Consequences of russet mite-induced tomato defenses for community interactions
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Tomato russet mites induce salicylic acid defenses but suppress jasmonic acid defenses in tomato


When attacked by herbivores, plants defend themselves by synthesizing toxins and defensive proteins. However, some herbivores have adapted to suppress induced defenses in plants. Little is known on the mechanism underlying defense suppression, and about its effects on defense responses induced by other attackers on the same plant. Here, we report on an eriophyoid mite, the tomato russet mite *Aculops lycopersici*, which induces salicylic acid (SA) defenses but suppresses jasmonic acid (JA) defenses in tomato (*Solanum lycopersicum*). Phytohormone measurements revealed that russet mites induce the accumulation of SA as well as JA in leaflets. However, several of the key JA-responsive defense genes were not or only marginally upregulated by russet mites, despite the upstream phytohormone accumulation. In contrast, the expression of the same JA-responsive defense-genes was strongly upregulated by spider mites (*Tetranychus urticae*), a natural food competitor of russet mites, whereas SA and defenses downstream of SA were induced to levels similar to those induced by russet mites. Strikingly, plants infested with the two mite species together displayed strongly reduced JA-responses, yet a doubled SA-response. To test if suppression of JA-defenses could be attributed to negative cross-talk via SA-signaling, we assessed induced defenses in SA-deficient *nahG* plants as well. The spider mite-induced JA-response in the presence of russet mites was no longer significant on *nahG*, but russet mites alone still did not induce JA-responses on *nahG* plants. This shows that russet mites suppress JA-defenses independent from SA. Moreover, once sharing a leaflet with spider mites, the russet mite-induced SA-response adds on to the spider mite-induced SA-response thereby antagonizing the spider mite-induced JA-response as a secondary effect. Taken together, we conclude that suppression of JA-defenses and simultaneous induction of SA-defenses may evoke secondary cross-talk when competing herbivores induce both responses simultaneously.

In nature, plants are exposed to multiple biotic agents, such as insect herbivores, mites and/or microbial pathogens. To protect themselves against these, they possess a wide variety of defensive chemicals, many of which are rapidly produced after an attacker has been detected and correlate with increased plant resistance (Howe & Jander, 2008). The activation of these inducible defenses is regulated by a set of phytohormones that mediate between herbivore signal (elicitor) recognition and
defense activation. Three phytohormones play a main role in regulating defense responses: jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Pieterse et al., 2009). Typically, feeding by herbivores and infection by necrotrophic pathogens induces the accumulation of JA, as well as that of its active derivative jasmonic acid-isoleucine (JA-Ile), whereas defenses against biotrophic pathogens (Glazebrook, 2005) and phloem-feeders (Kaloshian & Walling, 2005) are mediated by SA. ET is known to modulate both JA- and SA-dependent defenses (Adie et al., 2007). Additionally, other phytohormones, like abscisic acid (ABA), brassinosteroids, gibberellic acid (GA) and cytokinins, play important regulatory roles in plant defense as well (Pieterse et al., 2012). Importantly, the defense-signaling pathways regulated by these phytohormones interact within a complex network and have often been found to operate not simply additive but non-linearly via cross-talk in antagonistic or synergistic manners. It is frequently suggested that cross-talk allows plants to fine-tune the balance between different defensive strategies and growth (Koornneef & Pieterse, 2008), depending on the type of attacker that is encountered, or in the case that the plant is attacked by multiple attackers, depending on the timing and sequence of infestation (Pieterse & Dicke, 2007; Pieterse et al., 2012). Yet, the biological necessity for prioritizing one defense over the other is not always immediately evident. Of all signal interactions that can occur between hormonal pathways, cross-talk between the JA and SA pathway has received most of the experimental attention. SA has frequently been observed to antagonize JA-signaling, and these effects may take place upstream as well as downstream of JA-biosynthesis (Leon-Reyes et al., 2010). Although less often documented, it is known that JA-signaling can negatively impact the SA-pathway as well (Niki et al., 1998; Thaler et al., 1999). Notably, the net outcome of JA/SA signal interactions depends on the relative concentrations of each hormone, as synergistic, rather than antagonistic, interactions have been observed under low concentrations of JA and SA (Mur et al., 2006).

Plant defensive measures may sometimes fail to fend off herbivores, and this depends on the nature and magnitude of the response as well as the degree of susceptibility of the herbivore to this response. For instance, herbivores may avoid host plants that are potentially harmful (Bleeker et al., 2011), or develop resistance to plant-produced allelochemicals (Després et al., 2007). Alternatively, some herbivores can suppress the plant’s induced response to which they are susceptible. For instance, Kant et al. (2008) reported on a ‘suppressor’ strain of the two-spotted spider mite Tetranychus urticae which suppresses JA-defense responses in tomato, resulting in a higher performance of ‘inducer’ mites co-inhabiting the same leaflet. Suppressor mites performed equally well on wild-type (WT) and def-1 mutant plants, but had a lower performance on transgenic plants that overproduced the otherwise suppressed JA-defenses (Kant et al., 2008). Similarly, the red tomato spider mite
Tetranychus evansi was found to suppress both the JA- and SA-pathways in tomato (Sarmento et al., 2011), and this suppression was shown to occur downstream of phytohormone accumulation and independent from JA-SA cross-talk (Alba et al., manuscript submitted). Although it is not known how spider mites establish this suppression, there is reason to believe that, similar to plant pathogens (Abramovitch et al., 2006) and some herbivores (Musser et al., 2002; Bos et al., 2010; Wu et al., 2012), they produce and secrete effector proteins via their saliva that interfere with the establishment of induced responses (C. Villarroel, J. Liu & T. Van Leeuwen, unpublished data).

An alternative strategy for herbivores to block/manipulate induced plant defenses is to take advantage of cross-talk between defense-signaling pathways. Several studies suggest that herbivores can manipulate the plant’s JA-SA antagonism in a manner that benefits them (Zarate et al., 2007; Weech et al., 2008; Zhang et al., 2013). A well-known example is that of whiteflies which induce SA-defenses in Arabidopsis thaliana thereby suppressing JA-responses and this improves their performance (Zarate et al., 2007; Zhang et al., 2013). Yet, cross-talk may operate differently in different plant species (Halim et al., 2009; Rayapuram & Baldwin, 2007) which might explain why Zhang et al. (2012) found that on tobacco (Nicotiana tabacum) plants suppression of JA-defenses by whiteflies was mediated independently from SA. Furthermore, Zhang et al. (2009) reported that while co-infesting spider mite-infested Lima bean (Phaseolus lunatus) plants with whiteflies resulted in a decreased expression of two JA-associated genes and increased spider mite performance, this effect was not due to enhanced SA levels.

Taken together, although the JA/SA antagonism potentially allows plants to tailor their defenses to different attackers, and hence may be adaptive (Thaler et al., 2012), it may also be a target vulnerable for manipulation by herbivores. Moreover, in situations where the plant is attacked by multiple attackers, conflicts between SA-mediated resistance to biotrophic pathogens and JA-induced defense against herbivores can occur as a result of JA/SA-cross-talk (Bostock, 2005; Spoel et al., 2007; Verhage et al., 2010). For instance, induction of the SA pathway in Arabidopsis plants by the biotrophic pathogen Pseudomonas syringae lead to a suppression of JA-defenses and consequently leaves became more susceptible to the necrotrophic pathogen Alternaria brassicicola (Spoel et al., 2007). Hence, it is expected that in nature, where plants are exposed to a variety of attackers, such conflicts in plant defenses may occur frequently (Bostock, 2005; Thaler et al., 2010).

