The salivary proteome of Tetranychus urticae
Key to its polyphagous nature?
Jonckheere, W.S.A.

Citation for published version (APA):
Jonckheere, W. S. A. (2018). The salivary proteome of Tetranychus urticae: Key to its polyphagous nature?.

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Salivary proteins of spider mites suppress defenses in *Nicotiana benthamiana* and promote mite reproduction

Published as:
2.0. Abstract

Spider mites (Tetranychus sp.) are widely occurring arthropod pests on cultivated plants. Feeding by the two-spotted spider mite T. urticae, a generalist herbivore, induces a defense response in plants that is mainly depending on the phytohormones jasmonic acid (JA) and salicylic acid (SA). However, on tomato (Solanum lycopersicum), certain genotypes of T. urticae and the specialist species T. evansi were found to suppress these defenses. This phenomenon occurs downstream of phytohormone accumulation via an unknown mechanism. We investigated if spider mites possess effector-like proteins in their saliva that can account for this defense suppression. First we performed an in silico prediction of the T. urticae and the T. evansi secretomes, and subsequently generated a short list of candidate effectors based on additional selection criteria such as life stage-specific expression and salivary gland expression via whole-mount in situ hybridization. We picked the top five most promising protein families and then expressed representatives in Nicotiana benthamiana using Agrobacterium tumefaciens transient expression assays to assess their effect on plant defenses. Four proteins from two families suppressed defenses downstream of the phytohormone SA. Furthermore, T. urticae performance on N. benthamiana improved in response to transient expression of three of these proteins and this improvement was similar to that of mites feeding on the tomato SA-accumulation mutant nahG. Our results suggest that both generalist and specialist plant-eating mite species are sensitive to SA defenses but secrete proteins via their saliva to reduce the negative effects of these defenses.
2.1. Introduction

Phytophagous mites (Acari) comprise a diverse group of herbivores that constitute several species that are pests in crop plants. Within this group, the spider mites (Tetranychus sp.) are of special interest since they cover a broad host plant range and can develop into devastating outbreaks (Grbić et al., 2011b; Van Leeuwen et al., 2015). Adult spider mites feed from leaves by piercing mesophyll cells with their styles. Spider mites possess three pairs of salivary glands associated with these styles (Alberti and Crooker, 1985; Mothes and Seitz, 1981). Via their styles they inject saliva into pierced host cells and then probably retract the styles. Then they seal off the puncture wound with their mouth lobes after which they suck out the cytoplasm of these cells using their pharynx, which is a muscular food pump that transports the food to the oesophagus (Alberti and Crooker, 1985). For most mite genotypes, this mode of feeding induces in the plant an array of responses associated with an elevation of its defenses (Alba et al., 2015), and these include the increases in: the expression of genes associated with defenses, the activities of defense-related enzymes (Kant et al., 2004), and the accumulation of metabolites (Martel et al., 2015; Zhurov et al., 2014), some of which are released as volatiles that mediate indirect defenses (Ament et al., 2004). These defenses are primarily controlled by the phytohormone jasmonic acid (JA), whose downstream response is known to be effective against a wide range of arthropod herbivores including mites (Kant et al., 2008) and insects (Howe and Jander, 2008) but also necrotrophic pathogens (Glazebrook, 2005). Simultaneously with induction of the JA-pathway, spider mite feeding triggers the salicylate (SA) defense pathway (Kant et al., 2004), which is well characterized for its central role in controlling infections by (hemi-)biotrophic pathogens (Vlot et al., 2009). Not only spider mites (Ament et al., 2004; Matsushima et al., 2006; Glas et al., 2014; Alba et al., 2015) but also several insect herbivores induce such a mixture of JA and SA related defenses (Cao et al., 2014; Glas et al., 2014; Zhang et al., 2013a). Although the effect of SA-related defenses on herbivores is less well characterized than the effect of JA defenses, they are known to play an important role against some hemipterans such as aphids (Avila et al., 2012; Li et al., 2006; Thompson and Goggin, 2006). Finally, the JA and SA signaling pathways were found to antagonize each other (Robert-Seilaniantz et al., 2011; Thaler et al., 2012) via distinct regulatory hubs in signaling networks, probably in order to fine-tune the collective
defense responses (Gimenez-Ibanez and Solano, 2013). As a consequence, several species of pathogens and insect herbivores have adapted to exploit this hormonal antagonism to their own benefit by inducing a harmless defense at the expense of the harmful defense (Kazan and Lyons, 2014; Thaler et al., 2012).

Distinct types of spider mite adaptations have been reported that enable mites to counteract a host plant’s induced defense responses (Kant et al., 2015; Wybouw et al., 2015). The generalist spider mite species *Tetranychus urticae* harbors traits that allow individuals or local populations to either resist JA- and SA-related plant defenses or to suppress these to levels at which they are less detrimental (Kant et al., 2008). However, such traits can be rare and most mite individuals are sensitive to the plant defenses they induce given the fact that they perform better on mutant plants lacking distinct defenses (Alba et al., 2015). In addition, the mite species *T. evansi*, specialized on Solanaceae, was shown to reduce tomato JA- and SA-related defenses down to levels below those of non-infested plants turning these into superior food for itself and conspecifics (Sarmento et al., 2011). However, defense manipulation by herbivores also has consequences for interspecific competition since leaves infested with defense-suppressing mites can promote the performance of defense-susceptible competing species that co-inhabit the plant (Alba et al., 2015; Kant et al., 2008) and this may affect the suppressor negatively (Glas et al., 2014). Defense suppression by phytophagous mites is established independent from the SA-JA antagonism, although it may influence the final magnitude of the remaining defense response, and it likely occurs downstream of JA- and SA-accumulation (Alba et al., 2015; Glas et al., 2014). How herbivores like spider mites accomplish manipulation of host plant defenses is yet to be determined (Kant et al., 2015).

Defense suppression is a common strategy of phytopathogens to establish disease. Such plant pathogens can interfere with the defense response of their host by secreting molecules, called effectors, which interact with host defensive components and modulate these to their benefit. Often effectors are secreted in mixtures together with other proteins that can perform diverse functions, such as facilitating the penetration processes, or detoxification and digestion of plant material, and some of these proteins are recognized by plants probably as the result of an evolutionary arms race (Göhre and Robatzek, 2008). Different definitions of ‘effector’ were pro-
posed in literature (Thomma et al., 2011), among which a broad definition that considers effectors to be any parasite-secreted protein or small molecule that alters host-cell structure and function (Hogenhout et al., 2009; Schneider and Collmer, 2010). Such parasite effectors include molecules, often proteins, that manipulate plant resource allocation (Walters and McRoberts, 2006), plant morphology (Caillaud et al., 2008) or defense responses (Thomma et al., 2011).

Secreted effectors of non-arthropod herbivores, such as nematodes, that manipulate plant tissues and interfere with defenses are well documented (Haegeman et al., 2012). However, secretion of effector proteins by herbivorous arthropods, which comprise the largest diversity of crop pests, is largely unexplored territory. Nevertheless, there is an increasing notion that also among phytophagous insects secretion of effectors may be a strategy to overcome host plant defenses (Hogenhout and Bos, 2011; Kant et al., 2015; Stuart, 2015). Several species of lepidopteran caterpillars were found to secrete saliva containing the enzyme glucose oxidase which modulates plant defense responses (Musser et al., 2012), and the Hessian fly *Mayetiola destructor* was found to secrete an avirulence factor called vH13, which triggers ETI-like (effector-triggered immunity-like) resistance in plant carrying the H13 resistance gene (Aggarwal et al., 2014). Finally, effector-mediated suppression of PTI (PAMP-triggered immunity) was reported for the green peach aphid (*Myzus persicae*) salivary protein Mp10, which affected components of PTI when expressed in *Nicotiana benthamiana* (Bos et al., 2010). Although ectopic expression of some putative salivary aphid effectors improved aphid performance (Bos et al., 2010; Elzinga et al., 2014; Naessens et al., 2015; Pitino and Hogenhout, 2013) the expression of others affected performance negatively (Bos et al., 2010; Chaudhary et al., 2014). Hence, functional validation of herbivore effectors and elicitors of plant defenses clearly requires herbivore performance assays to validate if changes have occurred in the plant that benefit the herbivore.

Mites and insects do not share a recent evolutionary history: they probably descended from an ancient aquatic arthropod ancestor and diverged already over 400 million years ago (Weygoldt, 1999). Since several species of phytophagous mites were found to be able to suppress host plant defenses (Alba et al., 2015; Glas et al., 2014; Kant et al., 2008; Sarmento et al., 2011; Wybouw et al., 2015) we hypothesized that mites, like pathogens, insects, and nematodes, may have evolved effector proteins which are secret-
Spider mite effectors promote mite reproduction

ed via their saliva into host cells during feeding to modulate the host’s defense responses.

