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Control of Western flower thrips through jasmonate-triggered plant immunity

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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

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

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.

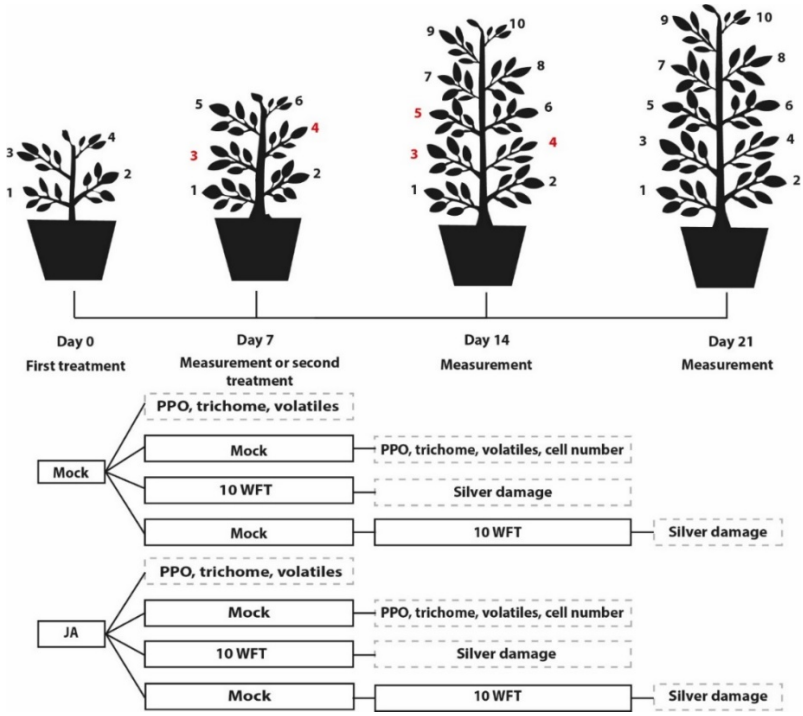

Fig. 1 Schematic representation of the experimental design. Four leaf-stage tomato plants were treated with 0.8% ethanol (mock treatment) or jasmonic acid (JA) exogenous treatment at day 0. Seven days after the initial treatment, mock- and JA-treated plants were sampled for PPO activity, type VI glandular 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.

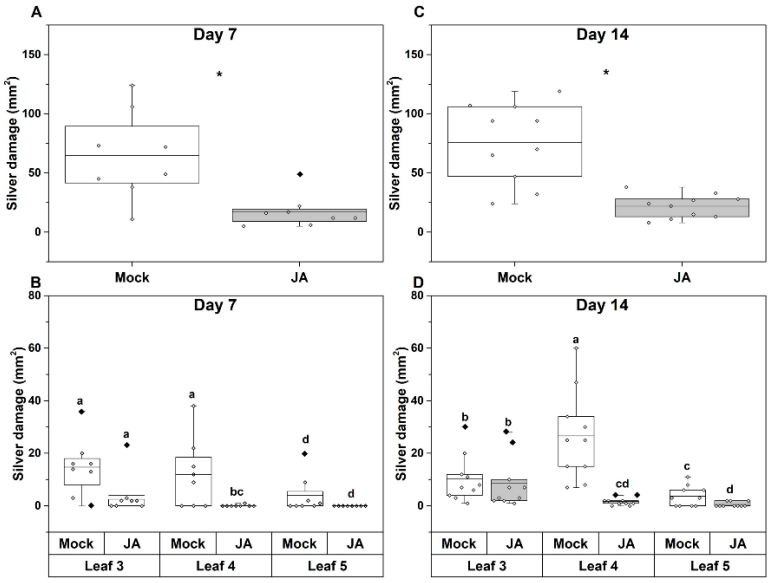

Fig. 2 Effect of JA exogenous treatment and leaf development stage on tomato resistance to Western flower thrips. Silver damage symptoms determined in **(A)** the whole plant and (**B**) leaf 3, 4 and 5 from bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants infested with 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 $P \leq 0.05$.

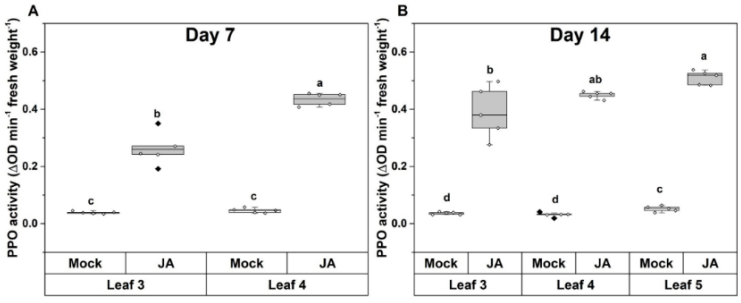

Fig. 3 Effect of JA exogenous treatment and leaf development stage on PPO activity in tomato. PPO activity determined on **(A)** leaf 3 and 4 from the bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants at 7 days after the initial hormone treatment, and on (**B**) leaf 3, 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.