Here, we investigated how the cultivated tomato (Solanum lycopersicum) responds to attack by two different mite (Acarí) species, i.e., the generalist two-spotted spider mite (Tetranychus urticae) and an eriophyoid mite, the tomato russet mite (Aculops lycopersici). Adult female spider mites are ca. 0.5 mm long, whereas adult
russet mites are about 125 μm long (Lindquist et al., 1996). Spider mites produce on average 4-10 eggs per day per adult female on tomato (Kant et al., 2004; Alba et al., submitted), depending on the temperature, which develop in reproducing adults themselves within a period of 2 weeks. Russet mites complete an entire life-cycle within 7 days, again depending on the temperature, and lay between 30-50 eggs during their life. The intrinsic rate of natural increase between A. lycopersici has been estimated to be nearly equal to that of T. urticae (Kawai & Haque, 2004). This means that both mite species display a rapid and exponential population buildup causing them to easily develop into pests on economically important crops, such as tomato (Lange & Bronson, 1981). Spider mites can be controlled relatively well in greenhouses and in the field using their natural enemy, the predatory mite Phytoseiulus persimilis (Helle & Sabelis, 1985). In contrast, russet mites are difficult to control as they hide in forests of glandular leaf hairs on leaves and stems (Van Houten et al., 2013).

Interestingly, in open fields and greenhouses, spider mites and russet mites have frequently been observed together on tomato plants (CHAPTER 3). Furthermore, it was found that spider mites have a higher reproductive performance on tomatoes pre-infested with russet mites as compared to clean plants (CHAPTER 3). This prompted the question if russet mites affect the tomato plant's induced defense response in such a way that spider mites may benefit. To test this we used a common genotype of the two-spotted spider mite which induces JA- and SA-dependent defenses in tomato and is vulnerable to the JA-defenses it induces (Kant et al., 2008; Alba et al., submitted). We hypothesized that russet mite infestations may alter the interaction between the spider mite and its host plant, thereby affecting spider mite reproductive performance. To investigate this, we first applied the ‘ask-the-plant’ approach, i.e., we studied defense responses in tomato plants that had been infested with spider mites, russet mites or the combination of spider mites and russet mites. As indicators for the defense response, we measured the accumulation of the phytohormones JA, JA-Ile and SA, the transcriptional responses of four established JA-marker genes as well as an SA-marker gene. Also, we determined the activity of the enzyme polyphenol oxidase (PPO), which is induced in tomatoes in response to wounding and treatment with systemin or methyl jasmonate (Constabel et al., 1995) and therefore often used as a marker for JA-regulated responses.

Material and methods

Plants and mites

Tomato seeds (Solanum lycopersicum cv. Castlemart (CM) and S. lycopersicum cv. Moneymaker (MM) as well as the transgenic line nahG, in the genetic background MM) were germinated in soil and grown in a greenhouse compartment at a temperature of 25°C and a 15/9 h light/dark regime. One week prior to each experiment,
plants were transferred to a climate room with day/night temperatures of 27ºC/25ºC, a 16/8 h light/dark regime and 60% RH. All experiments were performed on 21-day-old plants. nahG plants are transformed with the bacterial gene nahG encoding salicylate hydroxylase which removes endogenous SA by converting it into catechol (Brading et al., 2000). The nahG gene is under the control of the constitutively expressed CaMV 35S promoter. Experiments with WT and nahG plants were always carried out in parallel.

Tomato russet mites (A. lycopersici, also referred to as russet mites or abbreviated as RM) were obtained from Koppert Biological Systems (Berkel en Rodenrijs, The Netherlands), who in turn had obtained them in the summer of 2008 from a natural infestation in a greenhouse in the Westland area (The Netherlands), and was since reared in insect cages (BugDorm-44590DH, Bug Dorm Store, MegaView Science, Taichung, Taiwan) in a climate room on tomato plants (cv. CM) that were between 3 and 5 weeks old.

Two-spotted spider mites (T. urticae, also called spider mites or abbreviated as SM) were originally obtained in 2001 from a single European spindle tree (Euonymus europaeus), in the dunes near Santpoort (The Netherlands) (GPS coordinates: 52 26.503 N, 4 36.315 E). The strain we used has been identified as a JA-inducing mite genotype and as susceptible to these defenses (Kant et al., 2008). Since its collection from the field, the strain has been propagated on detached bean (Phaseolus vulgaris) leaves that were placed with the abaxial surface on wet cotton wool and maintained in a climate room (temperature of 25ºC, a 16/8 h light/dark and 60% RH).

Infestation and sampling of plants used for enzyme activity, gene expression, and phytohormone analyses

At the start of the experiments, 21-day-old tomato plants were infested with spider mites (SM), russet mites (RM) or with the two species together. RM infestations were done by transferring the mites on small pieces of leaflets (ca. 0.5 cm²) to the leaflets of uninfested plants. These leaflet pieces had been cut from leaves picked from a well-infested tomato plant and each piece contained ca. 250 mobile stages of RM as determined with a stereomicroscope. Plants with spider mites received five spider mites per leaflet on each of three leaflets per plant. Thus, each plant received 15 spider mites in total. RM and SM were always introduced at the same time. To prevent mites from dispersing we applied a thin barrier of lanolin (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) on the petioles of leaflets that were chosen for infestation. Uninfested control plants received lanolin, but no mites. In total, three leaflets per plant were infested which were pooled at the time of sampling. This sample was taken as one biological replicate. Sampled leaflets were flash-frozen in liquid nitrogen and stored at -80ºC until total RNA or phytohormones were extracted. We
always picked the same leaflets for infestation, \textit{i.e.}, one leaflet of the second compound leaf (counted from the bottom to the top of the plant), one from the third compound leaf and the terminal leaflet of the fourth compound leaf. Sampling was performed 7 days after infestation. Generally, starting with a density of ca. 250 RM per leaflet infection symptoms became visible 5-6 days after infestation. After 7 days of infestation (the time-point of sampling) symptoms of RM-infestation were clearly visible, but leaflets were not necrotic or senesced. The relative transcript levels presented in Figures 2.2 and 2.4 represent the mean of 11-13 biological replicates, obtained from three independent experiments that were carried out at different time-points. The PPO activity results are the mean of three biological replicates, from one experiment (Figure 2.3). The phytohormone levels presented in Figure 2.5 represent the mean of 9-10 biological replicates, obtained from two independent experiments that were carried out at different time-points.

Because RM infestations frequently start at the basis of the stem (Jeppson \textit{et al.}, 1975) and because RM densities are often higher on petioles and stems than on leaves (J.J. Glas, personal observation), we decided to assess phytohormone levels in stems as well. For this experiment, 21-day-old tomato plants were infested with ca. 1500 RM (mixed stages) in total. RM were released at the basis of the main stem and stem sections were sampled 11 days after infestation. At this time-point, clear symptoms of RM damage were visible, as evidenced by the degradation of the plant’s glandular trichomes (Van Houten \textit{et al.}, 2013). From each plant, the upper part of the stem was harvested (\textit{i.e.}, the section between the points where the third and sixth true leaf, counted from the bottom of the plant, are attached to the stem) (Figure 2.1). After sampling, stem pieces were flash-frozen in liquid nitrogen and stored at -80°C until phytohormones were extracted.

