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Spider mites suppress tomato defenses downstream of jasmonate and salicylate independently of hormonal crosstalk

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Summary

- Plants respond to herbivory by mounting a defense. Some plant-eating spider mites (*Tetranychus* spp.) have adapted to plant defenses to maintain a high reproductive performance. From natural populations we selected three spider mite strains from two species, *Tetranychus urticae* and *Tetranychus evansi*, that can suppress plant defenses, using a fourth defense-inducing strain as a benchmark, to assess to which extent these strains suppress defenses differently.
- We characterized timing and magnitude of phytohormone accumulation and defense-gene expression, and determined if mites that cannot suppress defenses benefit from sharing a leaf with suppressors.
- The nonsuppressor strain induced a mixture of jasmonate- (JA) and salicylate (SA)-dependent defenses. Induced defense genes separated into three groups: ‘early’ (expression peak at 1 d postinfestation (dpi)); ‘intermediate’ (4 dpi); and ‘late’, whose expression increased until the leaf died. The *T. evansi* strains suppressed genes from all three groups, but the *T. urticae* strain only suppressed the late ones. Suppression occurred downstream of JA and SA accumulation, independently of the JA–SA antagonism, and was powerful enough to boost the reproductive performance of nonsuppressors up to 45%.
- Our results show that suppressing defenses not only brings benefits but, within herbivore communities, can also generate a considerable ecological cost when promoting the population growth of a competitor.

*These authors contributed equally to this work.

Introduction

Higher plants possess sophisticated means to prevent or hamper herbivore feeding (Walling, 2000; Wu & Baldwin, 2010). Such defenses can be constitutive and/or induced upon attack by herbivores. In general, induced defenses may include morphological reinforcements as well as the accumulation of toxins and inhibitors of herbivore digestion (Kessler & Baldwin, 2002), but may also involve hypersensitive responses (Klingler et al., 2009) and resource allocation (Gomez et al., 2012). The first critical step to mount antiherbivore defenses is the perception of herbivory, but how this takes place and whether receptors are involved is not well understood (Bonaventure et al., 2011). It is clear that some characteristics of the response can be attributed to mechanical feeding damage (Mithöfer et al., 2005) but others can only be attributed to herbivory-derived signals referred to as elicitors (Howe & Jander, 2008). Most of these emanate from herbivore saliva or regurgitant and, when applied as pure compounds, elicit defined herbivory-induced changes, such as phytohormone accumulation, transcription of defense genes, and emission of volatiles (Wu & Baldwin, 2010).

The central regulators of plant defense responses are a set of phytohormones that mediate between signal recognition and activation of defenses. Although most of the known plant hormones have been found to influence the establishment of defenses in one way or another (Pieterse et al., 2012), there are three, jasmonic acid (JA), salicylic acid (SA) and ethylene (Et), which play primary roles, as interference with their biosynthesis or perception results in strong defense deficiencies (Wu & Baldwin, 2010). While JA, SA and Et have distinct effects on the type of defenses a plant displays, they also modulate each other’s individual actions, that is, ‘crosstalk’ (Pieterse et al., 2009), in a nonlinear way (Mur et al., 2006). While SA is essential for defense against biotrophic pathogens...
(Vlot et al., 2009), JA and, in particular, its amino acid conjugate JA-isoleucine (JA-Ile) are essential for defenses against herbivores (Howe & Jander, 2008) and necrotrophic pathogens (Glazebrook, 2005), whereas Et most probably modulates these two (Diezel et al., 2009). Defense responses induced by stylet-feeding herbivores appear to involve a cocktail of JA and SA responses (Kaloshian & Walling, 2005). In tomato (Solanum lycopersicum) JA accumulation is upstream of the expression of several defense genes, commonly used as markers for JA defenses, such as Wound-Induced Proteinase Inhibitor I (WIPI-I) and II (WIPI-II) (Farmer et al., 1992), Threonine Deaminate 2 (TD-2) (Gonzales-Vigil et al., 2011) and the activities of defensive enzymes such as polyphenol oxidases (PPOs) and peroxidases (Felton et al., 1989). SA defenses, in turn, are marked by the expression of pathogenesis-related (PR) proteins (Van Loon & Van Strien, 1999) and, in many different plant species, by the accumulation of reactive oxygen species (ROS), sometimes followed by apoptosis (Walling, 2000). Collectively, these are referred to as direct defenses. In addition, JA regulates the biosynthesis and release of an induced blend of volatiles, in part depending on SA (Ament et al., 2004, 2010), which can attract foraging natural enemies of herbivores and is therefore referred to as indirect plant defense (Kant et al., 2009).

The guild of stylet-feeding arthropods can be divided into two subguilds, those that feed predominantly on vascular sap, usually phloem sap, and those that feed from cytoplasm only (Miles, 1972). The latter applies to spider mites (Tetranychus ssp.): the adults use stylets of c. 150 μm long for lacerate-and-flush feeding on mesophyll cells, predominantly parenchyma, of which they can empty up to 18–22 min⁻¹ (Jeppson et al., 1975), leading to c. 1 mm² of visible chlorotic leaf surface area per adult mite d⁻¹ on tomato (Kant et al., 2004). The two-spotted spider mite T. urticae is highly polyphagous and has been recorded to feed from over 1100 plant species, among them tomato (Dermauw et al., 2012). This mite species is endemic to Europe. The red spider mite, Tetranychus evansi, is a specialist on Solanaceae in Brazil and Africa and a recent invasive pest in Europe (Boubou et al., 2012), where it has extended its host range and has displaced T. urticae on several host plant species in southern Europe (Ferragut et al., 2013). Adult females of both species produce, on tomato, between five and 15 eggs d⁻¹ which will develop into fertile adults within c. 2 wk, resulting in exponential population growth and, subsequently, host-plant overexploitation (Sarmento et al., 2011a). Spider mites produce silk webbing across the host-plant surface which shields them and their eggs from natural enemies. However, while biological control of T. urticae is well feasible, that of T. evansi is troublesome, as the webbing it produces is extraordinarily dense while, in addition, many biological control agents have a poor reproductive performance when preying on it (Sarmento et al., 2011b; Navajas et al., 2013).

When feeding on tomato leaves, most genotypes of T. urticae simultaneously induce expression of the JA- and SA-dependent marker genes WIPI-II and PR-P6, respectively (Li et al., 2002; Ament et al., 2004; Kant et al., 2004). However, some genotypes of T. urticae and T. evansi were found to suppress expression of these marker genes (Kant et al., 2008; Sarmento et al., 2011a). The use of the JA-perception mutant jasmonic acid-insensitive-1 (jai-1; Li et al., 2004) and of the biosynthesis mutant defenseless-1 (def-1; Li et al., 2002; Ament et al., 2004; Kant et al., 2008) has demonstrated that spider mites reach their maximal reproductive performance in the absence of JA signaling, while on 3SS::Prosystemin tomato, which is primed to display exceptionally strong JA defenses (Chen et al., 2006; Kandoth et al., 2007), reproductive performance is minimal. Although this strongly suggests that JA defenses are key anti-mite defenses for tomato, it appears that some spider mites have acquired resistance to them (Kant et al., 2008). However, such direct resistance against JA defenses was absent in the defense-suppressing T. urticae genotype (Kant et al., 2008). Taken together, these data suggest that the traits that enable some mite genotypes to suppress plant defenses are not likely to be allelic variants of the same traits that enable other mite genotypes to resist the same defenses.