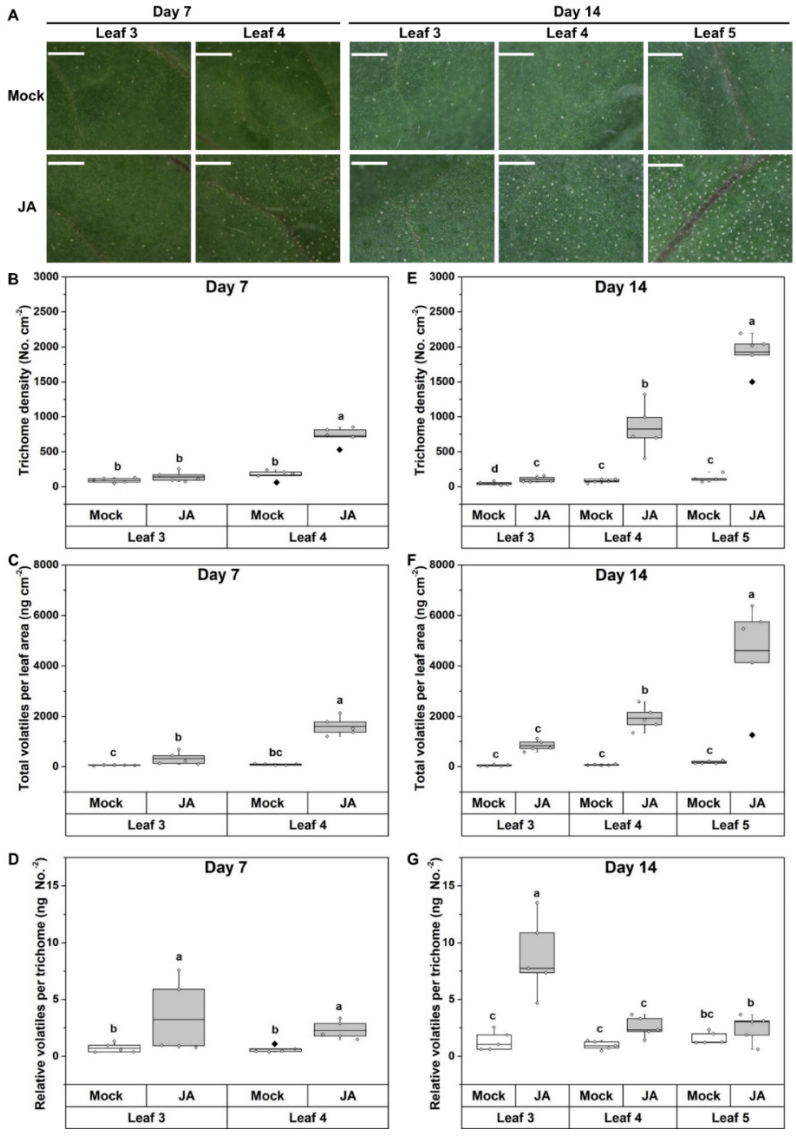

Fig. 4 Effect of JA exogenous treatment and leaf development stage on type-VI trichome density and their derived volatiles in tomato. (A) Representative photographs of adaxial leaf surface of leaflets taken from mock- and JA-treated tomato plants at 7 and 14 days after the initial treatment. **(B)** 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**).