**Enzyme activity analysis**

PPO activity was determined as described by Thaler \textit{et al.} (1996). Briefly, frozen and crushed leaf samples were weighed and enzymes were extracted by homogenizing approx. 200 mg leaf material in a 2 ml tube with 1.25 ml ice-cold 0.1M K Phosphate buffer (pH=8) containing 7% (w:v) polyvinylpolypyrrolidine and 400 μl of 10% Triton-X-100. The mixture was vortexed and centrifuged at 11,000 rpm at 4°C for 10 min. Of the resulting supernatant, 30 μl was added to 1 ml of a caffeic acid solution (2.92 mM in K Phosphate buffer, pH=8) and the increase in optical density (OD) at 470 nm was immediately measured, by means of a spectrophotometer (HITACHI-U2000, Tokyo, Japan). The change in absorbance was recorded every 15 s during a period of 2 min. The enzyme activity was recorded as ΔOD/min/g fresh leaf tissue but presented as relative to the mean activity of control plants (Figure 2.3).
Quantification of gene expression via qRT-PCR

Leaflets were cut at the base and three leaflets per plant were pooled in 50-ml tubes, flash frozen in liquid nitrogen, and stored at -80°C. Leaflets were ground in liquid nitrogen, and total RNA was extracted using a phenol-LiCl-based method as described (Verdonk et al., 2003). The integrity of RNA was checked on 1% agarose gels and subsequently quantified using a NanoDrop 100 spectrophotometer (Fisher Scientific, Loughborough, UK). DNA was removed with DNAse (Ambion, Huntingdon, UK) according to the manufacturer’s instructions, after which a control PCR was carried out to confirm absence of genomic contaminations. cDNA was synthesized from 2 μg total RNA using a poly(dT) primer and M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s instructions. cDNA dilutions (10×) were used as template in quantitative Real-Time PCR (qRT-PCR). Reactions were carried out in a total volume of 20 μl containing 0.25 μM of each primer, 0.1 μl ROX reference dye and 1 μl of cDNA template. Two technical replicates were performed per measurement. qRT-PCR was performed with Platinum SYBR Green qPCRSuperMix (Invitrogen, Paisley, UK) using an ABI 7500 (Applied Biosystems) system. The program was set to 2 min 50°C, 10 min 95°C, 45 cycles of 15 s at 95°C and 1 min 60°C, followed by a melting curve analysis. Target gene
expression levels were normalized to those of Actin. The normalized expression (NE) data were calculated by the ΔCt method NE = \[
\frac{1}{\text{PE}_{\text{target}} C_{\text{target}}_{\text{ct}}} / \frac{1}{\text{PE}_{\text{reference}} C_{\text{reference}}_{\text{ct}}}
\] (PE = primer efficiency; Ct = cycle threshold). The PEs were determined by fitting a linear regression line on the Ct-values of a standard cDNA dilution series. Specific amplification was ensured by melting curve analyses and generated amplicons were sequenced. For presenting the qRT-PCR data in the graphs we projected the data on a relative scale by dividing all values by the lowest average value (such that the lowest average is always 1).

As markers for the JA pathway we used four genes: (1) Polyphenol oxidase-F (PPO-F) which is one of the seven members of the PPO gene family and expressed in tomato leaves and also in type VI glandular trichomes (Thipyapong et al., 1997), (2) Throneine Deaminase-II (TD-II), involved in resistance against herbivores in tomato and tightly regulated by the JA-signaling pathway (Gonzales-Vigil et al., 2011), (3) Jasmonate-Inducible Protein 21 (JIP-21), also referred to as Cathepsin D inhibitor (CDI) or Tomato Chymotrypsin Inhibitor-21 (TCI-21) and inducible to high levels by wounding and methyl jasmonate in tomato leaves (Lisón et al., 2006) and (4) Wound-induced Proteinase Inhibitor II (WIPI-II) (Graham et al., 1985). JIP-21 and WIPI-II are induced in tomato by spider mites (Kant et al., 2004) and the latter two genes encode proteins that inhibit herbivore digestive proteinases (Hartl et al., 2011). As marker for the SA-pathway, we quantified expression levels of a fifth gene: Pathogenesis-related Protein 6 (PR-P6). Expression of PR-P6 is known to be induced in tomato by spider mites (Ament et al., 2004; Kant et al., 2004). The primers we used are listed in Table 2.1.

Quantification of phytohormones by means of LC-MS
Phytohormones were extracted by homogenizing frozen leaf material (approx. 250 mg) in screw cap tubes containing 1 ml of ethyl acetate spiked with 100 ng of D6-SA and D5-JA (‘C/D/N Isotopes’, Pointe-Claire, Quebec, Canada) as internal standards. Samples were ground twice, using a GenoGrinder (Precellys24 Tissue Homogenizer, Bertin Technologies, Aix-en-Provence, France), at 6500 rpm for 45 s and centrifuged at 13,000 rpm (8 g) for 20 min at 4°C. Supernatants from two extraction steps were pooled and evaporated until dryness in a vacuum concentrator (CentriVap Centrifugal Concentrator, Labconco, Kansas City, USA) at 30°C. The dried residue was dissolved in 250 μl 70% methanol, vortexed and centrifuged and the supernatant was transferred to LC-MS vials (Fisher Scientific, Hampton, USA).
Phytohormone measurements were conducted on a liquid chromatography tandem mass spectrometry system (Varian 320 Triple Quad LC/MS/MS). Twenty μl of each sample was injected onto a Pursuit 5 column (C18; 50 × 2.0 mm). 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), followed by 6 min in which solvent B increased till 98% (0.2 ml/min) 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), subsequently returning to 95% solvent A for 1 min until the end of the run. Compounds were detected in the electrospray ionization-negative mode. Molecular ions [M-H]\(^{-}\) at \(m/z\) 137 and 209 and 141 and 213 generated from endogenous SA and JA and their internal standards, respectively, were fragmented under 12V collision energy. The ratios of ion intensities of their respective daughter ions, \(m/z\) 93 and 97 and \(m/z\) 59 and 61, were used to quantify endogenous SA and JA, respectively. A standard dilution series of pure compounds of JA-Ile (OlChemIm, Czech Republic), JA and SA (Duchefa Biochemie, The Netherlands) was used to estimate the phytohormone concentrations and the retention time. The amounts were corrected for losses occurring during the extraction with a recovery rate, using the JA and SA internal standards.

### Statistical analysis

Gene expression data were statistically analyzed using a nested ANOVA. NE values were compared among treatments using “Treatment” (with the levels ‘Control’, ‘SM’, ‘RM’ or ‘Both mites’) as fixed factor and with the factors ‘Experimental replicate’, ‘Biological replicate’ and ‘Technical replicate’ included as random factors in the model. The factor ‘Technical replicate’ (with levels 1 and 2) was nested to the factor ‘Biological replicate’. Phytohormone data were log-transformed and analyzed using ANOVA, with ‘Treatment’ as fixed factor and ‘Experimental Replicate’ included as random factor in the model. Means of each group were compared using Fisher’s LSD (Least Significant Difference) post hoc test. Student t-test was performed in Excel (Microsoft, Redmond, WA, USA) and ANOVA followed by Fisher’s LSD tests in SPSS 20.0 (SPSS, Chicago, IL, USA).