Here we have identified several salivary-secreted candidate effector-proteins of spider mites and we have investigated the impact of transient *in planta* expression of these candidate effectors on the induced defense response of *N. benthamiana* and on spider mite reproductive performance. Using bioinformatics, *in situ* hybridization, gene-expression analysis and bioassays, we provide evidence that spider mites produce salivary proteins that have a strong negative effect on the plant’s SA response and we show that this suppression of SA defenses promotes the mite’s reproductive performance.

### 2.2. Material and methods

#### 2.2.1. High-throughput sequencing and *de novo* assembly

*Tetranychus evansi* Viçosa-1 (Alba et al., 2015) whole transcriptome was sequenced from cDNA using 454 GS+ Titanium technology at Eurofins (MWG, Germany). The raw reads were submitted to the Sequence Read Archive (SRA) at NCBI under the accession number SRR2127882. The final assembly produced 31,263 isotigs, from which a subset of 17,663 isotigs assembled from 5 or more reads were used to predict their coding regions and protein sequences using ORF-predictor (Min et al., 2005). Details on the sequencing and assembly can be found in METHODS S2.1.

#### 2.2.2. *In silico* prediction of the spider mite secretome

For *T. urticae* secretome prediction, the predicted proteins from *T. urticae* London genome (Grbić et al., 2011a) were used. The signal peptide prediction was done using SignalP 4.0 (stand-alone version; Petersen et al., 2011) and Phobious (Käll et al., 2004). Transmembrane domains were predicted using THMMM (standalone version; Krogh et al., 2001) and Phobious. Subcellular localization was predicted by TargetP (stand-alone version; Emanuelsson et al., 2007) and WoLF PSORT (stand-alone version; Horton et al., 2007). Default settings were used for all software parameters.

#### 2.2.3. Markov clustering and BLAST procedures

The pipeline to cluster candidate effector proteins by amino-acid similarity was described in Saunders et al. (2012); i.e., the predicted secreted proteins
of *T. urticae* and *T. evansi* were combined in one database (with their signal peptides removed). After a BLASTp search of the combined database against itself, the output was piped to TribeMCL (Enright et al., 2002) using default settings. To annotate the combined secretome, a database containing 88,616 tick’s reference proteins was created by obtaining protein sequences available at NCBI (using keyword ‘Ixodida’ [porgn:__txid6935]), subsequently a BLASTp search of the combined secretome to this database was performed (using an E-value cutoff of e-10).

### 2.2.4. RNA isolation and RT-qPCR

*Tetranychus urticae* and *T. evansi* main body parts (‘idiosoma’) were collected after removal of the anterior body part (‘gnathosoma’) using a scalpel on a glass Petri dish pre-cooled with liquid nitrogen. *N. benthamiana* agroinfiltrated or mock (i.e., the infiltration-buffer without bacteria) treated leaves were collected and immediately frozen in liquid nitrogen. This material was used for RNA isolation, cDNA synthesis and qPCR as described in Methods S2.2. In short, after grinding the material, total RNA was isolated using the Qiagen RNA extraction kit. For spider mite RT-qPCR assays, *T. urticae* 18S rRNA, and *T. evansi* Ribosomal Protein 49 were used as housekeeping genes and for *N. benthamiana* actin was used. All primer pairs used are listed in Tables S2.2 & S2.3. Statistical differences of transcript abundances shown in Figure 2.4 and in Figures S2.6 and S2.7 were calculated by using a General Linear Model in SPSS 20 (IBM). Statistical differences shown in Figure S2.1 were calculated using the Student’s *t*-test in Excel (Microsoft).

### 2.2.5. Cloning

The candidate genes were cloned from spider mite cDNA, i.e., from *T. urticae* Santpoort-2 or *T. evansi* Viçosa-1 (Alba et al., 2015), using primers designed to amplify the ORF but excluding the predicted signal peptide. Primers were also designed to include an ATG at the end of the forward primers and Att-B recombination sites for Gateway cloning (Invitrogen) (Table S2.1). Following recombination of the candidates into pDONR207, an LR reaction was done with the plant expression vector pSOL2092 (Zhang et al., 2013b), which contains the CaMV 35S promoter. All clones were sequenced and *T. evansi* candidate effector sequences were deposited at GenBank (Table 2.1). The final destination vectors were introduced into *A. tumefaciens* GV3101 cells by electroporation.
2.2.6. Plant material

*Nicotiana benthamiana* plants were grown in the greenhouse for 2-3 weeks and then transferred to a climate room (long day, 25°C, 70% RH). All *Agrobacterium*-mediated transient assays (Kapila et al., 1997), further referred to as agroinfiltration, were performed on plants 4-5 weeks old. *Tomato* *Lycopersicum esculentum* cv. Moneymaker and cv. Moneymaker *nahG* were grown as described in Glas et al. (2014). The two-spotted spider mite *T. urticae* Santpoort-2 had been obtained and propagated as described in Alba et al. (2015). For experiments we used adult female spider mites that were 2 (± 2) days old since turning adult via a method described in Kant et al. (2004). Four days after infestation, leaflets were detached and the eggs were counted using a stereo microscope. The experiment was repeated 4x on 10 plants per tomato genotype: per plant, three leaflets were infested. The results presented in FIGURE 2.6 represent the mean number of eggs per mite per day. Effect on performance was analyzed using a GLM in SPSS 20 (IBM) including plant genotype as main factor and experiment as random factor.

2.2.7. Transient expression assays

*Agrobacterium tumefaciens* transient transformation assays were done as described in Ma et al. (2012). The *A. tumefaciens* strain GV3101 carrying the candidate vectors, empty vector (pSOL2092) (EV, i.e., the expression vector

---

**TABLE 2.1. Overview of the final five candidate effectors**

<table>
<thead>
<tr>
<th>Family number</th>
<th>Number of family members</th>
<th>Cloned candidate</th>
<th>Gene model ID(^1)</th>
<th>Mature protein size (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>11 in <em>T. urticae</em></td>
<td>Tu19</td>
<td>tetur05g09110</td>
<td>198</td>
</tr>
<tr>
<td>7</td>
<td>in <em>T. evansi</em></td>
<td>Te19</td>
<td>KT182960</td>
<td>198</td>
</tr>
<tr>
<td>28</td>
<td>10 in <em>T. urticae</em></td>
<td>Tu28</td>
<td>tetur31g01040</td>
<td>266</td>
</tr>
<tr>
<td>1</td>
<td>in <em>T. evansi</em></td>
<td>Te28</td>
<td>KT182959</td>
<td>338</td>
</tr>
<tr>
<td>84</td>
<td>2 in <em>T. urticae</em></td>
<td>Tu84</td>
<td>tetur01g01000</td>
<td>227</td>
</tr>
<tr>
<td>2</td>
<td>in <em>T. evansi</em></td>
<td>Te84</td>
<td>KT182961</td>
<td>230</td>
</tr>
<tr>
<td>90</td>
<td>4 in <em>T. urticae</em></td>
<td>Tu90</td>
<td>tetur05g04560</td>
<td>287</td>
</tr>
<tr>
<td>0</td>
<td>in <em>T. evansi</em></td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>128</td>
<td>2 in <em>T. urticae</em></td>
<td>Tu128</td>
<td>tetur01g00940</td>
<td>235</td>
</tr>
<tr>
<td>1</td>
<td>in <em>T. evansi</em></td>
<td>Te128</td>
<td>KT182962</td>
<td>233</td>
</tr>
</tbody>
</table>

\(^1\) *T. urticae* gene models are available on the ORCAE genome portal (http://bioinformatics.psb.ugent.be/orcae/overview/Tetur).

---

Spider mite effectors promote mite reproduction
still containing the Gateway negative-selection cassette), or 35s:p19, were
grown from single colonies for 16 hours in 2 ml LB medium with the appro-
priate antibiotics. An aliquot of each pre-culture was then inoculated into
5-10 ml LB with 10 mM MES and 20 μM acetosyringone, using the same
antibiotics and grown until an OD between 1.0-1.5 was reached. After cen-
trifugation the bacteria pellets were re-suspended in MMAi (2% sucrose,
10mM MES, 0.2 mM acetosyringone) to a final O.D. of 0.6 and then incu-
bated for at least 1 h at room temperature. Bacterial suspensions were infil-
trated into the abaxial side of the 3rd-youngest-fully-expanded N. bent-
thamiana leaf using a needleless syringe.