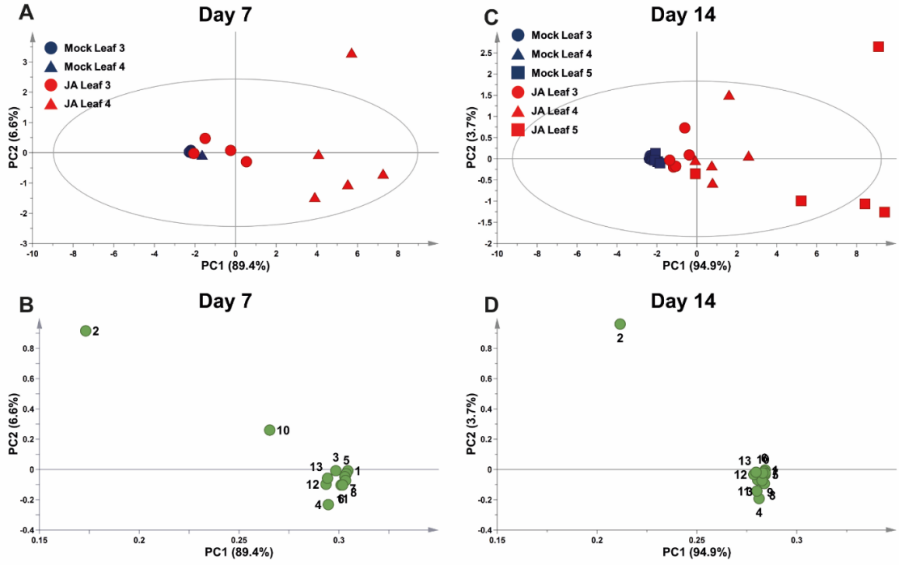

Fig. 5 Effect of JA exogenous treatment and leaf development stage on trichome-derived volatile profiles in tomato. Principal component analysis (PCA) of volatile compounds detected in leaf exudates of **(A** and **B)** leaf 3 and 4 and (**C** and **D)** of leaf 3, 4 and 5 of mock- and JA-treated plants 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**).

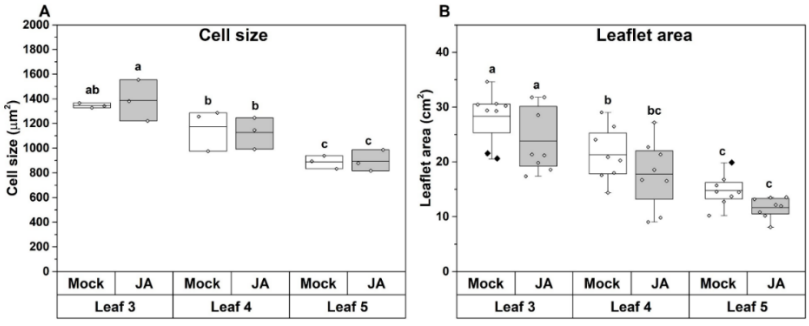

Fig. 6 Effects of JA and leaf development stage on epidermal cell size and leaflet area in tomato. (**A**) Epidermal cell size ($n = 3$) and (**B**) leaflet area ($n = 8$) determined on bottom leaf 3, 4 and 5 of mock- (white box plots) and JA-treated (grey box plots) tomato plants 14 days after the initial 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

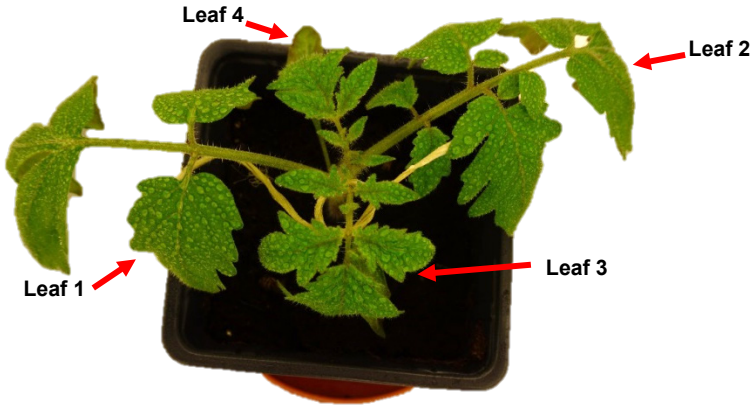

Fig. S1 Representative photograph of the tomato plants sprayed with JA at day 0. Tomato plants at four leaf stage were sprayed with JA or Mock solution until run off at day 0 and evaluated for type-VI trichome density, terpene content in trichome-derived leaf exudates, polyphenol oxidase activity and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

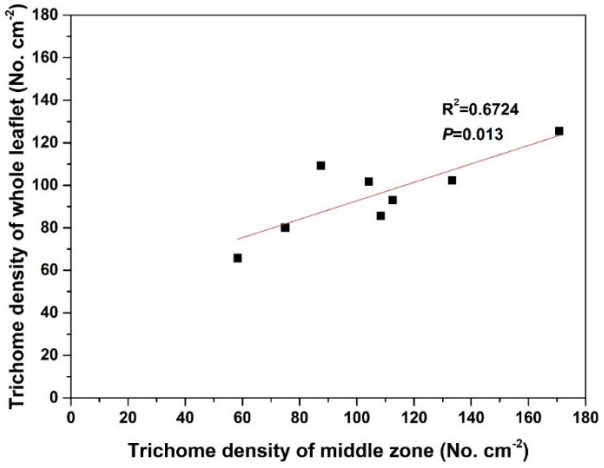

Fig. S2 Scatter plot depicting the relationship between the trichome density in the middle section of the tomato leaflet and in the whole leaflet. Type VI trichome density was determined in two areas of the middle section of a tomato leaflet and in 18 random areas of the same leaflet. Leaflets, each 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.