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<th>Target gene</th>
<th>GenBank (GB) ID</th>
<th>GenModel (ITAG2.3) Primer sequence</th>
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<td>PPO-F</td>
<td>AK247126.1</td>
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<td>M61915.1</td>
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<td>AJ295638.1</td>
<td>Soly03g098790.1 QF 5'-ACTCTGCTCTTGCTTTGTCC-3' QR 5'-CCCAAGAGATTTTCTGTTGA-3'</td>
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<td>PR-P6</td>
<td>M69218.1</td>
<td>Soly00g174340.1 QF 5'-GTACTGATCTTTGTTCTCCA-3' QR 5'-TAGAATAGTCTGTGTCC-3'</td>
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Results

Russet mites suppress expression of spider mite-induced JA-marker genes

By using qRT-PCR, we quantified the transcript levels of five key defense genes in leaflets infested with spider mites, russet mites and leaflets infested with spider mites plus russet mites. As markers for the JA-pathway we tested four genes, i.e., PPO-F, TD-II, JIP-21 and WIPI-II. As compared to uninfested control plants, spider mites induced a significant accumulation of all four transcripts (P<0.001 for all four comparisons; FIGURE 2.2A-D). In contrast, russet mites did not cause a significant up-regulation of these genes (PPO-F, P=0.96; TD-II, P=0.060; JIP-21, P=0.73; WIPI-II, P=0.92; FIGURE 2.2A-D).

It is important to note that the absence of gene induction can be due to several reasons, such as a low intensity of feeding, stealthy feeding (i.e., the herbivore feeds but somehow recognition by the plant is prevented; Walling, 2008), or to defense suppression. To discriminate among these possibilities, we decided to measure JA-marker gene expression also in plants co-infested with russet and spider mites. We reasoned that in the case of defense suppression additional infestation of spider mite-infested plants with russet mites should result in the inhibition of spider mite-induced JA-marker gene transcript levels, whereas we did this not expect to happen in the case that russet mites feed stealthy or do not feed at all.

We found that in co-infested plants, the expression levels of all four spider mite-induced JA-defense marker genes were reduced to levels half of those measured in leaflets infested with spider mites only (PPO-F, P<0.001; TD-II, P<0.001; JIP-21, P<0.001; WIPI-II, P=0.010; FIGURE 2.2A-D). Hence, this result suggests that in plants co-infested with both mite species JA-defenses are suppressed, rather than not-induced. In contrast, expression of the SA-marker PR-P6 was significantly induced by russet mites (P<0.001) as well as by spider mites (P<0.001; FIGURE 2.2E). Furthermore, in co-infested plants the expression of PR-P6 was doubled as compared to the levels in plants infested with spider mites only (P<0.001) or russet mites only (P<0.001; FIGURE 2.2E).

Spider mites induce PPO activity but russet mites not

First, a pilot experiment was carried out in which we treated 21-day old tomato plants with Methyl Jasmonate (MeJA). Overnight exposure of plants to a vapor of MeJA caused a significant increase in PPO activity as compared to untreated plants, thereby validating our method (data not shown). Subsequently, PPO activity was measured in mite-infested plants. Damage resulting from russet mite feeding did not lead to an increase in PPO activity, relative to uninfested control plants (P=0.33). In contrast, spider mite feeding caused a significant 4-fold increase in PPO activity.
FIGURE 2.2 Russet mites (RM) suppress spider mite (SM)-induced expression of JA-marker genes but induce expression of a SA-marker gene. Shown are the relative transcript levels of PPO-F (A), TD-II (B), JIP-21 (C), WIPI-II (D) and PR-P6 (E) in wild-type ("WT") (cv. MM) tomato leaflets infested with five SM, RM or both species ("Both mites") together. Values (+SEM) represent the mean of 11-13 plants from three independent experiments. Different letters above bars denote significant differences in expression levels between treatments (ANOVA followed by LSD test: \( P<0.05 \)).
(P=0.007). PPO activity in plants infested with both mite species was significantly induced compared to control and russet mite-infested plants (P=0.004 and P=0.02, respectively), but similar to that in plants infested with both mites together (P=0.77; Figure 2.3).

Suppression of spider mite-induced JA-marker gene expression is not significant anymore in nahG plants

In WT plants, JA-marker genes were not, or only slightly, up-regulated by russet mites. Notably, the presence of russet mites on spider mite-infested plants caused a significant inhibition of spider mite-induced JA-marker gene expression (Figure 2.2A-D). In contrast, both spider mites as well russet mites caused an increase in expression of the SA-marker PR-P6 and this response was doubled in plants infested with both species simultaneously (Figure 2.2E). Therefore, considering the well-described cross-talk between JA and SA, we subsequently wanted to test if suppression of JA-marker genes by russet mites could be attributed to negative JA/SA-cross-talk. For these experiments we used SA-deficient transgenic nahG plants, which are unable to accumulate SA.

On nahG plants, spider mite feeding caused a strong up-regulation of all four JA-marker genes (P<0.001 for all four comparisons; Figure 2.4), whereas the up-regula-

![Figure 2.3 Russet mites (RM) do not induce PPO activity. Shown are the relative mean activities (+SEM) of the protein polyphenol oxidase (PPO) in response to damage resulting from spider mite (SM) or RM feeding on tomato (cv. CM). Enzyme activities were determined after 7 days of infestation (n=3). Activities are expressed relative to the mean activity of control plants. Mean activities were divided by the mean activity of the control to obtain relative activities. Values presented are untransformed data. Different letters above bars denote significant differences in expression levels between treatments (ANOVA followed by Fisher’s LSD test: P<0.05).](image-url)
tion of these genes by russet mites was not significant (PPO-F, P=0.092; TD-II, P=0.22; JIP-21, P=0.71; WIPI-II, P=0.87; Figure 2.4), similar to the pattern in WT plants (Figure 2.2A-D). In contrast to WT plants, spider mite-induced PPO-F and TD-II expression was restored in nahG plants that had been co-infested with russet mites (comparison ‘SM’-‘Both mites’, PPO-F, P=0.98; TD-II, P=0.53; Figure 2.4A,B). Also, suppression of spider mite-induced JIP-21 expression was not significant anymore in nahG plants that had been infested with both mite species simultaneously (comparison ‘SM’-‘Both mites’, JIP-21, P=0.068; Figure 2.4C). Spider mite-induced expression of WIPI-II was down-regulated in nahG plants infested with both mite

**Figure 2.4** Russet mite (RM)-mediated suppression of the spider mite (SM)-induced JA-response depends on SA. Shown are the relative transcript levels of PPO-F (A), TD-II (B), JIP-21 (C) and WIPI-II (D) in nahG plants infested with five SM, RM or both species (‘Both mites’) together. Values (+SEM) represent the mean of 12-13 plants from three independent experiments. Different letters above bars denote significant differences in expression levels between treatments (ANOVA followed by LSD test: P<0.05).
species simultaneously (comparison ‘SM’-‘Both mites’, \( WPI-II, P=0.012; \) FIGURE 2.4D), similar to the pattern in WT plants.

**Quantification of JA, JA-Ile and SA in WT and \( nahG \) plants**

Subsequently, to obtain further insight in defense responses induced by russet mites and spider mites we quantified phytohormone levels in plants infested with both species. To explore the possible role of SA in the inhibition of JA-defenses, we measured phytohormones in WT plants as well as in SA-deficient \( nahG \) plants. Leaflets were sampled 7 days after infestation, as in the gene expression experiments.

In WT plants, spider mites induced a ca. 2.5-fold increase in accumulation of JA (\( P=0.001 \)), whereas the amount of JA-Ile was 7× higher compared to uninfested control leaflets (\( P<0.001 \); FIGURE 2.5A,B). Russet mites also induced the accumulation of JA and JA-Ile, up to levels that were, respectively, 1.5 and 6× higher compared to uninfested leaflets (\( P=0.03 \) and \( P<0.001 \), respectively). In co-infested WT plants, levels of JA were significantly higher than in leaflets infested with spider mites only (\( P=0.013 \)) or russet mites only (\( P<0.001 \)). Similarly, JA-Ile content was significantly higher in co-infested plants than in plants infested with spider mites only (\( P=0.02 \)) or russet mites only (\( P=0.001 \)).