Suppression of plant defenses is a phenomenon that is especially well known from plant pathogens (Abramovitch et al., 2006; Kamoun, 2006), but also herbivores, such as nematodes (Haegeaman et al., 2012) and insects (Musser et al., 2002, 2005; Will et al., 2007; Zarate et al., 2007; Weech et al., 2008; Zhang et al., 2009, 2011; Bos et al., 2010; Consales et al., 2012; Stuart et al., 2012; Wu et al., 2012), were found to manipulate plant defenses. Spider mites and insects do not share a recent history: the Chelicerates (among which the mites evolved) and the Uniramians (among which the insects evolved) diverged early in the arthropod lineage, probably well over 400 million yr ago, from an aquatic ancestor (Weygoldt, 1998), suggesting that traits that allow some of the current plant-eating insect and mite species to suppress host defenses may have evolved independently. Hence we reasoned that the distinct intraspecific and interspecific variation among Tetranychid mites (Matsushima et al., 2006; Kant et al., 2008; Sarmento et al., 2011a) forms an ideal basis for assessing some of the ecological costs and benefits of defense suppression within herbivore communities and for determining which processes are targeted by suppression. Therefore, we selected several putative defense-suppressing spider mites from natural populations, determined how tomato plants responded to them, and to what extent these responses modulate the mite’s interactions with its natural defense-inducing competitors within two species communities.

### Materials and Methods

#### Plants

Tomato (Solanum lycopersicum L. cv Castlemart, 35S::prosystemin and def-I) and bean (Phaseolus vulgaris L. cv Speedy) were germinated and grown in a glasshouse (16 : 8 h, 25 : 18°C, day : night, 50–60% relative humidity (RH)). Experiments involving plants were carried out in a climate room (25°C, 16 : 8 h, light : dark, 60% RH, 300 μmol m⁻² s⁻¹), to which plants were transferred 3 d in advance.
Spider mites

_Tetranychus evansi_ Baker & Pritchard Viçosa-1 (Supporting Information, Notes S1; Fig. S1a; Sarmento et al., 2011a), _T. evansi_ Algarrobo-1 (Fig. S1b; this paper), _T. urticae_ Koch DeLier-1 (Fig. S1c; this paper, see the section ‘Selection of _T. urticae_ DeLier-1’) and _T. urticae_ Santpoort-2 (Fig. S1d; ‘KMB’ in Kant et al., 2008) were reared on detached leaves of _S. lycopersicum_ cv Castlemart (for _T. evansi_) or _P. vulgaris_ cv Speedy (for _T. urticae_) in a climate room (25°C in 16 h:8 h, light: dark, 60% RH, 300 μmol m⁻² s⁻¹). The species identity of all four strains was confirmed on the basis of a phylogenetic reconstruction using their mitochondrial cytochrome oxidase subunit 1 (COI) sequences (Fig. S2).

For all infestation experiments and performance assays, we used adult female mites (3 ± 1 d old).

**Selection of _T. urticae_ DeLier-1**

Adult _T. urticae_ females were collected from three natural populations in the Netherlands in 2009: 125 individuals from spindle tree (_Euonymus europaeus_ L.), 64 from deadnettle (_Lamium album_ L.) and 50 from castor oil (_Ricinus communis_ L.) plants. Mites were individually transferred to def-1 leaves. Their virgin female offspring (F₁) were separated again and allowed to produce eggs on def-1. Mothers with a high reproductive performance (≥20 eggs per 4 d) were backcrossed with their sons for two more generations to F₃ (hereafter referred to as ‘strains’). The fecundity of adult females of all strains was subsequently assessed on def-1, wild-type (WT) and 35S::Proystemin tomato plants to identify JA defense-suppressing mites (Kant et al., 2008). This yielded one putative suppressor strain from the spindle tree population; three from the deadnettle population and one from the castor oil population (Fig. S3a). After comparing the expression levels of _Proteinase Inhibitor IIf_ (PI-IIf) induced by these strains on tomato with those induced by the benchmark inducer strain _T. urticae_ Santpoort-2 and in uninfested controls, we selected the strain that gave the smallest increase in PI-IIf transcript abundances for further experiments; this was _T. urticae_ DeLier-1 (Fig. S3b).

**Performance assays for individual spider mite strains**

To establish whether our spider mite strains are affected by JA-mediated defense responses, we assessed their performance on WT and def-1 tomato plants. Adult females were transferred to 21-d-old tomato plants (Methods S1): five mites per leaflet; three leaflets per plant; six plants per treatment. After 4 d, the number of eggs was recorded using a stereo microscope. This experiment was repeated three times. The total number of eggs per female were analyzed for each tomato genotype, and statistically analyzed using the Student’s t-test (PASW Statistics 17.0; SPSS Inc., Chicago, IL, USA).

**Performance assay for two spider mite strains sharing a leaflet (coinfestation)**

To assess the extent to which one strain can influence the reproductive performance of another strain, we followed the setup used in Kant et al. (2008). Leaflets of 21-d-old intact tomato plants were divided into two using a lanolin barrier. Five _T. urticae_ Santpoort-2 females were transferred to the tip-half of the leaflet, whereas the petiolule-half was infested with 15 mites from one of the suppressor strains (five + 15 mites per leaflet; three leaflets per plant; six plants per treatment). After 4 d, the number of eggs laid by the five _T. urticae_ Santpoort-2 females at the tip was recorded. This experiment was repeated three times. The average number of eggs per female 4 d⁻¹ was analyzed using ANOVA and the means of each group were compared by least significant difference (LSD) post hoc test using PASW Statistics 17.0.

**Phytohormone and gene expression assay on leaflets infested with 15 mites (time course)**

Leaflets of 21-d-old tomato plants were infested with adult female spider mites: 15 mites per leaflet; three leaflets per plant; 12 plants per treatment. At 1, 4 and 7 d postinfestation (dpi), four infested plants from each treatment and four control plants were sampled: infested leaflets and corresponding noninfested leaflets of control plants excised (without petiolule), flash-frozen in liquid nitrogen and stored at −80°C until we extracted phytohormones and mRNA. The three leaflets obtained from the same plant were pooled to form one biological replicate. Under these standard experimental conditions (Kant et al., 2004), leaflets with _T. urticae_ Santpoort-2 enter senescence 8–9 dpi and die c. 11–12 dpi (J. M. Alba & B. C. J. Schimmel pers. obs.).

**Phytohormone and gene expression assay on leaflets simultaneously infested with mites from two different strains (coinfestation)**

Leaflets of 21-d-old tomato plants were infested with adult female spider mites: five to 30 mites per leaflet; three leaflets per plant; six to 10 plants per treatment. At 7 dpi, leaflets were harvested and stored as described earlier. The three leaflets obtained from the same plant were pooled. Two types of coinfestation experiments were carried out with different infestation regimes, using _T. urticae_ Santpoort-2 (TuSP-2), _T. evansi_ Viçosa-1 (TeV-1), and/or _T. urticae_ DeLier-1 (TuDL-1). The first experiment consisted of six treatments, in which leaflets were infested with: no mites (control); 15 TuSP-2; 15 TeV-1; 30 TuSP-2; 30 TeV-1; or 15 TuSP-2 + 15 TeV-1 (coinfestation). Ten plants were used per treatment. The second experiment consisted of eight treatments: no mites; five TuSP-2; 15 TuSP-2; 15 TuDL-1; 25 TuDL-1; 15 TuSP-2 + 15 TuDL-1; five TuSP-2 + 15 TuDL-1; and five TuSP-2 + 25 TuDL-1. Six plants were used per treatment. This experiment was repeated twice.
Isolation of phytohormones and analysis by means of LC-MS/MS

Phytohormone analysis was performed using the procedure of Wu et al. (2007) with some minor modifications (Methods S2; Table S1). Amounts were compared across treatments per time point independently using ANOVA with ‘spider mite strain’ as factor. Means of each group were compared by LSD post hoc test using PASW Statistics 17.0.