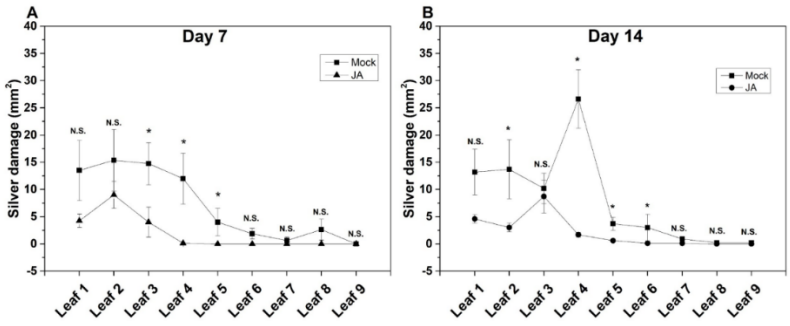

Fig. S3 Silver damage determined in tomato leaves at different development stage after JA application. Silver damage (mean ± SEM, n = 8) was determined in all leaves of tomato plants subjected to thrips infestation (**A**) at 7 and (**B**) 14 days after initial hormone treatment. Leaves were enumerated 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.

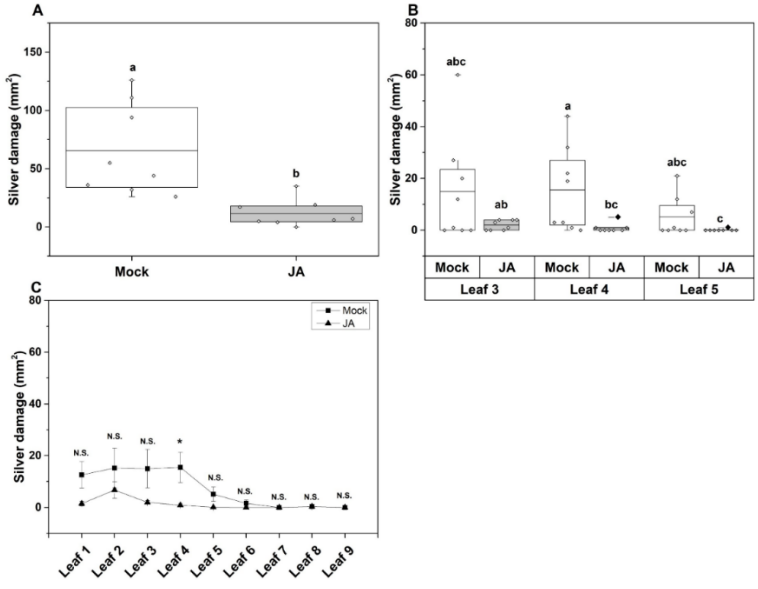

Fig. S4 Effect of JA exogenous treatment and leaf development stage on tomato resistance to thrips. Silver damage symptoms determined in **(A)** the whole plant, (**B**) leaf 3, 4 and 5 and (**C**) all specific leaves from bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants 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 $P \leq 0.05$.

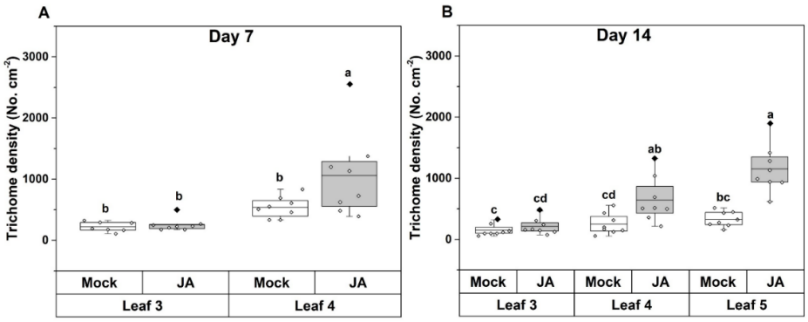

Fig. S5 Effect of JA exogenous treatment and leaf development stage on type-VI trichome density. Average (± SEM, $n = 8$) of type-VI trichome density determined on **(A)** leaf 3 and 4 from the bottom of mock- (white box plots) and JA-treated (grey box plots) tomato plants 7 days after the initial treatment 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	$P = 0.897$