Compared to uninfested control plants, the levels of SA were ca. 6× higher in spider mite- as well as in russet mite-infested tissue (\( P<0.001 \) for both comparisons; FIGURE 2.5C). In leaflets infested with both mite species, SA levels were ca. 8× higher than in uninfested control plants (\( P<0.001 \)) and also higher than in plants that had been infested with spider mites only (\( P=0.018 \)) or russet mites only (\( P=0.04 \)).

Both spider mites and russet mites induced a significant accumulation of JA (\( P=0.001 \) and \( P=0.025 \), respectively) as well as JA-Ile in \( nahG \) plants (\( P<0.001 \) and \( P=0.001 \), respectively). Spider mites induced significantly more JA and JA-Ile in \( nahG \) plants than russet mites (\( P=0.028 \) for JA and \( P=0.034 \) for JA-Ile). In co-infested \( nahG \) plants, levels of JA were significantly higher than in plants infested with spider mites only (\( P=0.027 \)) or russet mites only (\( P<0.001 \)). Similarly, the levels of JA-Ile were significantly higher in co-infested plants compared to \( nahG \) plants infested with spider mites only (\( P=0.003 \)) or russet mites only (\( P<0.001 \)). In general, \( nahG \) plants seemed to produce less JA than WT plants. In \( nahG \) plants, only marginal amounts of SA were detected (FIGURE 2.5C), which confirms that SA accumulation was effectively blocked due to degradation by the enzyme salicylate hydroxylase.

**Quantification of JA, JA-Ile and SA in WT tomato stems**

In a next experiment, we also determined phytohormone levels in tomato stems, which is the tissue where russet mites under natural circumstances begin to feed (Jeppson et al., 1975). In this experiment, spider mites were excluded as a treatment
Figure 2.5 Phytohormone levels in leaflets infested with spider mites (SM), russet mites (RM), and both species (Both mites) simultaneously. The figure shows the amounts of endogenous JA (A), JA-Ile (B) and SA (C) in uninfested and mite-infested wild-type (WT) (cv. MM) and nahG leaflets (7 days after infestation). Values in the bars represent the means (+SEM) in nanogram (ng) per g fresh weight (g FW) of 9-10 plants, obtained in two independent experiments. Different lowercase letters denote significant differences for WT plants; uppercase letters denote significant differences for nahG plants (ANOVA followed by Fisher’s LSD test; *P<0.05).
since they do normally not feed on stem tissue. Remarkably, russet mites caused an almost significant two-fold reduction in JA levels in the stem (Student t-test; \( P=0.069 \)) (FIGURE 2.6A). The amount of JA-Ile in stems was very low compared to leaves and did not differ between infested and uninfested stems (\( P=0.28 \); FIGURE 2.6B). In contrast to leaves, where russet mites caused a significant increase in SA-levels (FIGURE 2.5C), the SA concentration was not increased in russet mite-infested stems as compared to uninfested stems (\( P=0.45 \); FIGURE 2.6C).

Discussion

By combining assays to assess the activity of a defense enzyme, the accumulation levels of defense gene transcripts and phytohormone levels we have dissected the defense responses of tomato plants and their net dependency on JA and SA during infestation with spider mites, russet mites or the combination of spider mites plus russet mites. Even though both russet mites and spider mites induced the accumulation of JA and its active form JA-Ile in tomato leaflets (FIGURE 2.5A,B), we found that feeding by russet mites did not or only slightly upregulate the expression of JA-marker genes, whereas spider mites up-regulated these genes 3-30× more (FIGURE 2.2A-D). In contrast, both spider mites and russet induced a strong accumulation in transcript levels of the SA-marker gene \( PR-P6 \) and these levels were doubled in the presence of both mite species (FIGURE 2.2E). In line with this, the accumulation of SA was induced by each of the two mites as well (FIGURE 2.5C). Hence, this indicates that each of the two species activates the SA pathway, in spite of the fact that the two species feed from different tomato tissues.

![FIGURE 2.6 Phytohormone levels in tomato stems. The amounts of endogenous JA (A), JA-Ile (B) and SA (C) in uninfested (control) and mite-infested (RM) tomato (cv. CM) stems, after 11 days of infestation. Bars represent means (+SEM) in nanogram (ng) per g fresh weight (g FW) (n=3). Statistical analysis was performed with Student t-test. NS = not significant.](image-url)
Concerning this latter point: even though both spider and russet mites are cell content feeders, they feed from different cells. Spider mites feed from leaf spongy parenchyma and the palisade parenchyma cells, which they pierce with their ca. 150 μm stylets to suck the contents (Park & Lee, 2002), resulting in the formation of small, white-coloured lesions, which is a typical symptom of spider mite feeding on tomato. Probably because of their much shorter stylets (ca. 10 μm), russet mites do not damage cells in the mesophyll layer but instead they pierce epidermal cells on leaves, petioles and stems of tomato (Royalty & Perring, 1988). Russet mites have a particular mode of feeding which differs from that of spider mites. They start feeding on a single epidermal cell for a short time period, which usually rapidly collapses and dies in response to the attack. Subsequently, the mite moves away to attack distal cells, and this behavior causes a massive destruction of the epidermal cell layer. In response to this, a lignin layer is formed over the parenchyma, and oxidation of this layer causes the typical symptoms of damage resulting from russet mite feeding, visible as russetting, or silvering (Royalty & Perring, 1996). In conclusion, even though the two mite species attack different cell types and have different modes of feeding, this does not prevent the plant from activating SA-mediated defense in response to both species.

By measuring enzyme activity, we found that feeding by russet mites did not lead to a significant increase in total PPO activity, whereas activity of PPOs was significantly induced upon spider mite feeding (FIGURE 2.3). PPOs constitute a class of enzymes that use molecular oxygen for the oxidation of mono- and O-diphenols to O-dihydroxyquinones. In tomato plants, PPOs are stored apart from their phenolic substrates, most likely to prevent spontaneous reactions. When leaf tissue is injured, for instance during feeding by herbivores, PPOs will mix with their phenolic substrates and rapidly oxidize O-dihydroxyphenolics to the corresponding O-quinones (Felton et al., 1989). Quinones are highly reactive, and will bind to nucleophilic –NH₂ and –SH groups of molecules, like amino acids and proteins, which can have a negative impact on herbivores, for instance by reducing the availability of essential amino acids or the digestibility of proteins, and this reduces the nutritive quality of leaves to herbivores (Felton et al., 1989, 1992). Our finding that russet mite feeding does not up-regulate PPO activity confirms previous results by Stout et al. (1994) who reported that activity of JA-dependent enzymes, including PPOs and PIs, was not induced by russet mites. In contrast, the same authors reported an induction in the activity of the JA-biosynthesis enzyme lipooxygenase (LOX), which aligns with our finding that russet mites induce accumulation of JA-related phytohormones, but not that of the downstream defense genes.

In agreement with previous studies, spider mite feeding resulted in a strong activation of the JA-dependent defense response (Li et al., 2002; Kant et al., 2004), as
indicated by a significant increase in JA-Ile accumulation (Figure 2.5A,B), in JA-marker gene expression (Figure 2.2) and in PPO activity (Figure 2.3). However, in co-infested plants the expression of JA-defense marker genes was reduced to levels significantly below the levels in leaflets infested with spider mites only (Figure 2.2A-D). From this result, we conclude that russet mites suppress JA-mediated defense responses. Unexpectedly, the levels of JA-related phytohormones remained strongly up-regulated in co-infested plants (Figure 2.5A,B), suggesting that russet mites interfere with JA-mediated defenses downstream of JA-biosynthesis.