2.2.8. Phytohormone extraction and LC-MS
Phytohormone analysis was performed as described in Alba et al. (2015)
and its details can be found in METHODS S2.3. Statistical differences in the
amounts of phytohormones among samples were calculated using log-
transformed values by Fisher’s LSD test after ANOVA (SPSS 20, IBM).

2.2.9. Spider mite performance assays
Agroinfiltrated N. benthamiana leaflets were detached at 2 days post infil-
tration (DPI). From these detached leaves, glandular trichomes were gently
removed using filter paper soaked in water (FIGURE S2.10). Leaf discs (18
mm diameter) were placed on a cotton bed soaked in water. One female T.
urticae Santpoort-2 mite (2 days since turning adult) was placed on each leaf
disc and the number of eggs was counted at 2 and 4 days after introduction
of the mite, using a stereo microscope. Leaf discs with either a dead female
or a female that had drowned in the border of the wet cotton were discard-
ed from the analysis. Effect of the different effectors on mite performance
was evaluated per time point using ANOVA and means were compared
using Fisher’s LSD post hoc test (SPSS 20, IBM). To evaluate performance
on wild type and nahG tomato five adult female mites were placed on a sin-
gle leaflet and for each plant three leaflets were infested in total. Leaflets
were detached after four days and eggs were counted using a stereo micro-
scope. The experiment was repeated 4x using 10 plants per tomato geno-
type each time. The data was analyzed using a general linear model in SPSS
20 (IBM) using ‘plant genotype’ as main factor and ‘experiment’ as random
factor.
2.2.10. In situ hybridization

Tissue-specific expression of Te84 and Te28 was obtained via whole-mount in situ mRNA localization with DIG-labelled anti-sense RNA probe and anti-DIG-AP conjugate detection using NBT/BCIP or Fast Red substrate (Speel et al., 1992) using confocal microscopy as described in METHODS S2.4.

2.3. Results

2.3.1. In silico prediction of effector-like protein families in two spider mite species

We utilized the backbone of a broadly used effector-mining strategy (Bos et al., 2010) to generate a list of spider mite candidate-effector proteins (FIGURE 2.1) using two closely related mite species that can suppress plant

![Diagram of in silico pipeline](image)

**Figure 2.1. Overview of an in silico pipeline used to identify spider mite effectors.** Four selection steps were applied to obtain the combined dataset of putative secreted proteins from both the generalist *T. urticae* and the specialist *T. evansi*. Five protein families having effector potential were selected.
Chapter 2

defenses (Alba et al., 2015). First *T. urticae* for which we obtained the predicted transcriptome from the London strain (Grbić et al., 2011a). From its 18,414 predicted mRNAs (at November 2011) we inferred its proteome. Second *T. evansi* for which we obtained a transcriptome via sequencing its cDNA. A set of 1,558,090 high-quality reads (SRR2127882) with an average length of 456 nucleotides was assembled *de novo* using MIRA (Chevreux et al., 2004) into a total number of 31,263 putative mRNAs (N50 = 1461 and the average length = 1161 nucleotides.). We used only the 17,663 putative mRNAs that were assembled from five or more reads for protein prediction. The two predicted proteomes were processed in parallel for the subsequent data-mining steps.

Salivary proteins, like effector proteins, are secreted into the salivary duct by secretory cells. We utilized a conservative *in silico* pipeline to predict the secretome from the proteomes (Min, 2012). First, we identified proteins with signal peptides using SignalP 4.0 (Petersen et al., 2011) and Phobious (Käll et al., 2004). These proteins were screened via Phobious and TMHMM (Krogh et al., 2001) and excluded all proteins with a predicted transmembrane domain. Finally we used TargetP (Emanuelsson et al., 2000) and WoLF PSORT (Horton et al., 2007) for sub-cellular localization prediction and we continued only with those proteins for which extracellular targeting was predicted. After these filtering steps, the predicted *T. evansi* secretome consisted of 1121 proteins and that of *T. urticae* of 1493 proteins (FIGURE 2.1).

Next we applied two more filtering steps based on two common characteristics of pathogen effectors. First, Raffaele et al. (2010) reported effectors to be fast evolving and hence to occur in expanded gene families. Thus we clustered the proteins (Saunders et al., 2012) using TribeMCL (Enright et al., 2002) as described in the Materials and Methods. A total of 999 protein families were identified, with 193 families having 3 or more members (Group I), 276 families with two members (Group II), and 530 singleton clusters (Group III) (DATA S2.1). Serine proteases, represented by 35 proteins in *T. urticae* and 22 in *T. evansi*, constituted the largest family. For Group I there was only one unique family for *T. evansi*, family 193, while there were 20 families unique for *T. urticae*. We continued with Group I as this was the group most likely to contain effector-like protein families since effectors have been reported to be fast evolving and hence to occur in expanded gene families (Aggarwal et al., 2014; Pitino and Hogenhout, 2013).
Second, it has been reported that the majority of pathogen effector proteins identified so far are highly species- or genus-specific (Göhre and Robatzek, 2008; Thomma et al., 2011). Hence, we excluded all proteins with a functional BLAST annotation since there were no proteins in our data set with notable homology to known effectors. First, we selected those families from Group I that are unique for phytophagous mites (Acari) and are not found in Ixodida ticks (Acari). We used Ixodida because they do not eat plants and are phylogenetically closely related to mites with sufficient reference sequences available (88,616 protein sequences from at least 190 tick species by March 2014). In total, 79 families from Group I (40%) lacked any protein with similarity to Ixodida proteins (max. E-value 1e-10; DATA S2.1). Next, we submitted these 79 families to Blast2GO (nr database, BLASTp, max. E-value of 1e-10) (Conesa et al., 2005), and only four families could be annotated: families 6, 7, 10, and 39 (DATA S2.1). Family 10 contained proteins with similarity to fungal intradiol ring-cleavage dioxygenases and these proteins may play a role in the mite’s digestion or detoxification (Dermauw et al., 2013; Grbić et al., 2011b). Families 6, 7, and 39 included lipocalins, which have a wide range of functions and were shown to be differentially expressed when spider mites are challenged with xenobiotic stress (Dermauw et al., 2013). Hence these four families were excluded as well. The remaining 75 families were chosen for the next selection step.

2.3.2. Selection of the top 5 candidate effector families by gene expression analysis

We continued the selection procedure taking the expression characteristics of the remaining candidates into account assuming that: I) the expression of the genes encoding effector proteins should be higher in feeding stages (larva, nymph, or adult) than in a non-feeding stage (embryo); and II) effector genes should be typically expressed in the mite’s salivary glands.

Hence, first we analyzed the life-stage (egg, larva, nymph, and adult) specific gene-expression levels derived from the quantitative RNAseq data of *T. urticae* that was made publicly available together with the *T. urticae* genome (Grbić et al., 2011a; these data are included in DATA S2.1). To reduce the group of candidates, from the 75 families remaining, to a workable size we decided to arbitrarily select those that had at least one homolog expressed ≥10-fold in any of the feeding stages (larva, nymph, and adult) compared to the egg (embryo) stage, and only these 18 families were taken to the following step.
Second, since spider mites are too small (0.5 mm) for isolating salivary glands, we removed the 'head' part of adult mites (i.e., the anterior body region including the gnathosoma, which includes the salivary glands, but not the intestines and ovaries (Mothes and Seitz, 1981) from the main body and collected RNA from the remaining main body tissues as well as from intact mites for gene expression analysis. We selected from each of the 18 remaining families the member with the highest expression in the adult life stage (Data S2.1) and performed qRT-PCR for these 18 genes comparing the intact mite samples with the anterior body dissected samples. Five genes -Tu19, Tu28, Tu84, Tu90, and Tu128- showed a statistically significant and at least 10-fold lower expression in the anterior body dissected samples compared to the intact mite samples (Figure S2.1). We thus considered these five as the most likely expressed in salivary glands. Four of these have homologs in T. evansi (Te19, Te28, Te84 and Te128), while family 90 was unique for T. urticae. An InterProScan (Jones et al., 2014) search revealed that Tu28 and Te28 contained the structural domain Armadillo-type fold (IPR016024), known to facilitate protein-protein and protein-DNA interactions. Moreover, proteins of family 28 contain two 80-amino acid tandem repeats within this domain (Figure S2.2). No recognizable domains, motifs, or repeats were found in the other candidate effectors. An overview of these final five candidate effector families is shown in Table 2.1.