Gene expression analysis

Total RNA was isolated as described in Verdonk et al. (2003). Two micrograms of DNAse-treated RNA was used for cDNA synthesis and 1 μl of 10-times-diluted cDNA served as a template for a 20 μl quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using the Platinum SYBR Green qPCR-SuperMix-UDG kit (Invitrogen) and the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). To survey tomato defenses, we analyzed expression of the following genes: PPO-D, PPO-F, JIP-21, GAME-1, TD-2, THM27, LX, PR-1a, PR-P6, PI-IIe and PI-IIif. Actin was used as a reference gene. PCR-generated amplicons were sequenced to verify primer specificity. Gene identifiers, primer sequences and references are listed in Table S2. The normalized expression (NE) data were calculated by the ΔCt method NE = (1/(PE(target)/reference)) / (1/(PE(target)/reference)); in which PE is the primer efficiency and Ct is the cycle threshold. The NE of each target gene was compared per time point independently using a nested ANOVA with ‘spider mite strain’ as factor and ‘technical replicate’ (i.e. two for each reaction) nested into the corresponding biological replicate (cDNA sample). Means of each group were compared by Fisher’s LSD post hoc test using PASW Statistics 17.0. To plot the relative expression, NE values were scaled to the treatment with the lowest average NE.

Results

Selection of putative suppressor genotypes from natural populations

To identify and isolate putative JA defense-suppressing T. urticae, adult female spider mites were collected from natural populations found on three different host plants. We reasoned that the fecundity of JA-suppressor strains should be equally high on tomato (S. lycopersicum) WT and on the JA-biosynthesis mutant tomato def-1, as suppression will only be favored by natural selection when improving the reproductive performance of mites. Hence we tested the reproductive performance of each strain on these plants. Tetranychus urticae Santpoort-2 mites produced 34 ± 3 eggs on def-1, but only 22 ± 1 on WT (Fig. S4; Student’s t-test: P = 0.003), confirming its susceptibility to JA-mediated defenses (Kant et al., 2008). By contrast, mites from the putative suppressor strains T. urticae DeLier-1, T. evansi Viçosa-1, and Algarrobo-1 produced a similar number of eggs on both genotypes of plant (Student’s t-test, P > 0.05).

The reproductive performance of defense-susceptible T. urticae Santpoort-2 mites increases when sharing a leaflet with the putative suppressor strains

Using the performance test on def-1 and WT plants, we could not exclude the possibility that a high reproductive performance on WT plants is the result of direct resistance to induced tomato JA defenses. Hence we reasoned that only a genuine defense suppressor should be able to boost the reproductive performance of a defense-susceptible mite when both reside on the same leaflet. Indeed, all three strains clearly and significantly boosted the performance of T. urticae Santpoort-2 (Fig. 1). Compared with the control, where T. urticae Santpoort-2 ‘shared a leaflet with itself’, T. urticae DeLier-1 and T. evansi Algarrobo-1 improved the susceptible strains’ fecundity with > 25%, while T. evansi Viçosa-1 did so with over 45%.

The T. evansi strains suppress expression of tomato genes that mark JA, SA and senescence, but the suppressor T. urticae strain only suppresses that of genes induced late in the interaction

In order to narrow down the mechanisms that underlie the positive effect of putative suppressor strains on the fecundity of the susceptible strain, we assessed the magnitude and timing of defense-related phytohormone and transcript accumulation in response to each of the strains.

In general, T. urticae Santpoort-2 induced a significant increase of the oxylipins 12-oxo-phytodienoic acid (OPDA, the defense-related phytohormone and transcript accumulation in response to each of the strains.

Fig. 1 The reproductive performance of the jasmonic acid (JA)-defense-inducing and -susceptible Tetranychus urticae Santpoort-2 increases on tomato (Solanum lycopersicum) leaflets shared with spider mites from suppressor strains. The figure shows the average (+ SEM) number of eggs produced by adult female mites of strain T. urticae Santpoort-2 per 4 d on leaflets simultaneously coinfested with 15 adult females of T. urticae DeLier-1, T. evansi Viçosa-1, or T. evansi Algarrobo-1, or with T. urticae Santpoort-2 as a control. Numbers within the bars indicate the average egg production. Bars annotated with different letters were significantly different according to Fisher’s least significant difference (LSD) test (P ≤ 0.05) after ANOVA.
being significantly higher than uninoculated controls at 4 dpi (Fig. 2a–c). The accumulation of free SA upon infestation with *T. urticae* Santpoort-2 mites was even more rapid, that is, significantly higher than controls after 1 d, and appeared continuous (Fig. 2d). Notably, the basal SA concentration in control plants increased as they grew older (Fig. 2d: F_{2,8} = 6.010, P = 0.025). Phytohormone accumulation induced by suppressor *T. urticae* DeLier-1 followed the same temporal pattern, albeit consistently at c. two to five times lower levels (Fig. 2). Whereas concentrations of the defense-related phytohormones clearly peaked at 7 dpi with either of the two *T. urticae* strains, only minor, nonsignificant, increases were observed for any of these hormones after prolonged infestation with the *T. evansi* strains (Fig. 2), even though they induced SA to concentrations similar to those induced by *T. urticae* DeLier-1 at 1 dpi.

We then selected 10 genes related to plant defenses for a detailed time-course expression analysis via qRT-PCR using the same samples. We selected *Polyphenol-oxidase-D* (*PPO-D*) and *PPO-F* (Newman et al., 1993; Thippayong et al., 2004), *Glycoalkaloid metabolism-1* (*GAME-1*) (Ikin et al., 2011), the *AtMYB4/PhMYB4* homolog *THM27* (Mintz-Oron et al., 2008), the Cathepsin-D-inhibitor/chymotrypsin inhibitor encoding gene *Jasmonate-inducible Protein-21* (*JIP-21*) (Lisón et al., 2006), *Threonine Deaminase-2* (*TD-2*) (Gonzales-Vigil et al., 2011), the senescence-associated T2-like RNAse inhibitor encoding gene *Jasmonate-induced Protein-21* (*JIP-21*) (Tornero et al., 1997), *PR-P6* (Van Kan et al., 1992), and *Protease Inhibitor IIc* (*PI-IIc*) (Gadea et al., 1996).

Using *T. urticae* Santpoort-2 as a benchmark, the expression pattern of the selected genes clustered into three groups (Fig. 3, black bars): those with the highest transcript abundance at 1 dpi (referred to as ‘early’; *PPO-D* and *PPO-F*), 4 dpi (‘intermediate’; *GAME-1*, *THM27*, *JIP-21* and *TD-2*), or 7 dpi (‘late’; *LX*, *PI-IIc*, *PR-1a* and *PR-P6*). Except for *GAME-1*, *T. urticae* Santpoort-2 mites induced expression of all nine other genes in the tomato leaflets.

Rapid increased expression of the ‘early’ genes was evident after infestation with suppressor *T. urticae* DeLier-1 (Fig. 3). Expression levels of *PPO-F* were even higher in the DeLier-1 samples than in the Santpoort-2 ones. This was also observed for the ‘intermediate’ genes *JIP-21* and *TD-2*. Moreover, contrary to Santpoort-2-infested leaflets, transcript abundances of all ‘intermediate’ genes in DeLier-1-infested leaflets remained above control values at 7 dpi. The expression patterns of the ‘late’ genes resulting from DeLier-1 and Santpoort-2 feeding, respectively, were similar, but in general DeLier-1 mites induced lower transcript abundances.