Russet mites did not cause the accumulation of PPO-F transcript levels (Figure 2.2A), and neither of total PPO-activity. In contrast, spider mites induced a significant increase of both. In the co-infestation treatment, however, PPO-F transcript levels were intermediate while the total activity was as high as during spider mite infestation. In tomato, PPOs are encoded by a seven member gene family (Thipyapong et al., 1997) so possibly in the combination treatment other PPOs were differentially regulated as well. For example, apart from PPO-F, at least one other PPO, i.e., PPO-D, is known to be induced by spider mites as well (Alba et al., submitted), but we did not measure the expression of this gene. Since we determined total PPO activity in our enzyme activity assay, it could be that other PPOs, for instance PPO-D, are induced by spider mites but not suppressed by russet mites, thereby augmenting the intermediate PPO-F levels and explaining why PPO activity was not significantly different between spider-mite infested and co-infested plants (Figure 2.3). Expression analysis of the different PPO genes could reveal whether other PPOs compensate for the decrease in PPO-F activity or maybe are expressed to much higher levels and responsible for the total activity altogether.

It is important to note that the sampling point of day 7 after infestation was deliberately chosen. In preliminary experiments, we observed that at earlier time-points (i.e., at day 1 and 4 after infestation) russet mites induced JA-responses, whereas these responses were not significantly different anymore from control plants at day 7 after infestation. More important, significant suppression of spider mite-induced responses was observed at day 7 after infestation, whereas no differences were observed in JA-responses between plants infested with spider mites alone and plants infested with both russet mites and spider mites in the earlier time points (data not shown). Hence, this suggests that the effect of russet mites on the plant's defense response strongly depends on the time that the mites have been feeding on the plant as well on mite density, i.e., using a higher or lower number of mites for the infestations at the start of the experiments may have resulted in a different outcome.

Russet mites induced a significant increase in levels of SA (Figure 2.5C) and PR-P6 expression (Figure 2.2E). Moreover, in leaflets co-infested with both mite species SA-mediated defenses appeared to be stronger compared to leaflets with spider
mites or russet mites only. Therefore, considering the well-described antagonistic cross-talk between JA and SA (Pieterse et al., 2009), we subsequently tested if suppression of JA-marker genes by russet mites could be attributed to the antagonistic interaction of SA onto JA. We observed that spider mite-induced JA-marker gene expression was restored in SA-deficient nahG plants that had been co-infested with russet mites (FIGURE 2.4), suggesting that indeed suppression of spider mite-induced JA-defenses is due to negative cross-talk with SA. However, in contrast, russet mites alone still only induced marginal JA-responses in nahG plants (FIGURE 2.4), similar to the situation in WT plants (FIGURE 2.2A-D). Hence, from this result we conclude that suppression of the downstream JA-response by russet mites is independent from SA, whereas in plants co-infested with both species suppression does depend on SA. Taken together, these findings imply that in leaflets infested with both mite species cross-talk is a consequence, and not the cause, of the primary suppression of JA-defenses by russet mites and that in plants infested with both species their induced SA-responses add up and antagonize the spider mite-induced JA-response as a secondary effect.

Most notably, spider mites induced SA themselves to similar levels as russet mites. This raises an intriguing question: why does the russet mite-induced SA-response suppress spider mite-induced JA defenses but the SA induced by spider mites alone does not? Recall that spider mites induce JA- and SA-responses simultaneously, as seen in Figure 2.2 and 2.5. We think that actually also spider mite-induced SA antagonizes JA, albeit only to intermediate levels while only the total SA-response induced by the two mites together is powerful enough to suppress these intermediate levels to levels significantly below those in plants infested with spider mites only. This hypothesis is supported by the spider mite-induced JA-marker gene expression data in nahG: the relative expression of three of the four JA-defense markers was much stronger in nahG than in WT, i.e., relative expression of TD-II, JIP-21 and WIPI-II was, respectively, ca. 40, 30 and 400% higher in nahG plants when compared to WT plants (FIGURE 2.2B-D vs. 2.4B-D), which suggests that the spider mite-induced JA-response may be partially suppressed down to a more moderate expression level by SA in WT plants. Hence, this also implies that on co-infested nahG plants JA-defenses could in fact be partly suppressed as well by russet mites (as also indicated by Figure 2.4D), although perhaps only to intermediate levels because of stronger JA-dependent defense responses in nahG plants.

We did not find evidence for cross-talk at the phytohormone level as absence of SA did not lead to an extra increase in spider mite-induced JA or JA-Ile. In fact, levels of JA-related phytohormones tended to be lower in nahG plants compared to WT plants (FIGURE 2.5A,B). This differs from the result of a previous study where similar levels of JA were measured in WT and nahG plants under control conditions (Runyon...
Related to this, it should be noted that nahG plants may be subject to considerable pleiotropy as it has been shown that, at least in Arabidopsis, accumulation of catechol may result in a loss of host resistance to the plant pathogenic bacterium Pseudomonas syringae, due to the inappropriate production of H2O2 (Van Wees & Glazebrook, 2003). Furthermore, it has been shown that the salicylate hydroxylase gene expressed in nahG plants may act on substrates other than SA (Heck et al., 2003), which can result in pleiotropic changes in the metabolism of nahG plants. Together, this suggests that data obtained from nahG plants should be handled with caution.

To solve this problem, we attempted to create an independent tomato SA-mutant by silencing the gene Isochorismate Synthase (ICS) via callus transformation and regeneration using an inverted-repeat construct (FIGURES S2.1-S2.3). Unfortunately, this attempt was not successful: making the T0 transformants to flower took 1.5 years, and only after treating them with the SA-analogue BTH. Furthermore, the fruits we harvested carried no seeds (FIGURE S2.3). This suggests that SA-mutants in general may be severely compromised in their physiology.

Insect herbivores employ different strategies to circumvent host defenses (reviewed by Alba et al., 2011). Suppression of JA defenses may occur via the induction of SA-related defenses, as observed for the fungus Botrytis cinerea (El Oirdi et al., 2011), for lepidopteran caterpillars (Weech et al., 2008; Bruessow et al., 2010) and whiteflies (Zarate et al., 2007; Zhang et al., 2013). Other herbivores accomplish suppression independent from SA (Musser et al., 2002, 2005; Soler et al., 2012; Wu et al., 2012) or even independent from both the JA- and SA-pathway (Consales et al., 2012). Zhang et al. (2009) showed that additional infestation of whiteflies on spider-mite infested lima bean plans lead to a reduction in the levels of not only JA but also SA. This suggests that whitefly-mediated interference with spider mite-induced plant responses is not due to negative cross-talk with SA. In contrast, in our system we found that SA is involved in the suppression of JA-responses induced by spider mites on co-infested plants. However, as our results also suggest, the primary suppression of JA-defenses by russet mites is independent from SA and, hence, other factors must be involved as well. This is also indicated by the fact that induction of JA-related phytohormones was not increased in russet mite-infested SA-deficient plants compared to WT plants infested with russet mites. Therefore we conclude that, unlike whiteflies on Arabidopsis (Zhang et al., 2013), russet mites suppress JA defenses in an SA-independent manner. In Figure 2.7, a schematic overview is provided of the JA and SA responses induced by russet and spider mites and how we think these interact in plants infested with both mite species.