Finally, to ensure that the remaining five candidate effectors are indeed expressed in the salivary glands, we performed whole-mount in situ hybridization using digoxigenin-labeled antisense RNA probes. For Te84, we observed mites to be stained exclusively in both anterior prosomal

**Figure 2.2. Whole-mount in situ hybridization of a putative salivary protein gene belonging to family 84.** A digoxigenin (DIG)-labeled antisense probe was used for hybridization, while signal was developed using anti-DIG-AP and FastRed as substrate. Signal development can be observed in the anterior salivary glands (arrows) with bright field microscopy (A) and confocal laser-scanning microscopy (B). Scale bars indicate 50 μm.
Spider mite effectors promote mite reproduction

glands (Figure 2.2), which are one of the three paired spider mite salivary glands (Mothes and Seitz, 1981). Staining in the stylet was also noticeable in some mites (data not shown) (This sentence was erroneous deleted in the published version of Villarroel et al., 2016, making it seem as if ISH did not result in specific staining). However, comparison with the sense control samples made clear that this can be considered as background staining. We did not observe any mites with stained salivary glands using a Te84 sense probe (negative control) (Figure S2.3). We also hybridized antisense probes for Te28 (Figure S2.3), Tu19, Tu28, Tu84, Tu90, and Tu128 and their respective sense probes were used as controls. All these candidate effectors were expressed specifically in the salivary glands (Jonckheere et al., 2016; Chapter 3).

2.3.3. Transient over-expression of proteins belonging to two candidate effector families causes chlorosis in Nicotiana benthamiana

Wroblewski et al. (2009) found a wide range of phenotypes when expressing effectors of Pseudomonas or Ralstonia in N. benthamiana leaves, varying from no visible symptoms through various degrees of chlorosis to extensive tissue damage and cell death in the infiltrated area. Hence we evaluated if the five putative effectors (without their signal peptides) could also cause such visible

Figure 2.3. Four spider mite candidate effectors cause chlorosis in Nicotiana benthamiana. (A) Agroinfiltrated N. benthamiana leaves transiently transformed with the candidate effectors Te28, Tu28, Te84, and Tu84 and with the EV control. Pictures were taken at 5 DPI. (B) RT-PCR showing the expression of the candidates in the agroinfiltrated leaves at 2 and 5 DPI. The data are representative for 2 experiments.
phenotypes in *N. benthamiana* when expressed under control of the 35S promoter using agroinfiltration. We cloned cDNAs from *T. urticae* strain Santpoort-2 that performs better on the JA-biosynthesis mutant def-1 (Kant et al., 2008) and hence is not a superior suppressor (Alba et al., 2015) and from *T. evansi*. Candidates were co-expressed with the viral silencing suppressor p19 to keep a high and long-lasting transcription (Voinnet et al., 2003) using the EV as a control. We observed tissue chlorosis after transient expression of Tu28 and Te28 as well as Tu84 and Te84. This chlorosis was clearly visible 5 DPI (FIGURE 2.3A). The expression of the transgenes in the infiltration zone was confirmed by RT-PCR at 2 and 5 DPI (FIGURE 2.3B). Expression of candidate Te28 occasionally induced necrosis in *N. benthamiana* 4–5 DPI (FIGURE S2.4), but whether this is related to a higher expression of Te28, as the RT-PCR results suggest (FIGURE 2.3B), needs to be investigated. We did not observe chlorosis, or any distinct phenotype, after overexpression of the other candidates: Tu19, Te19, Tu90, Tu128 and Te128 (FIGURE S2.5), and we thus continued with the four putative effectors that did.

![Figure 2.4](imageURL)  
**Figure 2.4.** Spider mite candidate effectors affect the SA response induced by *Agrobacterium*. (A) Levels of salicylic acid in ng/gFW in agroinfiltrated leaves at 2 and 5 DPI. Error bars represent SE. Data were log-transformed prior to statistical analysis. Different letters indicate statistical differences according to Fisher-LSD test (*p*<0.05). (B) Relative gene expression of the SA-related marker genes *PR1* and *PR4* after agroinfiltration, at 2 (upper panel) and 5 DPI (lower panel) of the candidates, the EV or infiltration of the mock (infiltration medium). Error bars denote standard errors. Statistical differences were analyzed using a General Linear Model, and are indicated as different letters (*p*<0.05). The data in A and B are representative for two experiments.
2.3.4. Candidates from families 28 and 84 suppress *Agrobacterium*-induced SA-related defenses

Since, as for pathogen effectors, chlorosis can be indicative of effector-like properties (Wroblewski et al., 2009) we tested if Te28, Tu28, Te84, and Tu84 altered plant defenses. To test this we measured the accumulation of the phytohormones SA, JA, and JA-Ile and assessed the relative expression of the SA-related marker genes *Pathogenesis Related 1 (PR1)*, *Pathogenesis Related 4 (PR4)*, and the JA-related marker *Trypsin Proteinase Inhibitor (TPI)* at 2 and 5 DPI.

At 2 DPI the concentration of SA was 8-fold higher in leaves agroinfiltrated with the EV than in mock-treated leaves (FIGURE 2.4A). At this time point, levels of SA did not differ between leaves expressing the candidates and the EV, but amounts of SA in leaves expressing Te28 and Tu28 were significantly lower than those expressing Te84 (FIGURE 2.4A). In contrast, at 5 DPI the levels of SA were 7-fold higher in agroinfiltrated leaves expressing the EV compared to 2 DPI while SA levels were significantly lower in leaves expressing any of the candidate effectors than the EV (FIGURE 2.4A). We did not detect any JA or its conjugate JA-Isoleucine in any of the samples. The EV induced the SA-responsive marker gene *PR1* 237- and 1530-fold at 2 and 5 DPI respectively (FIGURE 2.4B). At 2 DPI, *PR1*-expression was 6-fold lower in leaves expressing Te28 and Tu28 compared to the EV. Te28 and Tu28 suppressed *PR1* expression partially since transcript levels were still 37-fold higher than in mock-infiltrated leaves (FIGURE 2.4B). At 5 DPI, *Agrobacterium*-induced *PR1* expression was 60-fold lower in leaves expressing Te28 and Tu28 compared to the EV. Also Tu84 suppressed *PR1* induction by 3-fold (2 DPI) and 30-fold (5 DPI) relative to EV. However, at 2 DPI, leaves expressing its counterpart Te84, *PR1* expression was 2-fold higher than in leaves with the EV (FIGURE 2.4B). Since at 5 DPI all four candidate effectors partially suppressed *PR1* induction relative to the EV control, we repeated this experiment using 35S:GFP instead of the EV and compared this to leaves expressing GFP-fusions with the candidates and observed a similar pattern of *PR1* suppression except for Te84, which might have been due to the GFP tag (FIGURE S2.6).

Expression of another SA-related marker *PR4*, which is known to be co-regulated by JA (Maimbo et al., 2010), was 6- and 27-fold higher, at 2 and 5 DPI respectively, when comparing EV to mock-treated leaves (FIGURE 2.4B). Like *PR1*, also *PR4* expression was suppressed, albeit mild-
ly, by Te28 and Tu28 at 2 and 5 DPI, with levels being 2-fold and 6-fold lower than those of the EV. Candidate Tu84 significantly suppressed PR4 induction only at 5 DPI by 3-fold, while PR4 expression in leaves expressing Te84 was equal to those with EV at both time points.

We also measured the expression of the JA-related marker gene TPI. In contrast to the SA markers, TPI was down-regulated after 2 DPI in all leaves expressing a candidate effector or the EV compared to the mock treatment. However, this reduction was slightly stronger for the four candidates compared to the EV and for Te28 and Tu84 down-regulation was even significant (FIGURE S2.7). In contrast, at 5 DPI, TPI expression was not significantly different between the leaves expressing the candidate effectors and the EV, due to a high level of variation with the EV.

2.3.5. Candidate effectors from families 28 and 84 promote T. urticae performance

Since expression of Te28, Tu28, Te84 and Tu84 affected the induced SA response of N. benthamiana, we assessed the reproductive performance of spider mites on leaf-discs of leaves expressing these four candidates or the EV. Oviposition of T. urticae was 25% higher on leaf-discs expressing Tu28, Te84, and Tu84, when compared to the EV (Fisher-LSD p<0.001) (FIGURE 2.5).

![Figure 2.5](image-url)

**Figure 2.5. Three candidate effectors improve Tetranychus urticae performance on Nicotiana benthamiana.** The figure shows the average number of eggs laid by one female adult spider mite after feeding for four days on N. benthamiana leaf-discs expressing the candidate effectors or the EV control. Error bars represent SE. Different letters indicate statistical differences according to Fisher-LSD test (p<0.05). This experiment was repeated twice with similar results.
Spider mite effectors promote mite reproduction

2.5). In contrast, oviposition of *T. urticae* was 25% lower on leaf-discs expressing the candidate Te28, compared to the EV (Fisher-LSD p<0.05) (FIGURE 2.5). Discs from Te28-expressing leaves showed the strongest *Agrobacterium*-induced chlorotic symptoms during the oviposition test and after 4 days of infestation (6 DPI with *Agrobacterium*) chlorotic symptoms of these discs were markedly different from the discs expressing any of the three other candidates or the EV (FIGURE S2.8).

