-mediated induction of tomato defenses against and resistance to WFT is affected by the leaf developmental stage. For this, I measured how JA induced the defensive protein polyphenol oxidase (PPO), type-VI foliar glandular trichome density and accumulation of their associated volatiles in developing and fully-developed leaves. In addition, I assessed the feeding damage by WFT on those leaves. Our results demonstrated that the capacity of tomato leaves to induce JA-associated defenses against WFT is constrained by the leaf development stage, and positively correlated with the levels of WFT resistance along the tomato canopy. Importantly, I also demonstrated that the production of type-VI trichome associated volatiles was differently regulated in developing and fully-developed leaves. These findings have important implications for agriculture, as type-VI trichomes constitute important physical and chemical defenses in tomato against WFT (Escobar-Bravo *et al.*, 2018).

In **chapter 3** I explored the potential use of novel bacteria-derived defense elicitors to activate JA-associated defenses against WFT in tomato. I determined how infiltration with the bacterial pathogen *P. syringae* pv. tomato *(Pst*) strain DC3000, the *Pst*-derived phytotoxin coronatine (COR) or *Pst*-derived medium affected tomato defenses and resistance against WFT. For this, I determined how COR and *Pst* influenced feeding damage by WFT, activation of the JA and SA defenses, type-VI foliar glandular trichome density and leaf chemistry. In addition, I investigated the action of *Pst*-derived culture medium with and without COR, and their interactive effect with pure COR, on tomato resistance to WFT. Our results showed that infiltration of plants with *Pst*, COR or *Pst*-derived culture medium without COR all increased tomato resistance against WFT through the induction of JA-associated defenses, suggesting the presence of non-identified defense elicitors in *Pst*-derived medium. Furthermore, I showed that the *Pst*- or COR-mediated enhancement of tomato resistance against WFT was not explained by the reinforcement of type VI leaf trichome densities, but rather the induction of other JA-associated chemical defenses.

In **chapter 4** I explored the phenotypic diversity in constitutive and inducible defenses against WFT in chrysanthemum. I determined whether variations in constitutive levels of leaf trichome density and oxidative defenses among different chrysanthemum cultivars correlated with the degree of WFT resistance. In addition, I explored whether differences in WFT resistance among a subset of chrysanthemum varieties could be explained

by the JA-mediated induction of trichome densities and a defense-related enzyme, PPO. First, our data showed that exogenous application of the phytohormone JA enhanced resistance against WFT in chrysanthemum. However, the phenotypic variation in WFT resistance among chrysanthemum cultivars were not explained by the presence/induction of non-glandular and glandular trichome densities, nor the activity of the defensive protein PPO.

In additional experiments, we observed that local application of JA on chrysanthemum plants did not have a significant effect on WFT resistance. Thus, in **chapter 5** I investigated whether activation of local and systemic chemical responses upon exogenous application of JA varies along the plant canopy in chrysanthemum, and whether it correlates with resistance to WFT. For this, I performed a comprehensive untargeted metabolomic analysis to determine JA-mediated induced chemical responses in local and systemic leaves. Our results showed that local and systemic induction of JA-mediated chemical defenses in chrysanthemum is spatially variable and dependent on the site of the induction. Furthermore, our analyses on the distribution of WFT-associated feeding in the chrysanthemum plant canopy and the metabolomic profiles of basal and apical leaves suggest that higher levels of constitutive and inducible defenses in basal leaves might explain their higher degree of WFT resistance.

In **chapter 6** I summarized and discussed the findings described in this thesis. In addition, I discussed the implications of these findings for the management of WFT in agricultural systems.

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Chapter 2

Type VI glandular trichome density and their derived volatiles are differently induced by jasmonic acid in developing and fully developed tomato leaves: implications for Western flower thrips resistance

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Variation in the induction of plant defenses along the plant canopy can determine distribution and colonization of arthropod herbivores within the plant. In tomato, type VI glandular trichomes, which are epidermal defensive structures, and their derived volatiles are induced by the phytohormone jasmonic acid (JA). How JA-mediated induction of these trichome-associated chemical defenses depends on the leaf developmental stage and correlates with resistance against herbivory is unknown. We showed that application of JA reduced Western flower thrips (WFT)-associated damage, however the amplitude of this response was reduced in the fully developed leaves compared to those still developing. Although JA increased type-VI trichome densities in all leaf developmental stages, as well as JA-inducible defensive proteins, these increases were stronger in developing leaves. Remarkably, the concentration of trichome-derived volatiles was induced by JA to a larger degree in developing leaves than in fully developed leaves. In fully developed leaves, the increase in trichome-derived volatiles was explained by an enhanced production per trichome, while in developing leaves this was mainly caused by increases in type-VI trichome densities. Together, we showed that JA-mediated induction of trichome density and chemistry depends on leaf development stage, and it might explain the degree of WFT-associated leaf damage in tomato.

Keywords developmental stages; *Frankliniella occidentalis*; induced defenses; phytohormone; *Solanum lycopersicum*

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1 Introduction

Plants can mount an array of inducible defense responses against herbivorous arthropods which includes the synthesis of deterrent/anti-feeding metabolites (Bennett & Wallsgrove, 1994; War *et al.*, 2012), defensive proteins (Thaler *et al.*, 2001) and/or increases in leaf epidermal defensive structures such as trichomes (Traw & Dawson, 2002). These responses are mediated by endogenous signaling molecules, e.g. phytohormones, among which jasmonic acid (JA), salicylic acid (SA) and ethylene are central regulators of plant defenses against pathogens and herbivores (Pieterse *et al.*, 2012). In particular, activation of the JA signaling pathway has been reported to confer resistance against chewing-biting and cell-content feeding insects, as well as necrotrophic pathogens (Walling, 2000; Glazebrook, 2005), while induction of SA signaling increases the resistance against biotrophic pathogens (Koornneef & Pieterse, 2008). Moreover, artificial activation of these defense-associated signaling pathways by using natural or synthetic elicitors has proven to increase plant resistance against different insects and diseases and is, therefore, regarded as a valuable strategy to control pests in agriculture (Thaler, 1999).

Induction of plant defenses by herbivory or defense elicitors may vary along the plant canopy, being of higher magnitude in young leaves (Reifenrath & Müller, 2007; Köhler *et al.*, 2015). For instance, Constabel et al. (2000) described lower constitutive and MeJA-mediated induction of the defensive protein polyphenol oxidase (PPO) in old leaves of poplar saplings (*Populus trichocarpa* × *Populus deltoides*) when compared to younger ones. In addition, constitutive levels of secondary metabolites, such as phenolics, and trichome density are reported to be higher in younger leaves of tomato plants (Wilkens *et al.*, 1996; Stout *et al.*, 1998a; Scott-Brown *et al.*, 2016). Young plant leaves contribute most to plant fitness and, therefore, they are most relevant to be protected against herbivores from an ecological point of view (Harper, 1989; Iwasa *et al.*, 1996; Van Dam *et al.*, 1996; Ohnmeiss & Baldwin, 2000). Accordingly, this has been proposed to explain why many foliar chewing and cell content feeding generalist insect pests prefer old leaves (Meyer & Montgomery, 1987; Bodnaryk, 1991; Leiss *et al.*, 2009b).

In tomato, artificial application of JA or its volatile form methyl jasmonate (MeJA) has been reported to induce the production of the defensive enzyme PPO (Thaler *et al.*, 1996; Degenhardt *et al.*, 2010; Cevallos-Cevallos *et al.*, 2012; Dobritzsch *et al.*, 2015) and type-VI leaf glandular trichomes (Boughton *et al.*, 2005; Maes & Goossens, 2010; Tian *et al.*, 2012; Tian *et al.*, 2014; Escobar-Bravo *et al.*, 2017). PPO catalyzes the transformation of phenolics to quinones, which can decrease the nutritional quality of leaf tissues for herbivorous arthropods (Stout *et al.*, 1994). Tomato type-VI glandular trichomes provide an important physical and chemical barrier against herbivores and, accordingly, their role in plant defenses has been amply studied (Glas *et al.*, 2012; Tian *et al.*, 2012; Kang *et al.*, 2014; Balcke *et al.*, 2017). These epidermal hairy structures are reported to produce and secrete diverse compounds affecting survival (Frelichowski Jr & Juvik, 2001), growth (Kang *et al.*, 2010b) and fecundity (Bleeker *et al.*, 2012) of herbivorous arthropods. Such compounds include defensive proteins as PPO and proteinase inhibitors (Tian *et al.*, 2012), terpenoids, phenolics and acylsugars (Kang *et al.*, 2014). Among these, terpenes occupy a major role in tomato defenses, as they can be directly toxic or repellent to insect pests (Bleeker *et al.*, 2009; Kant *et al.*, 2009; Bleeker *et al.*, 2012). Notably, artificial induction of PPO, type-VI glandular trichomes and their associated volatiles has been related to increased levels of resistance against diverse herbivorous arthropods (Kang *et al.*, 2010b; Tian *et al.*, 2012; Escobar-Bravo *et al.*, 2017). However, while these studies described the induction of PPO activity, type VI trichome densities and their associated volatiles in young leaves, less is known about the

induction of these defenses in leaves of different development stages. This is of special importance, as some of the main tomato pests, with a preferential feeding for basal and older parts of the plant, are vectors of devastating virus diseases (Roselló *et al.*, 1996; Escobar-Bravo *et al.*, 2016).

In the present study we investigated how JA-mediated induction of tomato defenses, i.e. PPO activity, type-VI trichome density and their associated allelochemicals, against the Western flower thrips (WFT) *Frankliniella occidentalis* [Pergande] was dependent on the development stage of the leaf. WFT is one of the most serious greenhouse pests in agricultural and horticultural crops worldwide (Mouden *et al.*, 2017). This insect preferentially feeds on the epidermal/mesophyll tissues of old or fully developed plant leaves (Joost & Riley, 2008; Leiss *et al.*, 2009b; Mirnezhad *et al.*, 2010; Kos *et al.*, 2014), sucking up the cell content and causing the so-called silver damage scars. WFT damage can affect product appearance and market quality (de Jager *et al.*, 1995), but it is also the vector of tospoviruses, such as the economically important *Tomato spotted wilt virus* (Maris *et al.*, 2003). WFT feeding can activate JA signaling and induce the accumulation of JA in Arabidopsis (De Vos *et al.*, 2005; Abe *et al.*, 2008; Abe *et al.*, 2009) and tomato leaves (Abe *et al.*, 2011). Previously, we reported that activation of JA-associated defenses by *F. occidentalis* infestation negatively altered host suitability for conspecifics, which correlated with increased type-VI leaf trichome densities and overall leaf production of their associated volatile allelochemicals in tomato (Escobar-Bravo *et al.*, 2017). However, the magnitude of the induction of these defenses, and the mechanisms involved, along the plant canopy was not further investigated. Here we have determined how artificial application of JA affected WFT-associated feeding damage in developing and fully developed tomato leaves by performing whole plant non-choice assays. These assays were combined with analysis of PPO activity, type-VI trichome density and production of their volatile allelochemicals in leaf exudates of developing and fully developed tomato leaves.

2 Materials and methods

2.1 Plant material

Tomato seeds (*Solanum lycopersicum* cv. Moneymaker) were germinated on wet filter paper in a petri dish. Five days later, germinated seeds were transplanted to plastic pots (11 cm × 11 cm × 10 cm) filled with potting soil and placed in a climate room provided with 113.6 µmol m^{-2} s^{-1} of PAR, a photoperiod of 16L: 8D, 20ºC and 70% RH.

2.2 Experimental design

To determine the effect of JA on polyphenol oxidase (PPO) activity, type-VI trichomes and their derived volatiles as well as resistance to WFT in tomato leaves of different development stages, we carried out the following experimental design (see **Fig. 1**): tomato plants at four leaf-stage were subjected to two treatments at day 0: a) mock-treatment or b) JA exogenous application. For this, JA-treated plants were sprayed with 1 mM of JA (Cayman, Ann Arbor, Michigan, USA) in 0.8% aqueous ethanol solution until runoff (Fig. S1) as described in Thaler *et al*. (2002). Control plants were sprayed with a mock solution consisting of 0.8% aqueous ethanol. Thereafter, plants were randomly placed in a climate room provided with 113.6 µmol m^{-2} s^{-1} of PAR, a photoperiod of 16L: 8D, 25°C and 70% RH. Seven days after the initial JA or mock treatment, plants were sampled for determination of PPO activity, type-VI trichome density and volatile content in trichome-derived leaf exudates on leaf 3 and 4 from the bottom. Leaf 3 was fully developed and leaf 4 was developing at the time of the hormone application (day 0), while both were fully developed at day 7. Therefore, leaf 3 was

referred to as fully developed leaf while leaf 4 and the later formed leaf 5 were referred as developing leaves. In addition, half of the remaining JA- and mock-treated plants were then subjected to: a) WFT infestation or b) no WFT infestation. For this, individual plants were placed into WFT-proof cages consisting of transparent plastic cylinders (50 cm height and 20 cm diameter) covered at one side with a displaceable lid made of WFT-proof gauze (Leiss *et al.*, 2009b). Then, 10 (8 females and 2 males) adult WFT obtained from a mass rearing on chrysanthemum, were released into each cage. Fourteen days after the initial hormone treatment, again half of the non-infested mock- and JA-treated plants were sampled for PPO activity, type-VI trichome density, trichome-derived volatiles, as well as epidermal cell size and leaf area on leaf 3, 4 and 5 from the bottom, while the other half was infested with 10 adult WFT following the procedure described above. Mock- and JA -treated plants infested at day 7 and day 14 after the initial hormone treatment were evaluated for WFT feeding damage symptoms at seven days after WFT infestation.

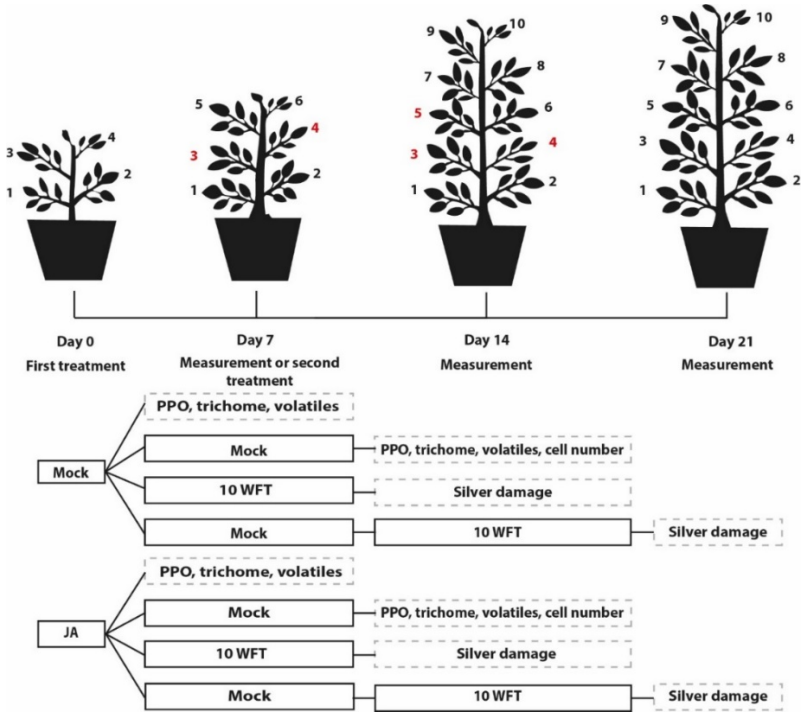

Fig. 1 Schematic representation of the experimental design. Four leaf-stage tomato plants were treated with 0.8% ethanol (mock treatment) or jasmonic acid (JA) exogenous treatment at day 0. Seven days after the initial treatment, mock- and JA-treated plants were sampled for PPO activity, type VI glandular trichome density, and volatiles content in trichome-derived exudates on leaf 3 and 4 from the bottom. Half of the remaining mock- and JA-treated plants were infested with 10 Western flower thrips (WFT). At 14 days after the initial hormone treatment, half of the remaining mock- and JA-treated were sampled for determination of epidermal cell size and leaflet area, PPO activity, type VI glandular trichome density, and volatiles content in trichome-derived exudates on leaf 3, 4 and 5 from the bottom. The remaining mock- and JA-treated plants were infested with 10 WFT. Mock- and JA-treated plants infested with WFT at 7 and 14 days after initial hormone treatment were evaluated for silver damage symptoms at 7 days after WFT infestation. Dark solid frames represent treatments, while dashed frames stand for measurements.

2.3 Measurement of silver damage

WFT feeding damage, hereafter referred as 'silver damage' was evaluated in WFT infested mock- or JA-treated plants by visually scoring each plant leaf. Silver damage was expressed as damaged leaf area in mm^2. Whole plant silver damage was calculated by adding up the damage of each individual leaf.

2.4 Determination of PPO activity

PPO activity was determined in two whole leaflets taken from leaf 3 and 4 from the bottom 7 days after the initial treatment, and from leaf 3, 4 and 5 from the bottom 14 days after the initial treatment, following the procedure described in Stout *et al*. (1998b). In brief, 0.150 g of frozen and ground plant material was homogenized in a 2 ml tube with 1.25 ml ice-cold 0.1 M pH 7.0 potassium phosphate buffer containing 7% polyvinylpolypyrolidine and 0.4 ml of 10% Triton X-100. The extracts were vortexed for 2 min and centrifuged at 11,000 × g for 10 min at 4ºC. Five microliters of the enzyme extract were added to 1 ml of 2.92 mM chlorogenic acid solution in pH 8.0 potassium phosphate buffer. The optical density (OD) at 470 nm was recorded in a spectrophotometer (UV-1800, Shimadzu) every 10 s for one min. PPO activity was expressed as changes in OD values per min per gram of fresh weight.

2.5 Determination of type VI trichome density and total number

Density of type-VI glandular trichomes in mock- and JA-treated plants was determined on the adaxial leaf side of leaflets taken from leaf 3 and 4 from the bottom 7 days after the initial treatment, and on leaflets taken from leaf 3, 4 and 5 from the bottom 14 days after the initial treatment. For this, the second terminal leaflet of the leaf was used. An area of 12 mm^2 of the adaxial leaflet side, in the middle section of the leaflet, was photographed using a Leica stereomicroscope (MZ16, Leica Microsystems, Wetzlar, Germany). Trichome number was counted on two pictures taken at both sides of the midrib of the leaflet by using the software 64-bit Fiji ImageJ (http://fiji.sc/Fiji). The average of these two measurements was calculated for each leaflet and expressed as number of type-VI trichomes per cm^2. Type VI trichome density measurements at 7 and 14 days after hormone induction was performed in two independent experiments. Estimation of total number of type-VI trichomes per leaflet were obtained by multiplying trichome density (No. cm^{-2}) by leaflet area (cm^2). In preliminary experiments, we have tested whether type-VI trichome density obtained from measurements in the middle section of the leaflet represents the averaged type-VI trichome density per leaflet. For this, type VI trichome densities determined in 18 randomly selected areas of tomato leaflets taken from the third/fourth leaf were used to calculate the type-VI trichome density of the whole leaflet. Next, type-VI trichome density was also determined in the middle section of the same leaflets. Linear regression analysis showed that the averaged trichome density obtained from measurements in the middle section of the leaflet constitutes a good predictor of the averaged density per leaflet ($R^2 = 0.6724$, $P = 0.013$), and thus, also for the total trichome number (Fig. S2).

2.6 Measurement of cell size and leaf area

To determine whether increased type-VI trichome density after JA application resulted from changes in epidermal cell size or leaf area, the top leaflet of leaf 3, 4 and 5 was analyzed 14 days after the initial treatment in plants that were subjected to mock or JA treatments. Detached leaflets were scanned (EPSON PERFECTTON 4990 PHOTO, Indonesia) and total leaflet area was measured by using the software Fiji ImageJ. Thereafter, pictures of the leaflet epidermal surface were obtained by using the same Leica stereomicroscope as used in trichome density measurement. For this, one or two drops of water were dripped on the adaxial surface of the leaflet on which a microscopy glass slide was placed to flatten the leaf

surface. Two pictures were taken in the middle of the leaflet at both sides of the midrib which were used to count the number of epidermal cells using the Fiji ImageJ. The cell size was obtained by dividing the scanned leaf area by cell number.

2.7 Determination of volatile content in trichome-derived exudates

Volatile content in type-VI trichome-derived leaf exudates was evaluated using the leaf dip method. This protocol was chosen because the terpenoid profile detected in individually collected type VI glands has been shown to be nearly identical to that observed with the leaf dip procedure (Kang *et al.*, 2010b; Kang *et al.*, 2014). It should be also noted that Akhtar *et al*. (2013) reported that a small proportion (~10%) of the major terpene component of type-VI glandular trichomes, β-phellandrene, might be produced by non-trichome leaf tissues. In that study, however, the authors extracted the volatile components of the whole leaf by grinding and thus, disrupting, all the leaf plant material. By contrast, in our study we used a very different extraction method, i.e. leaf dipping, that allows to extract the volatile content of the leaf surface exudates only, as it does not disrupt the underneath tissues. Hence, by dipping and gently shaking the leaf in pentane, only the content of type-VI glands was extracted and accounted here. Accordingly, trichome-derived volatiles were measured in two leaflets belonging to leaf 3 and 4 from the bottom at 7 days after the initial hormone treatment, and to leaf 3, 4 and 5 from the bottom at 14 days after the initial treatment. Leaf area of these two leaflets was measured before extraction by scanning and analyzing the images using the software Fiji ImageJ. Thereafter, leaf exudates were obtained by dipping these two leaflets in 2 ml pentane (Sigma-Aldrich) containing 10 µg of n-Tetradecane (Sigma-Aldrich) as internal standard (Sallaud *et al.*, 2012; Escobar-Bravo *et al.*, 2017), followed by a 2 min gentle shaking. The two leaflets were then discarded, and the extracts were analyzed by gas chromatography-mass spectrometry. One microliter from the resulting pentane leaf extract was injected into an Agilent model 7890 gas chromatograph fitted with a 5975C inert XL MSD Triple Axis Detector using a split ratio of 20:1. Compounds were separated using a DB-5MS column (30 m × 0.25 mm, 0.25 µm film thickness), and Helium as carrier gas at a flow rate of 1.6 ml min^{-1}. The oven temperature was programmed to rise from 40°C to 150°C at a rate of 15°C min^{-1}, followed by an increase to 220°C at a rate of 6°C min^{-1}. Terpenes were identified by comparing the detected spectrum with authentic standards if possible or with spectral information available in Agilent GC/MSD ChemStation. Quantification was performed on the basis of the internal standard procedure described in Escobar-Bravo et al. (2017). Terpene content was expressed as ng per cm^2 of leaf area, or ng per type-VI trichome by dividing the terpene content by the total number of trichomes estimated in the adaxial leaf sides in two leaflets. Terpene content per type-VI trichome was referred as relative volatile content per trichome.

2.8 Statistical analysis

All statistical analyses were performed using the SPSS software package (version 23; SPSS Inc., Chicago, IL, USA). Whole plant silver damage determined in mock- and JA-treated plants infested with WFT at 7 and 14 days after initial hormone treatment were analyzed by student-t tests. Data on silver damage determined in plants infested at 7 days was Log transformed prior to analysis. Generalized Linear Models (GLM) using linear distribution and identity link functions were used to analyze the effect of JA, leaf development stage (i.e. represented by leaf 3, 4 at 7 days, and leaf 3, 4 and 5 at 14 days) and their interaction on (1) silver damage symptoms, (2) PPO activity, (3) type-VI trichome density, (4) terpene content in trichome-derived exudates, (5) relative volatile content per trichome, (6) epidermal cell size and (7) leaflet area. Differences among groups were tested by Fisher´s least significant

difference (LSD) post-hoc test. Data on silver damage and PPO activity determined at 14 days after hormone treatment were Log transformed prior to analysis. Similarly, relative volatile content per trichome and content of individual terpene compounds measured at 7 and 14 days after the initial hormone treatment were log transformed prior to analysis. In addition, data on silver damage per leaf determined at 14 days after initial hormone treatment (i.e. infestation performed at 7 days after JA or mock solutions treatment) was analyzed using binominal GLM with logit as the link function. For this, data were transformed to 0 (0 damage symptoms) or 1 (damage symptoms > 1) prior to analysis, because zero silver damage was observed on most of the JA-treated leaves, especially leaf 5. Differences among groups were tested by LSD post-hoc test. Patterns of terpene compounds detected trichome-derived exudates of mock- and JA-treated plants at 7 and 14 days after initial hormone treatment were subjected to Principal Component Analysis (PCA) using the SIMCA-P 13 software package (Umetrics, Sweden). Silver damage determined on each leaf in mock- and JA-treated plants infested with WFT at 7 and 14 days after initial hormone treatment was analyzed by Mann-Whitney U test. All detailed statistics are shown in Table S1.

3 Results

3.1 Effect of JA exogenous treatment and leaf development stage on tomato resistance to WFT

Whole plant silver damage was significantly reduced in JA-treated tomato plants that were infested at 7 or 14 days after the initial hormone treatment and evaluated at 7 days after infestation (Student t-tests, $P < 0.01$) (**Fig. 2A, C**). Yet, WFT resistance significantly differed between leaves independent of the treatment, being fully developed leaves more susceptible than developing ones (Binominal GLM, $P \leq 0.001$) (**Fig. 2B, D** and **Fig. S3A, B**). At 7 days after the hormone treatment, JA reduced silver damage by 78%, 94% and 99% in leaf 3, 4 and 5, respectively, when compared to the leaves of mock-treated plants (Binominal GLM, $P < 0.001$). However, although the reduction in silver damage symptoms in leaf 3 was lower, the effect of JA did not significantly depend on the leaf development stage (Binominal GLM, $P = 0.587$ for the interaction). At 14 days after the hormone induction, JA treatment reduced silver damage by 36%, 94% and 82% on leaf 3, 4 and 5, respectively, when compared to the leaves of mock-treated plants (GLM, $P < 0.001$). This effect was dependent of leaf development stage with a higher reduction in silver damage symptoms detected on leaf 4 and 5 than on leaf 3 (GLM, $P < 0.001$ for the interaction). A similar result was obtained in a repeated experiment (**Fig. S4**).

3.2 Effect of JA exogenous treatment and leaf development stage on PPO activity

Levels of PPO activity were markedly influenced by leaf development stage (GLM, $P < 0.001$) and JA (GLM, $P < 0.001$) at 7 days after the initial hormone treatment, being significantly induced on both leaf 3 and 4 of JA-treated plants compared to their controls (**Fig. 3A**). Moreover, the magnitude of PPO induction was significantly higher in leaf 4 than in leaf 3 after JA application (GLM, $P = 0.002$ for the interaction).

Basal levels of PPO activity were higher in leaf 5 than in leaf 3 and 4 for control plants at 14 days after the initial hormone treatments (GLM, $P < 0.001$) (**Fig. 3B**). JA treatment significantly induced PPO activity in leaf 3, 4 and 5 (GLM, $P < 0.001$). However, the magnitude of this induction was affected by leaf development stage (GLM, $P = 0.040$ for the interaction) being the highest in leaf 5.

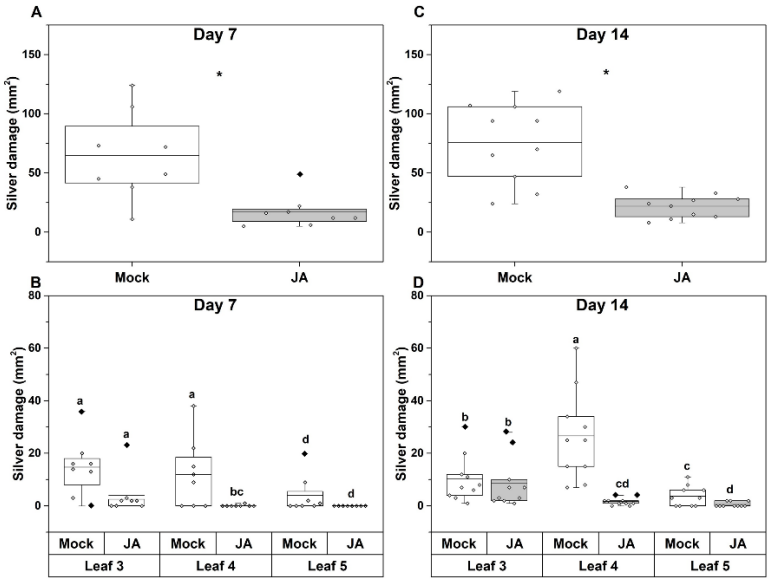

Fig. 2 Effect of JA exogenous treatment and leaf development stage on tomato resistance to Western flower thrips. Silver damage symptoms determined in **(A)** the whole plant and (**B**) leaf 3, 4 and 5 from bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants infested with 10 adult **Western flower thrips (WFT)** at 7 days after the initial hormone treatment. Silver damage symptoms determined in (**C**) the whole plant and (**D**) on leaf 3, 4 and 5 from bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants infested with WFT at 14 days after the initial hormone treatment. Silver damage symptoms were evaluated at 7 days after WFT infestation. Boxes and whiskers denote the 25th – 75th percentile and minimum–maximum observations, respectively; group mean values are indicated by the horizontal line. Diamonds denote individual values (n = 8) and the filled diamonds denote outliers. Asteriks denote significant differences as tested by student-t test at $P \leq 0.05$. Different letters indicate significant differences among groups compared by Fisher's least significant differences (LSD) test at $P \leq 0.05$.

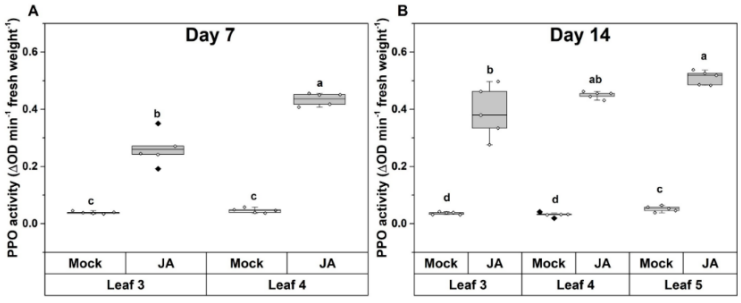

Fig. 3 Effect of JA exogenous treatment and leaf development stage on PPO activity in tomato. PPO activity determined on **(A)** leaf 3 and 4 from the bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants at 7 days after the initial hormone treatment, and on (**B**) leaf 3, 4 and 5 from the bottom of mock- (white box plots) and JA-treated (grey box plots) plants at 14 days after the initial hormone treatment. Boxes and whiskers denote the 25th – 75th percentile and minimum–maximum observations, respectively; group mean values are indicated by the horizontal line. Diamonds denote individual values (n = 5) and the filled diamonds denote outliers. Different letters indicate significant differences among groups compared by Fisher's least significant differences (LSD) test at $P \leq 0.05$.

3.3 Effect of JA exogenous treatment and leaf development stage on type-VI trichome density

Type-VI trichome density was significantly affected by leaf development stage (GLM, $P < 0.001$) (**Fig. 4A, B**). When treated with JA, tomato plants increased type VI trichome densities in leaf 4 (GLM, $P < 0.001$), but no induction was observed in leaf 3 (GLM, $P < 0.001$ for the interaction) at 7 days after the initial treatment. Similar results were observed in a repeated experiment (Fig. S5A).

Fourteen days after the initial hormone treatment, type-VI trichome density also differed among tomato leaves, being higher on leaf 4 and 5 when compared to leaf 3 (GLM, $P < 0.001$) (**Fig. 4A, E**). JA treatment strongly induced type-VI trichome density on leaf 4 and 5 (GLM, $P < 0.001$), but only a slight induction was observed on leaf 3 (GLM, $P < 0.001$ for the interaction). Similar results were obtained in a repeated experiment (Fig. S5B).

3.4 Effect of JA and leaf development stage on type-VI trichome-associated volatiles

Fourteen major volatiles were detected in the trichome-derived exudates of leaf 3, 4 and 5 obtained from mock- or JA-treated plants at 7 and 14 days after the initial treatment. Among these, 13 were identified as the monoterpenes α-pinene, *p*-cymene, myrcene, δ-carene, α-phellandrene, α-terpinene, limonene, β-phellandrene, trans-ocimene, γ-terpinene, terpinolene and the sesquiterpenes β-caryophyllene and α-caryophyllene (**Table 1**).

JA significantly increased the total content of terpenes in the trichome-derived exudates of leaf 3 and 4 at 7 days after the initial hormone treatment (GLM, $P < 0.001$ for leaf development stage; $P < 0.001$ for JA treatment; $P < 0.001$ for the interaction) (**Fig. 4C**). The magnitude of this induction was affected by leaf development stage. We detected a 4.6- and 16.2-fold increase in leaf 3 and 4, respectively. JA also induced the production of total terpenes per trichome in these leaves, and this induction was similar in both leaf 3 and 4 (GLM, $P = 0.977$ for leaf development stage; $P < 0.001$ for JA treatment; $P = 0.713$ for the interaction) (**Fig. 4D**).

At 14 days after the initial hormone treatment, a significant increase in the total terpene content of trichome-derived exudates was observed in JA-treated plants when compared to mock-treated plants (GLM: $P < 0.001$ for leaf development stage; $P < 0.001$ for JA treatment; $P = 0.287$ for the interaction) (**Fig. 4F**). Total terpene content in trichome-derived exudates from leaf 3, 4 and 5 of JA treated plants was 14, 25 and 25 times higher than in their equivalent control leaves of mock-treated plants. Hence, relative production of terpenes per trichome was significantly higher in leaf 3 than in leaf 4 or 5 in JA-treated plants (GLM, $P < 0.001$ for leaf development stage; $P < 0.001$ for JA treatment; $P < 0.001$ for the interaction) (**Fig. 4G**). Hence, the higher content of terpenes detected in the trichome-derived exudates of leaf 4 and 5 of JA -treated plants seemed to be explained by the greater density of type-VI glandular trichomes. Conversely, in leaf 3, trichome density was less affected by JA, thus accumulation of higher volatiles in leaf exudates can be mainly explained by an increased biosynthesis of the trichome-derived volatiles per trichome.

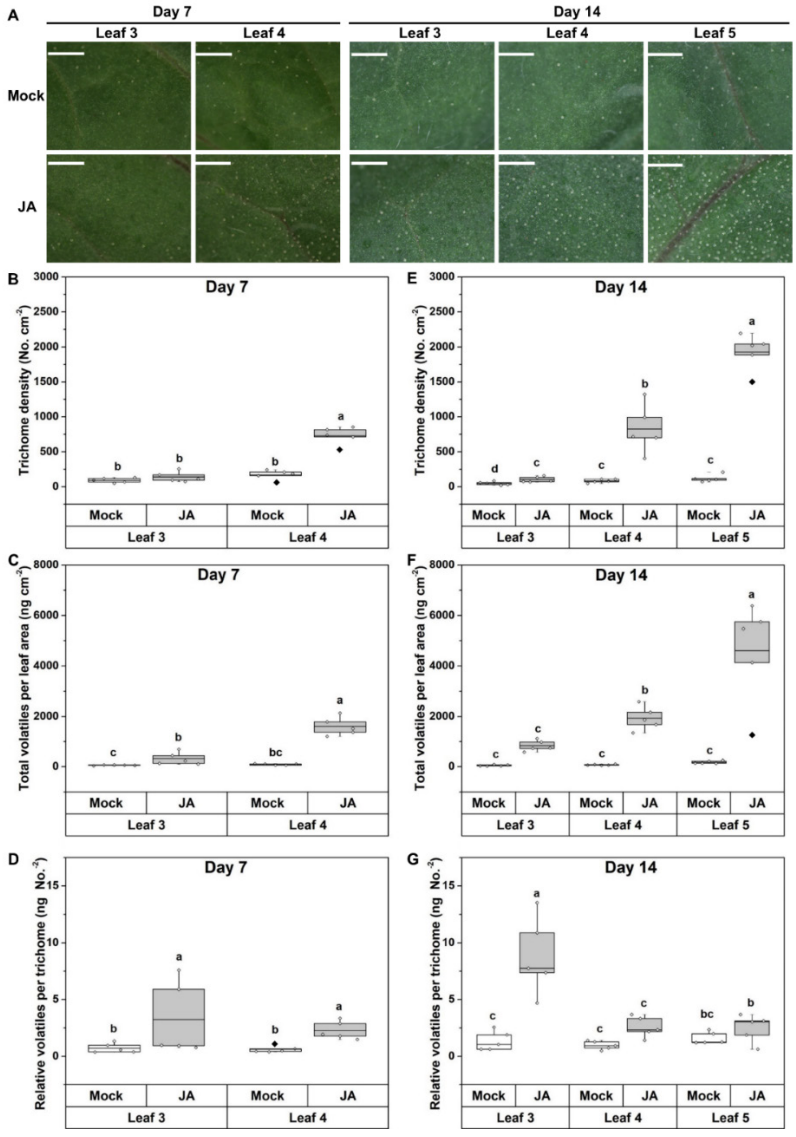

Fig. 4 Effect of JA exogenous treatment and leaf development stage on type-VI trichome density and their derived volatiles in tomato. (A) Representative photographs of adaxial leaf surface of leaflets taken from mock- and JA-treated tomato plants at 7 and 14 days after the initial treatment. **(B)** Type-VI trichome density. White bars represent 1 mm. **(C)** total volatiles content in trichome-derived leaf exudates and **(D)** relative total volatile content per trichome determined on leaf 3 and 4 from the bottom of mock- and JA-treated tomato plants 7 days after the initial treatment. **(E)** type-VI trichome density, **(F)** total volatiles content in trichome-derived leaf exudates and **(G)** relative volatile content per trichome determined on leaf 3, 4 and 5 from the bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants at 14 days after the initial treatment. Boxes and whiskers denote the 25th – 75th percentile and minimum–maximum observations, respectively; group mean values are indicated by the horizontal line. Diamonds denote individual values (n = 5) and the filled diamonds denote outliers. Different letters indicate significant differences among groups compared by Fisher's least significant differences (LSD) test at $P \leq 0.05$.