The two *T. evansi* suppressor strains both significantly induced the ‘early’ defense marker genes (Fig. 3). Timing and magnitude of suppression and subsequent induction of *GAME-1* and *THM-27* by *T. evansi* were similar to that of the *T. urticae* DeLier-1 strain, but the levels of induction differed considerably for *JIP-21* and *TD-2*, as the *T. evansi* strains induced both genes only slightly after 4 and 7 d. When compared with levels induced by *T. urticae* Santpoort-2 at 7 dpi, *T. evansi* inhibited *JIP-21*, but induced *TD-2*. Of the ‘late’ genes, analogous to *T. urticae* DeLier-1, only *PI-IIc* (a JA marker gene; Fig. 5; Notes S2) was induced at 1 dpi, while the three other genes were suppressed. At later time points, transcript abundances of *PI-IIc* remained elevated, albeit to a far lower extent than with the *T. urticae* strains, and those of *LX* (senescence), *PR-1a*, and *PR-P6* (both SA markers) returned to control values, or slightly higher, that is, for *LX* after infestation with Viçosa-1 (Fig. 3).

Based on these phytohormone and gene expression studies, we conclude that each mite strain triggers a unique defense response.
pattern in tomato leaflets, but that, at a particular time and compared with inducer *T. urticae* Santpoort-2, the two *T. evansi* strains suppressed ‘early’, ‘intermediate’, and ‘late’ genes, while the *T. urticae* suppressor strain DeLier-1 only suppressed the ‘late’ defense genes.

Induction and suppression of defenses do not correlate with feeding intensity

We explored the mites’ feeding intensities as an alternative explanation for differences in the magnitude of defense
induction. To do so, we assessed the total area of chlorotic lesions as a result of mite feeding (Kant et al., 2004). Notably, on leaflets infested with either of the suppressor mites, this typical white-on-green feeding damage pattern persisted at least until 7 dpi (Fig. S6a), while on leaves with T. urticae Santpoort-2 the lesions got increasingly surrounded by small areas of white-yellowish senescence and sometimes small oedema and russetting (Fig. S6b). To only assess feeding damage, we visually excluded these senesced areas as much as possible. The two T. evansi strains produced c. 100 mm² of feeding damage (Fig. S6c), corresponding to c. 9% of the total leaflet area. The two T. urticae mite strains produced a total feeding damage of c. 40 mm², corresponding to c. 3.5% of the leaflet area. The T. evansi strains thus inflicted twice as much feeding damage as the T. urticae strains. When including the senesced areas, damage inflicted by T. urticae Santpoort-2 equaled that of the T. evansi strains (data not shown). Hence, there was no positive relationship between the extent to which defenses were induced and the total leaf area that was damaged.

Tetranychus evansi suppresses the T. urticae-induced expression of JA and SA marker genes but not the upstream accumulation of JA-Ile or SA

As the most clear-cut evidence for defense suppression by spider mites is demonstrated by the increased reproductive performance of the JA-defense-susceptible T. urticae Santpoort-2 on coinfested leaflets (Fig. 1), and differences in JA and SA defense-related phytohormone (Fig. 2) and transcript abundances (Fig. 3) between inducer and suppressor strains in 'single strain-infested' leaflets were most apparent at 7 dpi, we combined both experiments to determine whether suppressor mites still manage to inhibit these defense signaling pathways when sharing a leaflet with inducer Santpoort-2.

We first selected the most potent suppressor strain, T. evansi Viçosa-1 (Fig. 1), and introduced 15 adult females to a leaflet to which, simultaneously, 15 adult T. urticae Santpoort-2 mites were also introduced. Seven days later we compared the concentrations of JA-Ile and SA plus expression levels of PI-IIc and PR-1a from these coinfested leaflets with those of uninfested leaflets (negative control), as well as with leaflets with only 15 adult T. urticae Santpoort-2 mites or only 15 T. evansi Viçosa-1 mites (positive controls). Finally, infestations with only 30 adult T. urticae Santpoort-2 mites or only 30 T. evansi Viçosa-1 were included to control for density-dependent effects.

In line with the previous results, infestation with 15 T. urticae Santpoort-2 mites resulted in strongly induced JA and SA defenses (Fig. 4a,b). The plant’s defense responses to Santpoort-2 mites increased in a density-dependent manner (Fig. S7). The 15 T. evansi Viçosa-1 mites caused minor increases in JA-Ile and SA concentrations, but higher densities of T. evansi Viçosa-1 did not further elevate hormone concentrations or PI-IIc expression, while even lowering that of PR-1a (Fig. S7).

In leaflets coinfested with 15 T. urticae Santpoort-2 mites and 15 T. evansi Viçosa-1, concentrations of JA-Ile and SA were equal to those only infested with Santpoort-2 (Fig. 4a,b). However, expression levels of PI-IIc and PR-1a were intermediate, that is, significantly lower than in leaflets with 15 T. urticae Santpoort-2, but still higher than in the leaflets with 15 T. evansi Viçosa-1 mites. Taken together, in coinfested leaflets, T. evansi Viçosa-1 does not suppress phytohormone accumulation, but only the expression of the downstream marker genes. Hence, suppression by T. evansi Viçosa-1 probably occurs downstream of phytohormone accumulation.

To test whether defense suppression within T. urticae species operates in the same way, we repeated the coinfestation experiment with T. urticae DeLier-1 as the suppressor. Here we used only five T. urticae Santpoort-2 mites, as the magnitude of suppression by DeLier-1 appeared lower than that of the T. evansi strains (Figs 2,3). The tomato JA-defense response induced by the two T. urticae strains together appeared to be additive (Figs 4c,d,S8). By contrast, the SA concentrations of coinfested leaflets equaled those infested only with Santpoort-2 and the PR-1a transcript abundances were suppressed down to intermediate values by DeLier-1 (Fig. 4c,d). Using 15 instead of five Santpoort-2 mites, or when using 25 DeLier-1 individuals, we did not observe significant cosuppression of defenses (Fig. S8). This indicates that DeLier-1 is a less potent suppressor of SA defenses than T. evansi Viçosa-1 and, although it induced a significantly lower JA response even at higher densities than Santpoort-2, it is unable to significantly suppress the Santpoort-2-induced JA-defense response.

Discussion

Suppression of plant immunity is especially well known from plant pathogens (Abramovitch et al., 2006; Burguyán & Havelda, 2011; De Jonge et al., 2011) and nematodes (Haegeman et al., 2012). In recent years, some herbivorous insects were also found to suppress plant defenses (Hogenhout & Bos, 2011), but
defense suppression by Chelicerates is still poorly documented (Kant et al., 2008; Sarmento et al., 2011a). Hence, we have characterized three JA defense-suppressing spider mite strains for the extent to which they are able to lower tomato defenses and to promote the reproductive performance of a JA defense-sensitive competing mite strain. We showed that *T. urticae* DeLier-1 is a moderate suppressor of induced defenses that improves the reproductive performance of competing *Santpoort-2* mites by 25%. Furthermore, we showed that suppression by the strains *T. evansi* Viçosa-1 and Algarrobo-1 inhibits JA and SA responses simultaneously and, hence, is not depending on the JA–SA antagonism. Moreover, suppression by *T. evansi* Viçosa-1 most likely occurs downstream of phytohormone accumulation and is powerful enough to cosuppress the expression of defense genes induced by *T. urticae* Santpoort-2, thereby boosting the reproductive performance of its competitor by 45%.