2.3.6. Spider mites produce more offspring on the SA accumulation mutant *S. lycopersicum nahG*

Spider mites induce (Kant et al., 2004) and suppress (Alba et al., 2015) both JA and SA responses at the same time. However, while JA is well-established as defense hormone that constrains mite performance (Ament et al., 2004; Kant et al., 2008; Li et al., 2002; Zhurov et al., 2014), the role of SA remains elusive. Hence, we tested to which extent SA defenses are detrimental to *T. urticae* Santpoort-2 (Alba et al., 2015) by using a tomato transgenic line expressing the bacterial gene *nahG*, a salicylate hydroxylase that renders plants unable to accumulate SA due its conversion into catechol (Brading et al., 2000). This catechol accumulation leads to dark green plants when they get older and leaves will start to fall off as well. However,

**Figure 2.6. Spider mite performance is higher on SA-deficient tomato *NahG* plants than on wild type plants.** The figure shows the average number of eggs per female adult mite feeding for 4 days on tomato plants expressing the transgene *nahG* or on the WT control Moneymaker. The experiment was performed 4 independent times using 6-10 plants per genotype in each assay. Effect on performance was analyzed using a General Linear Model including ‘Plant Genotype’ as main factor and ‘Experiment’ as random factor. Asterisk indicates a significant effect of plant genotype on *T. urticae* performance (*p*<0.05).
within the time frame of our studies, with three to four week old plants, the plants have a normal phenotype. After four days of infestation, *T. urticae* mites had deposited 10% more eggs on nahG plants compared to the wild type Moneymaker (FIGURE 2.6) and this increase was statistically significant (Genotype effect, \( p = 0.047 \)). This establishes that SA defense response do have a negative effect on mite performance.

2.4. DISCUSSION

Previously we showed that the phytophagous mites *T. urticae* and *T. evansi*, two agronomical-relevant pest species, suppress JA- and SA-defenses in plants to their own benefit via an unknown mechanism (Alba et al., 2015; Kant et al., 2008; Sarmento et al., 2011). In this paper we have shown that these spider mites possess at least two families of effector-like salivary gland proteins that can account for suppression of SA defenses (FIGURE 2.4). Furthermore, we showed that *in planta* expression of these proteins, i.e., Tu28 and Tu84 and its homolog Te84, promoted the reproductive performance of *T. urticae* (FIGURE 2.5) similar as when expressing the nahG gene (FIGURE 2.6). Thus these three spider mite salivary proteins, called Tu28, Tu84 and Te84, act as effector proteins by suppressing mite-induced SA defenses and promoting mite performance.

The chlorosis phenotype observed after transient expression of Te28, Tu28, Te84, and Tu84 (FIGURE 2.3) indicated that these proteins could have effector-like properties (Wroblewski et al., 2009). Indeed, *in planta* expression of Te28, Tu28, and Tu84 suppressed the *A. tumefaciens*-induced SA response in *N. benthamiana* as indicated by the marker genes *PR1* and *PR4* (Figure 2.4). *PR1* is a well-established SA-related marker gene in *N. tabacum* (Uknes et al., 1993; Van Loon and Van Strien, 1999) and is induced after pathogen attack in *N. benthamiana* (Maimbo et al., 2010; Pasin et al., 2014). *N. benthamiana PR4* encodes a hevein-like chitinase that is induced by the SA-mimic BTH (Friedrich et al., 1996), yet it is mainly associated with the JA response (Kiba et al., 2014; Zhang et al., 2012). A similar suppression of *PR1* expression was observed after expressing the Cauliflower Mosaic Virus (CaMV) protein P6 transiently using agroinfiltration in *N. benthamiana*, although here this coincided with an increase in JA-responsive genes due to interference with the localization of the regulatory protein NPR1 (Love et al., 2012). Interestingly, 2 days after the start of the agroinfiltration when induction of *PR1* and *PR4* was suppressed by Te28, Tu28, and
Tu84, these leaves had accumulated similar levels of SA as leaves transformed with the EV or with Te84. This agrees with the defense-suppression model we proposed previously that postulates that spider mites suppress defenses downstream of phytohormone accumulation (Alba et al., 2015). However, despite of inducing chlorosis, *T. evansi* protein Te84 did not suppress *PR1* or *PR4* expression, which suggests that there is no direct causal relationship between the chlorosis phenotype and the suppression of SA-related defenses. Moreover, chlorosis during agroinfiltration in *N. benthamiana* usually coincides with stronger, not weaker, SA responses (Rico et al., 2010). Taken together, chlorosis may be indicative, yet not fool-proof, as a visible phenotype for selecting candidate herbivore-effector proteins.

Spider mites induce (Glas et al., 2014; Kant et al., 2004, 2008; Li et al., 2004) and suppress (Alba et al., 2015; Sarmento et al., 2011) both JA and SA responses at the same time. Hence, ideally effector-expression assays allow for screening these two defense responses simultaneously. However, *A. tumefaciens* strain GV3101 induces SA responses in *N. benthamiana* (Sheikh et al., 2014; this study) and accordingly, we observed that agroinfiltration down-regulated JA-responses (Figure S2.7). Although the *TPI*-expression data suggest that two of these effectors (Te28 and Tu84) may affect JA responses as well, the agroinfiltration was especially suitable for investigating the effects of candidate effectors on SA-mediated responses. Salicylate-related defenses, central in plant-pathogen interactions (Thomma et al., 1998), are induced by herbivorous mites (Glas et al., 2014; Kant et al., 2004), and insect herbivores such as aphids (Moran and Thompson, 2001), whiteflies (Zarate et al., 2007) and by the larvae of some lepidopteran species (Diezel et al., 2009; Musser et al., 2002). In this study we also showed that *T. urticae* Santpoort-2 mites (Alba et al., 2015) performed substantially better on the SA-deficient *nahG* tomato plants compared to wild type Moneymaker plants (Figure 2.6). Although a 10% increase in reproductive performance as such seemingly indicates only limited biological significance, the effect will amplify exponentially across the consecutive generations (Figure S2.11). Although this effect of SA on mite performance still has to be shown in other species such as *Arabidopsis* or *N. benthamiana*, it suggests that SA-related processes, connected to the hypersensitive response, senescence or defensive products such as chitinases, may have defensive functions against phytophagous mites (Kielkiewicz, 1999; McCafferty et al., 2006) as they have on some phloem-feeding herbivores (Pegadaraju et al., 2005; Villada et al., 2009).
Oviposition assays on *N. benthamiana* leaf-discs provided a strong evidence for three of the four candidates to be effector proteins. While candidates Tu28, Te84, and Tu84 improved the performance of *T. urticae* mites up to 25%, candidate Te28 decreased mite performance thus acting as an elicitor rather than an effector in *N. benthamiana*. However, the strong chlorotic symptoms that developed after expression of Te28 could explain this adverse effect (Figure S2.8). Similarly, Bos et al. (2010) reported a negative effect of the chlorosis-inducing candidate aphid-effector protein Mp10 on aphid performance. Here the authors suggested it could be the result of an effector recognition by a plant resistance protein (R protein), which mediated effector-triggered immunity. Together, this suggests that different homologs from within a family (from the same or different herbivore species) may have different effects on the defenses of different plant races or species. For the three mite fitness-promoting effectors Tu28, Te84, and Tu84, the increase in *T. urticae* performance was not perfectly correlated with the suppression of SA-related marker genes. Candidate Te84, which only suppressed *PR1* at 5 DPI, improved spider mite performance to the same level as Tu28 and Tu84 did. However, Te84 did suppress SA accumulation and *PR1* expression at 5 DPI, indicating that still it may have an effect on SA-related defenses albeit delayed. Te84 and Tu84 differ moderately in their protein sequences (they are only 62% identical) (Figure S2.9), and that could explain the different timing observed on their suppression of SA-related defenses. Nevertheless, we cannot rule out that Tu28, Tu84, and Te84 may affect other relevant plant processes as well that turn leaves expressing these proteins into better food.