To further investigate the effect of JA and leaf development stage on the volatile composition of trichome-derived exudates, an unsupervised multivariate PCA analysis was performed on the volatile profile of mock- and JA-treated plants at 7 and 14 days after the initial hormone treatment (**Fig. 5**). At day 7 volatile profiles of mock- and JA-treated plants were separated by the first principal component (PC1), which explained 89.4% of the variance (**Fig. 5A**). A second principal component (PC2), explaining 6.6% of the variance, also separated leaf 3 and 4 of JA-treated plants. Although in both leaves most of the terpenes were significantly induced (**Fig. 5B**), the induced levels of these compounds were higher in leaf 4 than in leaf 3, except for *p*-cymene (**Table 1**). Similarly, at 14 days after the initial hormone treatment, volatile profiles detected in trichome-derived exudates of mock- and JA-treated plants were separated by PC1 that explained 94.9% of the variance (**Fig. 5C**). However, while the volatile profiles of leaf 4 and 5 from JA-treated plants were clearly separated from their controls, the chemical profile detected in leaf 3 seemed to differ from the developing leaves (**Fig. 5D**). Hence, most of the detected volatiles were less induced by JA in leaf 3 when compared to leaf 4 and 5 (**Table 1**). Under non-induced conditions, similar amounts of terpenes were observed for leaf 3 and 4 (except for the monoterpene myrcene), while in leaf 5 higher amounts of α-pinene, myrcene, δ-carene, α-phellandrene, α-terpinene, β-phellandrene, β-caryophyllene and α-caryophyllene were detected (**Table 1**).

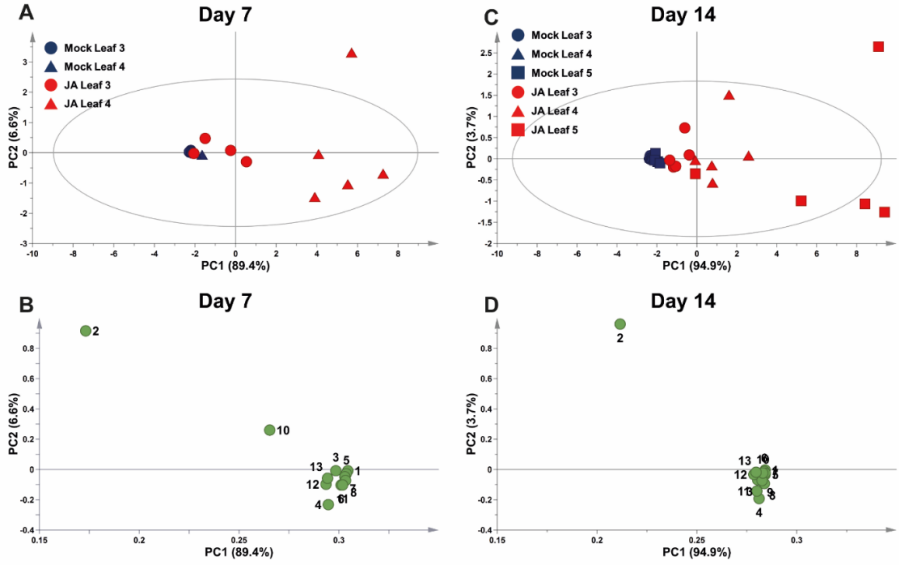

Fig. 5 Effect of JA exogenous treatment and leaf development stage on trichome-derived volatile profiles in tomato. Principal component analysis (PCA) of volatile compounds detected in leaf exudates of **(A** and **B)** leaf 3 and 4 and (**C** and **D)** of leaf 3, 4 and 5 of mock- and JA-treated plants at 7 and 14 days after hormone treatment, respectively. Score plot (A and C) and loading plot (B and D) of the first two principal components (PC) with the explained variance in brackets. The ellipse in (A) and (C) defines the Hotelling's T2 confidence region (95%). The numbers in (B) and (D) represent: 1, α-pinene; 2, *p*-cymene; 3, myrcene; 4, δ-carene; 5, α-phellandrene; 6, α-terpinene; 7, limonene & β-phellandrene; 8, trans-ocimene; 9, γ-terpinene; 10, terpinolene; 11, unknown; and 12, β-caryophyllene and 13, α-caryophyllene.

Table 1 Terpene content in leaves 3, 4 and 5 of mock- and JA-treated tomato plants at 7 or 14 days after thrips infestation

No.	Day 7					Day 14						
	Mock		JA		Interaction	Mock			JA			Interaction
	leaf 3 (ng cm^{-2})	leaf 4 (ng cm^{-2})	leaf 3 (ng cm^{-2})	leaf 4 (ng cm^{-2})		leaf 3 (ng cm^{-2})	leaf 4 (ng cm^{-2})	leaf 5 (ng cm^{-2})	leaf 3 (ng cm^{-2})	leaf 4 (ng cm^{-2})	leaf 5 (ng cm^{-2})	
1	1.64 ± 0.21 c	2.72 ± 0.33 c	10.36 ± 3.44 b	58.02 ± 5.41 a	$P < 0.001$	1.81 ± 0.31 e	2.03 ± 0.27 e	5.36 ± 0.85 d	24.42 ± 2.94 c	63.51 ± 7.19 b	165.36 ± 31.86 a	$P = 0.015$
2	0.25 ± 0.25 b	0 ± 0 b	4.23 ± 2.10 ab	20.74 ± 13.33 a	$P = 0.296$	0.43 ± 0.43 c	0.59 ± 0.59 c	1.15 ± 1.15 c	13.01 ± 6.45 b	29.03 ± 13.16 ab	56.12 ± 28.80 a	$P = 0.305$
3	0.33 ± 0.33 c	0.67 ± 0.67 c	3.69 ± 1.43 b	22.17 ± 1.17 a	$P < 0.01$	0.16 ± 0.16 f	0.93 ± 0.25 e	1.98 ± 0.23 d	6.44 ± 0.42 c	12.08 ± 1.40 b	22.8 6± 4.49 a	$P = 0.946$
4	12.51 ± 1.52 c	18.55 ± 1.88 bc	65.32 ± 21.94 b	283.56 ± 40.79 a	$P < 0.001$	12.50 ± 2.70 e	14.43 ± 2.34 e	30.59 ± 4.31 d	162.97 ± 16.04 c	342.15 ± 31.48 b	760.94 ± 147.57 a	$P = 0.103$
5	1.77 ± 0.15 c	3.08 ± 0.33 c	10.82 ± 3.64 b	52.52 ± 5.60 a	$P < 0.001$	1.74 ± 0.33 e	2.27 ± 0.29 e	5.36 ± 0.75 d	28.93 ± 3.64 c	67.52 ± 7.19 b	160.16 ± 31.66 a	$P = 0.016$
6	0 ± 0 c	0 ± 0 c	3.24 ± 1.40 b	19.16 ± 2.21 a	$P < 0.001$	0 ± 0 e	0.58 ± 0.26 e	1.82 ± 0.25 d	11.36 ± 1.57 c	24.34 ± 2.57 b	53.78 ± 10.93 a	$P = 0.366$
7	29.76 ± 3.76 c	45.33 ± 4.06 c	190.34 ± 63.72 b	940.99 ± 106.13 a	$P < 0.001$	30.96 ± 6.18 e	39.18 ± 4.65 e	87.86 ± 12.57 d	543.96 ± 68.00 c	1248.73± 133.08 b	2898.86± 573.01 a	$P = 0.134$
8	0 ± 0 c	0 ± 0 c	2.16 ± 1.37 b	19.64 ± 1.80 a	$P = 0.002$	0± 0 d	0± 0 d	0.31 ± 0.31 d	4.30 ± 0.38 c	10.24 ± 1.51 b	28.75 ± 5.41 a	$P < 0.001$
9	0 ± 0 b	0 ± 0 b	0 ± 0 b	1.94 ± 1.19 a	$P = 0.068$	0 ± 0 d	0 ± 0 d	0 ± 0 d	1.62 ± 0.46 c	4.09 ± 0.49 b	9.59 ± 1.84 a	$P < 0.001$
10	0 ± 0 b	0 ± 0 b	1.15 ± 1.15 b	9.41 ± 2.46 a	$P = 0.004$	0 ± 0 d	0 ± 0 d	0 ± 0 d	2.39 ± 0.27 c	5.21 ± 0.63 b	11.42 ± 2.51 a	$P = 0.001$
11	2.55 ± 0.67 c	5.91 ± 1.31 b	11.37 ± 3.77 b	66.08 ± 2.99 a	$P = 0.006$	0.60 ± 0.38 d	1.88 ± 0.36 d	4.83 ± 0.66 c	6.25 ± 0.68 c	22.45 ± 4.32 b	87.68 ± 17.42 a	$P = 0.013$
12	7.85 ± 0.95 b	16.19 ± 4.24 b	18.55 ± 7.18 b	99.79 ± 9.30 a	$P = 0.004$	5.33 ± 1.72 d	9.66 ± 1.44 d	30.44 ± 5.03 c	21.10 ± 1.53 c	81.49 ± 16.83 b	289.67 ± 65.02 a	$P = 0.146$
13	0.41 ± 0.41 b	0.84 ± 0.84 b	2.16 ± 1.45 b	16.30 ± 1.62 a	$P < 0.001$	0.44 ± 0.44 d	2.06 ± 0.65 d	6.13 ± 1.00 c	5.22 ± 0.44 c	18.08 ± 3.53 b	58.96 ± 13.18 a	$P = 0.543$

Data were expressed as mean ± SEM (n = 5). Different letters denote significant differences among groups compared by LSD test at $P \leq 0.05$ within the same day measurement. P value for the interactive effect between treatment and leaf age was shown. The numbers represent: 1, α-pinene; 2, ρ-cymene; 3, myrcene; 4, δ-carene; 5, α-phellandrene; 6, α-terpinene; 7, limonene & β-phellandrene; 8, trans-ocimene; 9, γ-terpinene; 10, terpinolene; 11, unknown; and 12, β-caryophyllene and 13, α-caryophyllene

3.5 Effect of JA exogenous treatment and leaf development stage on epidermal cell size and leaf area

Epidermal cell size significantly differed among leaf development stages with the developing leaves having smaller cells (GLM, $P < 0.001$) (**Fig. 6A**). No differences in epidermal cell size were observed between JA- and mock- treated plants for any of the leaf development

stages (GLM, $P = 0.989$ for JA treatment; $P = 0.744$ for the interaction). Leaflet area was smaller in leaf 3 when compared to leaf 4 and 5 (GLM, $P < 0.001$) (**Fig. 6B**). Application of JA significantly reduced leaflet area (GLM, $P = 0.003$) independent of the leaf development stage (GLM, $P = 0.897$ for the interaction). JA treatment led to a reduction of leaflet area of 17%, 18% and 21% for leaf 3, 4 and 5 respectively, while the corresponding induction of type-VI trichome density amounted to 114%, 903% and 1,539% respectively (**Fig. 6B**).

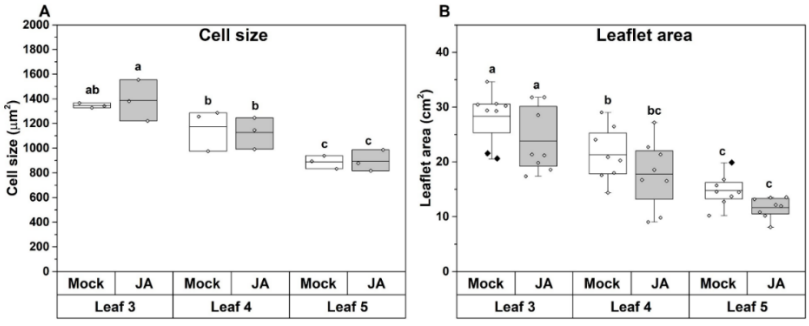

Fig. 6 Effects of JA and leaf development stage on epidermal cell size and leaflet area in tomato. (**A**) Epidermal cell size ($n = 3$) and (**B**) leaflet area ($n = 8$) determined on bottom leaf 3, 4 and 5 of mock- (white box plots) and JA-treated (grey box plots) tomato plants 14 days after the initial JA treatment. Plants received JA or mock treatment at day 0, followed by a mock treatment at day 7. Boxes and whiskers denote the 25th–75th percentile and minimum–maximum observations, respectively; group mean values are indicated by the horizontal line. Diamonds denote individual values and the filled diamonds denote outliers. Different letters indicate significant differences among groups compared by Fisher's least significant differences (LSD) test at $P \leq 0.05$.

4 Discussion

In the present study we demonstrated that the magnitude of the induction of anti-herbivore JA-associated defenses, such as PPO activity, type-VI trichome density and their associated allelochemicals, depend on leaf development stage in tomato plants. Our results showed differential induction of type-VI trichome densities and their associated volatiles in developing and fully developed leaves. Furthermore, we showed that the diminished induction of these defenses in fully developed tomato leaves coincided with a higher susceptibility to WFT when compared to developing leaves.

First, we showed that artificial application of JA reduced WFT-associated damage in tomato plants subjected to whole plant no-choice bioassay. This agrees with previous studies reporting the role of the activation of JA defenses (Li *et al.*, 2002; Abe *et al.*, 2009; Escobar-Bravo *et al.*, 2017) and induction of endogenous levels of JA upon WFT infestation (De Vos *et al.*, 2005; Abe *et al.*, 2008; Abe *et al.*, 2009; Abe *et al.*, 2011) in plant resistance against WFT. Yet, how induction of JA defenses varies along the plant canopy in tomato and how this correlates with the intensity of silver damage symptoms caused by WFT infestation has not been previously investigated. Our results showed that JA-mediated induction of tomato resistance against WFT was less strong in fully developed leaves, displaying more silver damage symptoms than in JA-induced developing tomato leaves at 7 and 14 days after the initial hormone treatment. This reduced JA-mediated induction of resistance to WFT might explain the higher susceptibility observed in fully developed tomato leaves of mock-treated plants when compared to developing leaves reported here, but also in other host plants as *Senecio* (Leiss *et al.*, 2009a), chrysanthemum (Kos *et al.*, 2014) and other wild and cultivated tomatoes (Mirnezhad *et al.*, 2010).

We have further demonstrated that JA-associated anti-herbivore defense responses were differently induced in developing and fully developed tomato leaves. Hence, the activity of the defensive enzyme PPO, which is reported to be enhanced by JA (Constabel *et al.*, 1995; Thaler *et al.*, 1996), was more induced in developing leaves than fully developed ones at 7 and 14 days after the initial hormone treatment. These results are in agreement with a previous study by Thipyapong and Steffens (1997). These authors reported that PPO was significantly induced in young leaves upon application of JA or its volatile form MeJA, but not in older tomato leaves. Similarly, Li and Steffens (2002) also showed that in prosystemin-transformed tomato plants, in which JA signaling was constitutively induced, expression of PPO was higher in young tomato leaves. This differential induction might affect tomato resistance against arthropod herbivores. For instance, a positive correlation between PPO activity and resistance to beet armyworm (*Spodoptera exigua*) and cotton bollworm (*Helicoverpa armigera*) (Bhonwong *et al.*, 2009), as well as cutworm (*S. litura*) (Mahanil *et al.*, 2008), has been demonstrated in tomato. This was explained by the role of PPO in the oxidation of phenolics to quinones upon leaf tissue disruption. Quinones can chemically interact with plant amino acids or proteins thus reducing their availability for herbivores (Felton & Duffey, 1991). Whether a stronger induction of PPO activity might increase tomato resistance against WFT was unknown but our data suggest that indeed a stronger induction of PPO is associated with a larger increase in WFT resistance across leaves of different ages. Interestingly, Leiss *et al*. (2009b) reported that resistance to WFT in chrysanthemum (*Dendranthema grandiflora*) leaves was strongly associated with elevated levels of chlorogenic acid. Because chlorogenic acid is one of the main substrates of PPO, a higher accumulation of this phenolic acid in chrysanthemum leaves might have resulted in higher oxidation rates and, therefore, augmented accumulation of highly reactive quinones.

In addition, we showed that, next to PPO, JA had a stronger inducing effect on type-VI trichome densities in developing leaves when compared to fully developed ones. This induction directly resulted from modifications in epidermal cells rather than changes in cell size. Likewise, Traw and Bergelson (2003) also reported that JA-mediated induction of trichomes in Arabidopsis resulted from direct epidermal cell transformations. Notably, although a number of other studies have described the induction of type-VI trichome densities by JA in tomato (Traw & Bergelson, 2003; Boughton *et al.*, 2005; Campos *et al.*, 2009; Peiffer *et al.*, 2009; Escobar-Bravo *et al.*, 2017), little is known about how this is affected by the leaf development stage. Here we showed that leaves that were present, but not fully developed at the time of JA application, i.e. developing leaves, responded with increased formation of type-VI trichomes, but those that were fully developed did not. This implies that the density of type-VI glandular trichomes is not fixed at the time of leaf emergence, but induction is no longer feasible when the leaves have reached a mature development stage. In line with this, induction of leaf trichomes by high sodium chloride, hydrogen peroxide or chitosan oligosaccharide treatments in *Artemisia annua* only occurred in leaves formed after the induction, but not in leaves that were already present at the time of the treatment application (Kjær *et al.*, 2012).

Production of the main type-VI trichome-associated volatile allelochemicals was also affected by JA treatment and leaf development stage. In agreement with previous studies (Li *et al.*, 2004; van Schie *et al.*, 2007; Spyropoulou *et al.*, 2014b; Escobar-Bravo *et al.*, 2017), we showed that JA application increased the total volatile content in trichome-derived leaf exudates of tomato plants. However, the magnitude of this induction, both for total volatile content and each of the identified volatile constituents (except for *p*-cymene), were dependent on the leaf development stage. Our results showed that developing leaves accumulated more type-VI trichome-associated volatiles in the leaf exudates of JA-treated plants at 7 and 14

days after the initial hormone induction. Notably, when the volatile content was expressed in terms of production per trichome, type-VI trichomes from fully developed leaves experienced the same (i.e. at 7 days) or even higher induction (i.e. at 14 days) in volatile production than developing leaves. The higher accumulation of volatiles in trichome-derived exudates of developing leaves at 14 days after the initial hormone treatment can be explained by the existence of higher trichome number. Conversely, the induction of volatiles in leaf 3 at 7 and 14 days after the hormone treatment might be due to the induction of the biosynthetic machinery of the glandular trichome. Interestingly, Tian *et al*. (2012) described that tomato leaf trichomes contain significantly more monoterpenes than stem trichomes, thus confirming the tissue-specific production of these volatile compounds. Yet, to the best of our knowledge, this is the first comparative study on how the leaf content in trichome-associated allelochemicals vary along leaves of different development stages in response to JA. Several type-VI trichome-specific genes coding for enzymes involved in the biosynthesis of terpene precursors, terpene synthases and for transcription factors responsible for their regulation have been described for tomato (van Schie *et al.*, 2007; Besser *et al.*, 2009; Spyropoulou *et al.*, 2014a; Spyropoulou *et al.*, 2014b). Developmental profiles of terpenoid accumulation along the tomato plant canopy has been generally performed for stem tissues, and attributed to the differential expression of these terpenoid-related biosynthesis genes. For instance, Besser *et al*. (2009) reported that transcript accumulation of genes involved in terpenoid biosynthesis were reduced in trichomes of the first elongating internode of the tomato stems, but increased in the subsequent internodes and decreased again in more mature sections. Interestingly, Falara *et al*. (2011) also described differential expression of several putative terpene synthases in trichomes on young and full developed tomato leaves. Induction of terpene-related biosynthesis genes by wounding, hormones or elicitors has been demonstrated in tomato trichomes (van Schie *et al.*, 2007; Falara *et al.*, 2011; Spyropoulou *et al.*, 2014b). Moreover, Spyropoulou *et al* (2014b) showed that some of these genes displayed differences in their JA-inducibility. Yet, whether the expression of these genes might be differentially modulated by both JA and the development stage of the leaf was not further discussed in this study.

Our results have important implications for the application of tomato resistance against herbivores, as trichome-derived volatiles play a prominent role in plant defenses against herbivorous arthropods (Kang *et al.*, 2010a; Kang *et al.*, 2010b; Tian *et al.*, 2012). An array of terpenes produced and stored in glandular trichomes have been reported to be directly toxic or repellent to diverse insect pests, like whiteflies, in tomato (Freitas *et al.*, 2002; de Azevedo *et al.*, 2003; Bleeker *et al.*, 2009; Bleeker *et al.*, 2012). Thus, a higher accumulation of these compounds in developing tomato leaves might have reinforced the defenses against WFT, as within plant or leaf distribution of secondary metabolites have a great impact on herbivore foraging. For instance, Shroff *et al*. (2008) showed that the differential distribution of glucosinolates within the *Arabidopsis thaliana* leaves strongly determined the feeding pattern of *Helicoverpa armigera* larvae. In the case of *F. occidentalis*, younger leaves of *Senecio* plants, which contain higher amounts of pyrrolizidine alkaloids, suffered less WFT damage than older leaves (Leiss *et al.*, 2009a). More recently, Scott-Brown *et al*. (2016) showed that as the leaves of *Rhododendron* plants matured, the trichome density and the leaf content of the diterpenoid grayanotoxin I decreased, while the number of *Heliothrips haemorrhoidalis* thrips and area of feeding damage increased. In addition, Köhler *et al* (2015) reported that a higher induction of the toxin 1,4-benzoxazin-3-ones in young maize leaves upon herbivory negatively correlated with feeding by the generalist *S. littoralis*. Hence, plants might increase the induction of defenses in those parts that contribute most to their fitness, i.e. young leaf tissues (Kishida & Nishimura, 2004; Moreira *et al.*, 2012).

How plants control the magnitude of their herbivore-mediated induced chemical defenses along the canopy is unknown. However, higher constitutive JA levels have been found in youngest tissues and flowers of soybean plants (Creelman & Mullet, 1995), which might enhance the pool of bioactive jasmonates after herbivory. Yet, Bosak *et al*. (2013) found that the increased plant susceptibility to *S. exigua* in old maize plants was not explained by variations in constitutive or herbivory-mediated induced JA levels. Hence, JA-associated plant responses along the plant canopy might also depend on the sensitivity of these tissues to JA as well (Ballaré, 2011).

In conclusion, our results showed that the differential induction of PPO activity, type-VI trichome densities and their associated volatiles in different leaves of tomato plants by exogenous application of JA also coincided with the capacity of these leaves to increase resistance against WFT. Importantly, JA did not increase the induction of type-VI trichome density in fully developed leaves, but it increased their biosynthetic capacity to produce more volatiles. This suggests that at certain leaf development stage the induction of trichome-associated chemical defenses might not be constraint, but only *de novo* production of these epidermal organs. We also concluded that protection of tomato plants against WFT by means of activation of the JA signaling pathway and, therefore, induction of PPO and trichome-mediated defenses, might be limited by the diminished capacity of fully developed leaves to generate JA-associated responses. This has important implications for the protection of crops against *F. occidentalis* in agricultural systems, as WFT can transmit *Tomato spotted wilt virus*, a devastating virus disease with a worldwide distribution preceded by the dispersal of *F. occidentalis* (Gilbertson *et al.*, 2015). Importantly, host plant resistance to virus-transmitting insects has been proposed as an important approach to reduce the virus dispersal (Nombela & Muñiz, 2009; Escobar-Bravo *et al.*, 2016). We hypothesize that the reduced capacity to artificially induce host plant resistance against WFT in older tomato leaves might, therefore, affect the effectiveness of this approach, yet this requires further research.

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Supplementary materials

Fig. S1 Representative photograph of the tomato plants sprayed with JA at day 0.
Fig. S2 Scatter plot depicting the relationship between the trichome density in the middle section of the tomato leaflet and in the whole leaflet.
Fig. S3 Silver damage determined in tomato leaves at different development stage after JA application.
Fig. S4 Effect of JA exogenous treatment and leaf development stage on tomato resistance to thrips.
Fig. S5 Effect of JA exogenous treatment and leaf development stage on type-VI trichome density.
Table S1 Results of the statistical analysis performed for each experiment.

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Supplementary Materials

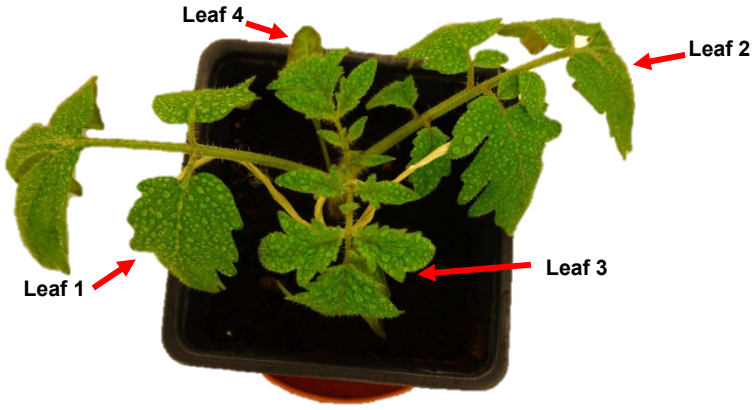

Fig. S1 Representative photograph of the tomato plants sprayed with JA at day 0. Tomato plants at four leaf stage were sprayed with JA or Mock solution until run off at day 0 and evaluated for type-VI trichome density, terpene content in trichome-derived leaf exudates, polyphenol oxidase activity and resistance to thrips at 7 and 14 days after the hormone application.

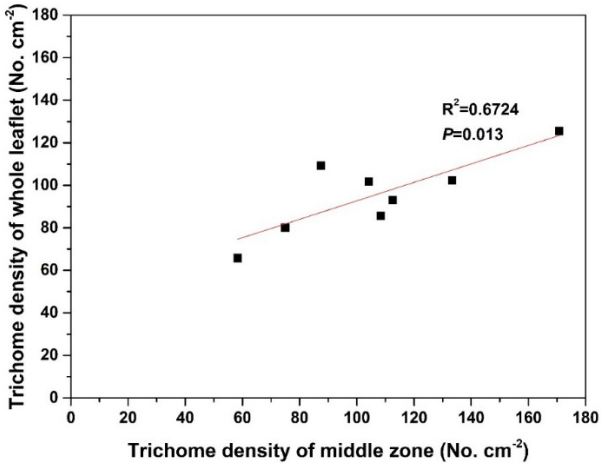

Fig. S2 Scatter plot depicting the relationship between the trichome density in the middle section of the tomato leaflet and in the whole leaflet. Type VI trichome density was determined in two areas of the middle section of a tomato leaflet and in 18 random areas of the same leaflet. Leaflets, each corresponding to an individual plant (n = 8 plants), were taken from the third/fourth leaf from the bottom. The Pearson's coefficient and *P* value are shown in the graph.

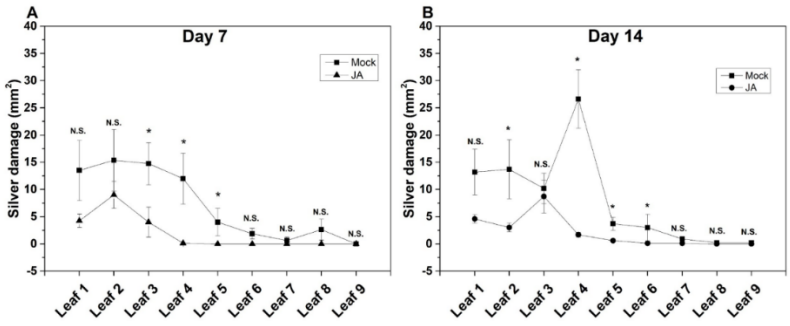

Fig. S3 Silver damage determined in tomato leaves at different development stage after JA application. Silver damage (mean ± SEM, n = 8) was determined in all leaves of tomato plants subjected to thrips infestation (**A**) at 7 and (**B**) 14 days after initial hormone treatment. Leaves were enumerated starting from the bottom, i.e. leaf 1 corresponds to the most basal part of the plant. Silver damage symptoms were evaluated 7 days after thrips infestation. Data were analyzed by Mann-Whitney U test. Asterisk denote significant differences at $P \leq 0.05$. NS: not significant.

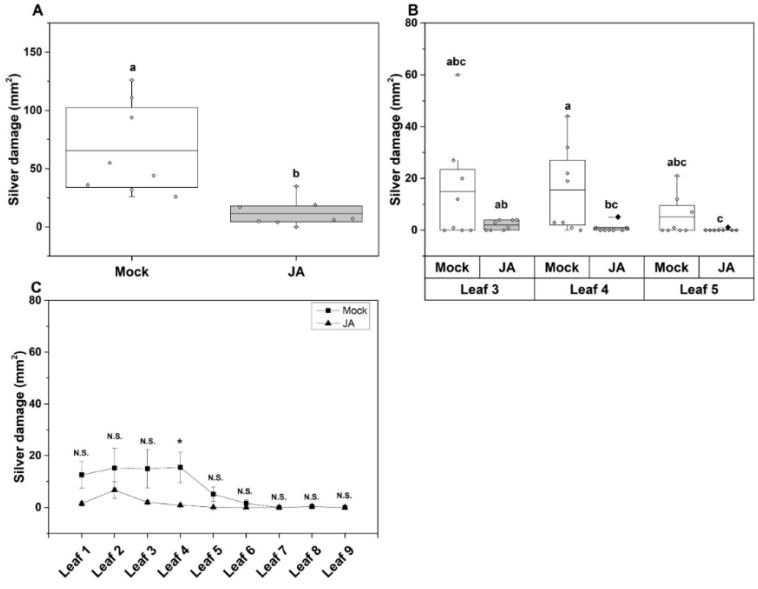

Fig. S4 Effect of JA exogenous treatment and leaf development stage on tomato resistance to thrips. Silver damage symptoms determined in **(A)** the whole plant, (**B**) leaf 3, 4 and 5 and (**C**) all specific leaves from bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants infested with 10 adult thrips at 14 days after the initial hormone treatment. Silver damage symptoms were evaluated at 7 days after thrips infestation. Data correspond to a repeated experiment. Boxes and whiskers denote the 25th–75th percentile and minimum–maximum observations, respectively; group mean values are indicated by the horizontal line. Diamonds in **(A)** and **(B)** denote individual values (n = 8) and the filled diamonds stand for outliers. Different letters indicate significant differences among groups compared by Fisher's least significant differences (LSD) test at $P \leq 0.05$.

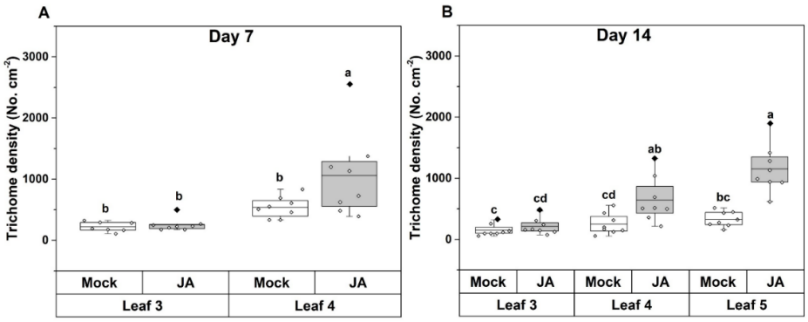

Fig. S5 Effect of JA exogenous treatment and leaf development stage on type-VI trichome density. Average (± SEM, $n = 8$) of type-VI trichome density determined on **(A)** leaf 3 and 4 from the bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants 7 days after the initial treatment and **(B)** on leaf 3, 4 and 5 from the bottom of mock- and JA-treated tomato plants at 14 days after the initial treatment. Different letters indicate significant differences among groups compared by Fisher's least significant differences (LSD) test at $P \leq 0.05$. Each diamond represeants an individual observation and the filled diamonds stand for outliers.

Supplementary table

Table S1 Results of the statistical analysis performed for each experiment.

Figure	Panel	Statistical test	Factor and statistic value	df	*P*
Fig. 2	A	Student t-test	JA; $t = 3.657$	14	$P = 0.003$
	B	Binominal GLM	Leaf development stage; *Wald* $\chi^2 = 13.867$	2	$P = 0.001$
			JA; *Wald* $\chi^2 = 13.333$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 1.067$	2	$P = 0.587$
	C	Student t-test	JA; $t=4.091$	18	$P < 0.001$
	D	GLM	Leaf development stage; *Wald* $\chi^2 = 40.139$	2	$P < 0.001$
			JA; *Wald* $\chi^2 = 34.610$	1	$P < 0.003$
			Interaction; *Wald* $\chi^2 = 20.093$	2	$P < 0.001$
Fig. 3	A	GLM	Leaf development stage; *Wald* $\chi^2 = 31.988$	1	$P < 0.001$
			JA; *Wald* $\chi^2 = 1157.450$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 9.748$	1	$P = 0.002$
	B	GLM	Leaf development stage; *Wald* $\chi^2 = 49.102$	2	$P < 0.001$
			JA; *Wald* $\chi^2 = 2477.067$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 10.048$	2	$P = 0.040$
Fig. 4	A	GLM	Leaf development stage; *Wald* $\chi^2 = 104.720$	1	$P < 0.001$
			JA; *Wald* $\chi^2 = 86.804$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 60.667$	1	$P < 0.001$
	B	GLM	Leaf development stage; *Wald* $\chi^2 = 55.940$	1	$P < 0.001$
			JA; *Wald* $\chi^2 = 101.699$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 49.973$	1	$P < 0.001$
	C	GLM	Leaf development stage; *Wald* $\chi^2 = 0.001$	1	$P = 0.977$
			JA; *Wald* $\chi^2 = 20.310$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 0.135$	1	$P = 0.713$
	D	GLM	Leaf development stage; *Wald* $\chi^2 = 156.640$	2	$P < 0.001$
			JA; *Wald* $\chi^2 = 231.744$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 43.369$	2	$P < 0.001$
	E	GLM	Leaf development stage; *Wald* $\chi^2 = 34.111$	2	$P < 0.001$
			JA; *Wald* $\chi^2 = 70.508$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 2.500$	2	$P = 0.287$
	F	GLM	Leaf development stage; *Wald* $\chi^2 = 14.072$	2	$P < 0.001$
			JA; *Wald* $\chi^2 = 44.230$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 17.241$	2	$P < 0.001$
Fig. 6	A	GLM	Leaf development stage; *Wald* $\chi^2 = 71.833$	2	$P < 0.001$
			JA; *Wald* $\chi^2 = 0.000$	1	$P = 0.0989$
			Interaction; *Wald* $\chi^2 = 0.590$	2	$P = 0.744$
	B	GLM	Leaf development stage; *Wald* $\chi^2 = 68.158$	2	$P < 0.001$
			JA; *Wald* $\chi^2 = 8.610$	1	$P = 0.003$
			Interaction; *Wald* $\chi^2 = 0.217$	2	$P = 0.897$

Chapter 3

Induced resistance against Western flower thrips by *Pseudomonas syringae*-derived defense elicitors in tomato

Gang Chen, Rocío Escobar-Bravo, Hye Kyong Kim, Kirsten A. Leiss, Peter GL Klinkhamer

Western flower thrips (WFT) *Frankliniella occidentalis* (Pergande) is a key agricultural pest of cultivated tomatoes. Induced host plant resistance by activating jasmonic acid (JA) signaling pathway constitutes a promising method for WFT control. The phytotoxin coronatine (COR), produced by *Pseudomonas syringae* pv. tomato DC3000 (*Pst*), mimics the plant hormone JA-Isoleucine and can promote resistance against herbivorous arthropods. Here we determined the effect of *Pst* and COR on tomato resistance against WFT, induction of JA and salicylic acid (SA) associated defenses, and plant chemistry. Additionally, we investigated the presence of other components in *Pst*-derived and filtered culture medium, and their interactive effect with COR on tomato resistance to WFT. Our results showed that infiltration of COR or *Pst* reduces WFT feeding damage in tomato plants. COR and *Pst* induced the expression of JA-associated gene and protein marker. COR also induced expression of a SA-related responsive gene, although at much less magnitude. Activation of JA defenses in COR and *Pst* infiltrated plants did not affect density of type VI leaf trichomes, which are defenses reported to be induced by JA. An untargeted metabolomic analysis showed that both treatments induced strong changes in infiltrated leaves, but leaf responses to COR or *Pst* slightly differed. Application of the *Pst*-derived and filtered culture medium, containing COR but not viable *Pst*, also increased tomato resistance against WFT confirming that the induction of tomato defenses does not require a living *Pst* population to be present in the plant. Infiltration of tomato plants with low concentrations of COR in diluted *Pst*-derived and filtered culture medium reduced WFT feeding damage in a greater magnitude than infiltration with an equivalent amount of pure COR indicating that other elicitors are present in the medium. This was confirmed by the fact that the medium from a COR-mutant of *Pst* also strongly reduced silver damage. In conclusion, our results indicate that induction of JA defenses by COR, *Pst* infection, the medium of *Pst* and the medium of a *Pst* COR^- mutant increased resistance against WFT. This was not mediated by the reinforcement of leaf trichome densities, but rather the induction of chemical defenses.