Induction of JA defenses by *T. urticae* Santpoort-2 parallels induction of SA defenses, while the other way around, suppression of JA defenses by the other three mite strains parallels suppression of SA defenses (Figs 2, 3). In fact, in tomato leaflets coinfested with *T. urticae* Santpoort-2 and *T. evansi* Viçosa-1 mites, *Pl-Ic* (JA-defense marker) and *PR-1a* (SA-defense marker) were both suppressed, even though JA-Ile and SA were induced to concentrations found in leaflets exclusively infested with Santpoort-2 (Fig. 4a,b). We therefore conclude that defense suppression by these spider mites acts downstream of phytohormones and independent of the JA–SA antagonism. By contrast, suppression by *T. kanzawai* (Ozawa et al., 2011) and some other herbivores (Zarate et al., 2007; Weech et al., 2008; Chung et al., 2013) were suggested to manipulate the JA–SA crosstalk mechanism to suppress JA defenses.

The low concentrations of phytohormones detected in leaflets infested with only suppressor mites thus do not seem to be the cause of suppression of downstream defenses, but rather are a consequence, possibly as a result of altered feedback regulation of hormone biosynthesis (Chini et al., 2007; Paschold et al., 2007 Serrano et al., 2013). The question remains as to why the simultaneous SA and JA responses induced by Santpoort-2 mites do not antagonize each other? One explanation is that these responses might be heterogeneous in space, for example, one may dominate at the feeding site and the other in surrounding tissues. Consequently, by harvesting complete leaflets we mix what in reality is an SA/JA response mosaic. Indeed, in wounded *Nicotiana attenuata* (Schittko et al., 2000; Wu et al., 2007), *Pseudomonas*-infected *Phaseolus vulgaris* (Meier et al., 1993) and elicitor-treated *N. tabacum* (Dorey et al., 1998) and *Zea mays* (Engelberth et al., 2012), defense responses were found to be stronger close to the wounded area or infection site. Not all defenses follow this pattern, as *Pl-I* transcript abundances were found to be highest distant to the wound site (Howe et al., 1996). Another explanation might be that simultaneous SA and JA responses actually do antagonize each other and what we observe are intermediate responses, as was also suggested to happen in *N. attenuata* infested with...
\textit{Manuca sexta} (Diezel \textit{et al.}, 2009). Thus, although JA and SA may crosstalk during induction of defenses by mites, their antagonistic interaction is not involved in defense suppression by mites.

Results from coinfection experiments of Santpoort-2 and DeLier-1 mites suggest that defense suppression by DeLier-1 functionally operates in the same way. The mechanistic evidence, though, is complex, as DeLier-1 triggers a defense response that is clearly distinct from that of the \textit{T. evansi} strains. The phytohormone accumulation data (Fig. 2) and the expression data on the 'late' defense genes (Fig. 3) suggest that DeLier-1 may delay defenses rather than fully block them. Despite the strong and fast induction of several JA-regulated defense genes, for example, \textit{PPO-F}, \textit{JIP-21}, and \textit{PI-IIc} (Fig. 3), suppression of JA-mediated defenses by DeLier-1 was shown to occur within the first 4 d of infestation (Fig. S3a). However, after 7 d of coinfection with inducer mites, suppression was clear for \textit{PR-1a}, but not for \textit{PI-IIc} (Fig. 4c,d). Moreover, this suppressive effect on \textit{PR-1a} was only observed when DeLier-1 outnumbered the inducer mites three to one, which confirms it is a less potent suppressor than Viçosa-1 (Fig. 1). Together, this suggests we may be overlooking the (more) relevant defenses and/or that SA defenses play a more important role in the defense response against mites than they do against herbivorous insects.

The defense suppression we observed does not act on the expression of all defense genes in a similar manner and magnitude (Fig. 3). Both \textit{PPO} genes were strongly and rapidly induced by all mite strains, including the suppressors, hence their classification as 'early'. For \textit{PPO-F}, \textit{T. urticae}, DeLier-1 even induced the overall highest transcription. PPOs are believed to act in the guts of herbivores where they may convert plant-derived flavonoids into quinones. These are highly reactive molecules that can make amino acids indigestible, can damage gut enzymes or DNA, and can form reactive oxygen species (Constabel & Barbehenn, 2008).

Two of the 'intermediate' response genes, \textit{GAME-1} and \textit{THM27}, are involved in regulation of the secondary metabolism, that is, the alkaloid and flavonoid metabolism, respectively. The same temporal (bell-shaped) expression pattern was observed for both genes in all mite-infested leaflets. \textit{GAME-1} is involved in the glycosylation of steroidal alkaloids, in particular aglycone tomatidine, presumably to reduce the autotoxicity of these metabolites (Ikin \textit{et al.}, 2011, 2013). The gene was down-regulated in all leaflets at 1 dpi but remained down-regulated only in \textit{T. urticae} Santpoort-2-infested leaflets. Tomatidine was found to be toxic to root-knot nematodes and, while most insects can cope with it, the potato aphid suffers from high concentrations (Müller \textit{et al.}, 2011). Hence, whether down-regulation of \textit{GAME-1} in \textit{T. urticae} Santpoort-2 infested leaflets reflects an effective defense response remains to be determined.

\textit{THM27} is an R2R3-MYB transcription factor that controls flavonoid metabolism (Adato \textit{et al.}, 2009; Dal Cin \textit{et al.}, 2011) and is homologous to \textit{AtMYB4} (Mintz-Oron \textit{et al.}, 2008) and \textit{PhMYB4} (Colquhoun \textit{et al.}, 2011). All mite strains down-regulated \textit{THM27} at 1 dpi, albeit not all significantly. At 4 dpi, however, it was significantly up-regulated, after which expression levels reduced again.

To put this into perspective, tomato plants might up-regulate the biosynthesis of lignins and flavonoids, including PPO substrates (Constabel & Barbehenn, 2008), especially early in the interaction, but then switch to alternative measures when the infestation progresses. Expression of \textit{JIP-21}, \textit{TD-2}, and \textit{PI-IIc} might be part of such alternative measures. They encode enzymes thought to interfere with the herbivore's digestive processes (Chen \textit{et al.}, 2005; Lisón \textit{et al.}, 2006; Gonzales-Vigil \textit{et al.}, 2011) and were induced at 4 and/or 7 dpi by all mite strains, although in a nonuniform way. For instance, after 7 d of infestation, suppressor mites had induced \textit{TD-2} to higher levels than did Santpoort-2, while this pattern was reversed for \textit{PI-IIc}. Some of the PR genes, which belong to a different class of defense genes (Van Loon & Van Strien, 1999), were sometimes found to be up-regulated upon infestation with DeLier-1, but never by the \textit{T. evansi} strains.

Using marker genes for drawing accurate conclusions regarding complex processes strongly depends on the correlation between the expression levels of such genes and the associated process. When investigating the correlation between the ‘classical’ tomato JA-marker gene \textit{PI-IIf} (Notes S2) and JA concentrations, we noticed that, especially at low JA concentrations, the gene was regularly highly expressed (Fig. S5). This suggests that not only JA but also other (hormonal) signals activated by spider mites influence its regulation. However, the correlation between the expression of another family member of this gene (\textit{PI-IIc}) and JA concentrations was much stronger and hence we used this gene as a marker for JA-related processes induced by spider mites. This underpins the fact that marker genes may require context-specific validation before being used as process indicators.

In summary, each mite strain affects the expression of tomato defense genes differently, but the putative negative effect of each of these genes on the spider mite performance remains largely unknown and is subject to future research. Furthermore, the expression pattern of the senescence-marker \textit{LX} (Lerts \textit{et al.}, 2006) at 7 dpi perfectly reflected the visual development of senescence in the infested leaflets. Possibly as a result of the induced defenses, leaflets infested with Santpoort-2 went into senescence early and in a density-dependent way before they died, while senescence in leaflets infested with DeLier-1 was less severe and came days later and \textit{T. evansi}-infested leaflets dried out without showing clear signs of senescence before dying.