To obtain the proteins presented here we cloned cDNAs from *T. urticae* strains that perform better on the JA-biosynthesis mutant *def-1* (Alba et al., 2015; Kant et al., 2008) and the SA-accumulation mutant *nahG* (this study) than on wild type plants and hence are not superior suppressors. This raises the question why poor suppressor mites, or maybe even non-suppressors, may yet possess genes encoding effector proteins. Possibly, like it was found for aphids, the proteins we report on here will affect defenses differently on different host plants (Pitino and Hogenhout, 2013). Alternatively, differences in expression levels, the amounts of protein in saliva, or the amounts of saliva secreted may render the efficiency of these proteins across different mite strains. However, the ability of effector proteins to suppress defenses may also depend on the presence of other substances in the saliva:
Spider mite effectors promote mite reproduction

also plant-pathogens secrete mixtures of effectors and non-effectors (some of which elicitors) and the effect of these on the host plant seems to depend on their combined action (Jones and Dangl, 2006; Kaloshian, 2004). In that view effectors serve to compensate for the plant-recognition of elicitors. Hence, not only differences in the effector-composition of mite saliva but also its elicitor-composition may determine the resulting plant response. In addition, the 322 \textit{T. evansi}-specific secreted proteins from group II (gene families with 2 copies) and III (singletons) that were not investigated may well contain effectors. These potential effectors could explain the mite’s superior ability to suppress tomato defenses below the levels of uninfested control plants (Sarmento et al., 2011).

Defense manipulation has been attributed to specialist herbivores (Schmelz et al., 2012) although it is doubtful if this trait is restricted to specialists (Ali and Agrawal, 2012). Our data show that specialist pests like \textit{T. evansi} and generalists like \textit{T. urticae} can produce homologous effectors that have a similar impact on host plant defenses. Possibly, the term generalist is misleading and such species are actually composed of a collection of host races more specialized to different plant species (Kant et al., 2008). In addition, many \textit{T. urticae} strains induce plant defenses (Alba et al., 2015; Martel et al., 2015; Zhurov et al., 2014) rather than suppressing these. This suggests that spider mites may secrete mixtures of elicitors and effectors and the extent to which these mixtures result in a stronger or weaker induction or suppression is probably context dependent - i.e., host plant genotype; growth conditions, etc.- and may vary across mite populations or within populations (Alba et al., 2015; Kant et al., 2008). Detailed knowledge on herbivore effector diversity, the plant target processes, and their mutual evolution may strongly increase our understanding of the forces that drive plant-herbivore interactions and explain the formation of pests. However, despite our detailed knowledge on plant defenses it may not be always obvious which plant response to use to screen for active effectors. This implies that for screening candidate effectors, herbivore performance assays may provide the only read-out with biological relevance.

Accession numbers

Sequence data from this article are available at the NCBI website (http:www.ncbi.nlm.nih.gov) and can be found under the following accession numbers: Te28, KT182959; Te19, KT182960; Te84, KT182961; and
Te128, KT182962. *Tetranychus evansi* RNA-seq data can be found under the SRA accession number SRR2127882.

Acknowledgments

The authors wish to thank Ludek Tikovsky, Harold Lemereis and Thijs Hendrix for taking care of the plants; Dr. Bart Thomma for providing the pSOL2092 vector; Vladimir Zhurov for advice in performing in situ hybridization assays. CAV was supported by CONICYT BECAS CHILE, MRK and JMA by NWO (STW-VIDI 13492), JIG by NWO (STW-13550) and WD is a postdoctoral fellow of the Fund for Scientific Research Flanders (FWO). The authors declare no conflict of interest.

2.5. REFERENCES


Spider mite effectors promote mite reproduction


Spider mite effectors promote mite reproduction


Spider mite effectors promote mite reproduction


Zhurov, V., Navarro, M., Bruinsma, K.A., Arbona, V., Santamaria, M.E., Cazaux, M.,

2.6. Supporting Information

**Data S2.1.** *Tetranychus evansi* and *T. urticae* clustered secretomes. See online submission at the *The Plant Journal* website (http://onlinelibrary.wiley.com/doi/10.1111/tpj.13152/full).
### Table S2.1. Primers sequences used for gateway cloning and in situ hybridization

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Tetur gene model identifier or NCBI accession number</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Te28-AttB</td>
<td>KT182959</td>
<td>GGGGACAAGTTTGTACAAAAAAAGCA</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCTTAATGGAATGAAATCAGTGGAC</td>
<td>GTATTAATTTGTCATTGATCTGCC</td>
</tr>
<tr>
<td>Tu28-AttB</td>
<td>tetur31g01040</td>
<td>GGGGACAAGTTTGTACAAAAAAAGCA</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCTTAATGGAATGAAATCAGTGGAC</td>
<td>GTATTAATTTGTCATTGATCTGCC</td>
</tr>
<tr>
<td>Te84-AttB</td>
<td>KT182961</td>
<td>GGGGACAAGTTTGTACAAAAAAAGCA</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCTTAATGGAATGAAATCAGTGGAC</td>
<td>GTATTAATTTGTCATTGATCTGCC</td>
</tr>
<tr>
<td>Tu84-AttB</td>
<td>tetur01g01000</td>
<td>GGGGACAAGTTTGTACAAAAAAAGCA</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCTTAATGGAATGAAATCAGTGGAC</td>
<td>GTATTAATTTGTCATTGATCTGCC</td>
</tr>
<tr>
<td>Tu19-AttB</td>
<td>tetur05g09110</td>
<td>GGGGACAAGTTTGTACAAAAAAAGCA</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCTTAATGGAATGAAATCAGTGGAC</td>
<td>GTATTAATTTGTCATTGATCTGCC</td>
</tr>
<tr>
<td>Te19-AttB</td>
<td>KT182960</td>
<td>GGGGACAAGTTTGTACAAAAAAAGCA</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCTTAATGGAATGAAATCAGTGGAC</td>
<td>GTATTAATTTGTCATTGATCTGCC</td>
</tr>
<tr>
<td>Tu90-AttB</td>
<td>tetur05g04560</td>
<td>GGGGACAAGTTTGTACAAAAAAAGCA</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCTTAATGGAATGAAATCAGTGGAC</td>
<td>GTATTAATTTGTCATTGATCTGCC</td>
</tr>
<tr>
<td>Tu128-AttB</td>
<td>tetur01g00940</td>
<td>GGGGACAAGTTTGTACAAAAAAAGCA</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCTTAATGGAATGAAATCAGTGGAC</td>
<td>GTATTAATTTGTCATTGATCTGCC</td>
</tr>
<tr>
<td>Te128-AttB</td>
<td>KT182962</td>
<td>GGGGACAAGTTTGTACAAAAAAAGCA</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCTTAATGGAATGAAATCAGTGGAC</td>
<td>GTATTAATTTGTCATTGATCTGCC</td>
</tr>
<tr>
<td>Te84-insitu</td>
<td>KT182961</td>
<td>AAATCTGGATTCGTTGTTTATG</td>
<td>GTGAGCATGGCTACTTATTAGCA</td>
</tr>
<tr>
<td>Te28-insitu</td>
<td>KT182959</td>
<td>CGAAGCTGCAAAACAAAAAC</td>
<td>GAATTTTCACCAATTTGTCATTG</td>
</tr>
</tbody>
</table>
## Chapter 2