Keywords: coronatine, *Frankliniella occidentalis*, induced plant defenses, jasmonic acid, *Pseudomonas syringae*, salicylic acid, *Solanum lycopersicum*, type VI glandular trichomes

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1 Introduction

The western flower thrips (WFT), *Frankliniella occidentalis* [Pergande], is one of the most serious greenhouse and field insect pests of vegetable and ornamental crops worldwide. It is a highly polyphagous insect that can feed on more than 200 wild and cultivated host species (Lewis, 1997) by piercing and sucking epidermal/mesophyll plant cells, which results in damaged areas of a silvery appearance ('silver damage'). WFT cause direct damage by feeding on leaves, flowers and fruits, or indirect damage through the transmission of plant viruses (de Jager, CM *et al.*, 1995; de Jager, KM *et al.*, 1995), being the main vector of tospoviruses, such as tomato spotted wilt virus (Maris *et al.*, 2003). Current control of WFT mainly depends on the use of pesticides and biological control. Use of pesticides leads to residue problems on marketable crops, human health risks, toxicity to non-target beneficial organisms, and environmental contamination (Bielza, 2008; Demirozer *et al.*, 2012; Gao *et al.*, 2012; Mouden *et al.*, 2017). Therefore, multiple and complementary tactics are necessary in the framework of integrated pest management (IPM) programs.

Enhancement of constitutive and/or inducible host plant defenses against WFT has recently been discussed as a promising alternative for thrips control (Steenbergen *et al.*, 2018). Plants defend themselves against herbivores by employing a plethora of physical and chemical arsenals, including trichomes, defensive enzymes and secondary metabolites that can be present in the plant before attack or induced after detecting the presence of the attacker. Induced plant defenses against herbivory are mainly controlled by the phytohormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET), and fine-tuned by other phytohormones such as abscisic acid, auxins, cytokinins and gibberellins (Pieterse *et al.*, 2012). Activation of JA-associated defenses has been reported to confer plant resistance against pierce-sucking arthropods such as spider mites and thrips (Li *et al.*, 2002; Ament *et al.*, 2004). In particular, WFT have been reported to be susceptible to JA-associated induced defenses in diverse plant species such as *Arabidopsis*, Chinese cabbage (*Brassica rapa*), cotton (*Aphis gossypii*) and tomato (*Solanum lycopersicum*) (Omer *et al.*, 2001; Abe *et al.*, 2008; Abe *et al.*, 2009; Escobar-Bravo *et al.*, 2017).

In tomato (*S. lycopersicum*), induction of JA-related defenses has been associated to increased levels of defensive type-VI glandular trichomes and their derived exudates, proteins such as proteinase inhibitors and polyphenol oxidases (PPO), and secondary metabolites (Boughton *et al.*, 2005; Degenhardt *et al.*, 2010; Escobar-Bravo *et al.*, 2017). Type-VI trichomes are important physical and chemical defense barriers, and their absence increases tomato susceptibility against herbivory (Kang *et al.*, 2010a; Kang *et al.*, 2010b). Overexpression of certain proteinase inhibitors has been reported to increase plant resistance against WFT (Annadana *et al.*, 2002; Outchkourov *et al.*, 2004), and enhanced PPO activities can confer enhanced resistance against beet armyworm (*Spodoptera exigua*), cotton bollworm (*Helicoverpa armigera*) (Bhonwong *et al.*, 2009) and cutworm (*Spodoptera litura*) (Mahanil *et al.*, 2008). Accordingly, application of natural or synthetic elicitors activating these JA-associated defenses can increase tomato resistance against various insects, including WFT (Thaler, 1999; Thaler *et al.*, 2002; Escobar-Bravo *et al.*, 2017).

The phytotoxin coronatine (COR) produced by several pathovars of *Pseudomonas syringae* acts as a virulence factor in *P. syringae* pv. tomato (*Pst*), allowing this pathogen to successfully develop high populations in the plant (Zhao *et al.*, 2001; Uppalapati & Bender, 2005; Zheng *et al.*, 2012). COR is a polyketide formed by the coupling of coronafacic acid (CFA) and coronamic acid (CMA) through an amide bond (Bender *et al.*, 1993). Its structure mimics a bioactive JA conjugate (JA-Isoleucine), thus having the ability to stimulate JA-

associated defense responses (reviewed by Geng *et al*., 2014), but also affecting ethylene and auxin signaling pathways (Uppalapati *et al.*, 2005). Both JA and COR can induce chlorosis, ethylene emission, inhibition of root elongation, volatile production, biosynthesis of stress-associated compounds and anti-herbivore proteins (Uppalapati *et al.*, 2005). Consequently, infiltration with COR-producing *P. syringae* or infiltration of pure COR in *Arabidopsis* enhanced plant resistance against arthropod herbivores, such as the caterpillar *Trichoplusia ni* (Cui *et al.*, 2005). In tomato, *P. syringae* infection (López-Gresa *et al.*, 2011) or COR application (Uppalapati *et al.*, 2005) also induces metabolomic changes in the plant. All these studies suggest that *Pst* may potentially be used to increase resistance against WFT in tomato. Yet, the effects of *Pst* and COR infiltration on tomato defenses against herbivory may differ. Activation of defense signaling pathways in *Pst*-infected plants is not only mediated by the phytotoxin COR, but also by an array of virulence factors such as exopolysaccharides effectors secreted by the type III secretion system, and cell-wall-degrading enzymes (Zhao *et al.*, 2003; He *et al.*, 2004). Thus, research on the possible use of other *Pst*-derived defense elicitors for their practical application in agricultural systems is crucial, as *Pst* is a plant pathogen.

Here we first investigated the effect of COR or *Pst* DC3000 infiltration on tomato defenses against WFT. In particular, we determined their effect on WFT-associated feeding damage, as well as variations in type-VI leaf trichome densities, leaf metabolome, expression of defense-associated genes and tomato growth. In a further attempt to test the possible role of other *Pst* DC3000 associated defense elicitors in tomato-WFT interaction, we also studied the effect of dilution series of the *Pst*-derived filtered medium and a COR-deficient *Pst* strain on tomato resistance against WFT and activation of JA signaling pathway.

2 Materials and methods

2.1 Plants, insect and bacterial strains

The tomato cultivar 'Moneymaker' (*Solanum lycopersicum*) was used in all experiments. Tomato seeds were germinated on filter paper soaked with MilliQ water and incubated at 20°C. Five days later, germinated seeds were planted in plastic pots (11 cm × 11 cm × 12 cm) filled with potting soil and maintained in a climate room at 20°C, 70% RH, 113.6 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR) and L16:D8 photoperiod.

Western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), were maintained on chrysanthemum flowers (cultivar Euro Sunny) in a climate room at 23°C, 60% RH and L12:D12 photoperiod.

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) NCPPB4369 was obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB, London, UK). *P. syringae* pv. *tomato* DB29 (*Pst* DB29, a *cmaA cfaA* double mutant of *Pst* DC3000) (Brooks *et al.*, 2004) was kindly provided by Prof. Barbara Kunkel from Washington University in St. Louis. Both *Pst* DC3000 and *Pst* DB29 were stored in 30% glycerol at -80°C for long term preservation.

2.2 Experimental design

To determine the effect of COR and *Pst* DC3000 on tomato defenses against WFT (Experiment 1), four-week old tomato plants were infiltrated with: (1) 5 µM COR solution, (2) a 10^8 cfu ml^{-1} of *Pst* DC3000 suspension or (3) a mock solution of sterilized MilliQ water. For this, four leaflets (two top leaflets of leaf 2 and 3 from the bottom) were pressure-infiltrated with 400 µl (100 µl for each leaflet) of one of the treatments on their abaxial leaf

sides using a 1-ml needleless syringe. Seven days after infiltration, plants were sampled for type VI trichome density, metabolomics, gene expression and polyphenol oxidase (PPO) activity analysis, or used for non-choice whole plant thrips bioassays.

With the aim to explore whether COR and other defense elicitors present in *Pst* DC3000-derived medium (without viable bacteria) increases tomato resistance against WFT, three additional experiments were conducted. First, to test if the COR present in *Pst* DC3000-derived medium can enhance tomato resistance against WFT (Experiment 2), tomato plants at four leaf-stage were infiltrated with 100 µl of: (1) mock solution (MilliQ water), (2) blank medium (described in Generation of *Pst*-derived and Blank Medium below), (3) blank medium supplemented with 0.68 µM COR (blank medium + COR), (4) 10^8 cfu ml^{-1} of *Pst* DC3000 suspension (*Pst* DC3000), or (5) *Pst* DC3000-derived medium (*Pst* DC3000 medium, without viable bacteria) containing 0.68 µM COR. The COR in the *Pst* DC3000-derived medium was produced by the bacteria during 6 d cultivation and the concentration was measured before the start of the experiment. Second, to test the existence of interactions of COR with other defense elicitors present in *Pst* DC3000-derived medium on tomato resistance against WFT (Experiment 3), four-week old tomato plants were infiltrated with 0.2x, 0.4x, 0.6x, 0.8x and 1.0x concentrations of: (1) blank medium, (2) 0.68 µM COR diluted with blank medium or (3) *Pst* DC3000-derived medium containing 0.68 µM COR. Third, to confirm the effect of other defense elicitors, present in *Pst* DC3000-derived medium, on tomato resistance against WFT (Experiment 4), four-week old tomato plants were infiltrated with: (1) blank medium, (2) 0.14 µM COR diluted with blank medium, (3) culture medium derived from *Pst* DB29, a COR^- mutant bacteria of *Pst* DC3000, diluted five-fold with blank medium, or (4) five-fold diluted *Pst* DB29-derived medium containing 0.14 µM COR. In these three experiments, four leaflets (two top leaflets of each of leaves 2 and 3 from the bottom) from one tomato plant were pressure-infiltrated on their abaxial leaf sides with about 400 µl in total of corresponding treatments as described above. Seven days after infiltration, part of the plants were sampled for PPO activity measurements and the other part was used for non-choice whole plant thrips bioassays.

2.3 *Pst* cultivation and suspension preparation

Pst DC3000 and *Pst* DB29 were cultured in a King's B medium plate (King *et al.*, 1954) supplemented with 100 µg ml^{-1} rifampicin and grown for 2 days at 28°C prior to use (Katagiri *et al.*, 2002). The activated *P. syringae* pv. *tomato* (*Pst*) was then transferred to King's B liquid medium supplemented with 100 µg ml^{-1} rifampicin in a shaking incubator (200 rpm) at 28°C for 8 to 12 h (Katagiri *et al.*, 2002).

To prepare *Pst* DC3000 suspension, the obtained *Pst* DC3000 King's B liquid culture was centrifuged at 4,000 rpm for 10 min at 4°C. The supernatant was discarded and the bacteria pellet was re-suspended in sterilized MilliQ water. The bacteria suspension was diluted with sterilized MilliQ water to reach a concentration of 10^8 colony-forming units (cfu) ml^{-1}, estimated by an optical density at 600 nm of 0.5, which was used for the experiments.

2.4 Generation of *Pst*-derived and blank medium

Pst-DC3000- and *Pst*-DB29-derived medium were obtained following the protocol of Palmer and Bender (1993) with some modifications. Briefly, 100 µl of the *Pst* King's B liquid culture obtained as described above was added to 20 ml Hoitink and Sinden medium optimized for COR production (also known as HSC) (nutrients per liter with a pH = 6.8: 1.0 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, 4.1 g KH_2PO_4, 3.6 g $K_2HPO_4 \cdot 3H_2O$, 0.3 g KNO_3, 20 µM $FeCl_3$, 20 g glucose) supplemented with 100 µg ml^{-1} rifampicin and grown in a shaking incubator (200 rpm) at

20°C for 6 days. The *Pst* culture (20 ml) was centrifuged at 4,000 rpm for 30 min at 4°C. The supernatants were filtered using 0.22 µm Regenerated Cellulose (RC) filters (Sartorius AG, Göttingen, Germany) to remove *Pst* from the medium. The absence of active bacteria in the *Pst*-derived medium was confirmed by spraying the filtered *Pst*-derived medium on plates of King's B medium and culturing the plates at room temperature. No colonies were detected at 3 d after the initial culture. Blank medium used as a control in Experiment 2, 3 and 4 was generated by incubating fresh HSC medium supplemented with 100 µg ml^{-1} rifampicin in a shaking incubator (200 rpm) at 20°C for 6 days. Thereafter, the HSC culture was centrifuged and the resulting supernatant was filtered using 0.22 µm RC filters. Presence of bacteria in the blank medium was also checked as described above for *Pst*-derived medium.

2.5 Measurements of coronatine concentration in *Pst* DC3000-derived medium

Concentration of coronatine (COR) in the *Pst* DC3000-derived medium was determined by HPLC as described by Palmer and Bender (1993) with slight modifications. In short, 20 ml of *Pst* DC3000-derived filtered medium was adjusted to pH = 9 and extracted twice with 20 ml of ethyl acetate. The aqueous phase was adjusted to pH = 2 and then extracted three times with 20 ml ethyl acetate. The ethyl acetate phase was dried in 45°C water bath in 50 ml tubes. The COR was recovered by re-dissolving twice with 250 µM 20% acetonitrile. Three samples were analyzed on a reverse-phase C-8 column (150 mm × 4.6 mm, 5 µm particle size, Agilent Zorbax Eclipse XDB) at 208 nm. The mobile phases, A and B, were MilliQ water and HPLC-grade acetonitrile, respectively. The flow rate was kept constant at 1 ml/min. The gradient elution was as follows: 0.00 min at 80 % A, 5.00 min at 35% A, 7.00 min/10% A, 8.00 min/ 10 % A, 8.10 min/80% A, 10.00 min/80 % A. The injection volume was 50 µl and the column temperature was 25°C. Calibration curves for quantification of COR were constructed by using dilution series of commercially available COR (Sigma-Aldrich, St. Louis, MO, USA).

2.6 Growth of *Pst* DC3000 in infiltrated tomato leaves

Bacteria growth in the leaflets of *Pst* DC3000-infiltrated plants was confirmed at seven days after infiltration. For this, one of the *Pst* DC3000-infiltrated leaflets was surface sterilized by placing it in a 70% ethanol solution for 1 minute, blotted briefly on paper towels and rinsed in sterile distilled water for 1 minute. Thereafter, a leaf disc (1.5 cm diameter) was punched and placed in a 3-ml microfuge tube with 100 µl sterile distilled water. The samples were ground and subsequently vortexed for 10 s. Ten µl of the leaf disc solution was plated on King's B medium supplemented with 100 µg ml^{-1} rifampicin and incubated at room temperature. Number of cfu was recorded at 3 days after incubation.

2.7 Non-choice whole pant thrips bioassay

A non-choice whole plant thrips bioassay was used to test tomato resistance against WFT (Leiss *et al.*, 2009b). For this, plants were placed inside individual WFT-proof cages consisting of transparent plastic cylinders (50 cm height, 20 cm diameter), closed at the top with displaceable lids made of nylon gauze of 120 µm mesh size. Ten adult WFT (8 females and 2 males) were released into each cage. Plants were maintained in a climate room with 113.6 µmol photons m^{-2} s^{-1} of PAR, 16L:8D of photoperiod, 25°C and 70% RH for 7 d. WFT feeding damage (hereafter referred as 'silver damage') was evaluated in all the leaves of the plant seven days after infestation, and expressed as the damaged area in mm^2. Evaluation of WFT-associated leaf damage in the whole plant has been proved to correlate well with resistance-associated parameters such as number of larvae, adult survival, adult abundance and preference (de Kogel *et al.*, 1997; Jiang *et al.*, 2005; Badenes-Pérez & López-Pérez, 2018), and it has been used in multitude of studies determining host plant resistance to WFT

(Leiss *et al.*, 2009a; Leiss *et al.*, 2009b; Mirnezhad *et al.*, 2010; Leiss *et al.*, 2013; Thoen *et al.*, 2016; Escobar-Bravo *et al.*, 2017; Badenes-Pérez & López-Pérez, 2018; Escobar-Bravo *et al.*, 2018). Silver damage symptoms caused by infestation with 10 adult WFT were very subtle at 7 days after infestation and it did not result in significant loss of leaf tissues (See Fig. S1). Thus, WFT development and feeding was not limited by the available plant material in the host plant.

2.8 Measurement of PPO activity

Polyphenol oxidase (PPO) activity was measured in one of the infiltrated leaflets belonging to the second leaf from the bottom using the protocol described in Stout *et al* (1998). In short, 0.150 g of frozen and ground plant material was homogenized in a 2 ml tube with 1.25 ml ice-cold 0.1 M pH 7.0 potassium phosphate buffer containing 7% polyvinyl polypyrolidine and 0.4 ml of 10% Triton X-100. The extracts were vortexed for 2 min and centrifuged at 11,000 × g for 10 min at 4ºC. Five microliters of the enzyme extract were added to 1 ml of 2.92 mM chlorogenic acid solution in pH 8.0 potassium phosphate buffer. The optical density (OD) at 470 nm was recorded in a spectrophotometer (UV-1800, Shimadzu) every 10 s for one min. PPO activity was expressed as changes in OD values per min per gram of fresh weight.

2.9 Gene expression analysis by RT-qPCR

Expression of the JA- and SA-associated marker genes, the *wound-inducible proteinase inhibitor II* (*WIPI-II*, also known as *PI-II)* and the *pathogenesis-related protein 6* (*PR-P6*, also known as *PR-1b*) (Alba *et al.*, 2015), respectively, were determined in mock-, COR- and *Pst* DC3000-treated plants at seven days after infiltration. The two infiltrated leaflets of leaf 3 from the bottom were flash frozen, homogenized and stored at -80°C until analysis. Around 100 mg of the leaf material was used for RNA isolation. Total RNA was extracted using a phenol/LiCl method (Verdonk *et al.*, 2003) followed by DNase (Ambion) treatment. Single strand cDNA was synthesized from 4 µg total RNA in a 20 µl reaction using a M-MuLV Reverse Transcriptase (Fermentas) according to manufacturer's recommendations. The quantity of targeted synthesized cDNAs was analyzed with real-time quantitative reverse transcription-PCR (qRT-PCR) in CFX96^{TM} Optics Module (BIO-RAD) using iQ^{TM} SYBR Green Supermix (BIO-RAD). The PCR protocol was set up in 20 µl reactions containing 0.25 µM of each primer and 1 µl of cDNA. The PCR program was as follows: 50°C for 5 min, 95°C for 2 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, followed by a melting curve analysis. Four biological replicates (i.e. individual plants) for each treatment were used for qRT-PCR analysis and two technical replicates were analyzed per treatment. *Actin* was used as internal standard for the normalization of expression levels for both targeted genes. The normalized expression (NE) of both genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). To illustrate the levels of gene expressions in plot, NE values were scaled to the treatment with the lowest average NE, which was set to 1. The gene specific qRT-PCR primers are shown in Method S1.

2.10 Trichome density measurement

Type-VI glandular trichome density was determined on non-infiltrated leaflets of mock-, COR- and *Pst* DC3000-treated plants at seven days after infiltration. For this, the second terminal leaflet of the third leaf from the bottom was used. Two pictures were taken in the middle section of the leaflet, at both sides of the midrib, in the adaxial and abaxial leaf sides by using a Leica stereomicroscope (MZ16, Leica Microsystems, Wetzlar, Germany). Each picture corresponded to an area of 12 mm^2. Trichome number was counted on the pictures

using the software 64-bit Fiji ImageJ (http://fiji.sc/Fiji). The average of these two measurements was calculated for each leaflet and expressed as number of type-VI trichomes per cm^2.

2.11 Nuclear Magnetic Resonance (NMR) analysis

NMR metabolomic analysis was performed on mock-, COR- or *Pst* DC3000-infiltrated leaflets at 7 days after infiltration. For this, plant material was freeze-dried and ground using a tissue lyser (Qiagen, Hilden, Germany). Twenty milligrams of fine powder were extracted with 1.5 ml of 80% methanol-*d4* in KH_2PO_4 buffer (90 mM, pH = 6.0) containing 0.02% (w/v) trimethyl silyl-3-propionic acid sodium salt-*d4* (TMSP). Plant extracts were vortexed for 1 min, ultra-sonicated for 15 min and centrifuged at 13,000 rpm for 15 min at room temperature. Eight hundred microliters of the supernatant were transferred to the NMR tubes for analysis.

The ^{1}H NMR spectra were acquired using a 600 MHz Bruker AV-600 spectrometer equipped with cryo-probe operating at a proton NMR frequency of 600 MHz at 25 °C, as described in López-Gresa *et al* (2012). Deuterated methanol served as internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min acquisition time with a digital resolution of 0.25 Hz/point, a pulse angle of 30° (10.8 µs), and a recycle delay of 1.5 s per scan. A pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the H_2O frequency during the recycle delay. Spectra were Fourier transformed with a 0.3 HZ line broadening and zero-filled to 32 K points. Phase and baseline correction of the resulting spectra were done manually, followed by a calibration to TMSP at 0.00 ppm using Topspin (version 2.1, Bruker). ^{1}H NMR spectra were then converted and saved as ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to the intensity of the internal standard TMSP and reduced to integrated regions, referred to as buckets, of equal width (0.04 ppm) corresponding to the region of δ 10.0 - 0.2. The Regions in the range of δ 4.92 - 4.70 and δ 3.33 - 3.28, corresponding to water and methanol, respectively, were removed prior to statistical analyses.

2.12 Statistical analysis

All statistical analyses were performed using the SPSS software package (version 23; SPSS Inc., Chicago, IL, USA). Effect of mock-solution, COR, *Pst* DC3000 or *Pst* DC3000-derived medium infiltration on silver damage symptoms, type VI trichome density, PPO activity and normalized expression of *WIPI-II* and *PR-P6* (Experiments 1 and 2) were analyzed by one-way ANOVA, followed by Fisher's Least Significant Difference (LSD) post-hoc test. Residuals of the data were first tested for normality and homogeneity of variance. Data on silver damage and *WIPI-II* and *PR-P6* expression obtained from Experiment 1, and PPO activity determined in Experiment 2 were Log transformed prior to analysis to meet ANOVA assumptions. Effect of treatments (blank medium, blank medium + COR and *Pst* DC3000-derived medium), concentration (0.2, 0.4, 0.6, 0.8 and 1.0x) and the interaction between these two factors (Experiment 3) on silver damage symptoms and PPO activity was determined by Generalized linear models (GLM) using linear distribution and identity link functions, followed by LSD post-hoc test. Data on silver damage were Log-transformed prior to analysis. Effect of COR, *Pst* DB29-derived medium and their interaction (Experiment 4) on silver damage and PPO activity was analyzed by GLM using linear distribution and identity link functions, followed by LSD post-hoc test. Data on silver damage were Log-transformed prior to analysis. Patterns of chemical shifts detected by NMR in leaf extracts of mock-, COR- or *Pst* DC3000-treated plants were subjected to multivariate analysis using the SIMCA-P 15 software package (Umetrics, Umeå, Sweden). Supervised partial least squares discriminant

analysis (PLS-DA) was applied to determine the variation in X variables (chemical shifts) modeled by the Y explanatory variable corresponding to mock, COR or *Pst* DC3000 treatments. R^2X and R^2Y is the cumulative variation explained by the PLS-DA model in variable X and Y, respectively. Q^2 is the cumulative predicted variation in Y, according to cross-validation. The final model was the one with minimum number of latent variables showing the highest value of Q^2. The chemical shifts with a variable importance in projection (VIP) > 1 were selected as the important X variables, some of which were identified and tested using a nonparametric analysis followed by non-parametric Kruskal-Wallis test. Detailed statistical results are shown in Table S1.

3 Results

3.1 Infiltration of COR or *Pst* DC3000 increases tomato resistance against WFT

Infiltration of tomato plants with COR or *Pst* DC3000 reduced silver damage by 47% and 37%, respectively, compared to the mock-treated plants (ANOVA: $P < 0.05$, **Fig. 1**). Overall, this reduction was evident in both infiltrated and non-infiltrated leaves of COR- and *Pst* DC3000-treated plants (ANOVA: $P < 0.05$, Supplemental Fig. S2).

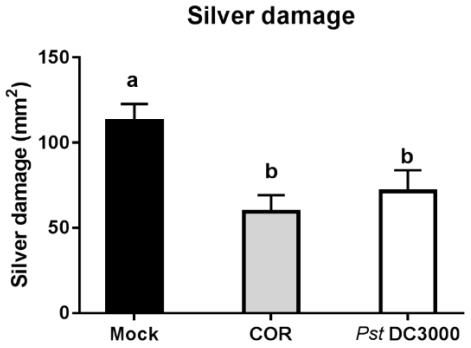

Fig. 1 Effect of COR and *Pst* DC3000 on tomato resistance against WFT. Silver damage symptoms (mean ± SEM, $n = 15$) in tomato plants infiltrated with mock solution (mock), coronatine (COR) or *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000). Plants were infested with western flower thrips (WFT) at 7 days after the initial treatment and evaluated 7 days after WFT infestation. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P \leq 0.05$.

3.2 COR and *Pst* DC3000 induced JA-signaling, and COR induced both JA and SA

To further determine the mechanism of COR and *Pst* DC3000-mediated induction of tomato defenses against WFT, expression of JA and SA-responsive genes, as well as the activity of the JA-associated defensive protein PPO, were analyzed at 7 days after infiltration. Both COR and *Pst* DC3000 infiltration strongly induced PPO activity in infiltrated tomato leaves (ANOVA: $P < 0.05$, **Fig. 2A**). Similarly, the expression of *WIPI-II,* a JA marker gene, was about 900 and 1,300 times higher in COR- and *Pst* DC3000-infiltrated plants, respectively, than in mock-treated leaves of control plants (ANOVA: $P < 0.05$, **Fig. 2B**). Interestingly, for *PR-P6*, a SA marker gene, a 28 times higher expression was observed in COR-treated plants, but not in mock and *Pst* DC3000-infiltrated plants (ANOVA: $P < 0.05$, **Fig. 2C**).

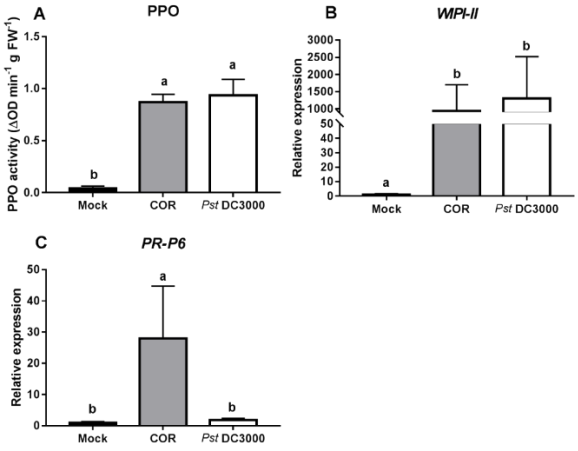

Fig. 2 Effect of COR and *Pst* DC3000 on jasmonic acid- and salicylic acid-associated responses. (**A**) Polyphenol oxidase (PPO) activity (mean ± SEM, $n = 5$) and relative transcript levels of (**B**) the JA-responsive gene *wound inducible proteinase inhibitor-II* (*WIPI-II*) and (**C**) the SA-responsive gene *pathogenesis related protein 6* (*PR-P6*) (mean ± SEM, $n = 5$) were measured in tomato plants at 7 days after infiltration with coronatine (COR), *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) or a mock solution (mock). The analysis was performed on infiltrated leaflets from the bottom second/third leaf. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P \leq 0.05$.

3.3 Infiltration of tomato plants with COR or *Pst* DC3000 does not increase type-VI trichome density

To determine whether COR and *Pst* DC3000 induce trichome-associated defenses against WFT, type-VI trichome density was determined on both adaxial and abaxial leaf sides of mock-, COR- and *Pst* DC3000-treated plants. Surprisingly, Type-VI trichome density in the adaxial leaf side was marginally decreased by COR or *Pst* DC3000 infiltration (ANOVA: $P = 0.071$, **Fig. 3A**). However, type-VI trichome density on abaxial leaf sides was slightly reduced in COR-treated plants in comparison to *Pst* DC3000- and mock-treated plants (ANOVA: $P < 0.05$, **Fig. 3B**).

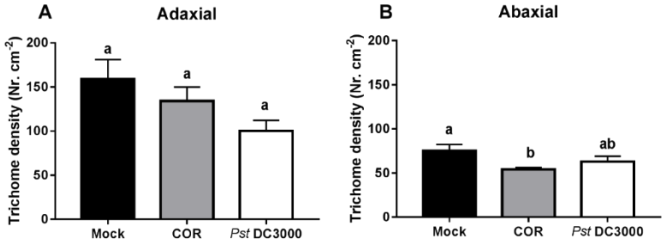

Fig. 3 Effect of COR and *Pst* DC3000 on type VI trichome density. Type VI trichome density (mean ± SEM, $n = 10$) on (**A**) adaxial or (**B**) abaxial leaf side was determined in tomato plants infiltrated with coronatine (COR), *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) or a mock solution (mock) at 7 days after the initial treatments. The analysis was performed in leaflets collected from the third youngest leaf. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P \leq 0.05$.

3.4 COR and *Pst* DC3000 induced similar metabolomic changes in infiltrated tomato leaves

A total of 244 signals were obtained from ^{1}H NMR measurement of the mock-, COR- and *Pst* DC3000-treated tomato plants. The multivariate PLS-DA analysis of the NMR signal profiles resulted in a model with five latent variables (LVs) that cumulatively explained 74.6% of the total metabolomic variation and 91.1% of the elicitor agent treatments, with a 40.7% total model predictability (model statistics: $R^2X = 0.746$, $R^2Y = 0.911$ and $Q^2 = 0.407$) (**Fig. 4**). The first LV explained 23.3% of the variance and separated mock- from both COR- and *Pst* DC3000-treated plants (**Fig. 4A**). The second LV explained 15.8% and separated COR- from *Pst* DC3000-treated plants. The discriminated patterns among mock-, COR- and *Pst* DC3000-treated plants were mainly explained by 80 signals with VIP scores higher than 1 (**Fig. 4B** and Fig. S3). Among these 80 NMR signals, twenty-two were identified, which corresponded to 16 different compounds (**Fig. 4C**), including isoleucine (δ 0.96), leucine (δ 1.00), valine (δ 1.04), alanine (δ 1.48), acetate (δ 1.92), glutamate (δ 2.04), malic acid (δ 2.48), aspartic acid (δ 2.64, 2.68, 2.80), citric acid (δ 2.72), gamma-aminobutyric acid (GABA, δ 3.00), ethanolamine (δ 3.12), sucrose (δ 5.40), chlorogenic acid (δ 6.40, 6.44, 6.88), rutin (δ 6.52, 7.00), fumaric acid (δ 6.56) and phenylalanine (δ 7.56). Both COR and *Pst* DC3000 treatments significantly increased leaf content of aspartic acid, ethanolamine and fumaric acid. However, increased GABA and rutin levels were only observed in *Pst* DC3000-treated plants (**Fig. 4D-H**). For the other identified compounds we did not find significant differences among treatments.

3.5 *Pst* DC3000-derived medium enhances tomato resistance against WFT

To confirm that *Pst* DC3000-derived compounds are responsible for the induced resistance against WFT in tomato, we infiltrated plants with the *Pst* DC3000-derived medium (containing COR) but no viable bacteria, and compared the effect on tomato resistance against WFT with those triggered by the infiltration with water mock, blank medium control, blank medium + COR and *Pst* DC3000. Silver damage symptoms were significantly reduced in tomato plants infiltrated with COR, *Pst* DC3000 or *Pst* DC3000-derived medium compared to water mock or blank medium-treated plants (ANOVA: $P < 0.05$, **Fig. 5A**). This reduction in silver damage was stronger in infiltrated leaves, when compared to systemic leaves (i.e. non-infiltrated leaves) (Fig. S4). In addition, PPO activity was induced in blank medium + COR-, *Pst* DC3000- and *Pst* DC3000-derived medium-treated tomato plants compared to water mock and blank medium controls (ANOVA: $P < 0.05$, **Fig. 5B**). This confirms the role of COR on the induction of tomato defenses against WFT, and that no bacterial infection is required to elicit WFT resistance.

3.6 Existence of other defense elicitors besides COR in *Pst* DC3000-derived medium

In the previous experiment, plants infiltrated with COR or *Pst* DC3000-derived medium (containing 0.68 μM COR as in the COR treatments) showed a similar reduction in silver damage symptoms. Yet, the effect of other defense elicitors present in *Pst* DC3000-derived medium might have been masked by the high concentration of COR in the medium. Thus, we further assessed the effect of serial dilutions of blank medium, blank medium + COR, and *Pst* DC3000-derived medium on tomato resistance against WFT and PPO induction (**Fig. 6A**). *Pst* DC3000-derived medium- and blank + COR-treated plants showed a significant reduction in silver damage symptoms (GML: $P < 0.05$ for treatment; $P = 0.078$ for dilution; $P = 0.383$ for the interaction). Notably, a stronger reduction in silver damage symptoms was observed in tomato plants treated with 0.2x concentration of *Pst* DC3000-derived medium when compared to 0.2x blank medium and 0.2x blank medium + COR. As these differences

were only found at 0.2x concentration, this might explain why the interaction factor between treatment and dilution was not statistically significant. These results suggest that there might be other plant defense elicitors in *Pst* DC3000-derived medium that, maybe in combination with COR, trigger stronger plant defense responses against WFT than COR alone (i.e. in blank medium + COR treatment). Indeed, at 0.2x concentration, induction of the PPO activity was significantly higher in *Pst* DC3000-derived medium-treated plants than in those infiltrated with blank medium + COR (GLM: $P < 0.05$ for interaction) (**Fig. 6B** and Table S1). No significant differences in PPO activity between *Pst* DC3000-derived medium- and blank + COR-treated plants were observed at 0.6, 0.8 and 1.0x concentration.

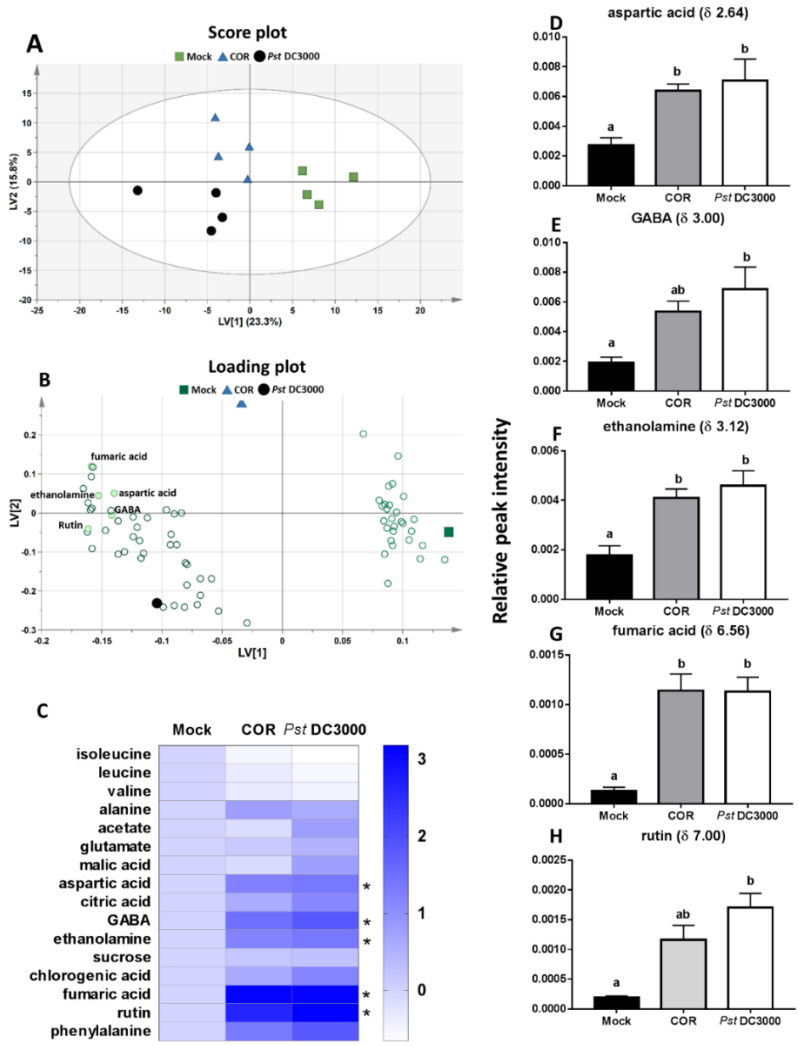

Fig. 4 Metabolome responses of tomato plants to COR and *Pst* DC3000 infiltration. Leaf metabolites were analyzed on tomato leaves infiltrated with coronatine (COR), *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) or a mock solution (mock) by NMR at 7 days after the initial treatment. Partial least square-discriminant analysis (PLS-DA) was performed based on ^{1}H-NMR spectra ($n = 4$ individual plants), and resulted in five latent variables (LVs) that cumulatively explained 74.6% of the total metabolomic variation and 91.1% of the treatment response, with a 40.7% total model

predictability. (**A**) Score plot showing the first two LVs. The ellipse represents the Hotelling T2 with 95% confidence in score plot. (**B**) Loading plot showing important metabolites contributing most to the model (VIP score > 1). (**C**) Heatmap of the identified sixteen compounds. Each of the three Heatmap columns represents the $\log_2$ fold change of relative peak intensity from one of the treatments Mock, COR or *Pst* DC3000 in comparison to Mock. Thus, all $\log_2$ fold change of compounds in mock treatment was 0 (fold change = 1) as shown in the first column. (**D-H**) Relative peak intensities (mean ± SEM, n = 4) of five metabolites (aspartic acid, GABA, ethanolamine, fumaric acid and rutin) identified in the ^{1}H NMR spectra that significantly differed among treatments. Different letters indicate significant differences among treatments tested by Mann-Whitney U test, $P \leq 0.05$.