The mechanism by which spider mites suppress host defenses is still unclear. Some phytopathogens, vectored by arthropods, have been implicated in the suppression of plant defenses, putatively to (indirectly) enhance their own fitness (Bellineur \textit{et al.}, 2005; Sugio \textit{et al.}, 2011; Casteel \textit{et al.}, 2012, 2014; Zhang \textit{et al.}, 2012; Chung \textit{et al.}, 2013). Preliminary data, though, indicate that spider mite-associated microbes do not mediate suppression of plant defenses (data not shown). Analogous to phytopathogens (Da Cunha \textit{et al.}, 2007; De Jonge \textit{et al.}, 2011), aphids (Rodriguez & Bos, 2013), and nematodes (Haegeman \textit{et al.}, 2012), spider mites may also secrete effectors via their saliva into plant tissues to interfere with host immune responses (Alba \textit{et al.}, 2011). The spider mite genome (Grbić \textit{et al.}, 2011) encodes at least 293 putative salivary proteins (with an \textit{E}-value < 1E–20), and thus mites are likely to
secrete a rich cocktail of proteins while feeding. Whether (some of) these predicted salivary proteins truly are involved in suppression (and/or induction) of plant defenses – and what their in planta targets are – remains to be demonstrated. Finally, the concurrent suppression of JA and SA defenses hints at manipulation of the redox homeostasis (Koornneef et al., 2008; Gruner et al., 2013), but as inhibition of phytohormone accumulation appears nonessential for suppression of the downstream response, other mechanisms are likely to be at play as well.

Our data suggest that defense-suppression traits are not very rare in natural populations of spider mites, especially not for T. evansi. Judging the effects that suppressors have on tomato, these traits may be diverse across and within species and be intertwined with (unrelated) traits that cause induction of plant defenses (Kant et al., 2008). However, the ecological costs and benefits of defense suppression are still unclear. Rationally, resistance (Kant et al., 2008) seems a ‘safer’ trait than the ability to suppress, as suppression can clearly benefit competing species as well (Fig. 1). We found putative suppressor genotypes within all three T. urticae populations we sampled (five putative suppressor strains among the 239 strains tested). This suggests that the trait is either maintained by frequency-dependent selection or results from genetic drift. Given the observation that suppression increases the fitness of these mites in the absence of competitors while – potentially – decreasing it in their presence suggests that competitor-associated fluctuating selection may be a driving force. By contrast, both T. evansi haplotypes suppressed defenses similarly and we did not observe intraspecific variation, suggesting that for this species the suppression trait got to fixation. The natural host range of the T. evansi haplotype from the Brazilian clade (such as T. evansi Viçosa-1; Fig. S2a) appears to be narrower than the ones from the Spanish clade (such as T. evansi Algarrobo-1; Fig. S2a), but both haplotypes are frequently found on several of the same solanaceous species as T. urticae in the same geographical regions (Navajas et al., 2013). Given our observation that T. urticae can increase its reproductive performance up to 45% when sharing a leaflet with T. evansi under laboratory conditions, we would not expect the displacement of natural T. urticae populations by T. evansi as is currently taking place on several host plants in southern Europe (Ferragut et al., 2013). Hence, the key question is how defense-suppressing herbivores manage to prevent or overcome the negative effects such that they themselves receive the largest net benefit from the manipulation? One of the answers may be that T. evansi monopolizes its feeding site by the production of extraordinarily large quantities of silken web, which not only shields the population from acaricides and natural enemies but also makes it hard for competing tetranychid mites to invade (Lemos et al., 2010; Sarmiento et al., 2011b). Although speculative, this trait may have been selected under pressure of competitors facilitated by the suppressed defenses. Interestingly, T. urticae DeLier-1 mites do not produce excessive amounts of webbing but do promote the reproductive performance of T. urticae Santpoort-2 and thus, if and how moderate plant-defense suppressors such as DeLier-1 protect their manipulated resources from competitors warrant more in-depth ecological research.

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References


**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Photos of adult female spider mites from each of the four strains used for this study.
Fig. S2 Phylogenetic trees based upon the cytochrome oxidase subunit 1 (COI) sequences from spider mites, including sequences from the mite strains used for this study.

Fig. S3 Fecundity of putative JA defense-suppressing *T. urticae* strains on *def-1*, wild-type (WT) and 35S::Prosystemin tomato and induction of Proteinase Inhibitor IIc (PI-IIc) by these strains upon infestation of WT plants.

Fig. S4 Reproductive performance of adult female spider mites on wild-type and *def-1* tomato.

Fig. S5 Regression analysis of jasmonic acid (JA) content and expression levels of Proteinase Inhibitor IIc (PI-IIc) and PI-IIf upon infestation of tomato leaflets with spider mites.

Fig. S6 Feeding damage inflicted by adult female spider mites on tomato leaflets.

Fig. S7 Concentrations of jasmonic acid-isoleucine (JA-Ile) and salicylic acid (SA), plus transcript abundances of Proteinase Inhibitor IIc (PI-IIc) and Pathogenesis-related protein 1a (PR-1a) in tomato leaflets after 7 d of infestation with spider mites from inducer strain *T. urticae* Santpoort-2, suppressor *T. urticae* DeLier-1, or both strains together.

Table S1 Parameters used for detection of phytohormones by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)

Table S2 qRT-PCR primer sequences

Methods S1 Protocol for infestation of tomato plants with spider mites.

Methods S2 Protocol for the extraction and quantification of phytohormones from tomato leaves.

Notes S1 Sampling and rearing of spider mites.

Notes S2 The Proteinase Inhibitor II (PI-II) gene family in tomato.

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Supporting Information Figs S1–S8, Tables S1 & S2, Methods S1 & S2 and Notes S1 & S2

Article title: Spider mites suppress tomato defenses downstream of jasmonate and salicylate independently from hormonal crosstalk