### Table S2.2. *Nicotiana benthamiana* primer sequences used for qPCR in this study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Primer efficiency</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NbPR1</td>
<td>CCTTCATTTCTTCT</td>
<td>AGGTTACAATCT</td>
<td>1.84</td>
<td>Yoon ea, 2009</td>
</tr>
<tr>
<td></td>
<td>CTGTTCTCT</td>
<td>GCAGCAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NbPR+</td>
<td>GGCCAAGATTCC</td>
<td>CACTGTTGTTTGG</td>
<td>1.82</td>
<td>Maimbo ea, 2010</td>
</tr>
<tr>
<td></td>
<td>TGTTGTAGAT</td>
<td>AGTTCCCTGTTCCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NbTPI</td>
<td>ACTTTGCAATGC</td>
<td>TCAACCACCTTGCG</td>
<td>1.80</td>
<td>Yoon ea, 2010</td>
</tr>
<tr>
<td></td>
<td>GATCCAG</td>
<td>TGGCATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NbActin</td>
<td>CGGAATCCACAGA</td>
<td>GGGAAGCCAAGA</td>
<td>1.82</td>
<td>Maimbo ea, 2010</td>
</tr>
<tr>
<td></td>
<td>GACTACATA</td>
<td>TAGAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table S2.3. *Tetranychus urticae* and *Tetranychus evansi* primer sequences used for RT-PCR and/or qPCR in this study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Te28</td>
<td>GCTAAGCACAACGGCTGAAGA</td>
<td>ATTTGCCGAGAAACCTGTATGG</td>
</tr>
<tr>
<td>Tu28</td>
<td>CGGAACACAGAATCTCATGCG</td>
<td>TGCCAGTTGCAATACACAGA</td>
</tr>
<tr>
<td>Te84</td>
<td>AACAAATGATTTGGCTGGCTGG</td>
<td>TCGAAAACATTTACCAGATGC</td>
</tr>
<tr>
<td>Tu84</td>
<td>GGCTTTATCTTTCTCATGGGT</td>
<td>ATGGCATTTGCAGAGATGGA</td>
</tr>
<tr>
<td>Tu18s</td>
<td>GGCTCACAGAGGTCTCTCTTC</td>
<td>ACAGTTGCCTCTCTCTGCCT</td>
</tr>
<tr>
<td>Tu14</td>
<td>CTCACATTGCTGTTGTGGCT</td>
<td>TGCTGATCACAGAGAGCTGTA</td>
</tr>
<tr>
<td>Tu16</td>
<td>GCTCGGCTAAATATGCTGCA</td>
<td>TGGCCAGTCAACAGTTCAA</td>
</tr>
<tr>
<td>Tu18</td>
<td>CCTGACTTCTGACATGACGCA</td>
<td>GATCTTTGGGCAACAGGAT</td>
</tr>
<tr>
<td>Tu19</td>
<td>CATGAAAATTGTGGATGATTGGG</td>
<td>TGGAGGTTCAACAGACACA</td>
</tr>
<tr>
<td>Tu23</td>
<td>CTCGATCTACTCCGATTTGGA</td>
<td>GCCATTCGGGTCTCAGTTTA</td>
</tr>
<tr>
<td>Tu29</td>
<td>ACTGGAATGCAATGCGAGAAA</td>
<td>TCCATCTGTCCTGCAATAG</td>
</tr>
<tr>
<td>Tu31</td>
<td>GCATTGTGTTTTGCTATATGG</td>
<td>CAGCGATTCTACCAACACA</td>
</tr>
<tr>
<td>Tu32</td>
<td>AATTTGGAAGGCCTTCTGATG</td>
<td>AAATCTAGCTGCTCTACCTAG</td>
</tr>
<tr>
<td>Tu33</td>
<td>TTGTAGTGACGCTCTATATGG</td>
<td>TGTTGCTTCCTCTCTACCTTG</td>
</tr>
<tr>
<td>Tu43</td>
<td>CCATTGGTTGCTTCTTCTTTT</td>
<td>TTTGTTGTCACAGCTTCAA</td>
</tr>
<tr>
<td>Tu57</td>
<td>TGCTGAAATCTGTTGCTATCG</td>
<td>CGCGGTTCCATTTATCTTTTG</td>
</tr>
<tr>
<td>Tu58</td>
<td>CAAAACCTTTTTCTCGTCAA</td>
<td>AGACGATTTGCACATTCAA</td>
</tr>
<tr>
<td>Tu65</td>
<td>CCGGCTCTCTCTCTCTCTGGAGAAG</td>
<td>GGTGTTGAGGCTAGGGAGTTTT</td>
</tr>
<tr>
<td>Tu80</td>
<td>TGGGCACGAAAAACCAAGAGA</td>
<td>TTTTGTGTTGATGCGCTTGG</td>
</tr>
<tr>
<td>Tu90</td>
<td>TGATGGTCAAACCATGTAACT</td>
<td>TGTTGCCATTGAAACAAAA</td>
</tr>
<tr>
<td>Tu128</td>
<td>AGCAGCTGATGCTTCTGTGACA</td>
<td>CATCGCTCTTTTACAGGTA</td>
</tr>
</tbody>
</table>
**Figure S2.1. Screening for potential *Tetranychus urticae* salivary-gland specific genes.** Expression values of 18 *T. urticae* genes in intact mites (grey bars) and in anterior body dissected mites (black bars). For each gene the bars were scaled to the lowest average, i.e., the lowest bar in each plot is 1. Candidates showing an at least 10-fold statistically-significant lower expression in dissected mites were considered as potential salivary-gland specific genes (Tu19, Tu28, Tu84, Tu90, and Tu128). Statistical differences were analyzed using Student’s *t*-test (*p*<0.05).
**FIGURE S2.2. Amino acid sequence alignment of Te28 and Tu28.** Alignment of mature amino acid sequences (minus predicted signal peptide) of Te28 and Tu28. Clustal Omega was used to align both sequences. Black and grey shading indicate identical and similar residues, respectively. Lines above the alignment indicate the ARMADILLO domain (IPR016024) in Te28 (above) and Tu28 (bottom). The conserved 80-amino acid repeats in Te28 and Tu28 are indicated with a line below the alignment.
Spider mite effectors promote mite reproduction

**Figure S2.3.** Detection of Te28 and Te84 gene expression in salivary glands of *Tetranychus evansi* using whole-mount *in situ* hybridization. DIG-labeled antisense riboprobes (left panel) were used for hybridization, while corresponding sense probes (right panel) served as negative controls. Signals were detected with alkaline phosphatase linked to an anti-DIG antibody. *In situ* hybridization experiments for Te28 and Te84 were repeated using both nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP) (top row) or Fast Red (bottom row) as substrate. The pictures show mites representative for the samples. Specific colorization (arrows) in salivary glands was only seen in antisense probe samples and never in sense probe samples. Some background staining in, e.g., stylet and legs was common in all samples. Scale bars indicate 0.5 mm. The black dark spots are spider mite midgut cells containing excretory material (e.g., chlorophyll) (first described by Wiesmann, 1968; Blauvelt, 1945).

**Figure S2.4.** Candidate effector Te28 causes tissue necrosis after agroinfiltration. Agroinfiltrated leaves expressing Te28 or with the EV. The arrow indicates a necrotic zone in the Te28-agroinfiltrated area. Necrosis after expression of Te28 occasionally occurred.
FIGURE S2.5. Agroinfiltration of candidate effectors from families 19, 90, or 128 does not induce chlorosis in *Nicotiana benthamiana*. Agroinfiltrated leaves after expression of candidates from families 19, 90, or 128. Infiltreated areas were delineated with a black marker. Leaves with the EV or expressing the candidate Te28 are shown as negative and positive control for tissue chlorosis, respectively. Pictures were taken at 5 DPI.

FIGURE S2.6. Relative gene expression of the SA-marker gene *PR1* after agroinfiltration of 4 candidate effectors or 35s:GFP as control. Relative expression of the SA-related marker *PR1* at 5 DPI after agroinfiltration of Te28, Tu28, Te84, Tu84, and 35s:GFP or infiltration of the mock control. Error bars denote SE. Statistical differences were analyzed using a General Linear Model, and are denoted as different letters (*p*<0.05).
Spider mite effectors promote mite reproduction

**Figure S2.7.** Relative gene expression of the JA-marker gene *TPI* after agroinfiltration of 4 candidate effectors. Relative expression of the JA-related marker *TPI* after agroinfiltration of Te28, Tu28, Te84, Tu84, and EV or infiltration of the mock control, at 2 and 5 DPI. Error bars denote SE. Statistical differences were analyzed using a General Linear Model, and are denoted as different letters (*p*<0.05). The data are representative for 2 experiments.

**Figure S2.8.** Leaf-discs expressing candidate Te28 show intense chlorotic symptoms. The picture shows leaf-discs from agroinfiltrated *Nicotiana benthamiana* leaves expressing the candidate effectors or the control EV at the end of a *Tetranychus urticae* performance assay (6 DPI, 4 days of spider mite infestation).
**Figure S2.9.** Amino acid sequence alignment of Te84 and Tu84. Alignment of predicted amino acid sequences (minus predicted signal peptide) of Te84 and Tu84. Clustal Omega was used to align both sequences. Black and grey shading indicate identical and similar residues, respectively. No known motifs were identified in Te84 or Tu84.