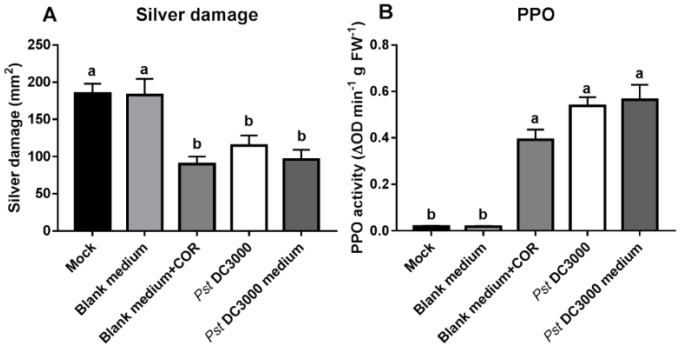

Fig. 5 Effect of *Pst* DC3000-derived medium on tomato resistance against WFT and JA-associated responses. (**A**) Silver damage symptoms (mean ± SEM, n = 10) in tomato plants infiltrated with mock solution (mock), blank medium, 0.68 µM coronatine (COR) dissolved in blank medium (blank medium + COR), *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) suspension or *Pst* DC3000-derived medium (containing 0.68 µM of COR). Plants were infested with western flower thrips (WFT) at 7 days after the initial treatment and evaluated 7 days after WFT infestation. (**B**) Polyphenol oxidase (PPO) activity (mean ± SEM, n = 5) was measured in the second leaf from the bottom of tomato plants pressure infiltrated with the above described treatments at 7 days after the initial treatment. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P \leq 0.05$.

3.7 Confirmation of the existence of other defense elicitors in *Pst* DC3000-derived medium

Our previous results showed that while the effect of COR on tomato defenses against WFT was concentration-dependent, the effect of the *Pst* DC3000-derived medium was not, thus pointing out to the existence of other defense elicitors in *Pst* DC3000-derived medium. To further investigate this, we tested the effect of medium obtained from a COR defective mutant of *Pst* DC3000 (*Pst* DB29), blank medium, or both treatments supplemented with a low concentration of COR (0.14 µM) on WFT resistance and PPO activity (**Fig. 7**). Silver damage symptoms did not significantly differ between plants infiltrated with blank medium and blank medium + COR (GLM: P = 0.994, for COR treatment) (**Fig. 7A)**, thus confirming our previous results. Yet, a small reduction in silver damage was observed in the infiltrated leaves of blank medium + COR (Fig. S5). Infiltration of plants with *Pst* DB29-derived medium without COR, however, significantly reduced silver damage symptoms when compared to blank medium and blank medium + COR treatments (GLM: $P < 0.05$ for the *Pst* DB29-derived medium; $P < 0.05$ for the interaction). This reduction was significant in both infiltrated and non-infiltrated leaves (Fig. S5). PPO activity was significantly induced by COR and *Pst* DB29-derived medium (GLM: $P < 0.05$ for COR treatment; $P < 0.05$ for the *Pst* DB29-derived medium). Furthermore, *Pst* DB29 + COR-treated plants showed a slight

higher PPO induction when compared to *Pst* DB29-derived medium and blank medium + COR treatments (GLM: $P = 0.104$ for their interaction) (**Fig. 7B**).

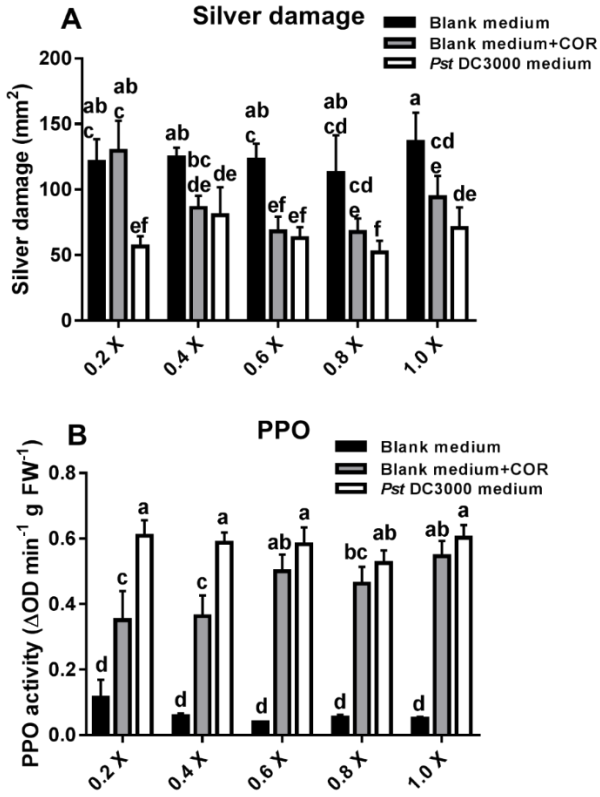

Fig. 6 Effect of different concentrations of COR in *Pst* DC3000-derived medium on WFT resistance and JA-associated responses. (**A**) Silver damage symptoms (mean ± SEM, $n = 7$) in tomato plants infiltrated with 0.2, 0.4, 0.6, 0.8 or 1.0x concentrations of: 1) blank medium, 2) blank medium + coronatine (COR) (0.64 μM), or 3) *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000)-derived medium (no viable bacteria, containing 0.64 μM of COR). Plants were infested with western flower thrips (WFT) at 7 days after the initial treatment and evaluated 7 days after WFT infestation. (**B**) Polyphenol oxidase (PPO) activity (mean ± SEM, $n = 5$) was measured in the second leaf from the bottom of plants infiltrated with the above described treatments at 7 days after the initial treatment. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P \leq 0.05$.

4 Discussion

Activation of defense-associated signaling pathways by using natural or synthetic defense elicitors has shown to increase plant resistance against different arthropod herbivores, and it might be regarded as a valuable strategy for pest control in agriculture (Thaler, 1999) in combination with other IPM techniques, such as biological control. Here, we have shown that infiltration with COR, *Pst* DC3000 or *Pst*-derived medium increased tomato resistance against WFT through the induction of enzymatic and chemical defenses.

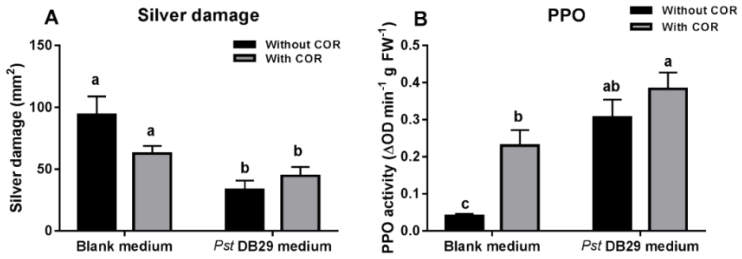

Fig. 7 Effect of COR and *Pst* DB29 medium on WFT resistance and JA-associated responses. (**A**) Silver damage symptoms (mean ± SEM, $n = 10$) determined in tomato plants infiltrated with blank medium, 0.14 µM coronatine (COR) in blank medium, *Pseudomonas syringae* pv. tomato DB29 (*Pst* DB29)-derived medium diluted five-fold with blank medium or 0.14 µM COR in *Pst* DB29-derived medium diluted five-fold with blank medium. Plants were infested with western flower thrips (WFT) at 7 days after the initial treatment and evaluated 7 days after WFT infestation. (**B**) Polyphenol oxidase (PPO) activity (mean ± SEM, $n = 5$) was measured in the second leaf from the bottom of tomato plants infiltrated with the above described treatments at 7 days after the initial treatment. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P \leq 0.05$.

Our results first showed that infiltration of tomato plants with the COR-producing bacteria *Pst* DC3000 or COR alone significantly reduced WFT-associated damage in non-choice whole plant bioassays (**Fig. 1**). This is in line with previous reports. Cui *et al*. (2005) described that the increased susceptibility to the caterpillar *Trichoplusia ni* in *Arabidopsis* plants infiltrated with virulent strains of *P. syringae* ES4326 was counteracted by COR, and that COR alone increased *Arabidopsis* resistance to this caterpillar. In tomato, Stout *et al*. (1999) described that infiltration with *P. syringae* pv. tomato significantly reduced *Helicoverpa Zea* larvae growth. Here we report on the effects of both *Pst* DC3000 and COR infiltration on tomato resistance against WFT. Furthermore, we show that not only COR, but that presence of other defense elicitors in *Pst*-derived medium can increase tomato resistance to WFT.

The enhancement of plant defenses against arthropod herbivores by infiltration of COR-producing *Pst* or COR itself has been explained by the strong induction of the JA-associated defense signaling pathway and suppression of the SA defense signaling (Cui *et al.*, 2005). Analysis of the effect of COR and *Pst* DC3000 infiltration on the activation of JA and SA signaling pathways showed that both treatments strongly induced the expression of the JA-associated gene marker *WIPI-II*, which encodes for a proteinase inhibitor II protein (PI-II), and increased activity of the JA-related defensive enzyme PPO at 7 days after the infiltration. This agrees with previous results described by Stout *et al*. (1999), who found that *Pst* infiltration increased PI-II and PPO activities in infiltrated tomato plants. *Pst* DC3000 is reported to activate JA signaling in tomato (Zhao *et al.*, 2003; Uppalapati *et al.*, 2005) and *Arabidopsis* (He *et al.*, 2004), which is proposed to be explained by the action of *Pst* DC3000-derived COR and type III effectors (He *et al.*, 2004). Our results showed that application of COR also induced the expression of the SA-associated gene marker *PR-P6*, a pathogen defense-related gene (PR) (**Fig. 2C**). Yet, the magnitude of the induction of *PR-P6* was approximately thirty times lower than that of *WIPI-II* in COR-treated plants. Both COR treatment and infection with *Pst* DC3000 lead to slight increases of SA levels in *Arabidopsis* (Uppalapati *et al.*, 2005) and tomato (Zhao *et al.*, 2003). In tomato this induction has been described to be stronger in COR deficient *Pst*, and thus it was suggested to be highly suppressed by the activation of JA signaling in COR-producing *Pst* (Zhao *et al.*, 2003). The

lack of induction of *PR-P6* in *Pst* DC3000-infected tomato plants (**Fig. 2B**) might be explained by our sampling time for gene expression analysis. Hence, induction of PRs has been generally observed 24 h after *Pst* infiltration (Zhao *et al.*, 2003; Uppalapati *et al.*, 2008; López-Gresa *et al.*, 2011). Overall, the strong activation of JA-associated defenses by *Pst* DC3000 and COR infiltration might explain the increased tomato resistance against WFT. Previous studies have shown that induction of JA defenses can reduce WFT-associated damage in tomato and other plant species (Li *et al.*, 2002; Abe *et al.*, 2009; Escobar-Bravo *et al.*, 2017). Activation of JA defense signaling is often associated with reduced plant growth (Guo *et al.*, 2018). Interestingly, our results showed that neither COR or *Pst* DC3000 infiltration significantly affected plant dry biomass or height of tomato plants (Fig. S6). Additionally, we did not detect any *Pst* DC3000 colonies in systemic leaves of *Pst* DC3000-infiltrated plants, but only in the local leaves (Fig. S7), confirming that even localized *Pst* DC3000 infections have a great impact on tomato defenses against other biotic stressors.

Activation of JA signaling pathway through exogenous application of jasmonates, such as the volatile form of JA methyl jasmonate (MeJA) (Boughton *et al.*, 2005; Maes & Goossens, 2010; Tian *et al.*, 2012; Escobar-Bravo *et al.*, 2017) is reported to increase type VI glandular trichome density in tomato leaves. We thus hypothesized that infiltration with *Pst* DC3000 or COR might induce these tomato defenses as well. Our results, however, showed that none of these treatments increased type VI trichome densities in newly formed leaves at 7 days after infiltration. This might be explained by differences in the magnitude of the induction of JA defenses when plants are treated with exogenous application of COR or *Pst* DC3000 infiltration, but also by the activation of different defense signaling pathways. Hence, although COR and MeJA application shared similar activities on tomato plants, some sets of genes are differently regulated by these two compounds (Uppalapati *et al.*, 2005; Tsai *et al.*, 2011). Both COR and *Pst* DC3000 are reported to induce JA, ethylene and auxin signaling pathways (O'donnell *et al.*, 2003; Cohn & Martin, 2005; Uppalapati *et al.*, 2005), and COR slightly induced SA signaling as well. Whether the induction of these signaling pathways explains the lack of induction of trichomes in COR and *Pst* DC3000 infiltrated plants would require further research. Alternatively, COR-mediated activation of SA signaling might have attenuated JA-mediated induction of trichomes (Traw & Bergelson, 2003), as both signaling are known to interact via antagonistic crosstalk (Pieterse *et al.*, 2012). Together, these results suggest that COR- and *Pst*-DC3000-mediated induction of tomato resistance against WFT is not explained by increased type-VI trichome densities.

An untargeted metabolomic analysis of tomato leaves infiltrated with COR or *Pst* DC3000 revealed that both treatments induced similar but not the same metabolomic changes. Both COR and *Pst* DC3000 increased the leaf content of organic acids, phenolics and amino acids (**Fig. 4**). These results are in agreement with those reported by López-Gresa *et al*. (2010; 2011), where higher concentrations of amino acids, organic acids, rutin and phenylpropanoids were detected in *Pst*-infected tomato plants. However, no comparison between the effects of COR and *Pst* infiltration on plant metabolome has been performed before. Interestingly, our results showed that the levels of the amino acid aspartic acid and the non-protein amino acid GABA, as well as the phenolic rutin, were slightly higher in *Pst* DC3000-infiltrated tomato leaves. Yet, these differences did not affect the levels of resistance of tomato plants against WFT, as both COR and *Pst* DC3000 significantly reduced silver damage symptoms in infiltrated plants (**Fig. 1**). The increase in some of these compounds might have influenced tomato defenses against WFT. For instance, high concentrations of the flavonoid rutin (quercetin-3-O-β-rutinoside) has been reported to deter herbivore feeding (reviewed by Simmons *et al*., 2001). On the other hand, increases in GABA levels are reported to occur in *Pst* DC3000-infected plants (Ward *et al.*, 2010), but also in response to

other biotic and abiotic stresses (Bouché *et al.*, 2003). Although all the functions of GABA in plants have not been completely elucidated, it is induced upon herbivory or insects crawling on the leaf surface (Bown *et al.*, 2002; Scholz *et al.*, 2015), and it has a negative effect on arthropod's performance when ingested by feeding in transgenic plants with elevated GABA levels or in GABA-enriched artificial diets (Ramputh & Bown, 1996; McLean *et al.*, 2003; Scholz *et al.*, 2015). Yet, whether its induction might affect tomato resistance against WFT would need further research.

Our results further showed that application of *Pst* DC3000-derived medium (without viable *Pst* bacteria and containing 0.68 μM of COR), COR (0.68 μM) or *Pst* DC3000, all increased tomato resistance against WFT (**Fig. 5A**). Moreover, these treatments increased PPO activities in infiltrated leaves, indicating the activation of JA signaling (**Fig. 5B**). Hence, infiltration of tomato plants with ca. seven times less COR than in our initial experiments (i.e. 5 μM, see **Fig. 1**) resulted in a similar reduction in silver damage symptoms. This suggested that COR has a strong impact on tomato defenses even at low concentrations, and that we might have overlooked the possible effect of other defense elicitors present in *Pst* DC3000-derived medium. This prompted us to further investigate whether infiltration with much lower concentrations of COR alone or in *Pst* DC3000-derived medium had the same effects on tomato resistance against WFT. Notably, infiltration of tomato plants with a 0.2x concentration of *Pst* DC3000-derived medium (containing 0.14 μM of COR) resulted in a stronger reduction of silver damage symptoms than application of COR (0.14 μM) alone dissolved in blank medium. Moreover, induction of PPO activity was higher in plants infiltrated with a 0.2x concentration of *Pst* DC3000-derived medium than with a 0.2x concentration of COR or blank medium. Hence, this suggests that the presence of other defense elicitors in *Pst* DC3000-derived medium might increase tomato resistance against WFT, and that this induction is also probably explained by a stronger activation of JA signaling. Indeed, further assays using a COR deficient mutant of *Pst* DC3000, *Pst* DB29, showed that tomato plants infiltrated with *Pst* DB29-derived medium displayed lower silver damage symptoms after WFT infestation and induced PPO activities as well (**Fig. 7A, B**). It should be noted that *Pst* DB29 is defective in the synthesis of COR precursors, CFA and CMA, both reported to induce some JA/wound associated plant responses in tomato, but at much less magnitude than COR (Uppalapati *et al.*, 2005). Thus, activation of tomato defenses against WFT could not be explained by the presence of CFA and CMA in the *Pst* DB29-derived medium. We hypothesize that these responses might be explained by changes in the culture medium composition in terms of (1) primary or secondary metabolites modified in the medium by the *Pst* growth, or (2) presence of *Pst*-derived effectors. For instance, He *et al*. (2004) described that effectors secreted by the type III secretion system of *Pst* DC3000 can augment the JA-signaling pathway to promote virulence. Nevertheless, this requires further research.

In summary, our study shows that infiltration with COR and *Pst* DC3000 increases tomato resistance against WFT by activating JA-associated defenses, but not type-VI leaf trichome densities. Our results also show that *Pst* DC3000-derived medium contains other defense elicitors that can increase resistance against WFT in infiltrated tomato plants, thus providing a potential treatment for WFT control in agriculture systems.

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Supplementary materials

Method S1 Gene expression analysis
Fig. S1 Representative photographs of leaves from thrips-infested (A) Mock-, (B) coronatine (COR)- or (C) *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC 3000)-treated plants.
Fig. S2 Effect of COR and *Pst* DC3000 on tomato resistance against WFT.
Fig. S3 Important NMR signals that contributed to the metabolome differentiation among treatments.
Fig. S4 Effect of *Pst* DC3000-derived medium on tomato resistance against WFT.
Fig. S5 Effect of COR and *Pst* DB29 medium on WFT resistance.
Fig. S6 Effect of mock solution, COR, or *Pst* DC3000 on plant growth.
Fig. S7 Bacteria growth and symptoms of tomato plants infiltrated with COR, *Pst* DC3000 or *Pst* DC-3000 derived medium.
Table S1 Results of the statistical analysis performed for each figure.

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Supplementary Materials

Methods S1

The gene-specific primers used for the RT-qPCRs are listed below.

***Wound inducible proteinase inhibitor II* (Solyc01g095200)**

WIPI II_F: 5′- GACAAGGTACTAGTAATCAATTATCC -3′

WIPI II_R: 5′- GGGCATATCCCGAACCAAGA -3′

***Pathogenesis related-protein 6* (Solyc00g174340)**

PR-P6_F: 5′- GTA CTG CAT CTT CTT GTT TCC A -3′

PR-P6_R: 5′- TAG ATAAGT GCT TGA TGT GCC -3′

***Actin* (Solyc03g078400)**

SlActin_F: 5′- TTAGCACCTTCCAGCAGATGT -3′

SlActin_R: 5′- AACAGACAGGACACTCGCACT -3′

Supplementary Figures and Tables

Supplementary Figures

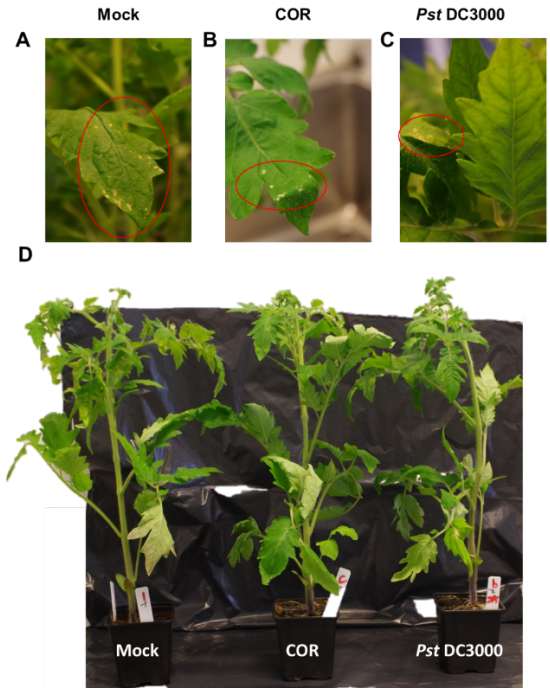

Fig. S1 Representative photographs of leaves from thrips-infested (A) Mock-, (B) coronatine (COR)- or (C) *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC 3000)-treated plants. The Red circles indicate the silver damage symptoms caused by western flower thrips (WFT) feeding. (D) Representative photographs of the WFT-infested plants subjected to mock-, COR- or *Pst* DC3000 treatments. Four leaflets of four-week old tomato plants were infiltrated with mock solution (water), 5 µM COR solution or 10^8 cfu ml^{-1} *Pst* DC3000. Seven days after treatments plants were subjected to non-choice whole plant thrips bioassays. Silver damage symptoms were determined at 7 days after infestation.

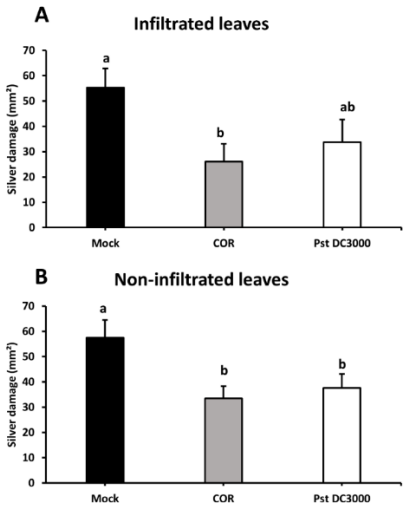

Fig. S2 Effect of COR and *Pst* DC3000 on tomato resistance against WFT. Silver damage symptoms (mean ± SEM, n = 15) were determined in (**A**) infiltrated and (**B**) non-infiltrated leaves of mock-, coronatine (COR)- and *Pseudomonas syringae* pv. tomato DC3000 *(Pst* DC3000)-treated tomato plants. Plants were infested with (Western flower thrips) WFT at 7 days after the initial treatment and evaluated 7 days after WFT infestation. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P \leq 0.05$.

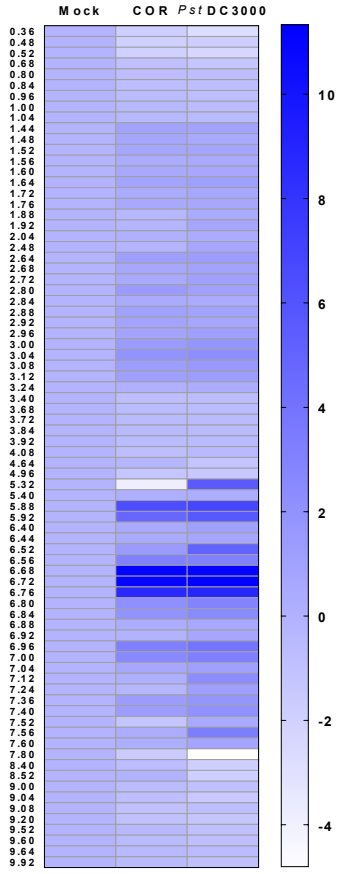

Fig. S3 Important NMR signals that contributed to the metabolome differentiation among treatments. Heatmap of the 78 out of 80 signals detected by NMR and displaying VIP scores > 1 based on PLS-DA analysis. Each heatmap column displays the log_2 fold change of relative peak intensity of the compounds differentially induced in Mock, COR or *Pst* DC3000 samples in comparison to Mock. Log_2 fold change of compounds in the mock treatment was 0 (fold change = 1). The other 2 out of 80 NMR signals cannot be shown in the Heatmap, because the mean relative peak intensity of Mock was 0.

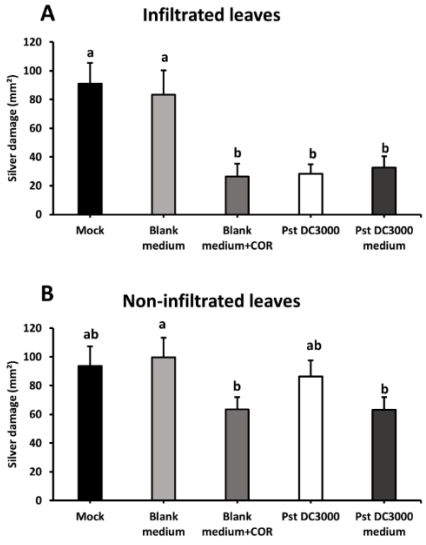

Fig. S4 Effect of *Pst* DC3000-derived medium on tomato resistance against WFT. Silver damage symptoms (mean ± SEM, $n = 10$) determined in (**A**) infiltrated and (**B**) non-infiltrated leaves of tomato plants treated with a mock solution (mock), blank medium, 0.68 µM coronatine (COR) dissolved in blank medium (blank medium + COR), *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) suspension or *Pst* DC3000-derived medium (containing 0.68 µM of COR). Plants were infested with Western flower thrips (WFT) at 7 days after the initial treatment and evaluated at 7 days after WFT infestation. Different letters indicate significant differences among treatments tested by Fisher's LSD test at $P \leq 0.05$.

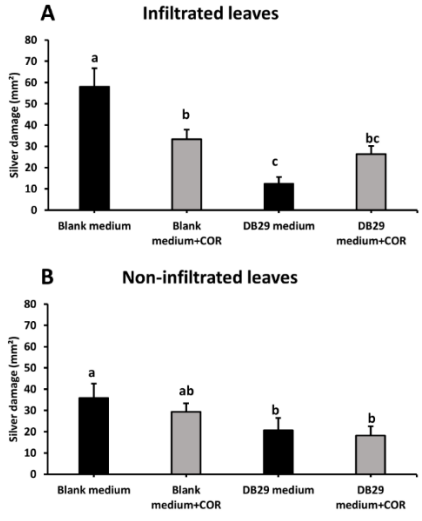

Fig. S5 Effect of COR and *Pst* DB29 medium on WFT resistance. Silver damage symptoms (mean ± SEM, $n = 10$) determined in (**A**) infiltrated and (**B**) non-infiltrated leaves of tomato plants treated with blank medium, 0.14 µM COR in blank medium, *Pseudomonas syringae* pv. tomato DB29 (*Pst* DB29)-derived medium diluted five-fold with blank medium or 0.14 µM COR in *Pst* DB29-derived medium diluted five-fold with blank medium. Plants were infested with Western flower thrips (WFT) at 7 days after the initial treatment and evaluated 7 days after WFT infestation.

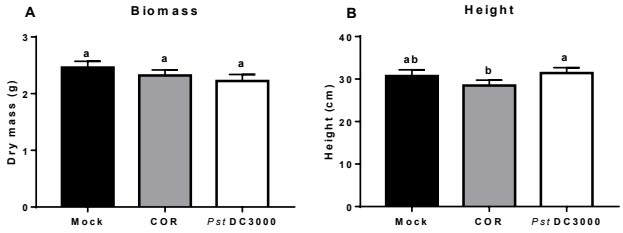

Fig. S6 Effect of mock solution, COR, or *Pst* DC3000 on plant growth. (**A**) Dry biomass of the above ground plant material and (**B**) stem height were determined in tomato plants infiltrated with a mock solution (mock), 5 µM of coronatine (COR) or 10^8 cfu ml^{-1} of *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) suspension. Measurements were performed at 7 days after the initial treatments. Depicted are the average (± SEM) of fifteen replicates. Different letters indicate significant differences among treatments (One way ANOVA followed by Fisher's LSD test, $P \leq 0.05$).

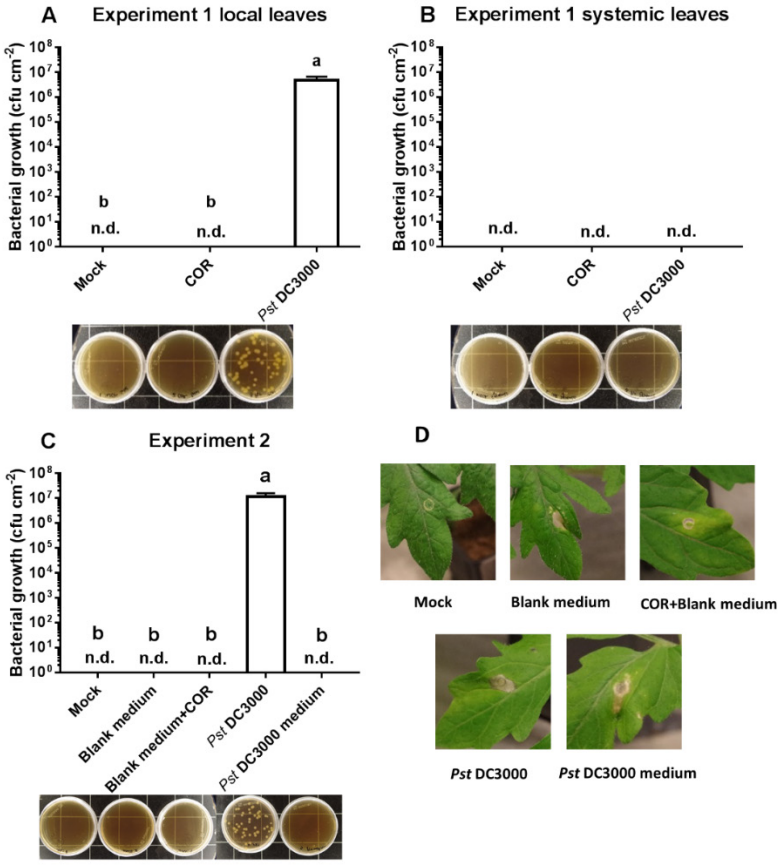

Fig. S7 Bacteria growth and symptoms of tomato plants infiltrated with COR, *Pst* DC3000 or *Pst* DC-3000 derived medium. Four leaflets of four-week old tomato plants were infiltrated with mock solution (water), 5 µM coronatine (COR) solution or 10^8 cfu ml^{-1} *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) suspension for experiment 1; or with mock solution (water), blank medium, 0.68 µM COR + blank medium, 10^8 cfu ml^{-1} *Pst* DC3000 suspension or *Pst* DC3000-derived medium diluted five-fold with blank medium for experiment 2. (**A**) *Pst* DC3000 growth was determined in mock-, COR- and *Pst* DC3000-infiltrated (i.e. local) leaves and (**B**) in non-infiltrated systemic leaves at 7 days after the initial treatments in experiment 1. (**C**) *Pst* DC3000 growth was determined in mock-, blank medium, blank medium +COR, *Pst*-DC3000, and *Pst* DC3000-derived medium-infiltrated (i.e. local) leaves at 7 days after the initial treatment in experiment 2. Differences in bacteria growth among the treatments were tested by non-parametric Kruskal-Wallis rank-sum test followed by Wilcoxon rank-sum test for multiple comparisons. n.d.: not detected. Below each graph, representative photographs of the bacterial colonies visually detected in the 10,000x dilution are shown. **D**) Representative photographs of the symptoms in local leaves observed 7 days after infiltration in the experiment 2 are shown.

Supplementary tables

Table S1 Results of the statistical analysis performed for each figure.

Figure	Panel	Statistical test	Factor and statistic value	df	*P*
Fig. 1	**None**	One-way ANOVA	COR or *Pst* DC3000; $F = 8.610$	2	$P = 0.001$
Fig. 2	**A**	One-way ANOVA	COR or *Pst* DC3000; $F = 25.494$	2	$P < 0.001$
	B	One-way ANOVA	COR or *Pst* DC3000; $F = 8.943$	2	$P = 0.007$
	C	One-way ANOVA	COR or *Pst* DC3000; $F = 7.038$	2	$P = 0.014$
Fig. 3	**A**	One-way ANOVA	COR or *Pst* DC3000; $F = 2.927$	2	$P = 0.071$
	B	One-way ANOVA	COR or *Pst* DC3000; $F = 3.744$	2	$P = 0.037$
Fig. 4	**D**	Kruskal-Wallis test	COR or *Pst* DC3000; $\chi^2 = 7.385$	2	$P = 0.025$
	E	Kruskal-Wallis test	COR or *Pst* DC3000; $\chi^2 = 8.000$	2	$P = 0.018$
	F	Kruskal-Wallis test	COR or *Pst* DC3000; $\chi^2 = 7.731$	2	$P = 0.021$
	G	Kruskal-Wallis test	COR or *Pst* DC3000; $\chi^2 = 7.538$	2	$P = 0.023$
	H	Kruskal-Wallis test	COR or *Pst* DC3000; $\chi^2 = 8.769$	2	$P = 0.012$
Fig. 5	**A**	One-way ANOVA	COR, *Pst* DC3000 or *Pst* DC3000 medium; $F = 9.790$	4	$P < 0.001$
	B	One-way ANOVA	COR, *Pst* DC3000 or *Pst* DC3000 medium; $F = 156.551$	4	$P < 0.001$
Fig. 6	**A**	GLM	Dilution; *Wald* $\chi^2 = 8.400$	4	$P = 0.078$
			COR or *Pst* DC3000 medium; *Wald* $\chi^2 = 55.789$	2	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 8.537$	8	$P = 0.383$
	B	GLM	Dilution; *Wald* $\chi^2 = 4.472$	4	$P = 0.346$
			COR or *Pst* DC3000 medium; *Wald* $\chi^2 = 429.336$	2	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 17.691$	8	$P = 0.024$
Fig. 7	**A**	GLM	*Pst* DB29 medium; *Wald* $\chi^2 = 31.481$	1	$P < 0.001$
			COR; *Wald* $\chi^2 < 0.001$	1	$P = 0.994$
			Interaction; *Wald* $\chi^2 = 7.104$	1	$P = 0.008$
	B	GLM	*Pst* DB29 medium; *Wald* $\chi^2 = 36.311$	1	$P < 0.001$
			COR; *Wald* $\chi^2 = 14.623$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 2.642$	1	$P = 0.104$

Chapter 4

Phenotypic variation in constitutive and jasmonic acid-mediated induced defenses against Western flower thrips in chrysanthemum

Gang Chen, Rocío Escobar-Bravo, Peter G. L. Klinkhamer

Western flower thrips (WFT), *Frankliniella occidentalis*, is a severe insect pest of Chrysanthemum [*Chrysanthemum* × *morifolium* Ramat. (Asteraceae)]. Here we have explored whether variations in constitutive and inducible levels of trichome density and activity of the defensive enzyme polyphenol oxidase (PPO) correlate with WFT resistance in chrysanthemum. First, our results showed that both non-glandular and glandular leaf trichome densities significantly varied among 95 different chrysanthemum cultivars. Additional analyses in a subset of 12 of those cultivars, displaying contrasting levels of trichome densities, showed significant variations in PPO activities as well. Yet, constitutive levels of trichome density and PPO activity did not correlate with chrysanthemum resistance to WFT. Exogenous application of the phytohormone jasmonic acid (JA) to six selected cultivars further showed that activation of JA defenses increased chrysanthemum resistance to WFT, but this effect was cultivar-dependent. JA-mediated induction of WFT resistance was not explained by variations in non-glandular leaf trichomes nor PPO activity. Taken together, our results show that trichome density and PPO might not play a relevant role on chrysanthemum defenses against WFT, however, this does not exclude the existence of other glandular trichome-associated chemical defenses that were not addressed in our study.