It is tempting to speculate that the presence of russet mites on a spider mite-infested plant may have affected the performance of these spider mites not only via the plant but also as a result of direct competition between the two herbivores.
such interactions would occur it could, in theory, cause a decrease in spider mite feeding intensity, which, in turn, could (partly) explain the reduced JA-defenses we observe in the presence of russet mites. However, there are several data that contra-

Spider mites induce SA and JA defenses in WT plants. Probably, during this dual-response, SA antagonizes JA since in nahG the JA-markers are induced to higher levels (compare the “SM” bars of Fig. 2.2B-D with those in Fig. 2.4B-D). Alternatively, JA may antagonize SA in WT plants since suppression of JA-responses via russet mites gives rise to a stronger SA-response (see the “both mites” bar in Fig. 2.2E).

Russet mites induce SA, JA and SA-dependent responses. SA-marker gene transcript levels are similar to those induced by spider mites (bar “SM” vs “RM” in Fig. 2.2E). In contrast, russet mites suppress JA defenses since they, like spider mites, induce JA-related phytohormones (white bars “SM” vs “RM” in Fig 2.5A,B) but not the downstream JA-marker genes, like spider mites do (bar “SM” vs “RM” in Fig. 2.2E). This suppression is independent from SA (bar “control” and “RM” in Fig. 2.2A-D vs. Fig. 2.4).

Both russet mites and spider mites induce SA but the SA-dependent response is doubled in co-infested plants (Fig. 2.2E). This increases the antagonistic effect of SA on the spider mite-induced JA-responses, since in nahG, the intermediate response observed in WT plants (Fig. 2.2A-C) disappears (Fig. 2.4A-C). In conclusion, russet mites suppress JA-defenses independently from SA but the SA-response they induce augments the SA-response induced by spider mites on the same leaf, thereby antagonizing the spider mite-induced JA-response via JA/SA-crosstalk as a secondary effect.

**Figure 2.7** Schematic overview of the JA and SA responses induced by spider mites (A), russet mites (B) and the two species together (C) on tomato. AU=Artificial Unit; WT = wild-type.
dict this direct interference hypothesis. First, we observed that spider mites laid more eggs on WT tomato plants that had been pre-infested with russet mites as compared to uninfested plants (Chapter 3), suggesting they eat equally much or more, not less, in their presence. Second, expression of PR-P6 was doubled in WT plants co-infested with both species compared to plants infested with either of the species alone, suggesting that the feeding damage is equal or more in the presence of both species. Third, for three of the four JA-marker genes significant suppression was not observed in co-infested nahG plants, which implies that spider mites keep on eating and damaging these plants, also in the presence of russet mites. Finally, we have no indications of direct interactions between russet mites and spider mites suggesting that other, plant-mediated, factors must have been responsible for the observed effects.

Russet mites often prefer to colonize the stems of tomatoes before the population expands to the leaves. Remarkably, in stems infested with russet mites, levels of JA were reduced twofold as compared to the levels measured in uninfested control stems (Figure 2.6A). Before jumping to conclusions, it is important to keep in mind here that russet mite infestation leads to a rapid degradation of trichomes on tomato stems (Chapter 5) and that trichomes from tomato stem tissue have been reported to contain significant amounts of JA (Peiffer et al., 2009). Hence, it is possible that the reduction in JA-content in russet mite-infested stems is (partly) due to the loss of trichomes on this tissue, rather than to a suppression of endogenous JA biosynthesis. To investigate if this induced morphological alteration stands at the basis of the JA-suppressed phenotype in stems, it would be relevant to measure phytohormone levels in trichome mutants as well. For instance, the odorless-2 mutant could be a good candidate for this since it has less type VI trichomes than WT plants, while it is unaffected in its JA-regulated defenses in the leaves (Kang et al., 2010).

In general, JA-Ile amounts (per g fresh weight) in tomato stems were very low as compared to the levels in leaves. In tomato, JA is produced primarily in the companion cells of the phloem (Hause et al., 2003; Stenzel et al., 2003) while biosynthesis of JA-Ile has been reported to occur in leaves, in a conjugation reaction carried out by the enzyme Jasmonate Resistant 1 (JAR1) (Suza et al., 2010). Matsuura et al. (2011) confirmed that biosynthesis of JA-Ile from JA and Ile occurs in wounded tomato leaves. Furthermore they found, by applying radiolabeled JA-Ile in relatively high amounts to the wounded tissue, that JA-Ile was transported throughout the plant via the stems and, hence, that it could be a mobile signal. It is unknown at present if JAR1 expression is restricted to particular tissues, such as the phloem where JA is produced, or to the mesophyll where JA-Ile binds to the SCFCOI1 complex (Wasternack et al., 2006). Possibly, conjugation of JA and Ile occurs predominantly in non-stem tissue, which could explain the low JA-Ile amounts in the stem.
Alternatively, there could also be a trivial explanation for the observed difference, i.e., tomato stems may contain relatively more water than leaves and, hence, comparing phytohormones on the basis of tissue fresh weight may be inappropriate. However, the amounts of SA in uninfested stems were in the same range as those observed in leaves, when calculated per g tissue fresh-weight, suggesting the comparison may actually hold. Unexpectedly, SA levels did not increase in russet mite-infested stems (Figure 2.6C), as they did in russet mite-infested leaflets (Figure 2.5C). This suggests that suppression of JA accumulation in stems is independent from a concomitant increase in SA. Taken together, induced phytohormone accumulation in stems may differ from that in the leaves and a more detailed analysis of defense gene expression in these tissues is necessary to determine if the same applied for induced defenses. Since the stems are the primary habitat of tomato russet mites, these data could provide important additional insights in how russet mites deal with host plant defenses.

On the possible mechanisms along which russet mites interfere with the plant defense response we can only speculate. Yet, concerning this, first it is important to note that the saliva of some gall-inducing eriophyoids may contain phytohormonal substances, which, upon feeding, can be secreted into epidermal cells where they initiate development of (abnormal) plant tissues. For instance, eriophyoid galls were found to contain more indole acetic acid (IAA) than non-attacked leaf tissue (Balasubramanian & Purshotaman, 1972; in: Krantz & Lindquist, 1979). De Lillo & Monfreda (2004) suggested that saliva of Aceria caulobia contains IAA and cytokinin-like compounds, which probably is related to the ability of this mite species to induce galls on its host plant, Suaeda fruticosa. To the best of our knowledge, nobody ever reported on substances present in the saliva of vagrant eriophyoid species, such as russet mites. Yet, technically it is possible to collect ‘salivary secretions’ from this species (De Lillo & Monfreda, 2004; Figure 2.8), which opens up exciting new possibilities to analyze the content of these fluids and their (potential) impact on host plant defenses.

Second, increasing evidence suggests that herbivores produce so-called effectors, i.e., proteins that are secreted in the saliva to suppress plant defenses, reminiscent of bacterial and fungal pathogens (Nomura et al., 2005). The first effector protein characterized from a herbivore was Glucose oxidase (GOX), which is widely present in the saliva of caterpillars, like Helicoverpa zea (Musser et al., 2002) and, as was discovered later, also in the saliva of some phloem feeding insects such as aphids (Harmel et al., 2008). The presence of GOX in caterpillar saliva was found to suppress wound-induced accumulation of nicotine in tobacco (Nicotiana tabacum) plants (Musser et al., 2002, 2005). Furthermore, it was found that also H. zea saliva contains ATP hydrolyzing enzymes (i.e., an apyrase, ATP synthase and ATPase
John A1), which could suppress JA- and ET-regulated defense genes in tomatoes (Wu et al., 2012). As in caterpillars (Musser et al., 2002, 2005; Schmelz et al., 2012) and aphids (Bos et al., 2010), it is tempting to consider the possibility that russet mites produce and secrete effectors in their saliva, targeting specific components in defense signaling pathways. Later on in this thesis (CHAPTER 6) I will go in more detail into this. At this point, it suffices to mention that we have obtained a complete genome and transcriptome of russet mites and, from the transcriptome data, we have identified several candidate effector proteins (CHAPTER 6), which we are planning to test for their effect on host plant defenses.