**Figure S2.10.** Agroinfiltrated *Nicotiana benthamiana* leaves after leaf-surface washing. (A) Picture of an intact agroinfiltrated *N. benthamiana* leaf showing high density of glandular trichomes (arrows) on the adaxial side. (B) Glandular trichomes were removed with a tissue paper soaked in water.
**Spider mite effectors promote mite reproduction**

**Figure S2.11.** Exponential growth of two hypothetical populations growing on the SA impaired nahG tomato plants or Moneymaker (MM) tomato plants. Despite the moderate differences in oviposition rate observed on both genotypes, this has a big impact on population level. The intrinsic rate of increase ($r_m$) was estimated using the regression model proposed by Jansen and Sabelis (1992) for *Tetranychus urticae*: $r_m = 0.131 + 0.011x$; where $x$ is the peak oviposition rate (i.e., number of eggs per female and per day). The intrinsic rates of increase for mites reared on Moneymaker (MM) and nahG plants were: $r_{m_{MM}} = 0.212$; and $r_{m_{nahG}} = 0.220$. The values were used to parametrize the model: $n = 10^{(t \times r_m)}$, where $n$ is the number of individuals and $t$ is the time in days.
Methods S2.1. High-throughput Sequencing and de novo assembly

*Tetranychus evansi* whole transcriptome was sequenced from cDNA. Total RNA was isolated from mites of all stages using the Qiagen RNA extraction kit and 24 μg (S28/S18 = 1.71) was used for cDNA synthesis and library preparation. From the total RNA sample poly(A)+ RNA was isolated, which was used for cDNA synthesis. First strand cDNA synthesis was primed with a N6 randomized primer. Then 454 adapters A and B were ligated to the 5' and 3' ends of ds-cDNA. The cDNA was finally amplified with PCR using a proof reading enzyme. Normalization was carried out by one cycle of denaturation and reassociation of the cDNA, resulting in N1-cDNA. Reassociated ds-cDNA was separated from the remaining ss-cDNA (normalized cDNA) by passing the mixture over a hydroxylapatite column. After hydroxylapatite chromatography, the ss-cDNA was PCR amplified. For sequencing, the cDNA in the size range of 500–1100 bp was eluted from a preparative agarose gel and sequenced using 454 GS+ Titanium technology at Eurofins (MWG, Germany). The raw reads were submitted to the Sequence Read Archive (SRA) at NCBI under the accession number SRR2127882. After quality checks and filters a total of 1,558,090 high quality reads were subsequently used for transcriptome de novo assembly using MIRA (settings: denovo, est, accurate, 454, -SK:acrc=no, -CL:msvs) (Chevreux et al., 2004). The final assembly produced 31,263 isotigs, from which a subset of 17,663 isotigs assembled from 5 or more reads were used to predict their coding regions and protein sequences using ORF-predictor (Min et al., 2005).

Methods S2.2. RNA isolation and RT-qPCR

*Tetranychus urticae* and *T. evansi* main body parts (‘idiosoma’) were collected after removal of the anterior body part (‘gnathosoma’) using a scalpel on a glass Petri dish pre-cooled with liquid nitrogen. *N. benthamiana* agroinfiltrated or mock (i.e., the infiltration-buffer without bacteria) treated leaves were collected and immediately frozen in liquid nitrogen. After material grinding, total RNA was collected using the Qiagen RNA extraction kit and treated with Turbo-DNase (Ambion) as described by the manufacturer. Subsequently, DNA-free samples were used for cDNA synthesis using M-MuLV RT (Fermentas), as described by the manufacturer. Reverse Transcriptase qPCR (RT-qPCR) was performed using EVA green
Spider mite effectors promote mite reproduction

(Biotium) by means of the ABI 7500 Real-Time PCR system (Applied Biosystems). Reactions were performed in a volume of 10-μl, containing 0.25 μM of each primer, 0.1 μl ROX reference dye and 1 μl of cDNA template (20 ng/μl). The cycling program was set to 5 min of pre-cycling stage (50°C), 5 min at 95°C, 45 cycles of 15 sec at 95°C and 1 min at 60°C. The program was followed by a melting curve analysis. For spider mite RT-qPCR assays *T. urticae* 18S rRNA, and *T. evansi* Ribosomal Protein 49 were used as housekeeping genes. For *N. benthamiana* RT-qPCR assays, actin was used as housekeeping gene. All primer pairs used are described in Table S2.2. Statistical differences of transcript abundances shown in Figure 2.4, Figures S2.6 and S2.7 were calculated by using a General Linear Model (GLM) analysis in SPSS 20 (IBM). Statistical differences shown in Figure S2.1 were calculated using the Student’s t-test in Excel (Microsoft).

**METHODS S2.3. PHYTOHORMONE EXTRACTION AND LC-MS**

Phytohormone analysis was performed as described in Alba et al. (2015). Briefly, between 80 to 150 mg of frozen leaf material was homogenized in 1ml of ethylacetate, which had been spiked with D6-SA and D5-JA (C/D/N Isotopes Inc., Canada) as internal standards to a final concentration of 100 ng/ml. After centrifugation at 13,000 rpm for 10 min at 4°C, the supernatant was transferred to new tubes. The pellet was re-extracted with 0.5 ml of ethylacetate (without the two internal standards) and centrifuged for 10 min at 4°C at 13,000 rpm. Both supernatants were combined and then evaporated to dryness on a vacuum concentrator. The residue was resuspended in 0.5 mL 70% methanol (v/v), centrifuged and the supernatants were transferred to glass tubes and then analyzed by LC-MS/MS. Measurements were performed on a liquid chromatography tandem mass spectrometry system (Varian 320 Triple Quad LC/MS/MS). SA and JA were quantified by comparing their peak area with the peak area of the respective internal standard. Statistical differences in the amounts of phytohormones among samples were calculated using log-transformed values by Fisher’s LSD test after ANOVA (SPSS 20, IBM).

**METHODS S2.4. WHOLE-MOUNT IN SITU HYBRIDIZATION**

Primers for Te84 and Te28 probe-template amplification were designed using Primer3 (http://bioinfo.ut.ee/primer3/). The preferred amplicon length was about 300bp. The resulting primers are listed in Table S2.1.
RNA was extracted from *T. evansi* mites (Total RNA Isolation Mini Kit, Agilent), treated with TURBO DNA-free™ Kit to remove contaminating genomic DNA and used for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit. PCR products were cloned into pGEM-T plasmids (Promega) and transformed into *E. coli*. Plasmids of liquid cultures were purified after which insert orientation and nucleotide sequence were checked by sequencing (LGC Genomics, Germany). A PCR was performed on the plasmids using pUC/M13 primers. PCR products, containing inserts flanked by T7 and SP6 promoter sites from the plasmid backbone, were checked by agarose gel electrophoresis and purified using Cycle Pure Kit (EZNA). Depending on the orientation, sense or anti-sense DIG-labeled probes were generated using T7 or SP6 RNA polymerase (Roche), using the pUC/M13 PCR product and DIG-UTPs (Roche) in the in vitro labeling reaction. Probes were then purified using SigmaSpin™ Sequencing Reaction Clean-Up Columns (Sigma).

_Tetranychus evansi_ larvae and adults of both sexes were collected from tomato plants and fixed in a 1:1 mix of heptane and PTw (PBS with 0.1% Tween-20) containing 4% formaldehyde. The mites were then washed in methanol and PTw, followed by sonication in a sonic cleansing bath and treatment with 0.2 mg/ml Proteinase K during 10 min. The mites were then re-fixed with 4% formaldehyde in PTw. Mites were prehybridized in hybridization buffer [50% formamide (Sigma), 4x SSC (Sigma), 1x Denhardt’s solution (Sigma), 250 μg/ml tRNA (wheat germ type V, Sigma), 250 μg/ml ssDNA (boiled salmon sperm DNA, Sigma), 50 μg/ml heparin (sodium salt, Sigma), 0.1% Tween-20 (Sigma), 5% dextran sulfate (sodium salt, Sigma)] for 1h at 52°C. Hybridization buffer was refreshed and probe was added. The mites were then incubated overnight at 52°C. Washing occurred at 53°C (6x 25 min) with wash buffer consisting of 50% formamide, 2x SSC and 0.1% tween-20. After washing at room temperature (RT) with PBTw (PTw with 0.1% BSA; Sigma), the mites were incubated at RT for 2h with 1:1000 dilution of anti-digoxigenin-AP (Fab fragments, Roche) in PBTw. The mites were then washed with PTw (5x 20 min) and several times with AP buffer (100 mM Tris pH 9.5, 100mM NaCl, 1 M MgCl₂, 0.1% Tween-20) until precipitation had disappeared. AP buffer containing the NBT/BCIP (nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyolphosphate) (Roche) or FastRed substrate (SIGMAFAST™ Fast Red TR/Naphthol AS-MX tablets, Sigma) was added and mites were incubated
several hours at 4°C in the dark, until blue or red staining was visible. Methanol was used to eliminate background staining and mites were cleared in 70% glycerol in PTw (pH 8.5) after washing with pure PTw. Finally, mites were mounted on microscopy glasses for further investigation.

FastRed-labeled spider mites were visualized using a laser-scanning microscope LSM510 (Zeiss), by exciting samples sequentially with the 458 nm (spider mite autofluorescence) and 543 nm (FastRed fluorescence) lasers. Mite autofluorescence and FastRed fluorescence emissions were detected sequentially by using the long pass filter LP 560. Z-stacks were performed by imaging up to 25 slices every 1-1.5 μm. Z-stacks were superposed by using ImageJ z-project (max intensity).