Keywords: chrysanthemum; constitutive defense; *Frankliniella occidentalis*; induced defenses; jasmonic acid; polyphenol oxidase; trichome density

1 Introduction

Chrysanthemum [*Chrysanthemum* × *morifolium* Ramat. (Asteraceae)], which was first bred in China and Japan ca. 3000 years ago, is one of the economically most important ornamental crops worldwide (Fletcher, 1992). Its production, however, is negatively affected by a high susceptibility to multitude of arthropod pests. A major arthropod pest of chrysanthemum is the Western flower thrips (WFT), *Frankliniella occidentalis* [Pergande]. WFT is also one of the most serious greenhouse pests in agricultural and horticultural crops worldwide (Mouden *et al.*, 2017). This tiny insect has piercing-sucking mouth parts, and it can cause two types of damage: direct damage through feeding on leaves, flowers and fruits, thus reducing plant growth and affecting product appearance and market quality (de Jager, CM *et al.*, 1995; de Jager, KM *et al.*, 1995), and indirect damage through the transmission of devastating virus diseases (Maris *et al.*, 2003). Currently, the use of insecticides has been the most common strategy for WFT control. Determining chrysanthemum defense mechanisms against WFT would facilitate breeding for resistant cultivars and, therefore, reduce the application of pesticides (de Jager, CM *et al.*, 1995).

To defend themselves against arthropod herbivores, plants have evolved sophisticated constitutive and inducible defenses. Constitutive defenses are defined as physical structures or chemical components present in the plant prior to herbivory, and controlled by genetics or environmental factors (Rosner & Hannrup, 2004; Franceschi *et al.*, 2005). Induced defenses are initiated upon herbivore attack, and regulated by the type of herbivore-associated damage, environment, as well as the plant genetics and ontogeny (Karban & Myers, 1989; Franceschi *et al.*, 2005; Köhler *et al.*, 2015). In the framework of Integrated Pest Management, breeding for enhanced constitutive and inducible plant defenses against herbivorous pests is considered a relevant strategy to achieve pest control (Bottrell, 1979; Mirnezhad *et al.*, 2010; War *et al.*, 2012). Both constitutive and inducible defenses have been observed to vary within and among plant species. In some cases plants with high constitutive chemical or physical defenses display weaker inducible defenses (Agrawal *et al.*, 2002; Wittstock & Gershenzon, 2002). Yet, some authors have reported a positive correlation between the two types of resistance mechanisms as well (Zangerl & Berenbaum, 1990; Siemens & Mitchell-Olds, 1998), while others have reported no correlation at all (Brody & Karban, 1992; Thaler & Karban, 1997; English-Loeb *et al.*, 1998; Underwood *et al.*, 2000). Exploration of these variations and identification of resistant chrysanthemum genotypes that combine both defense strategies would be of fundamental importance for plant breeding and pest control.

Host plant resistance to arthropod herbivores is based on morphological and/or chemical traits that can confer antixenotic and/or antibiotic properties. Among the plant defense-associated morphological structures, leaf trichomes have been associated to plant resistance against arthropod herbivores in different plant species (Levin, 1973; Dalin *et al.*, 2008). Trichomes are hairy epidermal structures mainly found in leaves and stems, that can be classified as non-glandular and glandular (Glas *et al.*, 2012). Non-glandular trichomes function as physical hurdles, hindering the ability of insects to access the leaf surface and thus to feed and/or oviposit (Duffey, 1986). Glandular trichomes provide a physical barrier in plants, but they can also chemically repel or poison arthropod herbivores (Wagner, 1991). In chrysanthemum, the presence of non-glandular and glandular leaf trichomes has been described by several authors (Vermeer & Peterson, 1979; Deng *et al.*, 2010; He *et al.*, 2011; Sun *et al.*, 2013). Furthermore, density of non-glandular trichomes and the size of glandular trichomes have been positively correlated with enhanced chrysanthemum resistance to aphids

in three chrysanthemum cultivars (He *et al.*, 2011). However, the study of He *et al*. (2011) used a rather low number of cultivars, and a greater number of genotypes would be needed to determine the role of chrysanthemum trichomes on pest resistance. Furthermore, whether the density of both glandular and non-glandular leaf trichomes is important for chrysanthemum resistance to other insect pests, such as WFT, is also unknown.

We have previously reported that leaf content in chlorogenic acid was positively correlated with chrysanthemum resistance to WFT (Leiss *et al.*, 2009b). Disruption of plant tissues by herbivory triggers the oxidation of chlorogenic acid by plant polyphenol oxidases (PPOs) and peroxidases. This can result in the production of highly reactive quinones that inhibit the digestion of plant proteins by herbivores (Stout *et al.*, 1994; War *et al.*, 2012). Notably, higher activities of PPO have been associated to increased resistance to diverse arthropod herbivores in tomato (Mahanil *et al.*, 2008; Bhonwong *et al.*, 2009). In chrysanthemum, He *et al* (2011) reported higher constitutive levels of PPO activity in an aphid-resistant cultivar, suggesting a possible role of this defensive enzyme in chrysanthemum resistance to herbivory.

Although both trichome density and PPO activity are constitutively expressed in plants, their expression can be modulated by abiotic and biotic factors (Biesiada & Tomczak, 2012; Hauser, 2014; Escobar-Bravo *et al.*, 2017; Escobar-Bravo *et al.*, 2018), as well as by defense elicitors. For instance, application of the phytohormone jasmonic acid (JA) has been reported to induce trichome densities in tomato (Boughton *et al.*, 2005; Escobar-Bravo *et al.*, 2017) and Arabidopsis (Traw & Bergelson, 2003) among other plant species. Similarly, JA can also induce PPO in diverse plant species (Thaler *et al.*, 1996; Constabel & Ryan, 1998; Chen *et al.*, 2018).

In the present study we investigated whether constitutive and inducible levels of non-glandular and glandular trichome densities, as well as PPO activity correlate with chrysanthemum resistance to WFT. For this, we first determined trichome density in 95 chrysanthemum cultivars. Thereafter, we selected 12 of these cultivars to further test whether constitutive levels of trichome densities and PPO correlate with WFT resistance. Finally, we also determined whether JA-mediated induction of plant defenses against WFT varies among chrysanthemum cultivars, and whether this might be explained by the differential expression of trichomes and PPO-associated defenses.

2 Materials and methods

2.1 Plants and insects

A total of 95 chrysanthemum cultivars provided by three Dutch chrysanthemum breeders [Dekker Chrysanten (Hensbroek), Deliflor Chrysanten (Maasdijk) and Dümmen Orange (De Lier)] were used in our study (**Table 1**). Cuttings were individually planted in plastic trays (4 cm × 4 cm × 6 cm) filled with potting soil. At 14 d after planting, plants were transplanted to plastic pots (9 cm × 9 cm × 10 cm) containing the same potting soil. Plants were randomly placed in a climate room provided with 20°C, 70% RH, 113.6 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR) and L16:D8 photoperiod.

Western flower thrips (WFT), *Frankliniella occidentalis* (Pergande) were obtained from a colony reared on chrysanthemum flowers (cultivar 'Euro Sunny') in a climate room at 23°C, 60% RH and L12:D12 photoperiod.

Table 1 Breeding IDs of the 95 chrysanthemum cultivars used in this study. Cultivars can be identified according to breeding ID at the different breeding companies: Dekker Chrysanten (cultivars 1–28), Deliflor Chrysanten (cultivars 29–63) and Dümmen Orange (cultivars 64–95).

Cultivar	Breeding ID	Cultivar	Breeding ID	Cultivar	Breeding ID	Cultivar	Breeding ID
1	DC-1	25	DC-25	49	48837	73	56072
2	DC-2	26	DC-26	50	48639	74	56168
3	DC-3	27	DC-27	51	9403	75	56701
4	DC-4	28	DC-28	52	41475	76	56703
5	DC-5	29	26741	53	45644	77	56713
6	DC-6	30	31563	54	45785	78	56817
7	DC-7	31	8713	55	48286	79	57352
8	DC-8	32	7688	56	40931	80	57709
9	DC-9	33	30600	57	43339	81	57773
10	DC-10	34	21697	58	90753	82	57993
11	DC-11	35	13185	59	36318	83	58498
12	DC-12	36	8578	60	43110	84	59209
13	DC-13	37	8393	61	44339	85	64952
14	DC-14	38	4875	62	48942	86	65001
15	DC-15	39	48864	63	9361	87	37511
16	DC-16	40	47287	64	42215	88	37577
17	DC-17	41	57067	65	42377	89	37630
18	DC-18	42	55229	66	42629	90	42415
19	DC-19	43	55238	67	42909	91	25533
20	DC-20	44	46885	68	50223	92	22898
21	DC-21	45	55223	69	50858	93	48015
22	DC-22	46	55115	70	51643	94	20880
23	DC-23	47	45728	71	56068	95	49230
24	DC-24	48	90633	72	56069		

2.2 Experimental design

To investigate phenotypic variations in constitutive and inducible chrysanthemum defenses associated to WFT resistance, we performed three different experiments. First, we determined constitutive levels of non-glandular and glandular trichome densities on leaves of 95 chrysanthemum cultivars at 35 d after planting (Experiment 1). Second, we selected 12 out of the 95 chrysanthemum cultivars to further determine whether constitutive levels of trichome density and polyphenol oxidase (PPO) activity correlated with WFT resistance (Experiment 2). For this, chrysanthemum plants were sampled for determination of non-glandular and glandular trichome density and PPO activity, or used for non-choice whole plant thrips bioassays, at 35 d after planting. Third, we tested whether application of the phytohormone jasmonic acid (JA) enhanced WFT resistance in 6 selected cultivars, and whether this was explained by the induction of trichome- and PPO-associated defenses (Experiment 3). For this experiment, each JA-treated chrysanthemum plant was sprayed with approximately 5 ml of 3 mM JA (Cayman, Ann Arbor, Michigan, USA) in 2.4% aqueous ethanol solution as described by Redman *et al* (2001). Each control plant was sprayed with a similar volume of a mock solution consisting of 2.4% aqueous ethanol. Seven days after the hormone treatment, mock- and JA-treated plants were sampled for determination of non-glandular and glandular trichome density, PPO activity, or used for non-choice whole plant thrips bioassays. We selected this sampling time because previous studies in tomato and *Arabidopsis* have determined that a significant increment of trichome density can be observed at 7 days after the hormone application (Boughton *et al.*, 2005; Yoshida *et al.*, 2009). In addition, higher PPO activities can be detected up to 7 days of JA induction in tomato (Thaler *et al.*, 2001).

2.3 Non-choice whole plant thrips bioassay

Non-choice whole plant bioassays were performed as described by Leiss *et al* (2009a). For this, individual plants were placed into WFT-proof cages consisting of perspex plastic cylinders (50 cm height and 20 cm diameter) closed at the top with a displaceable ring of nylon gauze (120 µm mesh size). Ten adult WFT (8 females and 2 males) were added to each plant. Plants were maintained in a climate room provided with 113.6 µmol photons m^{-2} s^{-1} of PAR, 16L:8D of photoperiod, 25°C and 70% RH. WFT feeding damage, hereafter referred to as 'silver damage', was visually scored for each leaf of the plant, and expressed as the damaged area in mm^2, at 7 days after infestation. Whole plant silver damage was calculated by adding up the damage of each individual leaf.

2.4 Analysis of trichome density and morphology

In all the experiments, densities of glandular and non-glandular trichomes were determined on the adaxial leaf surface of the third leaf from the apex. Two pictures were taken in the middle part of the leaf at both sides of the main vein, each covering an area of 12 mm^2, using a stereomicroscope (MZ16, Leica Microsystems, Wetzlar, Germany). Trichome number was counted in both pictures using the software 64-bit Fiji ImageJ (http://fiji.sc/Fiji), and the average of the two measurements was expressed as number of trichomes per cm^2.

2.5 Scanning electron microscopy (SEM)

SEM analysis was conducted on the third leaf from the apex. Leaves were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (PBS), pH 7.2, at room temperature. Samples were then dehydrated in acetone series of 50, 70, 90, 96, and 100% (v/v) and then dried in a Bal-Tec CPD 030 Critical Point Dryer with liquid CO_2 (Leica Microsystems). The samples were coated with gold in a Polaron SEM coating unit E5100. SEM images were taken with a JEOL 6400 scanning electron microscope at the Microscopy Unit of the Institute of Leiden (The Netherlands).

2.6 Determination of PPO activity

PPO activity was determined in the third leaf from bottom following the methodology described in Stout *et al*. (1998). Briefly, 0.150 g of leaf tissue without midrib flash frozen in liquid nitrogen, ground with a tissue lyser (Qiagen, Hilden, Germany) and homogenized in a 2 ml tube with 1.25 ml ice-cupper 0.1 M pH 7.0 phosphate buffer containing 7% polyvinylpolypyrolidine and 0.4 ml of 10% Triton X-100. The homogenate was vortexed for 2 min and centrifuged for 10 min at 11,000 × g and 4°C. Five microliters of the supernatant were added to 1 ml of 2.92 mM chlorogenic acid solution in pH 8.0 phosphate buffer. The optical density (OD) at 470 nm was recorded in a spectrophotometer (UV-1800, Shimadzu) every ten seconds for one minute. PPO activity was defined as the increment of OD values per min per gram of fresh weight.

2.7 Statistical analysis

Normality and homogeneity of data residuals were first checked using Kolmogorov-Smirnov and Levene's tests, respectively. We used one-way ANOVA to test significant differences in non-glandular trichome densities (Experiment 1) and PPO activity (Experiment 2) among cultivars. Data were square root transformed for non-glandular trichome density to meet the requirements of the ANOVA. Differences in glandular trichome density (Experiment 1) and silver damage symptoms (Experiment 2) among cultivars were analyzed by Kruskal-Wallis test. As a measure for the phenotypic variation across cultivars, the coefficient of variation (CV) was calculated. CV is a standardized measure of the dispersion of a sample. CV is expressed as the ratio of the standard deviation (δ) to the mean (μ), i.e. $CV = \delta/\mu$ (Sokal &

Rohlf, 1995). A high CV value means high phenotypic variation for the studied trait. The relationships between non-glandular and glandular trichome densities (Experiment 1), silver damage and PPO activity or trichome density (Experiment 2), and between constitutive and induced resistance indexes (Experiment 3) were determined by Pearson or Spearman correlation tests. In the Experiment 3, the effects of the hormone treatment, plant genotype and their interaction on silver damage, PPO activity and glandular trichome density were analyzed by Generalized Linear Models (GLM) using linear distribution and identity link functions. Differences among groups were tested by Fisher´s least significant difference (LSD) post-hoc test. The constitutive resistance index (CRI) was calculated for each cultivar in Experiment 3 by dividing the silver damage symptoms by the silver damage symptoms of a "reference cultivar". The "reference cultivar" was selected based on its lowest silver damage symptoms. Accordingly, the cultivar with the lowest silver damage has the highest constitutive resistance index, set as 1. The induced resistance index (IRI) was calculated for a given cultivar as the percent reduction in silver damage symptoms in JA-treated plants respect to controls: [(average of silver damage detected on JA-treated plants – average silver damage detected on mock-treated plants) / average of silver damage in mock-treated plants] (Brody & Karban, 1992). Statistical analyses were performed by using the SPSS software package (version 25; SPSS Inc., Chicago, IL, USA). All detailed statistics are included in Table S1.

3 Results

3.1 Non-glandular and glandular trichome densities vary among chrysanthemum cultivars

The morphological analysis of the chrysanthemum leaves revealed two types of leaf trichomes, non-glandular and glandular trichomes, the latter having a bean-shape structure and coinciding with the description reported by He *et al*. (2011) (**Fig. 1**).

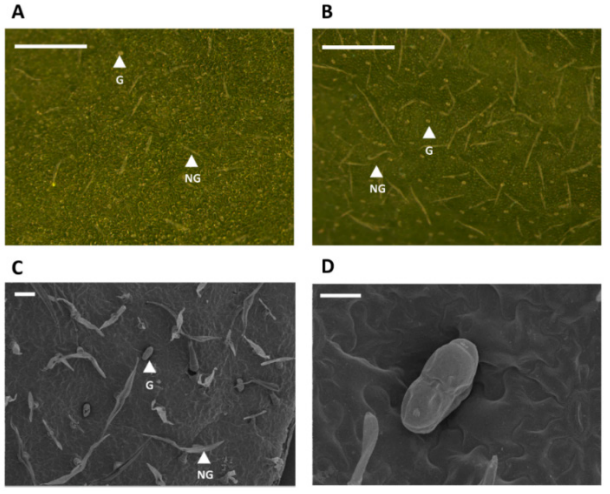

Fig. 1 Representative micrographs of non-glandular and glandular trichomes on chrysanthemum leaves. Light micrographs of trichomes in the adaxial leaf surface of (**A**) a chrysanthemum cultivar displaying low trichome density and (**B**) a cultivar displaying high trichome density. Scanning electron microscopic images of the adaxial leaf surface of chrysanthemum leaves (**C** and **D**). (**D**) Glandular trichome. White arrows indicate the position of non-glandular (NG) and glandular (G) trichomes. The white bars represent 100 μm in (A), (B) and (C), and 30 μm in (D).

Density of non-glandular trichomes differed strongly among cultivars (ANOVA, $P < 0.001$), ranging from 33 trichomes/cm² (cultivar 83) to 350 trichomes/cm² (cultivar 24) (**Fig. 2A**). The CV of non-glandular trichomes density was 37%. Similarly, glandular trichome densities varied strongly among cultivars (Kruskal-Wallis, $P < 0.001$), with a coefficient of variation of 113% (**Fig. 2B**). Out of the ninety-five cultivars analyzed, twenty of them had no glandular trichomes, and the highest density of glandular trichomes was 211 trichomes/cm². Densities of both non-glandular and glandular trichomes were positively correlated (**Fig. 3**; two-tailed Spearman, $P = 0.008$).

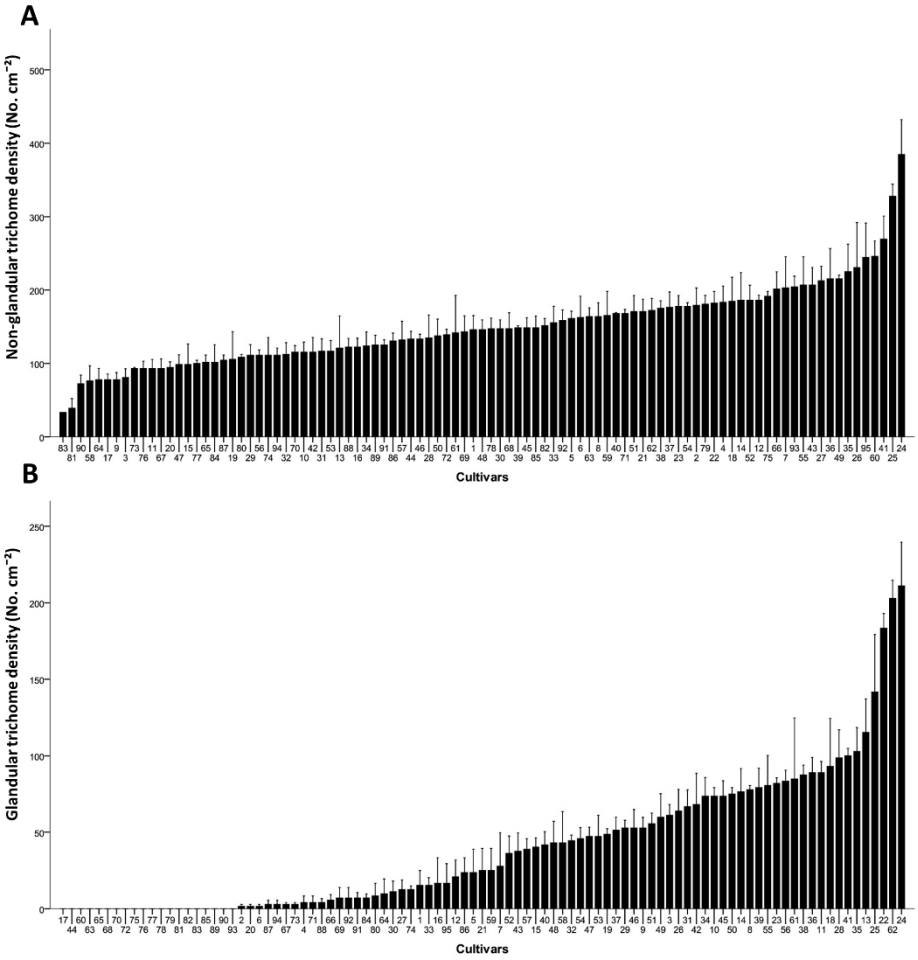

Fig. 2 Phenotypic variation in non-glandular and glandular trichome densities. Bars depict the mean (± SEM, $n = 3$) of leaf (**A**) non-glandular and (**B**) glandular trichome density analyzed in 95 chrysanthemum cultivars at 35 days after planting. Trichome density was determined on the adaxial side of the third leaf from the apex.

3.2 Trichome density and PPO levels do not correlate with chrysanthemum resistance to WFT

To determine whether trichome density was correlated with chrysanthemum resistance to WFT, we selected 12 chrysanthemum cultivars differing in trichome densities and tested their levels of WFT resistance using non-choice whole plant bioassays. Silver damage symptoms

significantly differed among the chrysanthemum cultivars (Fig. S1A; Kruskal-Wallis test, $P < 0.001$). No significant correlations between silver damage and non-glandular (**Fig. 4A**; two-tailed Pearson, $P = 0.564$) or glandular trichome density (**Fig. 4B**; two-tailed Pearson, $P = 0.715$) were observed.

To further test whether variation in WFT susceptibility correlated with differences in leaf chemical defenses, we also determined constitutive levels of PPO activity in the selected twelve cultivars (**Fig. 4C**). PPO activity significantly differed among the chrysanthemum cultivars (Fig. S1B; ANOVA, $P = 0.002$). However, the CV of PPO (i.e. 15%) was smaller than that of silver damage, i.e. 38%. PPO activity did not correlate with the silver damage symptoms (**Fig. 4C;** two-tailed Pearson, $P = 0.355$).

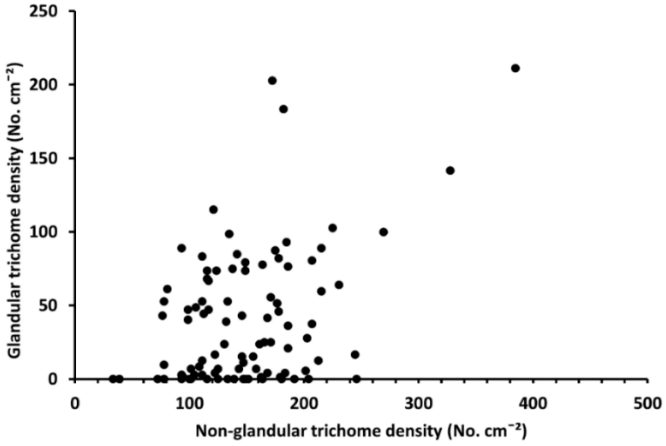

Fig. 3 Scatter plot depicting the relationship between non-glandular and glandular trichome densities. Non-glandular and glandular trichome density were measured in 95 chrysanthemum cultivars at 35 days after planting. Each dot corresponds to the mean of three plant replicates per cultivar. Trichome density was determined on the adaxial side of the third leaf from the apex.

3.3 Induction of chrysanthemum resistance to WFT differed among chrysanthemum cultivars, and it is not explained by variations in PPO and trichome levels

Constitutive levels of non-glandular and glandular trichomes, as well as PPO activity, were not correlated with chrysanthemum resistance to WFT. Thus, we further explored whether a higher inducibility of these defenses might explain the differences in WFT susceptibility among chrysanthemum cultivars. For this, we selected six cultivars differing in WFT resistance levels, PPO and trichome densities, and determined the induction of these defenses after application of JA. Silver damage symptoms significantly differed among the cultivars (**Fig. 5A**; GLM, $P < 0.001$ for genotype). Application of JA significantly reduced silver damage symptoms (GLM, $P < 0.001$ for hormone treatment) and this effect was depended on the chrysanthemum cultivar (GLM, $P < 0.001$ for the interaction).

JA significantly induced PPO activity in all the chrysanthemum cultivars (**Fig. 5B**; GLM, $P < 0.001$ for hormone treatment), and this induction was also dependent on the cultivar (GLM, $P < 0.001$ for interaction). Non-glandular trichome density varied among cultivars (GLM, $P < 0.001$ for genotype) (**Fig. 5C**), and it was significantly induced by JA (GLM, $P = 0.025$ for hormone treatment) depending on the cultivar (GLM, $P = 0.027$ for interaction). Glandular trichome density significantly varied among cultivars as well (**Fig.**

5D, GLM, $P < 0.001$). However, JA did not increase glandular trichome densities in any chrysanthemum cultivars (GLM, $P = 0.922$ for hormone treatment; $P = 0.541$ for interaction).

Induced production of non-glandular trichome (**Fig. 6A**; two-tailed Pearson, $P = 0.190$) or PPO activity (**Fig. 6B**; two-tailed Pearson, $P = 0.824$) did not correlate with the silver damage symptoms in JA-treated plants. Finally, CRI did not correlate with IRI for these 6 cultivars (**Fig. 6C**; two-tailed Pearson, $P = 0.944$).

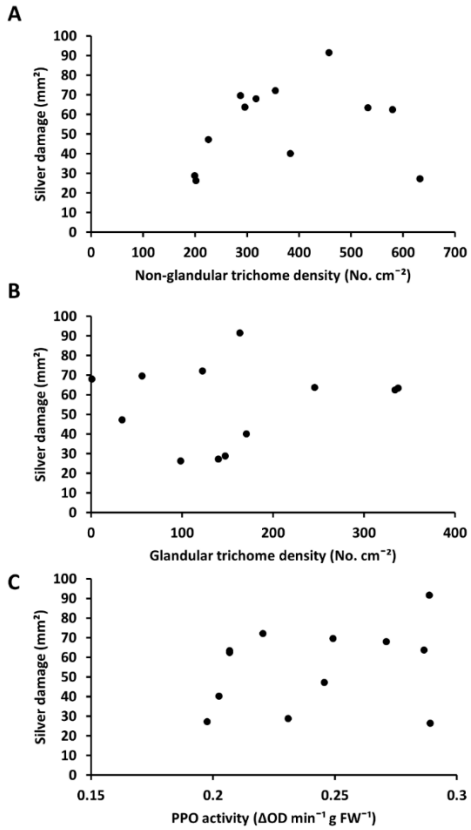

Fig. 4 Relationship between Western flower thrips resistance and putative defense-related traits in chrysanthemum. Scatter plots depicting the relationship between (**A**) silver damage and polyphenol oxidase (PPO) activity levels, (**B**) silver damage and non-glandular trichome density, and (**C**) silver damage and glandular trichome density. Plants were sampled for PPO activity and trichome density, or subjected to non-choice whole plant thrips bioassays, at 35 days after planting. Silver damage symptoms were evaluated at 7 days after Western flower thrips infestation. The plots display data obtained from 12 chrysanthemum cultivars. Each dot corresponds to the mean of five plant replicates per cultivar for PPO and trichome density, and of ten plant replicates per cultivar for silver damage symptoms.

4 Discussion

In the present study, we have demonstrated that constitutive and inducible resistance to WFT strongly differ among chrysanthemum cultivars. However, this variation could not be explained by the presence or induction of non-glandular and glandular trichomes, nor by the activity of the defensive protein PPO.

First, we showed that constitutive levels of non-glandular and glandular trichome densities varied considerably among chrysanthemum cultivars. A few studies have described the presence of trichomes in chrysanthemum leaves and the variation of trichome densities among cultivars (Stavrinides & Skirvin, 2003; He *et al.*, 2011; Sun *et al.*, 2013). However, those studies included only two or three chrysanthemum genotypes. Here we present a comprehensive analysis on trichome density using a large and significant representation of chrysanthemum cultivars. Further analyses on twelve selected chrysanthemum cultivars, differing in trichome densities, showed that these genotypes also displayed differences in their chemical defenses, i.e., PPO activity. However, our results revealed that variation in constitutive levels of non-glandular and glandular trichome densities, as well as PPO activities, were not correlated with WFT resistance in chrysanthemum.

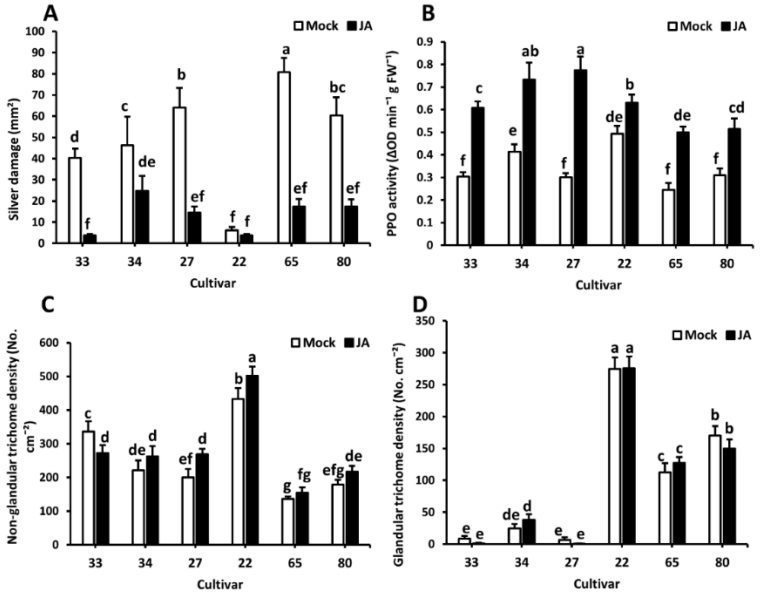

Fig. 5 Phenotypic variation in jasmonic acid-mediated induction of Western flower thrips resistance among chrysanthemum cultivars. Six different chrysanthemum cultivars were treated with mock or jasmonic acid (JA) solutions at 28 d after planting. Plants were sampled for determination of PPO activity and trichome density or subjected to non-choice whole plant bioassays at 7 days after the hormone treatments. (**A**) Silver damage symptoms (mean ± SEM, $n = 7$) evaluated in mock- and jasmonic-acid treated chrysanthemum plants at 7 days after Western flower thrips infestation. (**B**) PPO activity, (**C**) non-glandular trichome density and (**D**) glandular trichome density (mean ± SEM, $n = 5$-7) determined in mock- and JA-treated chrysanthemum plants. Different letters indicate significant differences among groups compared by Fisher's least significant differences (LSD) test at $P \leq 0.05$.

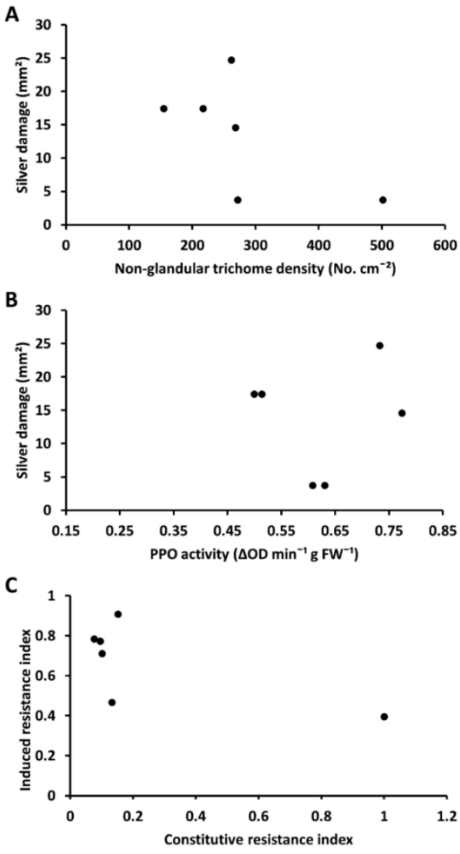

Fig. 6 Relationship between JA-associated induced defenses and chrysanthemum resistance to Western flower thrips. Scatter plots depicting: (**A**) the relationship between silver damage and non-glandular trichome density, and **(B)** silver damage and polyphenol oxidase (PPO) activity in jasmonic acid (JA)-treated plants corresponding to six different chrysanthemum cultivars. Each dot corresponds to the mean of five plant replicates per cultivar for PPO activity, and of seven plant replicates per cultivar for silver damage symptoms. Plants were treated with a JA solution at 28 days after planting and sampled for PPO activity, trichome density or subjected to non-choice whole plant thrips bioassays at 7 days after the hormone treatments. Silver damage symptoms was evaluated at 7 days after Western flower thrips infestation. And (**B**) relationship between constitutive resistance index (CRI) and induced resistance index (IRI).

Positive correlations between non-glandular or glandular trichome density and plant resistance to arthropod herbivores have been documented for many plant species (Mauricio, 1998; Handley *et al.*, 2005). Furthermore, in chrysanthemum, He *et al* (2011) observed a higher density of non-glandular trichomes in an aphid-resistant cultivar. Yet, trichome-mediated effects on herbivore performance might depend on the herbivore species. For instance, Tian *et al.* (2012) reported that high density of non-glandular trichomes in cultivated tomato (*Solanum lycopersicum*) leaves negatively influenced the feeding behavior and growth of the Colorado potato beetle (*Leptinotarsa decemlineata*), while it stimulated the growth of the moth *Helicoverpa zea*. The same authors described that the presence of tomato glandular trichomes hampered *H. zea* growth, but it did not have any effect on the Colorado potato beetle. In chrysanthemum, the chemical composition of leaf glandular trichomes has not been previously reported, nor it was characterized in our study. Therefore, we do not rule

out the possibility that differences in the production of trichome-derived allelochemicals, rather than the density, might still play a role in chrysanthemum resistance to WFT. Indeed, differences in the profiles and/or abundance of glandular trichomes-derived allelochemicals determine the levels of plant resistance in many plant species. For instance, production of methyl ketones by type-VI glandular trichomes in the wild tomato species *S. hirsutum* fam. *glabratum*, which are absent in the glandular trichomes of cultivated tomatoes, confers resistance to multitude of arthropod pests (Antonious *et al.*, 2005). Moreover, within tomato species, variations in the amount of glandular trichome-derived compounds are also reported (Ghosh *et al.*, 2014; Kim *et al.*, 2014). Interestingly, He *et al*. (2011) reported that the gland size of the glandular trichomes present in the leaves of an aphid-resistance chrysanthemum genotype were larger than in the susceptible genotypes. This might be associated to a higher production and storage of trichome-derived chemicals. In addition, Levin (1973) described that the trichome morphology, i.e., height, might be associated to cotton (*Gossypium hirsutum*) resistance to herbivores. Additional studies are thus needed to determine 1) whether glandular trichomes in chrysanthemum are biochemically active, 2) the compounds they produce, and 3) whether these putative compounds might confer anti-herbivory properties and explain differences in WFT susceptibility among cultivars.

PPO activity has been observed to correlate with the resistance of several plant species against herbivorous insects, e.g., in alfalfa (*Medicago sativa*) (Wei *et al.*, 2007) and in eggplant (*Solanum melongena*) (Bhattacharya *et al.*, 2009). The lack of correlation of PPO with chrysanthemum resistance to WFT might be explained by the absence of other chemical defenses. In a previous study, we reported that chlorogenic acid levels positively correlate with chrysanthemum resistance to WFT (Leiss *et al.*, 2009b). Variations in the levels of foliar chlorogenic acid, one of the main enzymatic substrates of PPO, might determine the effectivity of PPO-associated defenses. It would be interesting to perform additional chemical analysis to test the potential correlation between PPO levels and chlorogenic acid levels in future studies.

Finally, our results further showed that exogenous application of the phytohormone JA reduced WFT damage in chrysanthemum. Activation of JA signaling has been reported to confer resistance to WFT in diverse plant species (Li *et al.*, 2002; Abe *et al.*, 2009; Escobar-Bravo *et al.*, 2017; Chen *et al.*, 2018). Yet, our study constitutes the first report on the effects of JA on chrysanthemum defenses against WFT. Moreover, we showed that the effect of JA on chrysanthemum resistance to WFT was genotype-dependent (**Fig. 4A**). Phenotypic variation in the inducibility of plant defenses within plant species has been previously reported (Brody & Karban, 1992; English-Loeb *et al.*, 1998; Agrawal, 1999; Underwood *et al.*, 2000; Sauge *et al.*, 2006). This can be explained by differences in the chemical profiles and/or the presence of potentially inducible defense traits among genotypes within a plant species. In this sense, exogenous application of JA or its volatile form methyl jasmonate (MeJA) has been found to increase non-glandular and glandular trichome densities on newly formed leaves in tomato (Boughton *et al.*, 2005) and Arabidopsis (Traw & Bergelson, 2003), which in some cases resulted in an enhanced resistance to arthropod herbivores (Escobar-Bravo *et al.*, 2017). Additionally, JA is also reported to induce the expression of the defensive protein PPO, an effective defense against some herbivore species (Constabel *et al.*, 1995; Thaler *et al.*, 1996; Cipollini *et al.*, 2004; Chen *et al.*, 2018). We hypothesized that induction of these defenses might increase chrysanthemum resistance to WFT. However, our results demonstrated that, despite the positive effect of JA on non-glandular trichome density and PPO activity, the induction of these defenses could not explain the increased resistance to WFT. Furthermore, JA did not affect glandular trichome density in chrysanthemum. There might be several explanations for this: first, JA is not

associated with or it needs to cooperate with other phytohormones in glandular trichome formation in chrysanthemum (Hare & Walling, 2006; Xue *et al.*, 2018). Alternatively, chrysanthemum might need longer times (> 7 days) to generate new trichomes, as the period needed to observe changes in trichome densities in plants after herbivory or JA induction ranges from days to weeks (Dalin *et al.*, 2008). Taken together, what contributes to the differentially JA-mediated induction of chrysanthemum resistance to WFT is still unknown. Additional research to determine variations in leaf chemical responses to JA treatment among chrysanthemum cultivars might help to answer this question.