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Fig. S1 Adult female spider mites (*Tetranychus* spp.) from each of the four strains used for this study. (a) *T. evansi* Viçosa-1 with two eggs; (b) *T. evansi* Algarrobo-1; (c) *T. urticae* DeLier-1; and (d) *T. urticae* Santpoort-2. Bar, 0.5 mm.
Fig. S2 Maximum likelihood tree of the genus *Tetranychus* based upon mitochondrial *cytochrome oxidase subunit 1* (COI) sequences. Phylogenetic reconstruction based on the
Neighbor-Joining method of the COI sequences of (a) the *T. evansi* strains and (b) the *T. urticae* strains used for this study. Codes in the tree refer to GenBank accessions. Numbers at nodes indicate bootstrap values. Nucleotide divergence was estimated by the Kimura 2-parameter model. The *T. urticae* Houten-1 COI sequence (GB: KF447572) was used as outgroup in the *T. evansi* phylogenetic tree. The *T. evansi* Viçosa-1 COI sequence (GB: KF447575) was used as outgroup in the *T. urticae* phylogenetic tree. Evolutionary distance of 0.01 is indicated by the scale bar below each tree. The phylogenetic analysis was performed using MEGA version 5 (Tamura et al. 2011). The species identity of *T. urticae* and *T. evansi* was confirmed on the basis of the aedeagus (the male reproductive organ) morphology (not shown).
Fig. S3 Fecundity of adult females from each of the putative suppressor strains and *Tetranychus urticae* Santpoort-2 on *defenseless-1* (*def-1*), wild type (WT) and 35S::Prosystemin (PS) tomato
(Solanum lycopersicum cv Castlemart) and induction of Proteinase Inhibitor II (PI-II) by these strains upon infestation of WT plants. (a) Average (+ SEM) number of eggs produced by adult female mites of the putative suppressor strains and negative control T. urticae Santpoort-2 per 4 d on def-1, WT and 35S::Prosystemin. Suppressors of induced JA-defenses produce an equal number of eggs on WT and def-1, but their oviposition rate is reduced on 35S::Prosystemin, since the exceptionally strong JA-defenses employed by these plants cannot be suppressed by mites (Kant et al., 2008). (b) Relative transcript levels (mean + SEM) of PI-II in tomato leaflets after 4 d of infestation with 15 adult females from each of the putative suppressor strains. Uninfested- and T. urticae Santpoort-2-infested plants were used as negative and positive controls, respectively. Transcript levels were normalized to actin and scaled to the treatment with the lowest average normalized expression. The names of the putative suppressor strains as well as the host plant they were sampled from, i.e. Euonymus europaeus, Lamium album, or Ricinus communis, are indicated. Bars annotated with different letters were significantly different according to Fisher’s LSD test (P ≤ 0.05) after ANOVA.
Fig. S4 Reproductive performance of adult female spider mites on wild type and defenseless-1 (def-1) tomato (Solanum lycopersicum cv Castlemart) plants. The figure shows the average number of eggs (+ SEM) produced by adult female mites of strains *Tetranychus urticae* DeLier-1, *T. evansi* Viçosa-1, *T. evansi* Algarrobo-1 and *T. urticae* Santpoort-2 per 4 d on wild type and def-1 tomato leaflets. Numbers within the bars indicate the average egg production. Reproductive performance on wild type vs def-1 was evaluated by means of a Student’s t-test: **, *P* < 0.01; ns, no significant difference was found (*P* > 0.05).
Fig. S5 Regression between the jasmonic acid (JA) content and transcript levels of two JA marker genes, i.e. Proteinase Inhibitor IIc (PI-IIc) and PI-IIf, obtained from the same spider mite-infested tomato (Solanum lycopersicum) leaflets. The figure shows a scatter plot and trendline of JA-levels in nanogram (ng) per gram fresh leaf weight (gFW) vs (a, c, e, g) Log(PI-IIc) transcript levels or (b, d, f, h) vs Log(PI-IIf) transcript levels measured in leaflets 7 d post infestation with 15 adult (a, b) Tetranychus urticae Santpoort-2; (c, d) T. urticae DeLier-1; (e, f) T. evansi Viçosa-
1; or (g, h) *T. evansi* Algarrobo-1. Transcript levels (NE) were normalized to actin. The regression analysis was performed on all possible scale combinations of dependent/independent variables and shown are the scale combinations with the best fit between observed and predicted values, i.e. having the highest coefficient of determination ($R^2$). Each regression was tested by ANOVA (Sokal and Rohlf, 2012) and $P$-values (P) are presented within each plot.
Fig. S6 Feeding damage on tomato (Solanum lycopersicum) leaflets inflicted by adult female spider mites from each of the suppressor strains and Tetranychus urticae Santpoort-2. The lacerate-and-flush feeding on mesophyll cells by suppressor mites and inducer T. urticae Santpoort-2 results in distinct damage phenotypes. (a) Speckled chlorotic lesions are characteristic for all mite species. (b) However, after prolonged feeding by T. urticae Santpoort-2 the lesions get increasingly surrounded by areas of white-yellowish senescence and micro-oedema, but not on plants infested with suppressor strains. (c) Total feeding damage on tomato leaflets produced by 15 adult female mites after 7 d of infestation. Bars represent the
means (+ SEM), which are given as numbers within the bars. Bars annotated with different letters were significantly different according to Fisher’s LSD test ($P \leq 0.05$) after ANOVA. Bars (a, b), 1 mm.
The amounts of JA-Ile and SA, along with transcript levels of PI-IIc and PR-1a in tomato (Solanum lycopersicum) leaflets after 7 d of infestation with inducer Tetranychus urticae Santpoort-2, suppressor T. evansi Viçosa-1, or both strains together. The figure shows (a) the levels of jasmonic acid-isoleucine (JA-Ile) and Proteinase Inhibitor IIc (PI-IIc) transcript and (b) the levels of free salicylic acid (SA) and Pathogenisis-related 1a (PR-1a) transcript. Leaflets were infested with T. urticae Santpoort-2 (TuSP-2), T. evansi Viçosa-1 (TeV-1) or simultaneously with TuSP-2 + TeV-1 (both). Uninfested leaflets were used as controls. The numbers below the x-axis
indicate the number of adult female mites used to infest the leaflets with. The bars show the mean (+ SEM) which are given as numbers within or above the bars. Phytohormone amounts are presented as nanogram (ng) per gram fresh leaf weight (gFW). Transcript levels were normalized to actin and scaled to the lowest mean per panel. Bars annotated with different letters (lowercase for JA-Ile and SA; uppercase for PI-IIc and PR-1a) were significantly different according to Fisher’s LSD test ($P \leq 0.05$) after ANOVA. The boxed data is also presented in Fig. 4a, b.
Fig. S8 The amounts of JA-Ile and SA, along with transcript levels of PI-IIc and PR-1a in tomato (Solanum lycopersicum) leaflets after 7 d of infestation with inducer Tetranychus urticae Santpoort-2, suppressor T. urticae DeLier-1, or both strains together. The figure shows (a) the levels of jasmonic acid-isoleucine (JA-Ile) and Proteinase Inhibitor IIc (PI-IIc) transcript and (b) the levels of free salicylic acid (SA) and Pathogenisis-related 1a (PR-1a) transcript. Leaflets were infested with T. urticae Santpoort-2 (TuSP-2), T. urticae DeLier-1 (TuDL-1) or simultaneously
with TuSP-2 + TuDL-1 (both). Uninfested leaflets were used as controls. The numbers below the $x$-axis indicate the number of adult female mites used to infest the leaflets with. The bars show the mean (+ SEM) which are given as numbers above the bars. Phytohormone amounts are presented as nanogram (ng) per gram fresh leaf weight (gFW). Transcript levels were normalized to actin and scaled to the lowest mean per panel. Bars annotated with different letters (lowercase for JA-Ile and SA; uppercase for PI-IIc and PR-1a) were significantly different according to Fisher’s LSD test ($P \leq 0.05$) after ANOVA. The boxed data is also presented in Fig. 4c, d.
**Table S1** Parameters used for detection of phytohormones and related compounds by LC-MS/MS

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<th>Compound</th>
<th>Capillary CID(^1) (V)</th>
<th>Molecular ion [M-H] (m/z)</th>
<th>Fragment ion (m/z)</th>
<th>CE(^2) (V)</th>
<th>Reference</th>
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<tr>
<td>OPDA</td>
<td>-35</td>
<td>291</td>
<td>165</td>
<td>18</td>
<td>Koo <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>D(_5)-JA (IS)</td>
<td>-35</td>
<td>213</td>
<td>61</td>
<td>12</td>
<td>this paper</td>
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<tr>
<td>JA-Ile</td>
<td>-35</td>
<td>322</td>
<td>130</td>
<td>19</td>
<td>Wu <em>et al.</em> (2007)</td>
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<tr>
<td>SA</td>
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<td>137</td>
<td>93</td>
<td>15</td>
<td>Wu <em>et al.</em> (2007)</td>
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<tr>
<td>D(_6)-SA (IS)</td>
<td>-35</td>
<td>141</td>
<td>97</td>
<td>15</td>
<td>this paper</td>
</tr>
</tbody>
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\(^1\)collision-induced dissociation; \(^2\)collision energy; IS, internal standard.
### Table S2 Nucleotide sequence of primers used for qRT-PCR analysis

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<th>Target Gene</th>
<th>Name</th>
<th>GenBank (GB)</th>
<th>Gen Model</th>
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<th>Reverse Primer 5’→3’</th>
<th>References</th>
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<td>PPO-F</td>
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<td>AK247126.1</td>
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<td>CCCAAGAGGATTTTCGTG</td>
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<td>Solyc07g043</td>
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<td>TGTTTCAAGAGGATTTG</td>
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<td>Van Kan et al. (1992)</td>
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**Methods S1** Protocol for infestation of tomato plants with spider mites.