In conclusion, we have shown that russet mites suppress JA-responses, since they induce accumulation of SA and JA-related phytohormones, while only the downstream SA-response, and not the JA-response, is up-regulated by russet mites. Furthermore, since russet mites also did not induce JA-responses on SA-deficient nahG plants, we could show that russet mites suppress JA-defenses independent from SA. However, since the JA-response was restored on co-infested nahG plants, we conclude that induction of SA by russet mites does antagonize the JA-response induced by spider mites, when sharing the same leaflet.

The fact that suppression of defenses affects responses induced by competing species prompts the question if such indirect interactions can modify a herbivore’s community structure. Cultivated tomato can be attacked by as many as 100-200 dif-

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**Figure 2.8** Light micrograph of an *Aculops lycopersici* specimen and its “salivary secretion” at the basis of the mouthparts (see arrow). Mites were collected by spinning an infested tomato leaflet in an Eppendorf tube. Subsequently, they were mounted in a droplet of microscope immersion oil on a slide and observed under a light microscope (magnification of 400×) (following the method of De Lillo & Montedìa, 2004).
ferent arthropod herbivores (Lange & Bronson, 1981), and russet mites are a world-
wide pest of tomatoes (Perring, 1996). Hence, it is likely that in nature as well as in
greenhouses russet mites often co-occur on plants with other pest species. Probably
because infestations are often recognized too late, russet mites densities in green-
houses have been reported to be much higher than those used in the present study
(Kawai & Haque, 2004). Therefore it is possible that, under these (semi-natural) con-
ditions, the effect of russet mite-induced responses on spider mite-induced defense
responses may be much more pronounced as the effects observed in the present
study, i.e., the intermediate responses we have observed during co-infestation may
escalate to full suppression. The consequences of russet mite-induced (SA) and
-suppressed (JA) defenses for natural competitors of russet mites are described in
CHAPTER 3.

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CHAPTER 2 | INDUCTION AND SUPPRESSION OF DEFENSES IN TOMATO


SOCIAL NETWORKS


Creating the ICS RNAi construct
To investigate the role of SA in the plant-mediated interactions between russet mites, spider mites and the phytopathogen Pst DC3000, we have made use of transgenic nahG plants. However, since these plants may give rise to unwanted side-effects due to the accumulation of catechol (Van Wees & Glazebrook, 2003), we also took a different approach by attempting to create transgenic lines in which expression of Isochorismate Synthase (ICS) was knocked down using RNA-interference (RNAi). ICS encodes an enzyme involved in SA biosynthesis in tomato plants (Uppalapati et al., 2007).

A gene construct was designed to simultaneously silence ICS and the β-glucuronidase (GUS) reporter gene, via the production of short interfering hairpin RNAs (hpRNA). The reason to include GUS is that it can be transiently expressed in leaves of stably transformed plants using ATTAs, thereby making it easier to screen for plants that show strong silencing (Ament et al., 2010). This method was successfully applied before for the silencing of SAMT and two interactors of a NBS-LRR-encoding resistance protein in tomato (Ament et al., 2010, and Lukasik-Shreepathy et al., 2012, respectively), as well for the silencing of NBS-LRR-encoding genes in lettuce (Wroblewski et al., 2007). The construct was made as follows: a 352-bp fragment of the 3’ part of the mRNA (Solyc06g071030; 1497-1850 bp downstream from the ATG codon) (Figure S2.1) was amplified with primers on which SfiI cleavage restriction sites had been introduced: F-Sfi-ICS = ATGGCCATGTAGGCC-GAAGAGAGTGAATTTGCGGTTG and R-Sfi-ICS = ATGGCCAGAGGCC-AAATTGCATATGACAGGTTGATG (15 bp-adapters containing the Sfi restriction sites are indicated in bold), using a proofreading polymerase (Phire Hot Start, Thermo Scientific, Landsmeer, The Netherlands). After sequencing the fragment, it was digested with restriction enzyme SfiI and ligated in the pre-made binary vector pGollum, which contained already a 344-bp part of the GUS gene (U12639, bases 3514-3858) fused to an intron (~700 bp) of the pyruvate orthophosphate dikinase (pdk) gene from Flaveria trinervia, which separated the two arms of the inverted repeat (Figure S2.2). The complete ICS fragment was sequenced in this construct, including its borders, using the Sfi-ICS primers, the pGreenF2 primer (ACTATCCTTCGCAAGACCC), annealing to the 35S promoter, a primer for the GUS fragment (CCAACTCCTACCGTAC) and the OSCtermRev primer (TCATGCGATCATAGGCGTCT), annealing to the OCS terminator. SfiI was purchased from New England Biolabs (Ipswich, UK) and T4 DNA ligase was obtained from Fermentas (St. Leon-Rot, Germany).
Stable plant transformations

For tomato-stable transformation, the construct was introduced into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983), using electroporation. Transgenic plants were produced using explants derived from cotyledons of sterile seedlings of *S. lycopersicum* (cultivar CM), as previously reported (Cortina & Culiáñez-Macià, 2004).

Analysis of transgenic plants

Genomic DNA was isolated from leaves of the different T0 lines and PCR was used to determine whether plants had been transformed, using primers on the 35S pro-

FIGURE S2.1. Sequence of the 3041bp SlICS mRNA. Coding sequence is indicated in uppercase; 5’and 3’UTR are indicated in lowercase; start codon in bold. Primer sequences used to generate the RNAi construct are underlined.
motor sequence (35s_F: TTCAAAGCAAGTGGATTGATGTG and 35s_R2: GCTCTTAT-ACTCGAGC GTGTCC). The plasmid was included as a positive control. Three independent lines were obtained. Transformed plants were severely affected in their morphology, as they grew in a ‘bush-like’ structure, evidenced by the production of many

Figure S2.2 Sequence of the 35S:GUS-ICS construct. (A) Different parts of the sequence are indicated by different colours (35S promoter=green; ICS fragment=red; GUS fragment=blue; intron=black) Primers used for sequencing are underlined. (B) the same sequence but with the inverted repeat (as expressed in the plant).
side-branches and stunted growth (Figure S2.3). One year after transfer to the soil, the plants still had not produced a single flower. Hence, we started to spray them with the SA-analogue BTH (Syngenta, Basel, Switzerland) in a concentration of 0.2 g/l to rescue possible defects caused by SA-deficiency. Plants were sprayed every 2 days. This approach seemed successful, because the plants started to produce flowers and fruits, 8 weeks after the BTH treatment was started. Unfortunately, however, the fruits harvested did not contain any seeds (Figure S2.3). In a second rescue attempt, plants were grafted on rootstocks of WT plants (cv. CM) but unfortunately...
also this approach did not result in the production of seed-containing tomatoes. To test whether the silencing of ICS had an effect on SA levels, phytohormones were measured in the (un-induced) T0 plants. Phytohormone measurements were performed as described in the Material and methods section. Most transformed plants displayed 2-fold reduced SA-levels compared to the untransformed control plant. In contrast, JA-Ile levels were higher in transformed plants compared to the untransformed control plant, which might be due to the JA/SA-antagonism (Figure S2.4).

Figure S2.4 Phytohormone levels in ir ICS plants. Amounts of endogenous JA (A), JA-Ile (B) and SA (C) in a untransformed (Untransf.) as well as in several transformed plants that were either cut or grafted.
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