Theory predicts that constitutive defenses are negatively correlated with induced defenses in plants (Herms & Mattson, 1992). However, induced defenses did not correlate with constitutive defenses in our study (**Fig. 6C**). Moreover, some chrysanthemum genotypes displaying the lowest silver damage symptoms under control conditions (**Fig. 5A**; genotype 33) also experienced a stronger reduction in WFT-associated damage after JA application (tenfold reduction) than the most susceptible ones (e.g., genotype 80, three-fold reduction). Our findings agree with those described in in cotton (*G. hirsutum*), in which plant constitutive resistance to spider mites (*Tetranychus turkestan*) did not correlate with herbivory-induced resistance (Brody & Karban, 1992). Similar results were also obtained in soybean (*Glycine max*), where constitutive resistance to Mexican bean beetles (*Epilachna varivestis*) did not significantly correlated with herbivore-induced resistance (Underwood *et al.*, 2000). In another example, JA-mediated induced resistance to diamondback [*Plutella xylostella* (L.)] in crucifers did not correlate with their constitutive resistance to the same herbivore (Zhang *et al.*, 2009). The lack of correlation between induced and constitutive resistance in chrysanthemum suggests the possibility to breed for cultivars with high levels of both types of resistance.

In conclusion, our results showed that constitutive and induced chrysanthemum resistance to WFT varied substantially among chrysanthemum cultivars. However, neither constitutive and/or inducible levels of PPO activity and leaf trichome density can be considered as useful markers for the identification of WFT resistant genotypes. Yet, we identified chrysanthemum genotypes that displayed high basal and JA-mediated induced levels of WFT resistance, revealing the possibility to breed for both resistances.

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Supplementary materials

Table S1 Detailed statistical analysis performed for data displayed in each figure.
Fig. S1 Phenotypic variation in Western flower thrips resistance and polyphenol oxidase activity among chrysanthemum cultivars.

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Supplementary materials

Table S1 Detailed statistical analysis performed for data displayed in each figure

Figure	Panel	Statistical test	Factor and statistic value	Degree of freedom	Significance
Fig. 2	A	ANOVA	Genotype; $F = 6.59$	$df1 = 94$, $df2 = 190$	$P < 0.001$
	B	Kruskal-Wallis	Genotype; $\chi^2 = 250.1$	$df = 94$	$P < 0.001$
Fig. 3	-	Spearman correlation	$r = 0.269$; $N = 95$	-	$P = 0.008$
Fig. 4	A	Pearson correlation	$r = 0.186$, $N = 12$	-	$P = 0.564$
	B	Pearson correlation	$r = 0.118$; $N = 12$	-	$P = 0.715$
	C	Pearson correlation	$r = 0.293$; $N = 12$	-	$P = 0.355$
Fig. 5	A	GLM	Genotype; *Wald* $\chi^2 = 70.979$	$df = 5$	$P < 0.001$
			JA or Mock; *Wald* $\chi^2 = 111.912$	$df = 1$	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 33.160$	$df = 5$	$P < 0.001$
	B	GLM	Genotype; *Wald* $\chi^2 = 58.106$	$df = 5$	$P < 0.001$
			JA or Mock; *Wald* $\chi^2 = 195.343$	$df = 1$	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 25.740$	$df = 5$	$P < 0.001$
	C	GLM	Genotype; *Wald* $\chi^2 = 261.895$	$df = 5$	$P < 0.001$
			JA or Mock; *Wald* $\chi^2 = 5.034$	$df = 1$	$P = 0.025$
			Interaction; *Wald* $\chi^2 = 12.661$	$df = 5$	$P = 0.027$
	D	GLM	Genotype; *Wald* $\chi^2 = 1036.121$	$df = 5$	$P < 0.001$
			JA or Mock; *Wald* $\chi^2 = 0.010$	$df = 1$	$P = 0.922$
			Interaction; *Wald* $\chi^2 = 4.057$	$df = 5$	$P = 541$
Fig. 6	A	Pearson correlation	$r = -0.619$; $N = 6$	-	$P = 0.190$
	B	Pearson correlation	$r = -0.118$; $N = 6$	-	$P = 0.824$
	C	Pearson correlation	$r = -0.037$; $N = 6$	-	$P = 0.944$
Fig. S1	A	Kruskal-Wallis	Genotype; $\chi^2 = 48.8$	$df = 11$	$P < 0.001$
	B	ANOVA	Genotype; $F = 3.32$	$df1 = 11$, $df2 = 48$	$P = 0.002$

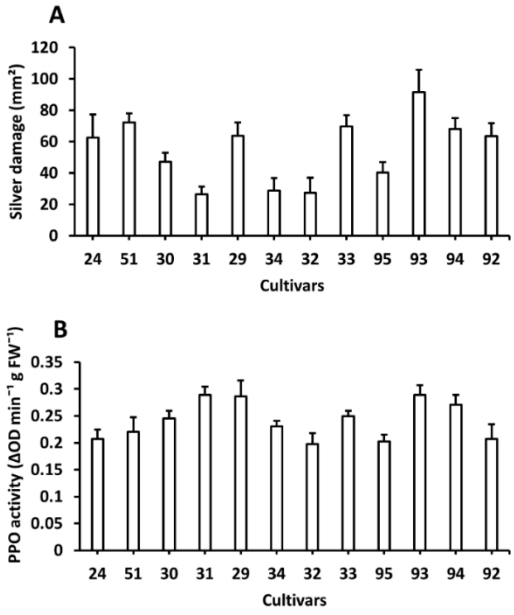

Fig. S1 Phenotypic variation in Western flower thrips resistance and polyphenol oxidase activity among chrysanthemum cultivars. (**A**) Silver damage symptoms (mean ± SEM, n = 10) and (**B**) polyphenol oxidase (PPO) activity (mean ± SEM, n = 5) were determined in 12 different chrysanthemum cultivars. Plants were sampled for PPO activity measurement or used for non-choice whole plant bioassays at 35 days after planting. Western flower thrips (WFT) leaf damage ('silver damage') was determined at 7 days after WFT infestation.

Chapter 5

Site-dependent induction of jasmonic acid-associated chemical defenses against Western flower thrips in chrysanthemum

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Plants have evolved numerous inducible defense traits to resist or tolerate herbivory, which can be activated locally at the site of the damage, or systemically through the whole plant. Here we investigated how activation of local and systemic chemical responses upon exogenous application of the phytohormone jasmonic acid (JA) varies along the plant canopy in chrysanthemum, and how these responses correlate with resistance to Western flower thrips (WFT). Our results showed that JA application reduced WFT damage per plant when applied to all the plant leaves or when locally applied to apical leaves, but not when only basal leaves were locally treated. Local application of JA to apical leaves resulted in a strong reduction in WFT damage in new leaves developed after the JA application. Yet, activation of a JA-associated defensive protein marker, polyphenol oxidase, was only locally induced. Untargeted metabolomics analysis further showed that JA increased the concentrations of sugars, phenylpropanoids, flavonoids and some amino acids in locally induced basal and apical leaves. However, local application of JA to basal leaves barely affected the metabolomics profiles of systemic non-treated apical leaves, and vice versa. Our results suggest that JA-mediated activation of systemic chemical defense responses is spatially variable and depends on the site of the application of the hormone in chrysanthemum.

Keywords: chrysanthemum, *Frankliniella occidentalis*, jasmonic acid, local and systemic induced defenses, metabolomics

This Chapter has been submitted to Planta.

1 Introduction

Plants defend themselves against herbivory by employing a plethora of physical and chemical arsenals. Chemical defenses can exert repellent, anti-nutritive, and/or toxic effects on herbivores, or attract their natural enemies (Howe & Jander, 2008). Physical defenses, such as leaf toughness and trichomes, can also increase plant fitness by negatively affecting herbivore performance and preference. Furthermore, these plant defenses can be classified according to their differential regulation as constitutive or inducible defenses (Agrawal & Karban, 1999). Constitutive defenses are defined as morphological or chemical-based defensive traits that are always expressed in the plant, irrespective of herbivore attack (Agrawal, 2007). Induced plant defenses, however, can be physical- or chemical-related traits that are initiated or elevated upon herbivory (Agrawal, 2007). Plant inducible defense responses to herbivory are mainly modulated by the phytohormones jasmonic acid (JA), salicylic acid (SA) and ethylene (Bari & Jones, 2009; Smith *et al.*, 2009). In general, chewing-biting and cell-content herbivores, certain phloem feeders and necrotrophic pathogens activate the JA signaling pathway (Walling, 2000; Glazebrook, 2005; Lazebnik *et al.*, 2014), while the SA pathway is generally activated by biotrophic pathogens and phloem feeders (Walling, 2000; De Vos *et al.*, 2005; Glazebrook, 2005).

Induction of plant defenses by herbivory can occur locally at the site of attack and systemically in undamaged parts of the plant located at a substantial distance from the challenged area (Pieterse *et al.*, 2014). Although these defense responses have been reported to occur within minutes in both local and systemic tissues, they often vary in their magnitude, space and time within and among plant species. This variation can be explained by the genetic background, the development plasticity, transmission of long-distance signals, and the vascular architecture of the plant (Van Dam *et al.*, 2001; Arnold & Schultz, 2002; Arimura *et al.*, 2004; Orians, 2005; Howe & Jander, 2008). For example, glucosinolates, which are important chemical defenses against biotic stresses, are reported to be locally and systemically induced by herbivore feeding in tap and lateral roots of several *Brassica* species, but not in fine roots (Tsunoda *et al.*, 2018). This has been explained by the capacity of plants to increase the protection of tissues that contribute most to plant fitness, such as primary roots. Importantly, these variations can affect herbivore distribution along the plant canopy, and modulate plant-mediated interactions among different herbivore species (Lee *et al.*, 2017).

Knowledge about variation in induced defenses against insect herbivores is important to develop strategies for plant protection in agri- and horticulture. In both Western flower thrips (WFT) [*Frankliniella occidentalis,* (Pergande)] is one of the most important insect pests (Steenbergen *et al.*, 2018). WFT feeding damage on flowers, fruits and plant leaves can reduce growth and yield, and affect product appearance and quality (De Jager, C *et al.*, 1995; de Jager, KM *et al.*, 1995). Western flower WFT infestation activates the JA signaling pathway in Arabidopsis (*Arabidopsis thaliana*) (Abe *et al.*, 2008; Abe *et al.*, 2011), turnip (*Brassica rapa*) (Abe *et al.*, 2009), and tomato (*Solanum lycopersicum*) (Li *et al.*, 2002; Escobar-Bravo *et al.*, 2017). Activation of JA-associated defenses play a prominent role in plant resistance against this pest (Steenbergen *et al.*, 2018). Previous experiments carried out in our laboratory have shown that exogenous application of JA enhances resistance against WFT in chrysanthemum [*Chrysanthemum* × *morifolium* Ramat. (Asteraceae)] as well (see Chapter 4 in this thesis). However, when this phytohormone was locally applied on basal chrysanthemum leaves it did not seem to have a significant effect on WFT resistance (Chen *et al*. unpublished data), suggesting possible constraints in the induction of systemic defenses against this pest.

Here we have investigated whether local and systemic chemical defense responses to the exogenous application of JA vary along the plant canopy in chrysanthemum. In addition, we have determined whether a differential JA-mediated induction of local and systemic chemical responses correlates with WFT susceptibility. For this, we have conducted insect bioassays to determine the effects of local and systemic JA application on WFT-associated feeding damage along the plant canopy. In addition, we have determined the activation of JA signaling upon local or systemic application of JA by analyzing the induction levels of a JA-responsive defensive protein marker, polyphenol oxidase (Thaler *et al.*, 1999). Finally, we have performed a comprehensive non-targeted metabolomic analysis to determine how JA application affects chrysanthemum chemical defenses upon local or systemic induction. Our study offers a comprehensive analysis of induced chemical defenses in chrysanthemum, one of the most important cultivated ornamental species for which WFT represent one of the most damaging insect pests affecting their production worldwide.

2 Materials and Methods

2.1 Plant material and insects

Chrysanthemum [*Chrysanthemum* × *morifolium* Ramat. (Asteraceae)] cuttings (cv. Baltica) were provided by Deliflor Chrysanten (Maasdijk, The Netherlands). The cuttings were individually planted in small plastic trays (2 cm × 2 cm) filled with potting soil and placed in a climate room provided with 20°C, 70% RH, 113.6 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) and L16:D8 photoperiod. At 10 days after planting, plants were transplanted to plastic pots (9 cm × 9 cm × 10 cm) containing the same potting soil.

The Western flower thrips (WFT) (*Frankliniella occidentalis*) [Pergande] were maintained on chrysanthemum flowers (cultivar Euro Sunny) in a climate room at 23°C, 60% RH and L12:D12 photoperiod.

2.2 Experimental design

To determine the effect of jasmonic acid (JA) on the induction of local and systemic chemical defenses against WFT we carried out the following induction treatments (Fig. 1): (1) application of JA or mock solution to all the plant leaves, (2) local application of JA or mock solution to leaves 4 and 5 from the bottom (basal leaves), or (3) local application of JA or mock solution to leaves 9 and 10 from the bottom (apical leaves). Leaves were sprayed with 3 mM of JA (Cayman, Ann Arbor, Michigan, USA) in 0.8% aqueous ethanol solution as described in Redman *et al*. (2001). Control plants were sprayed with 0.8% aqueous ethanol (mock) solution. Mock- and JA-treated plants were placed in separate climate rooms for 45 min after the treatment. Thereafter, both control and JA-treated plants were randomly placed in a climate room at 20°C, 70% RH, 113.6 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) and L16:D8 photoperiod. At 7 days after JA or mock solutions application, basal (4-5) and apical (9-10) leaves of 5 plants per treatment were sampled for metabolomics analyses by NMR, and leaves 5, 6, 8, 9, 13 and 14 of 5 plants of each treatment were sampled for polyphenol oxidase (PPO) activity. The remaining plants were subjected to non-choice whole-plant thrips bioassays (see below).

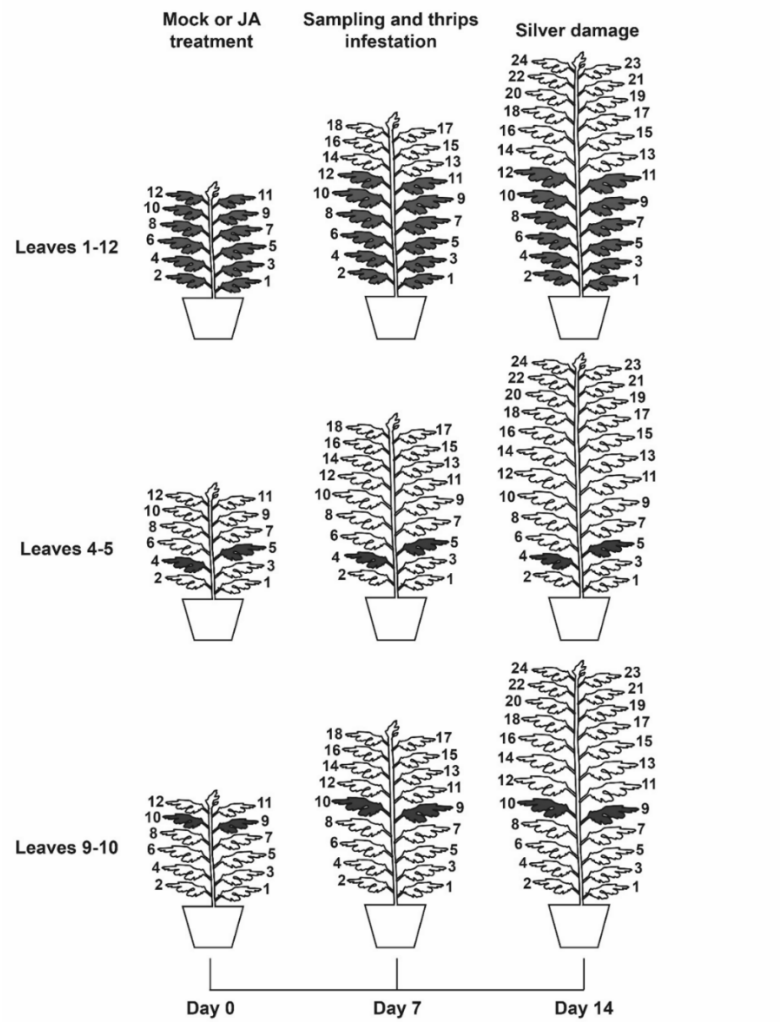

Fig. 1 Schematic representation of the experimental design. Jasmonic acid (JA) or mock solutions were applied to (1) all leaves, (2) basal leaves (4-5) or (3) apical leaves (9-10) of chrysanthemum plants at day 0. Seven days after the hormone treatments, JA- and mock-treated plants (n = 5) were sampled for determination of polyphenol oxidase (PPO) activity on leaves 5, 6, 8, 9, 13 and 14 from the bottom. Another set of plants were sampled for NMR analysis on leaves 4-5 and 9-10 from the bottom (n = 5). The remaining plants (n = 10 per treatment) were infested with Western flower thrips (WFT). Evaluation of WFT feeding damage ('silver damage') was carried out at 7 days after WFT infestation (day 14). The leaves filled with black were treated with JA or mock solutions on day 0.

2.3 Non-choice whole plant thrips bioassay

Plants were individually placed into WFT-proof cages as described in Leiss *et al*. (2009a) (n = 10 for each treatment). Ten adult WFT (8 females and 2 males) were added to each plant. All cages were randomly placed in a climate room provided with 113.6 µmol photons m^{-2} s^{-1} of PAR, 16L:8D of photoperiod, 25°C and 70% RH. Seven days after WFT infestation, WFT-associated feeding damage (hereafter referred as 'silver damage') was evaluated in all the leaves of the plant and expressed as the damaged area in mm^2 per plant or the silver damage

caused by WFT in four groups of leaves: i.e. leaf 1-6, leaf 7-12, leaf 13-18 and leaf 19-24 from the bottom.

2.4 Determination of polyphenol oxidase activity

Polyphenol oxidase (PPO) activity was determined following the methodology described in Stout *et al*. (1998). Briefly, 0.150 g of leaf tissue was flash-frozen in liquid nitrogen, ground in a tissue lyser (Qiagen, Hilden, Germany), and homogenized in a 2 ml tube with 1.25 ml ice-cold 0.1 M pH 7.0 phosphate buffer containing 7% polyvinyl-polypyrrolidone and 0.4 ml of 10% Triton X-100. The homogenate was vortexed for 2 min and centrifuged for 10 min at 11,000 g at 4ºC. Five microliters of the extract were added to 1 ml of 2.92 mM chlorogenic acid solution in pH 8.0 potassium phosphate buffer. The optical density (OD) at 470 nm was recorded in a spectrophotometer (UV-1800, Shimadzu) every 10 sec for one minute. PPO activity was calculated as the increment of OD values per min per gram of fresh weight.

2.5 Nuclear Magnetic Resonance (NMR) analysis

NMR analysis was performed on basal (leaves 4 and 5) and apical (leaves 9 and 10) leaves at 7 days after the hormone or mock treatments (n = 5). Leaves 4 and 5, and 9 and 10, were pooled prior to analysis. Plant material was freeze-dried and ground using a tissue lyser (Qiagen, Hilden, Germany). Twenty milligrams of fine powder were extracted with 1.5 ml of 80% methanol-*d4* in KH_2PO_4 buffer (90 mM, pH = 6.0) containing 0.02% (w/v) trimethyl silyl-3-propionic acid sodium salt-*d4* (TMSP). Plant extracts were vortexed for 1 min, ultra-sonicated for 15 min and centrifuged at 13,000 rpm for 15 min at room temperature. Eight hundred microliters of the supernatant were transferred to the NMR tubes for analysis. The ^{1}H NMR spectra were acquired using a 600 MHz Bruker AV-600 spectrometer equipped with cryo-probe operating at a proton NMR frequency of 600 MHz at 25°C, as described in López-Gresa *et al* (2012). Deuterated methanol served as internal lock. ^{1}H NMR spectrum consisted of 128 scans requiring 10 min acquisition time with a digital resolution of 0.25 Hz/point, a pulse angle of 30° (10.8 μs), and a recycle delay of 1.5 s per scan. A pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the H_2O frequency during the recycle delay. Spectra were Fourier transformed with a 0.3 Hz line broadening and zero-filled to 32 K points. Phase and baseline correction of the resulting spectra were done manually, followed by a calibration to TMSP at 0.00 ppm using Topspin (version 2.1, Bruker). ^{1}H NMR spectra was then converted and saved as ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to the intensity of the internal standard TMSP and reduced to integrated regions, referred to as buckets, of equal width (0.04 ppm) corresponding to the region of δ 10.0-0.2. The Regions in the range of δ 5.0-4.7 and δ 3.34-3.28, corresponding to water and methanol, respectively, were removed prior to statistical analyses.

2.6 Statistical analysis

Statistical analyses were performed using the SPSS software package (version 23; SPSS Inc., Chicago, IL, USA). Normality and homogeneity of the residuals were first checked using Kolmogorov-Smirnov and Levene's tests, respectively. Differences in silver damage symptoms per plant between JA- and mock-treated plants were analyzed by student-t tests. Effects of the main factors JA and groups of leaves based on their position in the plant (1-6, 7-12, 13-18 and 19-23 from the bottom) and their interaction on silver damage symptoms were analyzed by Generalized Linear Models (GLMs) using linear distribution and identity link function. Differences in PPO activity among leaves 5, 6, 8, 9, 13 and 14 detected in JA and mock-treated plants were analyzed by GLMs using linear distribution and identity link

function. Data on silver damage and PPO activity determined in plants receiving local application or systemic JA application were Log-transformed prior to analysis. Effects of JA, leaf position (4-5 and 9-10) and their interaction on levels of metabolites identified in the NMR analysis were analyzed by GLMs using linear distribution and identity link function. Differences among groups were tested by Fisher's least significant difference (LSD) post-hoc test. Patterns of chemical shifts detected by NMR in leaves 4-5 and 9-10 of mock- and JA-treated plants were analyzed by Partial Least Squares Discriminant analysis (PLS-DA) using the SIMCA-P 15 software package (Umetrics, Sweden). This analysis determines the variation in X variables (chemical shifts) modeled by the Y explanatory variable, i.e. mock and JA solution application on basal (leaves 4 and 5), apical (leaves 9 and 10) or on all leaves. The final model was selected according to the minimum number of latent variables showing the highest predicted variation in Y (Q^2). The chemical shifts with a variable importance in projection (VIP) > 1 were selected as the important X variables. Detailed statistical results are shown in Supplementary Table S1, S2 and S3.

3 Results

3.1 Systemic or local application of JA to apical leaves, but not local application to basal leaves, reduces silver damage per plant

Application of JA to all the leaves of chrysanthemum plants significantly reduced silver damage symptoms per plant (**Fig. 2A**, Table S1; student *t*-test, $P < 0.05$). This reduction was statistically significant for leaves 1-6, 7-12 and 13-18 (**Fig. 2B**). Local application of JA to basal leaves (4-5) did not significantly reduce the silver damage per plant (**Fig. 2C**; student *t*-test, $P = 0.592$), although there was a significant reduction in leaves 13-18 compared to their controls (**Fig. 2D**). Local application of JA on apical leaves (9-10) significantly reduced the silver damage symptoms per plant (**Fig. 2E**; student *t*-test, $P = 0.038$). This reduction was significant for leaves 13-18 and, although not significant, also evident for leaves 7-12. (**Fig. 2F**). Overall, silver damage symptoms were higher in leaves 7-12 and 13-18 compared to leaves 1-6 and 19-24 (**Fig. 2B, D,** and **F**).

3.2 JA induces polyphenol oxidase activity in local but not in systemic leaves

When JA was applied to all the leaves of chrysanthemum plants, PPO activity was significantly induced in leaves 5, 6, 8, 9 and 13, but not in leaf 14, at 7 days after the JA treatment (**Fig. 3A**, Table S1). Application of JA to basal leaves (4-5) significantly increased PPO activity in leaf 5, while in the other leaves there was a very small and non-significant increase (**Fig. 3B**). Likewise, application of JA to the apical leaves (9-10) induced PPO locally, i.e. on leaf 9, but not in non-treated leaves (**Fig. 3C**). Notably, PPO activity levels were higher in the youngest leaves (13 and 14) in both mock- and JA-treated plants.

3.3 JA effects on the leaf metabolome are locally but not systemic

All leaves treated with JA. A total of 246 signals were detected in the ^{1}H NMR analysis of leaves corresponding to mock- and JA-treated chrysanthemum plants. PLS-DA analysis of the metabolomics profiles of basal (4-5) and apical (9-10) leaves of plants from which all the leaves were treated with JA or mock solutions resulted in a model with five latent variables (LVs). This model explained 75.5% of the total metabolomics variation and 95.5% of the treatment variation, with a 77.3% total model predictability (model statistics: $R^2X = 0.755$, $R^2Y = 0.951$ and $Q^2 = 0.773$; CV-ANOVA, $P < 0.001$) (Fig. **4A**). The first LV separated JA-treated basal leaves (4-5) from JA-treated apical leaves (9-10) and mock-treated basal and apical leaves, explaining 40.1% of the metabolomic variation. The second LV explained 15.8% and separated basal leaves (4-5) from apical leaves (9-10) of both treatments. Differences

among treatments were mainly explained by 101 signals with variable importance for projection (VIP) scores higher than 1 (Fig. **4B** and Fig. S1). Among these, fourteen signals were identified corresponding to sugars (fructose, glucose and sucrose), amino acids (valine, threonine, alanine, arginine, glutamine, asparagine, adenine), organic acids (citric acid), phenylpropanoids (chlorogenic acid, 3,5-dicaffeoylquinic acid) and flavonoids (luteolin 7-*O*-glucoside). JA application significantly increased the levels of sucrose, glucose, threonine, asparagine, phenypropanoids (chlorogenic acid and 3,5-dicaffeoylquinic acid), the flavonoid luteolin 7-*O*-glucoside and camphor, and it reduced the levels of citric acid in basal leaves (4-5) at 7 days after the hormone treatment (**Fig. 4C**, Table S2 and S3). Overall JA application affected the metabolomics profile of apical (9-10) leaves less strongly than those of basal leaves, but a significant reduction in the leaf content of some amino acids (alanine and glutamine) and a significant induction of adenine, and phenylpropanoids (chlorogenic acid and 3,5-dicaffeoylquinic acid) were observed (**Fig. 4C**, Table S2 and S3). Apical leaves (9-10) showed lower levels of sugars (fructose and sucrose) and phenylpropanoids (chlorogenic acid and 3,5-dicaffeoylquinic acid), and higher levels of amino acids (valine, threonine, alanine, arginine, and adenine) than basal (4-5) leaves, independently of the treatment.

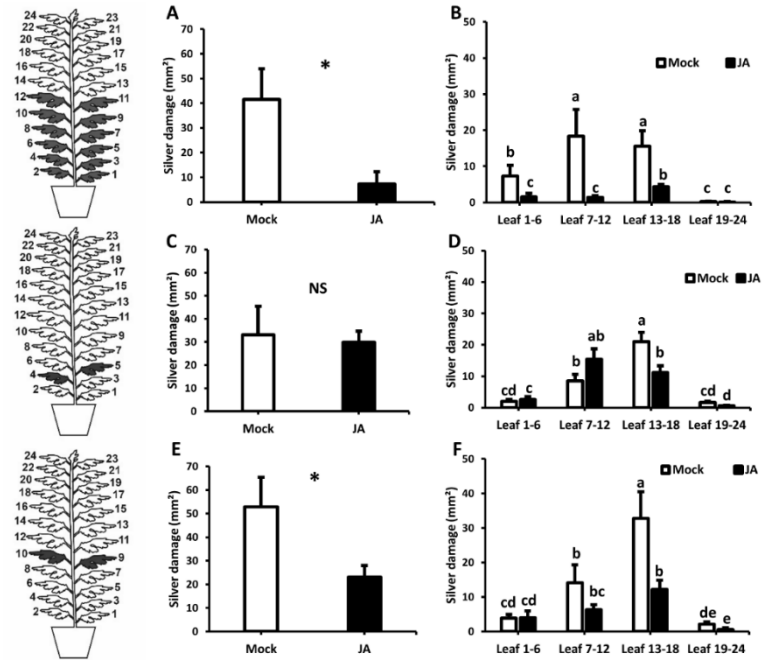

Fig. 2 Effect of systemic and local JA treatment on chrysanthemum resistance to Western flower thrips. Silver damage symptoms (mean ± SEM, n = 10) were determined for the whole plant or separately in four groups of leaves along the plant canopy in mock- and jasmonic acid (JA)-treated plants at 7 days after Western flower thrips infestation. Mock or JA solutions were applied to all the plant leaves (**A** and **B**), basal leaves (4-5 from the bottom; **C** and **D**) or to apical leaves (9-10 from the bottom; **E** and **F**). Asterisks denote significant differences determined by unpaired t-test at $P \leq 0.05$. Different letters indicate significant differences among groups compared by Fisher's LSD test at $P \leq 0.05$. n.s. = not significant.

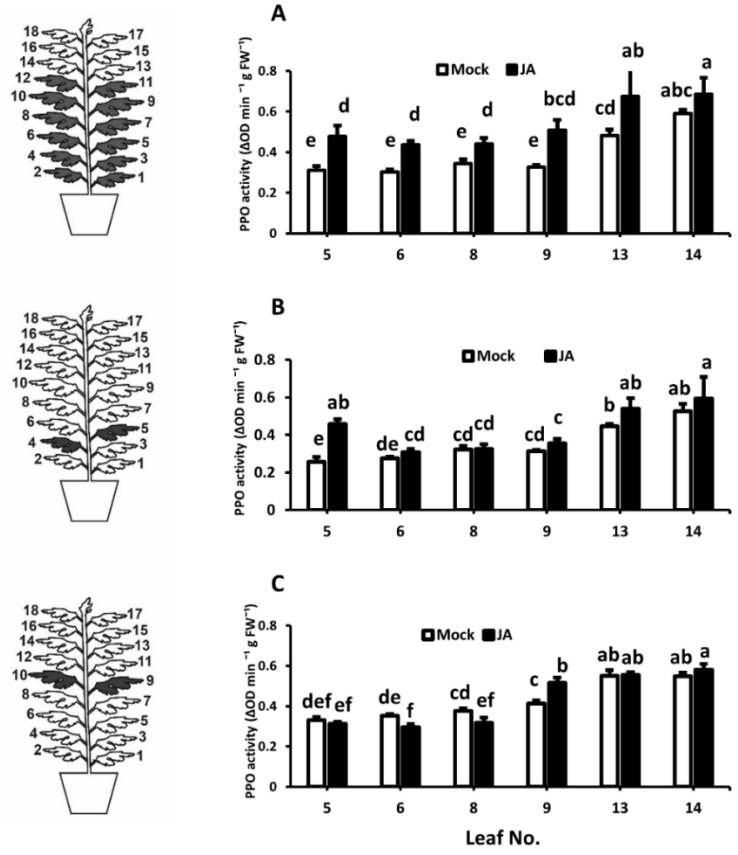

Fig. 3 Effect of systemic and local JA induction on polyphenol oxidase activity in chrysanthemum. Polyphenol oxidase (PPO) activity (mean ± SEM, $n = 5$) was determined on leaf 5, 6, 8, 9, 13 and 14 from the bottom of mock- and jasmonic acid (JA)-treated plants. Mock or JA solutions were applied to all plant leaves (**A**), basal leaves (**B**) or apical leaves (**C**). Plants were sampled at 7 days after the hormone treatments. Different letters indicate significant differences among groups compared by Fisher's LSD test at $P \leq 0.05$.

Basal leaves treated with JA. When plants were treated locally with JA on basal leaves (4-5), the metabolomic responses to the hormone treatment were only evident in those local leaves, while barely altering the chemistry of systemic apical leaves (9-10) (**Fig. 5A**). The PLS-DA analysis resulted in a model with three LVs explaining 63.1% of the total metabolomic variation and 80.9% of the treatment response, with a 49.4% total model predictability ($R^2X = 0.631$, $R^2Y = 0.809$ and $Q^2 = 0.494$; CV-ANOVA, $P = 0.025$). The first LV explained 42.7% of the variance and separated JA-treated basal leaves (4-5) from apical leaves (9-10) of mock- and JA-treated plants. The second LV explained 14.8% of the variance and separated mock-treated basal leaves (4-5) from the other leaves. These differences were mainly explained by 125 signals with VIP scores higher than 1 (**Fig. 5B** and Fig. S2). JA application to basal leaves (4-5) reduced the levels of sugars (fructose, glucose and sucrose) and the amino acid glutamine, while increasing the levels of the amino acid arginine, phenolic acids and flavonoids in these leaves (**Fig. 5C**, Table S2 and S3). No significant differences

in the levels of these compounds were observed for the apical leaves, except for a slight but significant reduction in citric acid and alanine levels (Table S2 and S3).

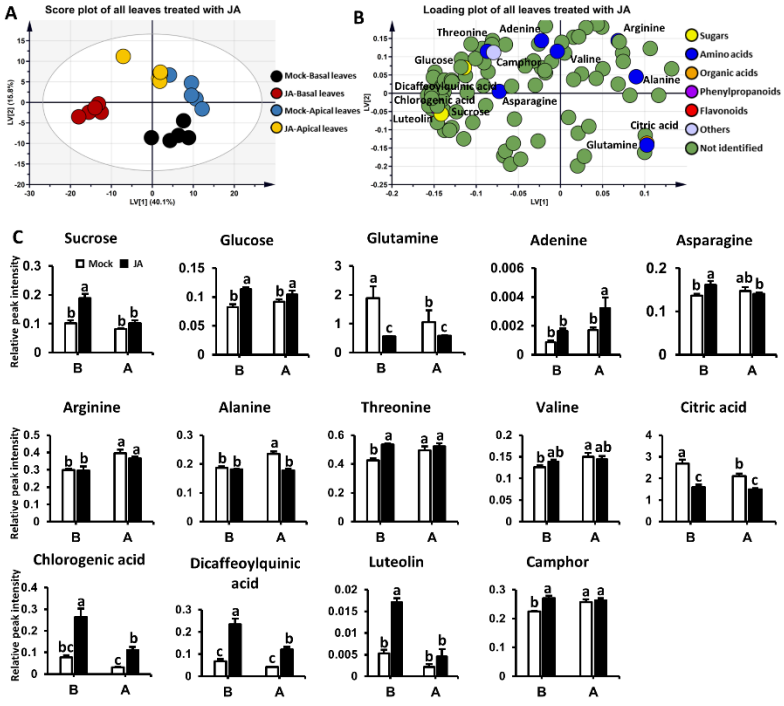

Fig. 4 Metabolic responses of basal and apical chrysanthemum leaves to systemic application of JA to all the plant leaves. Leaf metabolites were analyzed by NMR in basal (leaf 4-5) and apical (leaf 9-10) leaves of chrysanthemum plants at 7 days after the application of mock or jasmonic acid (JA) solutions to all plant leaves. Partial least square-discriminant analysis (PLS-DA) was performed on the obtained ^{1}H NMR spectra (n = 5). (**A**) Score plot showing the first two latent variables (LVs). The ellipse represents the Hotelling T2 with 95% confidence. (**B**) Loading plot showing important chemical shifts that contribute most to the model (variable importance in projection, VIP > 1). The identified compounds are shown in the plot. (**C**) Relative peak intensity (mean ± SEM, n = 5) of the identified compounds in basal (B) and apical (A) leaves of mock- and JA-treated plants are shown. Different letters indicate significant differences among groups compared by Fisher's LSD test at $P \leq 0.05$.

Apical leaves treated with JA. Finally, plants treated locally with JA on apical (9-10) leaves also displayed local metabolomic responses at 7 days after the hormone induction (**Fig. 6A**). The PLS-DA analysis resulted in a model with four LVs explaining 61.0% of the total metabolomic variation and 91.3% of the treatment response, with a 68.2% total model predictability (R^2X = 0.610, R^2Y = 0.913 and Q^2 = 0.682; CV-ANOVA, P = 0.018). The first LV explained 25.5% of the variance and separated basal leaves (4-5) from apical (9-10) leaves regardless of the hormone treatment. The second LV explained 23.0% of the variance and separated the JA-treated apical leaves from their controls. No clear metabolic separation was observed between basal leaves of mock- and JA-treated plants. Differences among treatments were explained by 100 signals with VIP scores higher than 1 (**Fig. 6B** and Fig. S3). JA significantly reduced the levels of fructose, glutamine and citric acid, and it increased the levels of glucose, adenine, phenylpropanoids (chlorogenic acid and 3,5-dicaffeoylquinic acid) and the flavonoid luteolin-7-*O*-glucoside in apical leaves (9-10). Notably, although the

local application of JA to apical leaves barely affected the overall metabolomic profiles of basal leaves, significant lower levels of sugars (fructose and sucrose), some amino acids (valine, alanine, and glutamine), camphor and *myo*-inositol were observed (**Fig. 6C**, Table S2 and S3). Levels of sugars (fructose, glucose and sucrose), phenylpropanoids (chlorogenic acid 3,5-dicaffeoylquinic acid), the flavonoid luteolin-7-*O*-glucoside and some amino acids (glutamine) were significantly lower in mock-treated apical leaves when compared to mock-treated basal leaves, while levels of some amino acids (threonine, alanine and arginine) were significantly higher (Table S2 and S3).