Unless otherwise indicated, we followed a standardized protocol for plant infestation experiments. We used adult female spider mites all of which within 48 h of the same age obtained from a so-called ‘egg-wave’. This egg-wave was generated by allowing random adult females from a base colony to produce eggs for 48 h on detached *Phaseolus vulgaris* cv Speedy (for *T. urticae*) or *Solanum lycopersicum* cv Castlemart (for *T. evansi*) leaflets which had been put flat on wet cotton wool, after which the adults are removed and the eggs allowed to hatch and mature in a climate room (25°C, 16 h : 8 h, light : dark, 60% RH, 300 µmol m⁻² s⁻¹). After 16 d, the 3 ± 1-d-old adult females were collected and transferred to 21-d-old tomato plants. Per plant, three leaflets were infested with mites: the terminal leaflet (at least 4 cm in length) of the youngest leaf possible; the second leaflet (i.e. non-terminal) of the second youngest leaf; and the second (i.e. non-terminal) leaflet of the third youngest leaf. A lanolin (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) barrier was made around the petiolule to prevent the mites from escaping. Uninfested control leaflets got the same lanolin barrier.

**Methods S2** Protocol for the extraction and quantification of phytohormones from tomato leaves.

Extraction and analysis of phytohormones from tomato (*Solanum lycopersicum* cv Castlemart) was performed using the procedure of Wu *et al.* (2007) with some minor modifications. In short, c. 200 mg of frozen leaf material was homogenized (Precellys 24, Bertin Technologies, Aix-en-Provence, France) in 1 ml of ethyl acetate which had been spiked with D₆-SA and D₅-JA (C/D/N Isotopes Inc, Canada) as internal standards with a final concentration of 100 ng ml⁻¹. Tubes were centrifuged at 13000 rpm for 10 min at 4°C and the supernatant (the ethyl acetate phase) was transferred to new tubes. The pellet was re-extracted with 0.5 ml of ethyl acetate without internal standards and centrifuged for 10 min at 4°C at 13000 rpm. Both supernatants were combined and then evaporated to dryness on a vacuum concentrator (CentriVap Centrifugal Concentrator, Labconco, Kansas City, MO, USA) at 30°C. The residue was re-suspended in 0.5 ml of 70% methanol (v/v), centrifuged, and the supernatants were transferred to glass tubes and then analyzed by means of LC-MS/MS. A serial dilution of pure standards of
OPDA, JA, JA-Ile and SA was run separately. Measurements were conducted on a liquid chromatography tandem mass spectrometry system (Varian 320-MS LC/MS, Agilent Technologies, Amstelveen, the Netherlands). We injected 20 µl of each sample onto a Pursuit XRs 5 column (C18; 50 × 2.0 mm, Agilent Technologies, Amstelveen, the Netherlands). The mobile phase comprised of solvent A (0.05% formic acid in water; Sigma-Aldrich, Zwijndrecht, the Netherlands) and solvent B (0.05% formic acid in methanol; Sigma-Aldrich). The program was set as follows: 95% solvent A for 1 min 30 s (flow rate 0.4 ml min⁻¹), followed by 6 min in which solvent B increased till 98% (0.2 ml min⁻¹) which continued for 2 min 30 s with the same flow rate, followed by 1 min 30 s with increased flow rate (0.4 ml min⁻¹), subsequently returning to 95% solvent A for 1 min until the end of the run. A negative electrospray ionization mode was used for detection. The parent ions, daughter ions, and collision energies used in these analyses are listed in Table S1. For all oxylipins we used D₅-JA to estimate the recovery rate and their in planta concentrations were subsequently quantified using the external standard series. For SA we used D₆-SA to estimate the recovery rate and it was quantified using the external standard. Phytohormone amounts were expressed as ng per gram fresh mass leaf material.

Note S1 Sampling and rearing of spider mites.

*Tetranychus evansi* Viçosa-1 was collected from tomato (*Solanum lycopersicum* cv Santa Clara) in a glasshouse on the campus of the Federal University of Viçosa, Brazil (GPS coordinates: 20 45.473 S 42 52.163 W) where it was maintained on detached leaves of the same tomato cultivar. It was previously shown to suppress JA- and SA-related defenses on these plants (Sarmento et al., 2011a). Ten heavily infested leaves from the Viçosa population were transferred to Amsterdam in 2009 and the mites were propagated on detached leaves of *S. lycopersicum* cv Castlemart ever since (see the Materials and Methods section). To explore whether suppression of defenses by *T. evansi* is a haplotype-specific trait we included a second haplotype, i.e. Algarrobo-1, which belongs to a different phylogenetic clade (Fig. S2a; Boubou et al., 2012). *Tetranychus evansi* Algarrobo-1 was collected in 2011 near Malaga, Spain (GPS coordinates: 36 45.487 N 4 02.407 W) from a single *S. nigrum* plant. These mites were transferred to Amsterdam in 2011 and propagated on detached leaves of *S. lycopersicum* cv
Castlemart ever since. The base population from which isofemale strain *T. urticae* DeLier-1 was selected was sampled from deadnettle (*Lamium album*) near De Lier, the Netherlands in 2009 (GPS coordinates: 51 57.124 N 4 13.108 E). After the indicated selection procedure on *def-1*, *WT* and *35S::Prosystemin* tomato plants (see the Materials and Methods section), the *T. urticae* DeLier-1 strain was not reared on tomato anymore, but on detached leaves of the common bean (*Phaseolus vulgaris* cv Speedy) to prevent selection for mites resistant to tomato-induced defenses. *T. urticae* Santpoort-2, was an isofemale strain described previously in Kant *et al.* (2008) where it was referred to as ‘KMB’. It was collected from spindle tree (*Euonymus europaeus*) near Santpoort, the Netherlands (GPS coordinates: 52 26.503 N 4 36.315 E) in 2001 and has been propagated on detached *Phaseolus vulgaris* cv Speedy leaves ever since. This was done to prevent selection for mites that either suppress, or are resistant to, tomato-induced defenses.

**Note S2** The Proteinase Inhibitor II (PI-II) gene family in tomato.

We noticed that the transcript levels of the ‘classical’ marker Proteinase Inhibitor II If (PI-IIf), also referred to as Wound-Induced Proteinase Inhibitor II (WIPII, Graham *et al.*, 1985; Farmer *et al.*, 1992; Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2004; Zhang *et al.*, 2004; Kant *et al.*, 2008) and which is highly induced by methyl jasmonate (MeJA)-treatment (Li *et al.*, 2004) and by spider mites (Kant *et al.*, 2004, 2008), did not always show a strong correlation with endogenous jasmonic acid (JA) levels (Fig. S5b,d,f,h), suggesting its expression may be regulated by additional signals. *WIPII*-II is one of six paralogous genes clustered adjacent to the genome (i.e. Solyc03g020030 until Solyc03g020080). We assessed the expression of the paralogs in correlation with endogenous JA-levels and selected Solyc03g020050 (Gadea *et al.*, 1996), which we designated as *PI-IIc* since it is the third locus in the cluster, as a more reliable quantitative JA-marker (Fig. S5a,c,e,g).
References