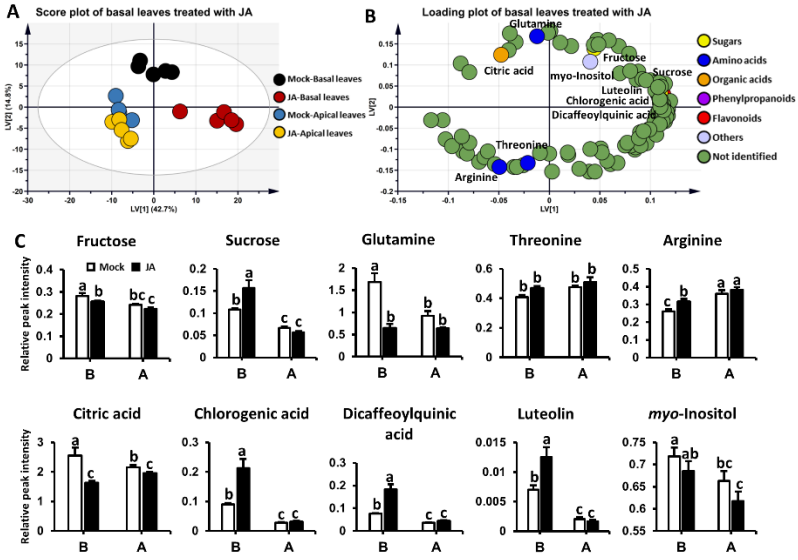

Fig. 5 Metabolomic responses of basal and apical chrysanthemum leaves to local application of JA to basal leaves. Leaf metabolites were analyzed by NMR in basal (leaf 4-5) and apical (leaf 9-10) leaves of chrysanthemum plants at 7 days after the local application of mock or jasmonic acid (JA) solutions to basal leaves (4-5 from the bottom). Partial least square-discriminant analysis (PLS-DA) was performed on the obtained ^{1}H NMR spectra (n = 5). (**A**) Score plot showing the first two latent variables (LVs). The ellipse represents the Hotelling T2 with 95% confidence. (**B**) Loading plot showing important chemical shifts that contribute most to the model (variable importance in projection, VIP > 1). The identified compounds are shown in the plot. (**C**) Relative peak intensity (Mean ± SEM, n = 5) of the identified compounds in basal (B) or apical (A) leaves of mock- and JA-treated plants are shown. Different letters indicate significant differences among groups compared by Fisher's LSD test at $P \leq 0.05$.

4 Discussion

In this study we have demonstrated that JA-mediated induction of local and systemic chemical defense responses varies along the plant canopy in chrysanthemum. We showed that either systemic or local application of JA to apical, but not to basal leaves, increased systemic plant resistance against the Western flower thrips (WFT) *Frankliniella occidentalis*. Variation in the systemic induction of chemical defense was not explained by the vertical alignment of local and systemic leaves, and thus their direct vascular connections, nor differences in the responses to the hormone treatment between apical and basal leaves. On the contrary, levels of constitutive and inducible chemical defenses were higher in basal leaves.

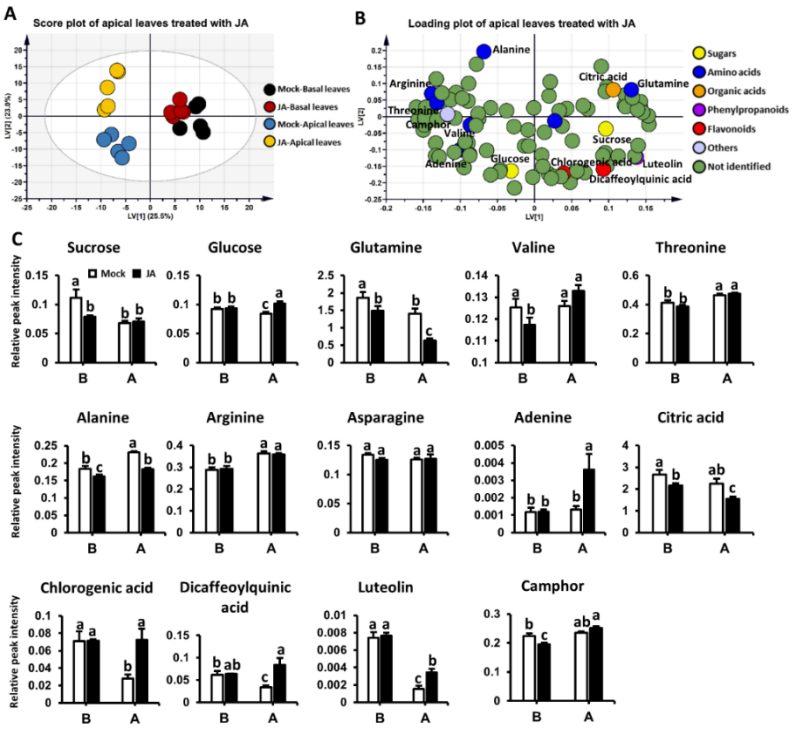

Fig. 6 Metabolomic responses of basal and apical chrysanthemum leaves to local application of JA to apical leaves. Leaf metabolites were analyzed by NMR in basal (leaf 4-5) and apical (leaf 9-10) leaves of chrysanthemum plants at 7 days after the local application of mock or jasmonic acid (JA) solutions to basal leaves (9-10 from the bottom). Partial least square-discriminant analysis (PLS-DA) was performed on the obtained ^{1}H NMR spectra (n = 5). (**A**) Score plot showing the first two latent variables (LVs). The ellipse represents the Hotelling T2 with 95% confidence. (**B**) Loading plot showing important chemical shifts that contribute most to the model (variable importance in projection, VIP > 1). The identified compounds are shown in the plot. (**C**) Relative peak intensity (Mean ± SEM, n = 5) of the identified compounds in basal (B) or apical (A) leaves of mock- and JA-treated plants are shown. Different letters indicate significant differences among groups compared by Fisher's LSD test at $P \leq 0.05$.

Our results first showed that WFT-associated feeding damage differed along the plant canopy in chrysanthemum, being basal leaves (leaves 1-6 from the bottom) more resistant than medium-apical leaves (leaves 7-12 and 13-18) (Fig. 2B, D and F). Likewise, van Haperen *et al*. (2019) described a higher susceptibility in young/apical leaves of a WFT-susceptible accession of sweet pepper (*Capsicum* spp.). These results, however, differ from previous studies reporting higher WFT susceptibility in basal/old leaves of *Rhododendron simsii* (Scott-Brown *et al.*, 2016), tomato (*S. lycopersicum*) (Chen *et al.*, 2018), *Senecio* (Leiss *et al.*, 2009a) and in a resistant accession of sweet pepper (van Haperen *et al.*, 2019). This might be explained by differences in nutrients and defense-related metabolites and their distribution within the plant, as these components are important determinants of herbivore performance (Behmer *et al.*, 2002; Köhler *et al.*, 2015).

Exogenous application of JA has been previously reported to confer plant resistance against WFT in Arabidopsis (Abe *et al.*, 2008), cabbage (Abe *et al.*, 2009), tomato (Escobar-Bravo *et al.*, 2017) and chrysanthemum (see chapter 4 in this thesis). Here we showed that

silver damage symptoms were significantly reduced in the chrysanthemum plants when all the leaves were treated with JA. It should be noted that leaves that were developing during WFT infestation (leaf 19-24) were barely damaged independently of the treatment (Fig. 2B), probably because their small size, a shorter period exposed to WFT and/or they were better defended. We further showed that local JA application to basal leaves (4-5) did not affect WFT resistance in systemic apical leaves (7-12), and it slightly reduced silver damage in leaves 13-18 only. Conversely, local JA induction of apical leaves (9-10) strongly reduced WFT damage in these and the adjacent leaves developed after the induction (13-18), which contributed most to the overall reduction in silver damage per plant (Fig. 2E, F). Systemic induction of defenses against pathogens and herbivores has been amply studied in different plant species (Hilleary & Gilroy, 2018). For instance, Cohen *et al* (1993) showed that local application of JA to basal tomato leaves triggered systemic resistance to a fungal pathogen. We thus hypothesized that diminished systemic induction of resistance against WFT when JA is locally applied to basal chrysanthemum leaves might be explained by a lower capacity of these leaves to respond to the hormone treatment and, therefore, activate JA signaling.

In a first attempt to investigate whether basal and apical leaves differ in their responses to JA, we determined the induction of the JA-associated marker enzyme polyphenol oxidase (PPO) along the chrysanthemum canopy. First, our results showed that PPO activity levels were higher in apical leaves (13-14) than in basal leaves (5-9) of mock-treated plants. Augmented PPO activity levels have been reported to confer enhanced plant resistance to arthropod herbivores (Wang & Constabel, 2004; Mahanil *et al.*, 2008). As young apical chrysanthemum leaves are more susceptible to WFT, our results suggest that differences in constitutive levels of PPO within the plant canopy might not explain the degree of susceptibility to WFT. Application of JA to all the plant leaves increased PPO levels in basal and apical leaves (5-9) (Fig. 3A), suggesting that both groups are responsive to the hormone treatment. Interestingly, application of JA to basal or apical leaves induced PPO levels only locally. This is in strong contrast with previous studies in tomato, where the activity of this defense-related protein has been reported to be induced in systemic leaves after local wounding, JA application or herbivory (Stout *et al.*, 1994; Stout *et al.*, 1996).

Despite the lack of systemic induction of PPO after local application of JA, we did observe an increased resistance to WFT in systemic leaves (Fig. 2C, D). Thus, we further investigated whether JA affected other local and systemic chemical defenses against WFT by analyzing changes in the leaf metabolome. First, our results showed that constitutive chemical defenses of basal and apical leaves significantly differed (Fig. 4). Basal (4-5) leaves of mock-treated plants contained higher levels of phenolics (i.e. chlorogenic acid, dicaffeoylquinic acid and flavonoid luteolin-7-*O*-glucoside), organic acids and sugars (sucrose and glucose), while levels of amino acids were overall reduced, when compared to apical (9-10) leaves. Essential amino acids like threonine and arginine are important nitrogen sources for herbivore growth and development (Chen *et al.*, 2005). Reduced concentrations of these primary metabolites might explain why basal leaves were less preferred by WFT. Furthermore, enhanced levels of phenolics and sugars might have contributed to the higher levels of WFT resistance observed in basal leaves. Both chlorogenic acid and caffeoylquinic acid have been reported to contribute to WFT resistance in chrysanthemum (Leiss *et al.*, 2009b). Similarly, enhanced levels of the flavonoid luteolin have been associated to WFT resistance in carrot (*Daucus carota* L.) (Leiss *et al.*, 2013). Sugars, including glucose, fructose and sucrose, are reported to be involved in plant development and defenses as well (Sheen *et al.*, 1999; Smeekens *et al.*, 2010; Trouvelot *et al.*, 2014), as they can act as signaling molecules and/or provide resources for the constitutive and inducible production of C-based compounds, such as phenolics (Arnold *et al.*, 2004; Guo *et al.*, 2013). Thus, a higher

concentration of sugars in basal leaves might have increased constitutive and hormone-mediated induced defenses. These findings, however, contradict the optimal defense theory (ODT) (McKey, 1974; Ohnmeiss & Baldwin, 2000). ODT predicts that within-plant allocation of defense-associated metabolites positively correlate with the fitness value of specific tissues. Younger leaves are generally of a greater relative fitness value than older/mature leaves (Iwasa *et al.*, 1996; Ohnmeiss & Baldwin, 2000) and they are reported to display higher levels of chemical and/or physical defenses (van Dam *et al.*, 1994; Zangerl & Rutledge, 1996; Scott-Brown *et al.*, 2016; Eisenring *et al.*, 2017; Chen *et al.*, 2018). Further comprehensive work is thus needed to evaluate the influence of these induction strategies on plant fitness in chrysanthemum.

Notably, when JA was applied to all the leaves of chrysanthemum plants, the metabolomic responses of basal (4-5) leaves were stronger than those of apical (9-10) ones (Fig. 4). JA increased the concentrations of sugars, phenylpropanoids, flavonoids and the amino acid asparagine, and reduced the levels of glutamine and citric acid in both basal and apical leaves, but these differences were slightly larger in basal leaves. Induction of phenolic acids (dicaffeoylquinic and chlorogenic acid) and luteolin by the volatile form of JA, methyl jasmonate (MeJA), has been previously reported in wild tobacco (*N. attenuata*), carrot (*Daucus carota* L.) and rice (*Oryza sativa* L.) (Keinänen *et al.*, 2001; Kong *et al.*, 2004; Heredia & Cisneros-Zevallos, 2009). Also, MeJA application has been reported to reduce the amino acid glutamine in *Arabidopsis* (Hendrawati *et al.*, 2006), which is a predominant amino acid constituent of the insect gut (Yoshinaga *et al.*, 2003). Taken together, induction of JA-associated chemical defenses (i.e. phenolics and sugars) and reduction in the nitrogen content of the hormone-treated leaves might have contributed to the observed enhanced resistance to WFT (Fig. 2A, B).

Our results also showed that local application of JA to basal leaves barely affected the metabolomic profiles of systemic apical leaves, and vice versa, at 7 days after the hormone application. This might explain why the local induction of basal leaves (4-5) did not alter WFT susceptibility in apical leaves (9-10) (Fig. 2D). Systemic defense responses are often found to be highly variable in space and time, and many studies have reported differences in local and systemic defense responses to herbivory or exogenous hormone application (Babst *et al.*, 2009; Moreira *et al.*, 2009; Lee *et al.*, 2017; Kundu *et al.*, 2018). Systemically induced resistance can be achieved by the systemic transport, through the plant vascular system, of defensive metabolites and/or signals from the induced tissues that activate *de novo* expression of resistance-associated traits (Heil & Ton, 2008). Distribution of defenses within plants is then often controlled by their vascular architecture, and the translocation of leaf compounds occurs mainly among leaves that are in an approximate vertical row (orthostichy) in many plant species (Orians, 2005). For instance, in Eastern Cottonwood (*Populus deltoides*) (Jones *et al.*, 1993), tobacco (*Nicotiana attenuata*) (Schittko & Baldwin, 2003), tomato (*Lycopersicon esculentum* Mill. `Moneymaker') (Rhodes *et al.*, 1999), cotton (*Gossypium* sp.) (Eisenring *et al.*, 2017) and Arabidopsis (Ferrieri *et al.*, 2015) leaves with direct vascular connections to the damaged leaf are reported to display stronger chemical defense inductions than leaves without these vascular connections. Our results showed that local application of JA to both basal or apical leaves increased plant resistance to WFT in leaves developed after the induction treatment (13-18) (Fig. 2C, D), albeit at different magnitudes. This suggests that they might share direct vascular connections. Alternatively, a stronger sink strength in these new developed leaves (13-18) might have attenuated the systemic responses in mature leaves (9-10). For instance, Arnold and Schultz (2002) showed that JA treatment enhanced sink strength in the developing leaves of hybrid poplar saplings, which resulted in a higher import of carbohydrates and production of

condensed tannins in those leaves. Additional analyses are needed to explore if the induced systemic resistance to WFT in leaves 13-18 correlates with increases in imported resources and chemical defenses from the adjacent leaves (9-10).

In conclusion, we showed that local and systemic induction of JA-mediated chemical defenses in chrysanthemum is spatially variable and dependent on the site of the induction. Furthermore, we showed that higher levels of constitutive and inducible defenses in basal leaves might explain the distribution of WFT-associated feeding within the chrysanthemum plant canopy. Yet, our data also demonstrate that apical leaves, which were preferred by WFT, induced a stronger systemic protection against WFT in leaves that were developed after the hormone induction, contributing most to the enhanced resistance to this insect. Our study has important implications for agriculture systems, as it highlights the variability in within-plant induction of chemical defenses in one of the most important cultivated ornamental species worldwide.

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Supplementary Materials

Table S1 Detailed statistical analysis performed for data displayed in Fig. 2 and 3.
Table S2 Relative intensity of the identified compounds detected by NMR in basal (leaf 4-5 from the bottom) and apical (leaf 9-10 from the bottom) leaves of chrysanthemum plants at 7 days after the application of mock or jasmonic acid (JA) solutions to all the plant leaves, basal or apical leaves.
Table S3 Statistical analysis of identified chemical compounds.
Fig. S1 Heatmap of important NMR signals detected in chrysanthemum basal and apical leaves of plants treated with mock or jasmonic acid (JA) in all the leaves.
Fig. S2 Heatmap of important NMR signals detected in chrysanthemum basal and apical leaves of plants treated with mock or jasmonic acid (JA) in basal leaves.
Fig. S3 Heatmap of important NMR signals detected in chrysanthemum basal and apical leaves of plants treated with mock or jasmonic acid (JA) in apical leaves.

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Supplementary Materials

Table S1 Detailed statistical analysis performed for data displayed in Fig. 2 and 3.

Figure	Panel	Statistical test	Factor and statistic value	df	*P*
Fig. 2	A	Student t-test	JA vs Mock; $t = 2.408$	18	$P = 0.027$
	B	GLM	Age group; *Wald* $\chi^2 = 68.352$	3	$P < 0.001$
			JA vs Mock; *Wald* $\chi^2 = 30.761$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 10.378$	3	$P = 0.016$
	C	Student t-test	JA vs Mock; $t = 0.545$	18	$P = 0.592$
	D	GLM	Age group; *Wald* $\chi^2 = 121.898$	3	$P < 0.001$
			JA vs Mock; *Wald* $\chi^2 = 0.798$	1	$P = 0.373$
			Interaction; *Wald* $\chi^2 = 9.285$	3	$P = 0.026$
	E	Student t-test	JA vs Mock; $t = 2.236$	18	$P = 0.038$
	F	GLM	Age group; *Wald* $\chi^2 = 81.662$	3	$P < 0.001$
			JA vs Mock; *Wald* $\chi^2 = 8.332$	1	$P = 0.004$
			Interaction; *Wald* $\chi^2 = 1.842$	3	$P < 0.606$
Fig. 3	A	GLM	Leaf age; *Wald* $\chi^2 = 83.272$	5	$P < 0.001$
			JA vs Mock; *Wald* $\chi^2 = 45.994$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 5.713$	5	$P = 0.335$
	B	GLM	Leaf age; *Wald* $\chi^2 = 113.699$	5	$P < 0.001$
			JA vs Mock; *Wald* $\chi^2 = 17.369$	1	$P < 0.001$
			Interaction; *Wald* $\chi^2 = 20.997$	5	$P = 0.001$
	C	GLM	Leaf age; *Wald* $\chi^2 = 406.573$	5	$P < 0.001$
			JA vs Mock; *Wald* $\chi^2 = 0.005$	1	$P = 0.942$
			Interaction; *Wald* $\chi^2 = 29.185$	5	$P < 0.001$

Table S2 Relative intensity of the identified compounds detected by NMR in basal (leaf 4-5 from the bottom) and apical (leaf 9-10 from the bottom) leaves of chrysanthemum plants at 7 days after the application of mock or jasmonic acid (JA) solutions to all the plant leaves, basal or apical leaves.

Categories	Compounds	Chemical shift	Hormone application											
			All leaves				Basal leaves				Apical leaves			
			Leaf 4-5		Leaf 9-10		Leaf 4-5		Leaf 9-10		Leaf 4-5		Leaf 9-10	
			Mock	JA	Mock	JA	Mock	JA	Mock	JA	Mock	JA	Mock	JA
Sugars	Fructose	4.08	0.2677± 0.0166 a	0.2882± 0.0078 a	0.2317± 0.0089 b	0.2370± 0.0107 b	0.2823± 0.0128 a	0.2561± 0.0046 b	0.2412± 0.0062 bc	0.2234± 0.0073 c	0.3193± 0.0214 a	0.2533± 0.0072 bc	0.2951± 0.0422 ab	0.2278± 0.0026 c
	Glucose	5.20	0.0825± 0.0048 b	0.1133± 0.0036 a	0.0916± 0.0045 b	0.1045± 0.0068 a	0.0819± 0.0069 b	0.1056± 0.0039 a	0.0798± 0.0037 b	0.0891± 0.0055 b	0.0924± 0.0026 b	0.0938± 0.0030 b	0.0845± 0.0030 c	0.1017± 0.0038 a
	Sucrose	5.40	0.1015± 0.0099 b	0.1882± 0.0149 a	0.0818± 0.0029 b	0.1015± 0.0097 b	0.1079± 0.0040 b	0.1567± 0.0179 a	0.0666± 0.0037 c	0.0567± 0.0023 c	0.1116± 0.0142 a	0.0789± 0.0024 b	0.0683± 0.0038 b	0.0709± 0.0056 b
Amino acids	Valine	1.04	0.1262± 0.0038 b	0.1401± 0.0036 ab	0.1497± 0.0095 a	0.1456± 0.0060 ab	0.1211± 0.0054 c	0.1269± 0.0029 bc	0.1387± 0.0076 ab	0.1433± 0.0086 a	0.1253± 0.0040 a	0.1173± 0.0034 b	0.1260± 0.0023 a	0.1329± 0.0027 a
	Threonine	1.32	0.4261± 0.0105 b	0.5343± 0.0068 a	0.4961± 0.0249 a	0.5226± 0.0220 a	0.4078± 0.0162 b	0.4707± 0.0123 a	0.4746± 0.0102 a	0.5114± 0.0312 a	0.4121± 0.0182 b	0.3872± 0.0093 b	0.4640± 0.0104 a	0.4744± 0.0070 a
	Alanine	1.48	0.1861± 0.0053 b	0.1813± 0.0019 b	0.2354± 0.0086 a	0.1773± 0.0054 b	0.1762± 0.0081 b	0.1705± 0.0036 b	0.2225± 0.0088 a	0.1824± 0.0078 b	0.1831± 0.0083 b	0.1623± 0.0043 c	0.2314± 0.0031 a	0.1821± 0.0041 b
	Arginine	1.72	0.2991± 0.0079 b	0.2974± 0.0213 b	0.3948± 0.0211a	0.3659± 0.0105 a	0.2597± 0.0137 c	0.3165± 0.0140 b	0.3603± 0.0208 a	0.3819± 0.0147 a	0.2871± 0.0122 b	0.2928± 0.0133 b	0.3634± 0.0099 a	0.3586± 0.0065 a
	Glutamine	2.44	1.8832± 0.1427 a	0.5525± 0.0451 c	1.0538± 0.1888 b	0.5830± 0.0442 c	1.6859± 0.2011 a	0.6535± 0.0844 b	0.9240± 0.1056 b	0.6381± 0.0299 b	1.8569± 0.1702 a	1.4902± 0.1378 b	1.4044± 0.1468 b	0.6249± 0.0706 c
	Asparagine	2.84	0.1363± 0.0046 b	0.1612± 0.0091 a	0.1474± 0.0087 ab	0.1405± 0.0035 b	0.1347± 0.0040 a	0.1409± 0.0056 a	0.1397± 0.0024 a	0.1361± 0.0053 a	0.1339± 0.0034 a	0.1250± 0.0032 a	0.1261± 0.0030 a	0.1273± 0.0070 a
	Adenine	8.20	0.0009± 0.0001 b	0.0016± 0.0002 b	0.0017± 0.0002 b	0.0032± 0.0007 a	0.0012± 0.0003 ab	0.0010± 0.0002 b	0.0016± 0.0002 a	0.0015± 0.0003 ab	0.0012± 0.0002 b	0.0012± 0.0001 b	0.0013± 0.0002 b	0.0036± 0.0009 a
Small organic acid	Citric acid	2.72	2.6841± 0.1854 a	1.5876± 0.1175 c	2.1100± 0.1058 b	1.4910± 0.0483 c	2.5476± 0.2692 a	1.6300± 0.0677 c	2.1578± 0.0686 b	1.9504± 0.0500 c	2.6584± 0.2234 a	2.1682± 0.0970 b	2.2466± 0.2354 ab	1.5565± 0.1000 c
Phenylpropanoids	Chlorogenic acid	6.36	0.0777± 0.0100 bc	0.2642± 0.0378 a	0.0320± 0.0013 c	0.1108± 0.0141 b	0.0903± 0.0041 b	0.2122± 0.0313 a	0.0271± 0.0034 c	0.0311± 0.0029 c	0.0713± 0.0109 a	0.0714± 0.0017 a	0.0279± 0.0047 b	0.0725± 0.0128 a
	3,5-Dicaffeoylquinic acid	6.48	0.0673± 0.0025 c	0.2339± 0.0269 a	0.0412± 0.0025 c	0.1205± 0.0125 b	0.0757± 0.0028 b	0.1835± 0.0220 a	0.0354± 0.0030 c	0.0443± 0.0034 c	0.0615± 0.0088 b	0.0635± 0.0015 ab	0.0339± 0.0041 c	0.0844± 0.0152 a
Flavonoids	Luteolin-7-*O*-glucoside	7.44	0.0053± 0.0008 b	0.0171± 0.0010 a	0.0022± 0.0007 b	0.0046± 0.0017 b	0.0070± 0.0007 b	0.0126± 0.0017 a	0.0020± 0.0004 c	0.0017± 0.0002 c	0.0074± 0.0007 a	0.0077± 0.0004 a	0.0016± 0.0003 c	0.0035± 0.0004 b
Others	Camphor	0.96	0.2241± 0.0036 b	0.2703± 0.0088 a	0.2573± 0.0102 a	0.2632± 0.0076 a	0.2162± 0.0069 a	0.2416± 0.0131 ab	0.2543± 0.0191 a	0.2409± 0.0155 ab	0.2236± 0.0094 b	0.1957± 0.0048 c	0.2357± 0.0046 ab	0.2511± 0.0063 a
	myo-Inositol	3.64	0.7137± 0.0367 ab	0.7632± 0.0233 a	0.6919± 0.0255 ab	0.6761± 0.0346 b	0.7185± 0.0197 a	0.6852± 0.0220 ab	0.6631± 0.0219 bc	0.6172± 0.0218 c	0.8158± 0.0407 a	0.7101± 0.0084 b	0.6813± 0.0107 bc	0.6426± 0.0202 c

The mean and SEM for each compound is shown. Different letters denote significant differences among groups compared by LSD test at $P \leq 0.05$.

Table S3 Statistical analysis of identified chemical compounds.

Categories	Compounds	Chemical shift	Hormone application								
			All leaves			Basal leaves			Apical leaves		
			JA treatment	Leaf position	Interaction	JA treatment	Leaf position	Interaction	JA treatment	Leaf position	Interaction
Sugars	**Fructose**	**4.08**	$P = 0.211$	$P < 0.001$	$P = 0.463$	$P = 0.003$	$P < 0.001$	$P = 0.571$	$P = 0.002$	$P = 0.247$	$P = 0.974$
	Glucose	**5.20**	$P < 0.001$	$P = 0.973$	$P = 0.049$	$P < 0.001$	$P = 0.044$	$P = 0.121$	$P = 0.001$	$P = 0.988$	$P = 0.004$
	Sucrose	**5.40**	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.021$	$P < 0.001$	$P < 0.01$	$P = 0.034$	$P < 0.001$	$P = 0.013$
Amino acids	**Valine**	**1.04**	$P = 0.384$	$P = 0.010$	$P = 0.107$	$P = 0.371$	$P = 0.003$	$P = 0.923$	$P = 0.840$	$P = 0.004$	$P = 0.008$
	Threonine	**1.32**	$P < 0.001$	$P = 0.067$	$P = 0.010$	$P = 0.004$	$P = 0.002$	$P = 0.451$	$P = 0.500$	$P = 0.001$	$P = 0.101$
	Alanine	**1.48**	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.009$	$P < 0.001$	$P < 0.001$	$P = 0.003$
	Arginine	**1.72**	$P = 0.296$	$P < 0.001$	$P = 0.352$	$P = 0.006$	$P < 0.001$	$P = 0.221$	$P = 0.961$	$P < 0.001$	$P = 0.586$
	Glutamine	**2.44**	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.091$
	Asparagine	**2.84**	$P = 0.148$	$P = 0.441$	$P = 0.010$	$P = 0.750$	$P = 0.978$	$P = 0.221$	$P = 0.330$	$P = 0.489$	$P = 0.205$
	Adenine	**8.20**	$P = 0.001$	$P = 0.001$	$P = 0.281$	$P = 0.395$	$P = 0.031$	$P = 0.822$	$P = 0.007$	$P = 0.003$	$P = 0.008$
Small organic acid	**Citric acid**	**2.72**	$P < 0.001$	$P = 0.003$	$P = 0.032$	$P < 0.001$	$P = 0.789$	$P = 0.006$	$P < 0.001$	$P = 0.001$	$P = 0.527$
Phenylpropanoids	**Chlorogenic acid**	**6.36**	$P < 0.001$	$P < 0.001$	$P = 0.004$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.004$	$P = 0.007$	$P = 0.005$
	3,5-Dicaffeolquinic acid	**6.48**	$P < 0.001$	$P < 0.001$	$P = 0.002$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.001$	$P = 0.677$	$P = 0.003$
Flavonoids	**Luteolin-7-*O*-glucoside**	**7.44**	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.002$	$P < 0.001$	$P < 0.001$	$P = 0.011$	$P < 0.001$	$P = 0.051$
Others	**Camphor**	**0.96**	$P < 0.001$	$P = 0.067$	$P = 0.005$	$P = 0.640$	$P = 0.146$	$P = 0.131$	$P = 0.285$	$P < 0.001$	$P < 0.001$
	***myo*-Inositol**	**3.64**	$P = 0.538$	$P = 0.046$	$P = 0.233$	$P = 0.039$	$P = 0.001$	$P = 0.742$	$P = 0.001$	$P < 0.001$	$P = 0.114$

The effects of the hormone treatment, leaf position and their interaction on the relative intensity of the identified compounds were tested by Generalized linear models.

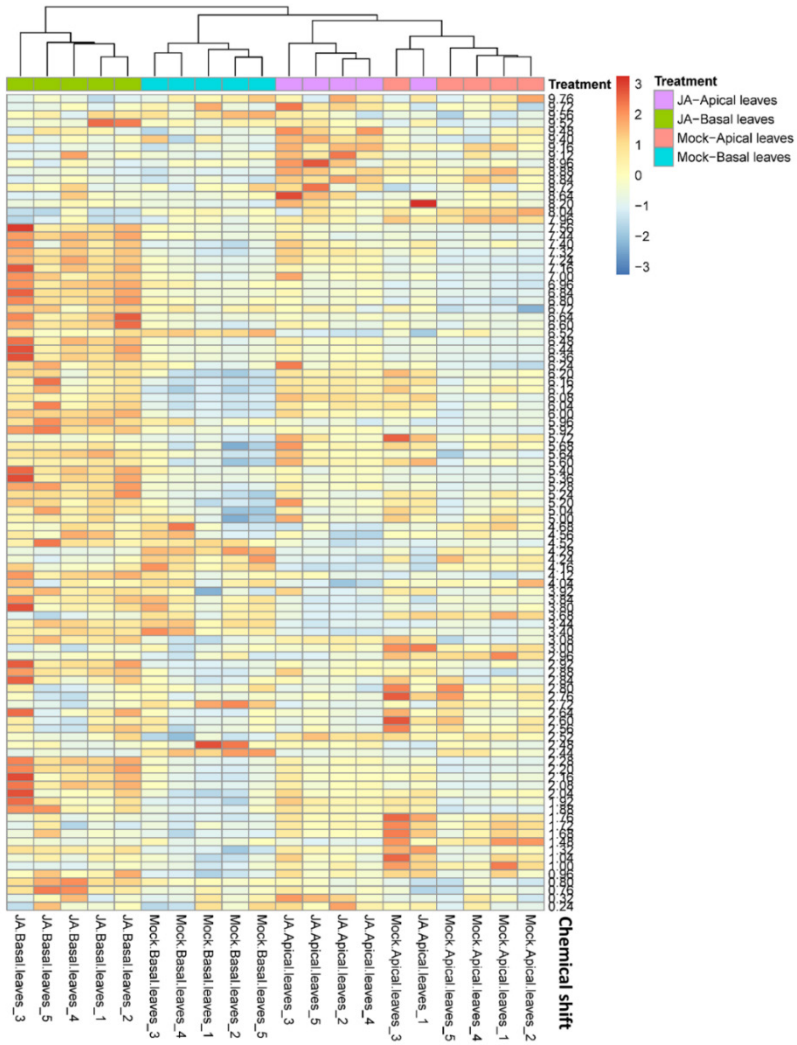

Fig. S1 Heatmap of important NMR signals detected in chrysanthemum basal and apical leaves of plants treated with mock or jasmonic acid (JA) in all the leaves. The NMR signals were selected according to their variable importance in projection (VIP > 1) obtained based on the partial least square-discriminant analysis (PLS-DA). The heatmap shows the standard relative peak intensity of the important chemical shifts of basal (leaf 4-5) or apical (leaf 9-10) leaves of chrysanthemum plants at 7 days after the hormone treatment. Hierarchical classifications with attributes (HCAs) were performed to group the chemical profiles in each treatment according to their Euclidean distance.

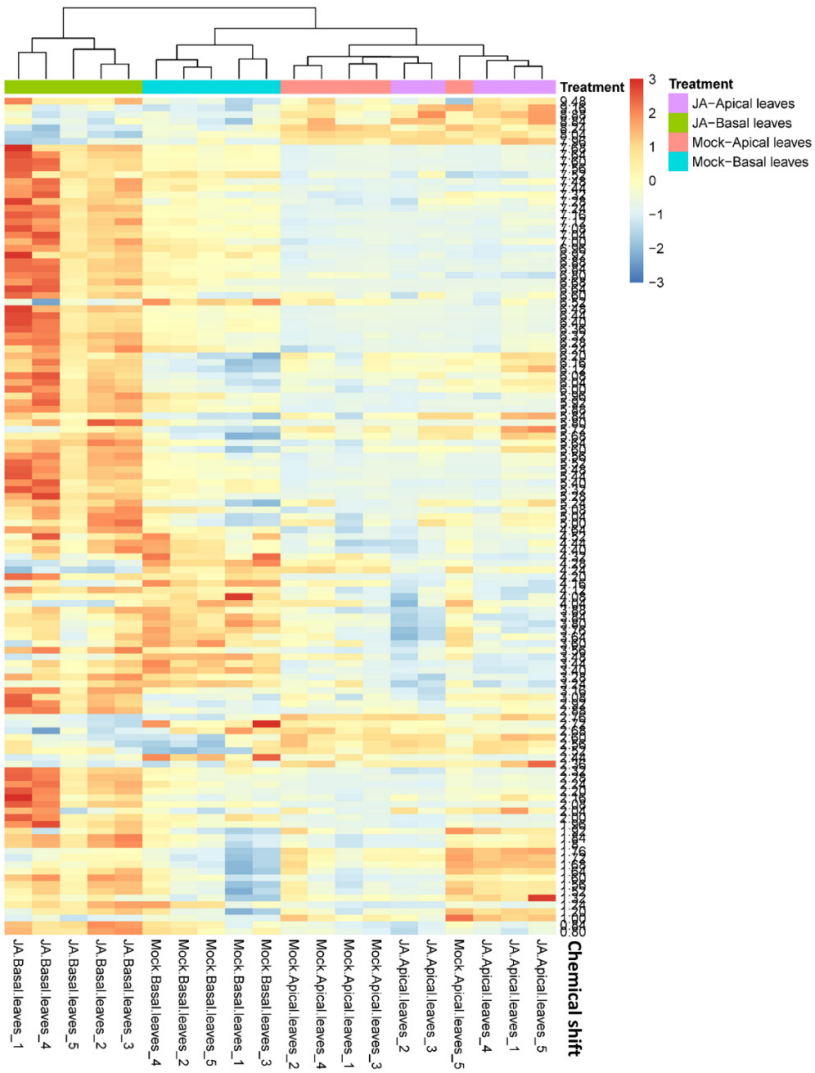

Fig. S2 Heatmap of important NMR signals detected in chrysanthemum basal and apical leaves of plants treated with mock or jasmonic acid (JA) in basal leaves. The NMR signals were selected according to their variable importance in projection (VIP > 1) obtained based on the partial least square-discriminant analysis (PLS-DA). The heatmap shows the standard relative peak intensity of the important chemical shifts of basal (leaf 4-5) or apical (leaf 9-10) leaves of chrysanthemum plants at 7 days after the hormone treatment. Hierarchical classifications with attributes (HCAs) were performed to group the chemical profiles in each treatment according to their Euclidean distance.

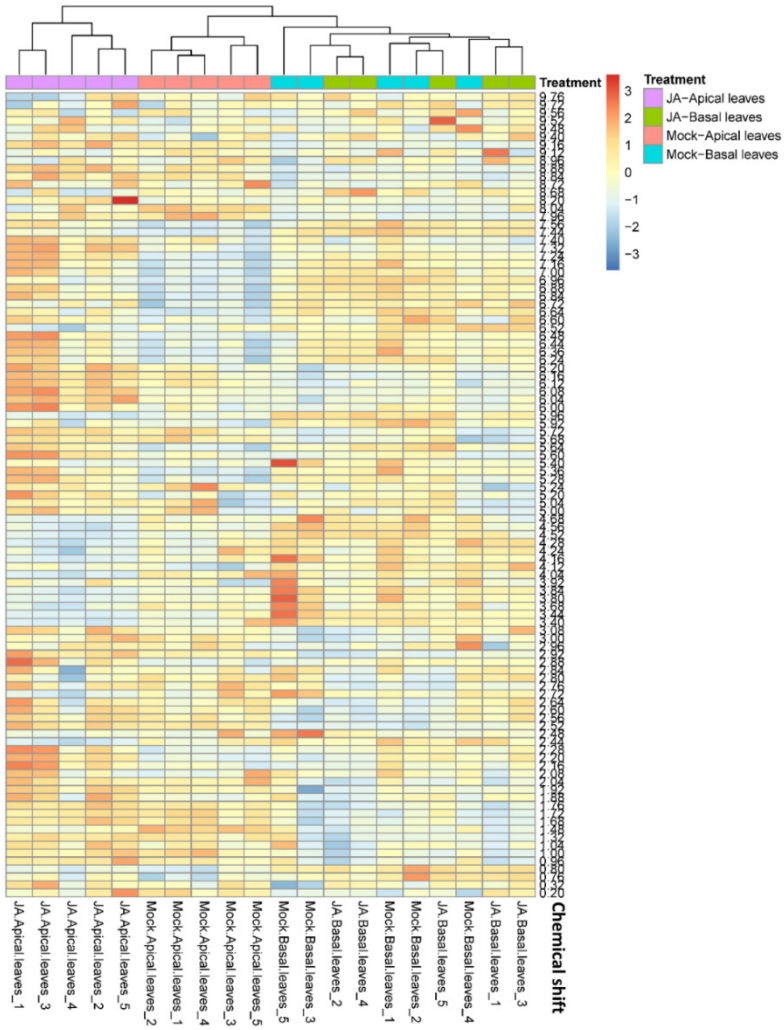

Fig. S3 Heatmap of important NMR signals detected in chrysanthemum basal and apical leaves of plants treated with mock or jasmonic acid (JA) in apical leaves. The NMR signals were selected according to their variable importance in projection (VIP > 1) obtained based on the partial least square-discriminant analysis (PLS-DA). The heatmap shows the standard relative peak intensity of the important chemical shifts of basal (leaf 4-5) or apical (leaf 9-10) leaves of chrysanthemum plants at 7 days after the hormone treatment. Hierarchical classifications with attributes (HCAs) were performed to group the chemical profiles in each treatment according to their Euclidean distance.

Chapter 6

Summary and discussion

Crop production is severely hampered by the attack of arthropod pests and the pathogens they transmit. Current pest control mainly depends on the use of pesticides, which entails a serious risk for the environment and the human health. An alternative strategy is to enhance host plant resistance to pests and pathogens using elicitors that increase the expression of defense-associated traits (Benhamou, 1996; Stout *et al.*, 2002). One of the most extensively studied defense elicitor is the phytohormone jasmonic acid (JA) (Campos *et al.*, 2014). JA controls both constitutive and inducible plant defenses (Li *et al.*, 2002; Li *et al.*, 2004). Artificial application of this phytohormone has been described to activate JA signaling and to induce a wide array of chemical and morphological responses in plants that, in many cases, increase their resistance to herbivorous arthropods (Thaler *et al.*, 1996; Abe *et al.*, 2009; Maes & Goossens, 2010). Nevertheless, both constitutive and inducible plant defenses against arthropod herbivores can vary within and among plant species. Furthermore, these defenses might differ in their nature and magnitude within the plant canopy, which can determine herbivore preference and performance (Lee *et al.*, 2017). In this thesis, I have investigated whether these variations in constitutive and inducible defenses within-plant and/or inter-genotypes correlate with differences in susceptibility to the Western flower thrips (WFT) *Frankliniella occidentalis* in cultivated tomato (*Solanum lycopersicum*) and commercial chrysanthemum (*Chrysanthemum* × *morifolium* Ramat). In addition to the effects of JA on plant defenses, I have explored whether the action of bacterial-derived defense elicitors might mimic the positive effects of JA on tomato and chrysanthemum defenses against this insect pest.

In Chapter 2 we investigated whether the induction of JA-associated defenses varied along the tomato plant canopy, and whether this explains the differential distribution of WFT-associated damage between developing and fully-developed leaves. Our results showed that JA treatment enhanced tomato resistance to WFT, but the magnitude of this induction was much stronger in developing leaves compared to already fully-developed ones at the time of application. Levels of the defensive-related protein polyphenol oxidase (PPO), type-VI trichome densities and the content in trichome-derived volatiles were all much highly induced in developing leaves than in fully developed ones after the hormone treatment. We hypothesized that the stronger induction of these anti-herbivore defenses in young developing leaves explains why these leaves were less preferred by WFT. Hence, type-VI trichomes and the production of their derived allelochemicals are important tomato defenses that can confer resistance to WFT as well (Escobar-Bravo *et al.*, 2018). From an ecological point of view, a stronger induction of these defenses in developing leaves can increase the protection of those plant tissues that contribute more to plant fitness (Constabel *et al.*, 2000). Indeed, young leaves are photosynthetically more active and, therefore, a rich source of nutrients for the plant but also the feeding target of herbivores. How plants can modulate the magnitude of JA-associated defense responses is not clear, but there are several hypotheses that might explain this phenomenon. For instance, developing leaves might act as sink tissues, where the carbohydrates are preferentially allocated and used for the production of chemical defenses (Arnold & Schultz, 2002; Arnold *et al.*, 2004). In addition, a higher light caption by apical developing leaves might increase their sensitivity to JA, and thus confer a higher capacity to display JA-associated defense responses (Constabel *et al.*, 2000; Ballaré, 2011). Interestingly, despite the reduced capacity of fully-developed leaves to increase trichome densities, the production of terpenes per trichome was higher than in developing leaves. This finding suggests tissue-specific responses of the trichome biosynthetic machinery to the phytohormone JA. Notably, differential expression of terpene synthases along the tomato canopy has been previously described (Besser *et al.*, 2009). Yet, it would be interesting to

determine how terpene-related biosynthetic genes respond to JA treatment in different tomato organs as well.

In Chapter 3, to explore the effect of other JA-mimic elicitors on tomato defenses against WFT, we investigated the action of *Pseudomonas syringae* pv tomato DC3000 (*Pst*) infection and the phytotoxin it produces, coronatine (COR). Furthermore, we investigated whether other *Pst*-derived defense elicitors might enhance tomato resistance to WFT. Our results showed that infiltration of *Pst* or COR reduced WFT-associated leaf damage, concomitant with the activation of JA-associated responses. Yet, COR also activated salicylic acid (SA) signaling in infiltrated leaves, while *Pst* did not. This suggests that tomato plants respond differently to *Pst* and COR to some degree, which was confirmed by the slightly different metabolome profiles of *Pst*- and COR-infiltrated leaves. Unexpectedly, activation of JA signaling in *Pst*- and COR-infiltrated plants did not induce the production of type VI leaf trichomes in newly formed leaves. This could be explained by the different COR and jasmonates effects on plant physiology (Uppalapati & Bender, 2005; Tsai *et al.*, 2011). Finally, our results showed that, besides COR, other defense elicitor/s present in *Pst*-derived culture medium can enhance tomato resistance to WFT as well. The nature of the defense elicitor/s present in the medium, however, is unknown and requires further research. Yet, our data showed that this induction was mediated by the activation of JA signaling. Whether *Pst*-derived culture medium affects another defense and growth-related signaling pathways was not tested, and it would require additional investigation. In line with this, it would be also interesting to test whether inoculation with *Pst*-derived culture medium might enhance plant resistance to other important pests and pathogens of tomato. Altogether, our findings highlight the potential use of defense elicitors derived from *Pst* DC3000 for tomato protection against WFT. Yet, the effect of *Pst*-derived elicitors on the production of flowers and fruits, and the fruit biomass of tomato plants needs further investigation.

Leaf trichomes and PPO activity have long been associated with plant resistance to arthropod herbivores in different plant species (Levin, 1973; Dalin *et al.*, 2008; Mahanil *et al.*, 2008; Bhonwong *et al.*, 2009). In Chapter 4 we investigated whether there are variations in constitutive and inducible levels of trichome density and PPO activity among different chrysanthemum cultivars, and whether this variation correlated with WFT resistance. Our results showed that both non-glandular and glandular trichome densities varied significantly among chrysanthemum cultivars. However, differences in trichome densities did not explain the levels of chrysanthemum susceptibility to WFT. Still, whether chrysanthemum glandular trichomes produce allelochemicals, and whether differences in plant susceptibility are associated to the production of these putative compounds was not further investigated and needs additional research. Constitutive levels of PPO activity did not correlate with chrysanthemum resistance to WFT either. We hypothesized that the lack of correlation between PPO activity and chrysanthemum resistance to WFT results from the insufficient expression levels of this enzyme or the deficiency in other chemical defenses. Previous work in our laboratory demonstrated that chlorogenic and feruloyl quinic acids levels positively correlated with chrysanthemum resistance to WFT (Leiss *et al.*, 2009). These phenolic compounds can be oxidized by PPO and peroxidases, which produces derived compounds that can alter the nutritional quality of plant tissues for herbivorous arthropods (Felton & Duffey, 1991). Additional studies to determine the possible correlation between PPO levels and phenolic acid leaf content, and chrysanthemum resistance to WFT are thus needed. Finally, using a subset of cultivars, we also showed that exogenous JA application significantly enhanced chrysanthemum resistance to WFT. Interestingly, this induction was cultivar-dependent, and it was not explained by increases in leaf trichomes nor PPO activity. Our results suggest the existence of other JA-induced defense mechanisms in chrysanthemum

responsible for this induced resistance. Furthermore, our data showed that WFT-resistant genotypes displayed both high constitutive and highly inducible defenses against WFT, which opens new venues for chrysanthemum breeding.

Having demonstrated that JA application can enhance chrysanthemum resistance to WFT (Chapter 4), we further investigated (Chapter 5) whether local and systemic defense responses to exogenous JA application vary along the plant canopy in chrysanthemum, and correlate with WFT resistance levels. First, our results showed that apical (leaf 9-10 from the bottom) chrysanthemum leaves were more susceptible to WFT than basal (leaf 4-5 from the bottom) ones. The metabolomic analyses revealed that basal leaves displayed higher content in phenolic compounds and lower concentrations of amino acids when compared to apical leaves. This can explain why basal leaves were less preferred by WFT, as they might be less nutritious for herbivorous arthropods (Behmer *et al.*, 2002). Furthermore, the higher content in phenolic compounds might have conferred increased deterrent properties against WFT (Leiss *et al.*, 2009; Demkura *et al.*, 2010; Leiss *et al.*, 2013). In addition, our data showed that variations in constitutive levels of PPO activity along the plant canopy could not explain the differences in WFT susceptibility. This is in line with previous results described in Chapter 4, where variations in PPO activities among different chrysanthemum cultivars did not correlate with WFT resistance levels. We also demonstrated that local application of JA can enhance WFT resistance in systemic chrysanthemum leaves, but that this effect depended on the site of the hormone application. While local application of JA on apical leaves reduced the silver damage symptoms per plant, local application of JA on basal leaves did not. Specifically, the leaves developed after the JA induction (leaves 13-18) experienced a stronger reduction in silver damage symptoms when the below and adjacent leaves 9 and 10 were locally induced. The metabolomic analysis, however, demonstrated that both basal and apical leaves responded to the JA treatment only locally. Thus, how local treatment of apical leaves enhanced WFT resistance in newly formed leaves is still unknown. Further metabolomic and hormonal analysis are needed to determine these systemic responses in chrysanthemum.

Tomato and chrysanthemum: Differences and similarities in constitutive and JA-associated defense responses

WFT is an important pest of tomato and chrysanthemum. Here, we have shown that the pattern of WFT-associated damage varies along the plant canopy in both plant species. As WFT is a generalist herbivore, we speculated that this pattern might be associated with the distribution of the chemical and physical defenses within the plant, and that WFT would feed more on less protected leaf tissues. Overall, our data supported this hypothesis. But the pattern of damage along the canopy was opposite in the two species, as the morphological and chemical defenses against WFT differed between tomato and chrysanthemum. We showed that a higher density of type-VI glandular trichomes in apical developing leaves coincided with a higher accumulation of trichome-associated volatiles per leaf and less silver damage symptoms in tomato. Notably, further analyses in our laboratory demonstrated that type-VI trichome-associated allelochemicals play a fundamental role in tomato defenses against WFT (Escobar-Bravo *et al.*, 2018). In chrysanthemum, however, densities of non-glandular and glandular trichomes was not associated to WFT resistance (Chapter 3). Furthermore, our data showed that in chrysanthemum, WFT caused less silver damage symptoms in basal leaves than in apical ones (Chapter 4). Interestingly, basal leaves presented higher levels of the phenolic compound chlorogenic acid, which has been positively associated with WFT resistance in chrysanthemum (Leiss *et al.*, 2009). When compared to tomato, however, previous experiments in our laboratory showed that a higher

production of chlorogenic acid in the leaves did not affect WFT resistance (Mirnezhad, 2011). It would be interesting to determine the within-plant distribution of other plant secondary and primary metabolites in tomato as well. The comparison with the chemical profiles of chrysanthemum might give some clues about common defense patterns against WFT in both plant species. Finally, whether basal chrysanthemum leaves might greatly contribute to plant fitness, and this is the reason they are better protected against WFT herbivory also needs further investigation.

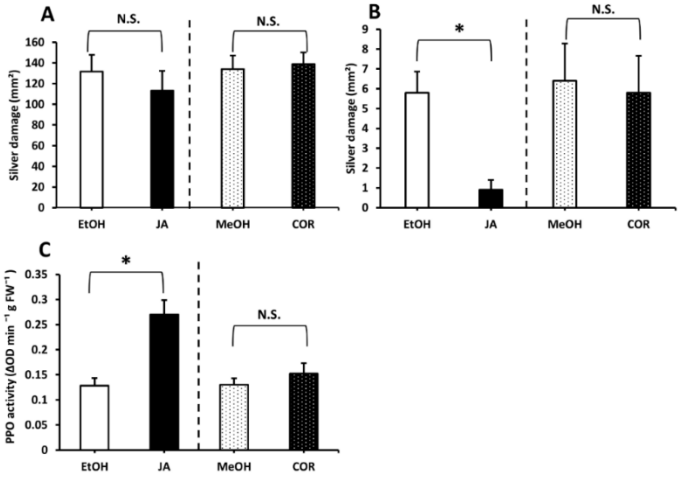

Fig. 1 Local chrysanthemum defense-associated responses to COR and JA infiltration. Chrysanthemum cuttings (cv. Morreno Pink) were grown in a climate room (20°C, 70% RH, 113.6 µmol m^{-2} s^{-1} of photosynthetically active radiation and L16:D8 photoperiod). At 19 d after planting, two leaves (leaf 3 and 4 from the bottom) were pressure-infiltrated with: 1) ethanol solution (EtOH, 0.6%, solvent for jasmonic acid dilution), 2) jasmonic acid (JA, 3 mM), 3) methanol solution (MeOH, 0.32%, solvent for coronatine dilution) or coronatine (COR, 10 µM). At 7 days after infiltration, plants were sampled for determination of polyphenol oxidase (PPO) activity or used for whole-plant non-choice thrips bioassays. Silver damage symptoms determined in (**A**) the whole plant and (**B**) the infiltrated leaves at 7 days after WFT infestation (mean ± SEM, $n = 10$). (**C**) Polyphenol oxidase activity (PPO) (mean ± SEM, $n = 5$) was determined in infiltrated leaves. Differences in PPO levels and silver damage symptoms between EtOH- and JA-treated plants, and MeOH and COR-treated plants, were determined by Student *t*-test. Asterisk indicates significant differences at $P \leq 0.05$. N.S. not significant. The methodology used for the PPO activity measurements and non-choice whole plant bioassays is the same as the described in Chapter 5. The methodology used for JA or COR infiltration is the same as the described in Chapter 3.

Exogenous application of the phytohormone JA enhanced both tomato and chrysanthemum resistance to WFT. Yet, while application of JA increased type-VI trichome densities in newly formed tomato leaves (Chapter 2), the application of this hormone did not affect the production of glandular trichomes in chrysanthemum (Chapter 4). In addition, we found that whereas local application of JA generally induces systemic chemical responses in tomato (Chapter 2), it failed to induce systemic responses in chrysanthemum leaves (Chapter 5). Moreover, JA-mediated enhancement of chrysanthemum resistance to WFT strongly depended on the site of the hormone application along the plant canopy (Chapter 5). In a further attempt to determine whether this was a specific response to JA, we have tested local responses to COR as well. Local application of COR did not affect chrysanthemum resistance to WFT (**Fig. 1A, B**), nor induced PPO activity in treated leaves (**Fig. 1C**). The molecular mechanisms that explain the differences in COR-mediated induced responses between

tomato and chrysanthemum are unknown. However, it might be explained by the capacity of the F-box protein coronatine insensitive1 (COI1) and JAZ complexes to recognize COR, as the binding of COR to COI-JAZs complexes is highly specific (Katsir *et al.*, 2008).

In conclusion, we showed that constitutive and inducible chemical and morphological defenses against WFT differ between tomato and chrysanthemum plants. Furthermore, we demonstrated that both plant species respond differently to bacteria-derived defense elicitors, such as the phytotoxin coronatine. This highlights the plant species-specificity of these interactions and the possible limitation for the use of pathogen-associated molecular patterns to enhance the plant immune system (Quintana-Rodriguez *et al.*, 2018). This study thus provides knowledge and novel strategies for WFT control. Yet, further comprehensive work is needed to evaluate the influence of these induction strategies on plant fitness.

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Samenvatting en discussie

Oogsten staan sterk onder druk door aantasting van pathogenen en herbivore insecten. De huidige manier van ongediertebestrijding is voornamelijk afhankelijk van synthetische pesticiden. Deze brengen een groot risico met zich mee voor de gezondheid van mens en milieu. Een alternatieve strategie om om te gaan met pathogenen en herbivoren is het verhogen van de natuurlijke resistentie van de waardplant. Dit is mogelijk door gebruik te maken van metabolieten die de expressie van afweer gerelateerde eigenschappen vergroten (Benhamou, 1996; Stout *et al.*, 2002). Een van zulke elicitoren om de afweer tegen insect herbivoren te verhogen is het phytohormoon Jasmonzuur (JA) (Campos *et al.*, 2014). JA reguleert zowel de constitutieve als de induceerbare afweer (Li *et al.*, 2002; Li *et al.*, 2004). Toediening van JA activeert een grote variatie aan chemische en morfologische reacties in de plant die de afweer tegen insect herbivoren vergroten (Thaler *et al.*, 1996; Abe *et al.*, 2009; Maes & Goossens, 2010). Het niveau van zowel constitutieve als induceerbare afweer varieert sterk tussen en binnen planten soorten. Ook kunnen deze afweer mechanismes verschillen in hun aard en omvang tussen bladeren van de plant, hetgeen de voorkeur en prestaties van de herbivoor sterk kan bepalen (Lee *et al.*, 2017). In mijn proefschrift heb ik de mechanismen onderzocht die de variatie in gevoeligheid voor Western flower thrips (WFT) *Frankliniella occidentalis* in gecultiveerde tomaat (*Solanum lycopersicum*) en commerciële chrysanten (Chrysanthemum × morifolium Ramat) kunnen verklaren.

Het afweer systeem van planten kan gekaapt worden door pathogene bacteriën, die elicitors van de JA signaal transductie kunnen produceren en zo de plant aanzetten tot het verhogen van hun afweer tegen herbivoren. Daarbij verlaagt de plant door interactie met de SA signaal transductie die die afweer tegen pathogenen ingang zet zijn afweer niveau tegen de pathogeen die deze elicitors produceert. Naast de effecten van JA op de afweer tegen trips heb ik het effect onderzocht van door bacteriën geproduceerde elicitors van de JA signaal transductie op resistentie tegen trips.

In Hoofdstuk 2 heb ik onderzocht of het effect van JA-toediening aan bladeren van tomaat afhangt van de ouderdom en de positie van het blad. ik vond dat toediening van JA de afweer tegen WFT verhoogde. De mate van inductie van deze afweer was sterk afhankelijk van het ontwikkelingsstadium van het blad. In bladeren die zich nog aan het ontwikkelen waren vonden we een veel sterkere inductie dan in bladeren die al volledig gevormd waren op het moment van de JA toediening. De activiteit van het aan afweer gerelateerde eiwit polyphenol oxidase (PPO), de dichtheid van type-VI trichomen en de concentratie van door deze trichomen geproduceerde vluchtige stoffen werden veel sterker geïnduceerd als de bladeren nog niet volledig gevormd waren. Trichomen en de vluchtige stoffen die ze produceren spelen een belangrijke rol in resistentie tegen WFT (Escobar-Bravo *et al.*, 2018).. We veronderstellen daarom dat de sterke inductie hiervan in jonge bladeren mede kan verklaren waarom deze minder aantrekkelijk waren voor WFT. Vanuit evolutionair perspectief is het te begrijpen dat jonge bladeren beter door de plant verdedigd worden. Op de eerste plaats dragen ze meer bij aan de fitness van een plant dan oudere bladeren die minder fotosynthetisch actief zijn en een kortere levensduur hebben en op de tweede plaats hebben jonge bladeren hogere stikstof concentraties waardoor ze veelal aantrekkelijker zijn voor insect herbivoren (Constabel *et al.*, 2000). Hoe planten de sterkte van de JA- gebonden afweer reguleren is niet duidelijk. Er zijn verschillende hypotheses die dit fenomeen mogelijk kunnen verklaren. De eerste is dat de zich nog ontwikkelende bladeren als sink functioneren voor koolhydraten die gebruikt worden voor de productie van afweer stoffen (Arnold & Schultz, 2002; Arnold *et al.*, 2004). De tweede hypothese is dat de apicale bladeren, die zich

nog ontwikkelen, meer licht opvangen en daardoor een grotere gevoeligheid voor JA hebben (Constabel *et al.*, 2000; Ballaré, 2011). Een interessant resultaat was dat, ondanks de verminderde capaciteit van de oudere bladeren om trichomen te produceren, de productie van terpenen per trichoom wel hoger was dan dat van zich nog ontwikkelende bladeren. Besser *et al.* (2009) hebben laten zien dat de terpeen synthese verschilt afhankelijk van de positie van het blad. Onze resultaten laten zien dat reacties als gevolg van toediening van JA met betrekking tot de productie van door trichomen terpenen ook afhankelijk zijn van de positie van het blad. Het zou interessant zijn dit onderzoek uit te breiden naar genexpressie in de verschillende organen en weefsels. In Hoofdstuk 3 hebben we het effect van andere elicitoren van afweer tegen WFT in tomaat bekeken. We het effect van *Pseudomonas syringae* pv tomato DC3000 (*Pst*) infectie en het phytotoxine dat deze bacterie produceert (coronatine, COR) onderzocht. Daarnaast hebben we onderzocht of andere *Pst*-gebonden elicitoren van de afweer de weerbaarheid van tomaat tegen WFT kon verhogen. Onze resultaten lieten zien dat zowel infectie met *Pst* en COR de schade door WFT verminderden, door activatie van JA-geassocieerde reacties. Verrassenderwijs activeerde COR ook de signaal transductie van salicylzuur (SA) in bladeren terwijl infectie met *Pst* dat niet deed. Ook vond ik verschillen in de metabolome profielen van de met *Pst* geinfecteerde bladeren en bladeren die met COR behandeld waren. In tegenstelling tot onze verwachting leidde de elicitering van de JA signaal transductie door beide behandelingen niet tot verhoogde productie van trichomen in nieuwe bladeren. Verschillen tussen de effecten van toediening van COR en jasmonaten op de fysiologie van de plant zijn eerder beschreven door Uppalapati & Bender (2005) en Tsai et al., (2011). Naast COR bleken er andere elicitoren van de afweer tegen WTF aanwezig in medium waarin *Pst* gekweekt was en waaruit de bacteriën gefilterd waren. Welke elicitoren dit betreft is nog onbekend en vraagt om verder onderzoek. Wel toondeik aan dat ook deze inductie via activatie van de JA signaal transductieverliep. Of hier ook nog andere regulatoren van de plant bij betrokken waren is niet duidelijk. Het zou interessant zijn om te onderzoeken of inoculatie met medium waarin *Pst* gekweekt is ook de resistentie tegen andere schadelijke insecten en pathogenen van tomaat kan vergroten. Alles bij elkaar laten onze bevindingen de mogelijkheden zien van toepassing van door *Pst* DC3000 geproduceerde elicitoren van de afweer tegen WFT. Er is echter wel meer onderzoek nodig naar de langere termijn effecten met name wat betreft de oogst.

Van verschillende planten soorten is bekend dat de afweer tegen insect herbivoren gecorreleerd is met trichoom dichtheid en de PPO activiteit (Levin, 1973; Dalin *et al.*, 2008; Mahanil *et al.*, 2008; Bhonwong *et al.*, 2009). In Hoofdstuk 4 heb ik onderzocht of er variatie was in constitutieve en induceerbare niveaus van beide tussen chrysanten cultivars en of deze variatie gecorreleerd was met de resistentie tegen WFT. Onze resultaten lieten verschillen tussen cultivars zien in de dichtheid van zowel non-glandulaire als glandulaire trichomen. Deze verschillen in trichoom dichtheid waren niet gerelateerd aan verschillen in gevoeligheid voor WFT. Opgemerkt moet worden dat verschillen in de productie door trichomen van metabolieten die betrokken kunnen zijn bij deze afweer niet onderzocht zijn. Constitutieve niveaus van PPO activiteit correleerde evenmin met de afweer van chrysanten tegen WFT. Eerder werk in ons laboratorium liet zien dat de resistentie van chrysant tegen WFT positief gecorreleerd was met de concentraties van chlorogeen zuur en o.a. 3 5-dicaffeoylquinic acid (Leiss *et al.*, 2009). Deze fenolische verbindingen kunnen geoxideerd worden door PPO en peroxidases, de verbindingen die hierdoor ontstaan, veranderen de voedingswaarde van de plant voor herbivore insecten (Felton & Duffey, 1991). Het lijkt veelbelovend om verder onderzoek te doen naar de relaties tussen PPO niveaus, concentraties van fenolische zuren en de resistentie tegen WFT. Als laatste hebben ik aangetoond dat exogene toediening van JA de afweer tegen WFT bij Chrysant significant verhoogde. Interessant was dat de mate van

inductie van de afweer per cultivar verschilde. Deze verschillen waren niet gerelateerd aan verhoogde productie van trichomen of aan verhoogde PPO activiteit. Blijkbaar spelen andere mechanismes Een rol. Nadat ik had aangetoond dat toediening van JA de afweer van chrysanten tegen WFT kon verhogen (Hoofdstuk 4) hebben ik in meer detail onderzocht (Hoofdstuk 5) of de lokale en systemische inductie van afweer door toediening van JA af hing van de ouderdom (en dus positie) van de behandelde bladeren. Verrassenderwijs vonden we dat apicale bladeren (blad 9-10 van beneden geteld) van chrysanten gevoeliger waren voor WFT dan basale bladeren (blad 4-5 van beneden geteld). Basale bladeren bevatten meer fenolische verbindingen en minder aminozuren in vergelijking met apicale bladeren. Dit kan verklaren waarom basale bladeren minder aantrekkelijk waren voor WFT, waarschijnlijk zijn ze minder voedzaam voor herbivore insecten (Behmer *et al.*, 2002) en leidt de aanwezigheid van hogere concentraties aan fenolische verbindingen tot verhoogde weerstand tegen WFT (Leiss *et al.*, 2009; Demkura *et al.*, 2010; Leiss *et al.*, 2013). Positie-afhankelijke verschillen tussen bladeren in WTF resistentie waren niet gekoppeld aan PPO activiteit. Dit laatste komt overeen met de resultaten van Hoofdstuk 4, waar de variatie in PPO activiteit tussen verschillende chrysant cultivars niet gecorreleerd was met afweer tegen WFT. Terwijl lokale toediening van JA op apicale bladeren de zilverschade per plant reduceerde, was dat niet het geval bij toediening van JA aan basale bladeren. Meer specifiek lieten de na de JA behandeling gevormde bladeren (bladeren 13-18) een sterkere reductie van zilverschade zien wanneer de bladeren daar in de buurt (bladeren 9 en 10) lokaal geïnduceerd waren. De metabolomische analyse daarentegen liet zien dat zowel de basale als de apicale bladeren alleen lokaal reageerden op de JA behandeling. Dus hoe lokale behandeling van apicale bladeren de resistentie van nieuwe bladeren kon verhogen is nog onbekend.

Tomaat en chrysant: Verschillen en overeenkomsten in constitutieve en JA-geassocieerdeafweer reacties.

WFT is een belangrijke plaag op tomaat en chrysant. In dit proefschrift heb ik aangetoond dat het patroon van door WFT veroorzaakte schade over de plant tussen beide soorten varieert. Omdat WFT een generalistische herbivoor is veronderstel ik dat dit patroon gerelateerd is aan de distributie van de chemische en fysieke afweer binnen de plant. Over het algemeen ondersteunde onze data deze hypothese. Het patroon van de schade was tegengesteld in beide soorten waarschijnlijk omdat ook het patroon van zowel morfologische als chemische factoren die mogelijkerwijs betrokken zijn bij deze afweer verschilden. Ik vond voor tomaat een hogere dichtheid van type VI glandulaire trichomen in apicale zich nog ontwikkelende bladeren alsmede een verhoogde concentratie van aan deze trichomen geassocieerde vluchtige stoffen in combinatie met minder zilverschade. Binnen het STW perspectief programma GAP is aangetoond dat type VI trichomen en de door deze trichomen geproduceerde vluchtige stoffen een belangrijke rol kunnen spelen in de resistentie van tomaat tegen WFT (o.a. Escobar-Bravo *et al.*, 2018). In chrysanten daarentegen waren de dichtheden van niet glandulaire en glandulaire trichomen niet geassocieerd met WFT resistentie (Hoofdstuk 3). Verder vonden we dat in chrysanten WFT minder schade veroorzaakte in basale bladeren ten opzichte van apicale bladeren (Hoofdstuk 4). Deze basale bladeren hadden hogere concentraties van fenolische verbinding zoals chlorogeen zuur, deze stof is positief geassocieerd met WFT resistentie in chrysanten (Leiss *et al.*, 2009). In tomaat daarentegen heeft verhoging van de concentraties van chlorogeen zuur niet geleid tot verhoogde resistentie tegen WFT (Mirnezhad, 2011). Het zou, vanuit evolutionair perspectief, interessant zijn te onderzoeken of bij chrysant de basale bladeren een grotere relatieve bijdrage leveren aan de fitness van de plant dan bij tomaat.

Toediening van het phytohormoon JA verhoogde de WFT resistentie in zowel tomaat als chrysant. Maar terwijl JA applicatie de hoeveelheid type VI trichomen verhoogde in nieuw gevormde bladeren van tomaat (Hoofdstuk 2), was er geen effect op de productie van glandulaire trichomen in chrysanten (Hoofdstuk 4). Daarnaast bleek dat hoewel lokale applicatie van JA over het algemeen systemische chemische reacties teweeg bracht in tomaat (Hoofdstuk 2), dit niet het geval was bij chrysant (Hoofdstuk 5). De effecten van toediening van JA op resistentie tegen WFT was bij chrysanten sterk afhankelijk van aan welke bladeren JA werd toegediend (Hoofdstuk 5). Om verder te onderzoeken of deze lokale response specifiek voor JA was hebben we ook de lokale response na toediening van COR bepaald. In tegenstelling tot onze verwachting had lokale toediening van COR geen effect op de resistentie van chrysanten tegen WFT (Fig. 1A, B), ook induceerde de PPO activiteit in behandelde bladeren niet (Fig. 1C). De moleculaire mechanismes die het verschil in COR response tussen tomaat en chrysant kunnen verklaren zijn onbekend. Misschien zijn deze verschillen te verklaren door het verschil in vermogen van het F-box eiwit coronatine insensitive1 (COI1) en dat van JAZ complexen om COR te herkennen. De binding van COR aan COI-JAZ complexen is zeer specifiek (Katsir *et al.*, 2008).

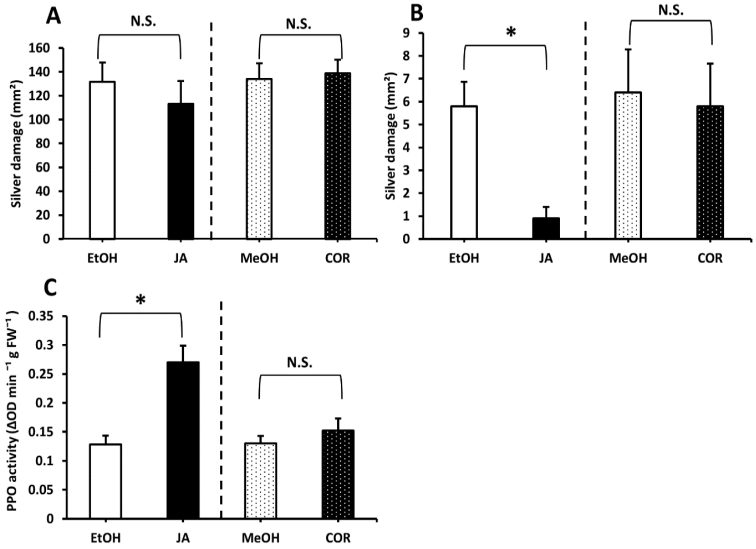

Fig. 1 De lokale effecten op zilverschade en PPO activiteit van toediening van COR en JA. Chrysanten stekken (cv Morreno Pink) werden opgegroeid in een klimaat kamer (20°C, 70% RH, 113.6 µmol m^{-2} s^{-1} PAR en L16:D8). Negentien dagen na oppotten werden twee bladeren (blad 3 en 4 van beneden geteld) onder druk geïnfiltreerd met 1) ethanol oplossing (0.6%) 2) JA(3 mM opgelost in ethanoloplossing), 3) methanol oplossing (0.32%), of 4) coronatine (10 µM opgelost in methanol oplossing). Zeven dagen na infiltratie werden planten gebruikt voor de bepaling van de polyphenol oxidase (PPO) activiteit en werden andere planten gebruikt voor een niet keuze proef met trips. Zilverschade werd gemeten in zowel de gehele plant (**A**) als voor de behandelde bladeren (**B**) zeven 7 dagen na WFT infestatie (mean ± SEM, n = 10). (**C**) PPO (mean ± SEM, n=5) activiteit werd bepaald in geïnfiltreerde bladeren. *= $P \leq 0.05$ student t-test. N.S. is niet significant. De gebruikte methodes zijn hetzelfde als die beschreven in hoofdstukken 3 en 5.

Concluderend hebben we aangetoond dat constitutieve en induceerbare chemische en morfologische afweer mechanismen tegen WFT verschillen tussen tomaat en chrysant planten. Ik heb aangetoond dat deze soorten verschillend reageren op door een bacterie geproduceerde elicitoren van de afweer zoals het phytotoxine coronatine. Dit laat de plant

soort-specifieke interacties voor deze soorten zien en daarmee de mogelijke beperkingen van het gebruik dergelijke stoffen voor het verhogen van het immuunsysteem van de plant (Quintana-Rodriguez *et al.*, 2018). Voor tomaat geeft dit onderzoek inzichten voor nieuwe strategieën voor WFT bestrijding. Er is echter meer werk nodig om de effectiviteit te evalueren mede in het licht van de effecten op de groei van de plant.

Curriculum vitae

Gang Chen was born on the 18th of December 1989 in Bazhong, Sichuan Province, China. After he finished his high school studies in Bazhong Middle School, he started the study of Forestry at the Sichuan Agricultural University in 2008 and obtained his bachelor's degree in 2012. He continued his Master study in Silviculture under the supervision of Prof. Lihua Tu and Prof. Tingxing Hu at the same university from 2012 to 2014. During the MSc, he studied the Allelopathic effects of *Cinnamomum japonicum* on the plant growth of *Impatiens balsamina* and the characteristics of soil carbon components in a secondary evergreen broad-leaved forest. In October 2014, he started his PhD project, as described in this thesis, in the research group Plant Ecology and Phytochemistry, cluster Plant Science and Natural Products, Institute of Biology Leiden under the supervision of Prof. Dr. Peter G. L. Klinkhamer and Dr. Rocío Escobar-Bravo, supported by the China Scholarship Council. His PhD research mainly involved induced and constitutive defenses in tomato and chrysanthemum and is supported by a grant to Prof. Dr. Peter G. L. Klinkhamer from the Technology Foundation STW, project 'Green Defense against Pests (GAP)'.

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