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Salivary proteins of spider mites suppress defenses in *Nicotiana benthamiana* and promote mite reproduction

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SUMMARY

Spider mites (*Tetranychidae* 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 mainly depends on the phytohormones jasmonic acid and salicylic acid (SA). On tomato (*Solanum lycopersicum*), however, 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.

Keywords: *Tetranychus urticae*, *Tetranychus evansi*, *Nicotiana benthamiana*, *Solanum lycopersicum*, effector, plant defense suppression, salicylic acid, jasmonic acid, *Agrobacterium tumefaciens* transient assay, *nahG* tomato.

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 (*Tetranychidae* sp.) are of special interest since they cover a broad host-plant range and can develop into devastating outbreaks (Grbic *et al.*, 2011; Van Leeuwen *et al.*, 2015). Adult spider mites feed from leaves by piercing mesophyll cells with their stylets. Spider mites possess three pairs of salivary glands associated with these stylets (Mothes and Seitz, 1981). Via their stylets they inject saliva into pierced host cells and then probably retract the stylets. 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 to food to the oesophagus (Albert 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 (Zhurov *et al.*, 2014; Martel *et al.*, 2015) 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 (Zhang *et al.*, 2013a; Cao *et al.*, 2014). 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 (Li *et al.*, 2006; Thompson and Goggin, 2006; Avila *et al.*, 2012). 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 (Thaler *et al.*, 2012; Kazan and Lyons, 2014).

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 (Sarmiento *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 (Kant *et al.*, 2008; Alba *et al.*, 2015) 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 (Glas *et al.*, 2014; Alba *et al.*, 2015). 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 (Gohre and Robatzek, 2008). Different definitions of 'effector' have been proposed in the 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 resistance in plants carrying the H13 resistance gene (Aggarwal *et al.*, 2014). Finally, effector-mediated suppression of PTI 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; Pitino and Hogenhout, 2013; Elzinga *et al.*, 2014; Naessens *et al.*, 2015) 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, 1998). Since several species of phytophagous mites were found to be able to suppress host-plant defenses (Kant *et al.*, 2008; Sarmiento *et al.*, 2011; Glas *et al.*, 2014; Alba *et al.*, 2015; Wybouw *et al.*, 2015) we hypothesized that mites, like pathogens, insects, and nematodes, may have evolved effector proteins which are secreted 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.

RESULTS

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 1) using two closely related mite species that can suppress plant defenses (Alba *et al.*, 2015). First *T. urticae* for which we obtained the predicted transcriptome from the London strain (Grbic *et al.*, 2011). 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 nts 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 nts). 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, 2010). First, we identified proteins with signal peptides using SignalP 4.0 (Petersen *et al.*, 2011) and Phobius (Kall *et al.*, 2004). These proteins were screened via Phobius and TMHMM (Krogh *et al.*, 2001) and excluded all proteins that had a predicted transmembrane domain. Finally we used TargetP (Emanuelsson *et al.*, 2000) and WolfPsort (Horton *et al.*, 2007) for subcellular localization

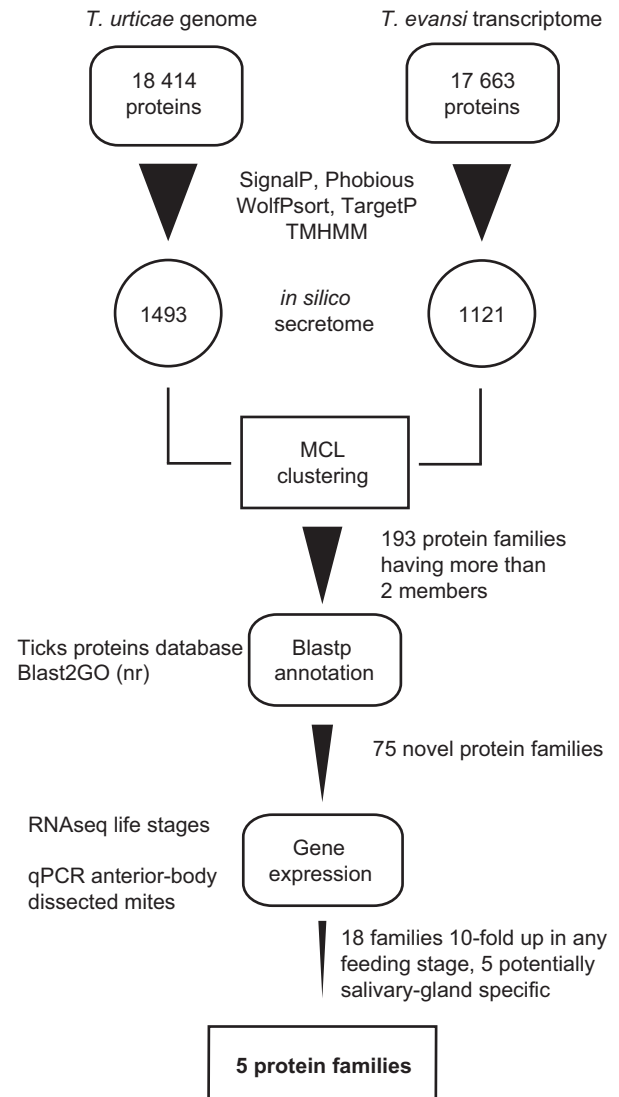


Figure 1. Overview of an *in silico* pipeline used to identify spider mite effectors.

Four selection steps were applied to obtain the combined dataset of putative secreted proteins from both the generalist *Tetranychus urticae* and the specialist *T. evansi*. Five protein families having effector potential were selected.

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 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 Experimental Procedures. In total, 999 protein families were identified, with

193 families having three or more members (group I), 276 families with two members (group II), and 530 singleton clusters (group III) (Data S1). 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 group was the 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 (Pitino and Hogenhout, 2013; Aggarwal *et al.*, 2014).

Second, it has been reported that the majority of pathogen effector proteins identified so far are highly species or genus specific (Gohre 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 S1). 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 S1). 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 (Grbic *et al.*, 2011; Dermauw *et al.*, 2013). 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.

Selection of the top five 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 (larvae, 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-specific (egg, larvae, nymph, or adult) gene-expression levels derived from the quantitative RNA-seq data of *T. urticae* that was made publicly available together with the *T. urticae* genome (Grbic *et al.*, 2011; these data are included in Data S1). 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 homologue expressed ≥ 10 -fold in any of the feeding stages (larvae, 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 S1) 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 S1). 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). 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 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 glands (Figure 2), which are one of the three paired spider mite salivary glands (Mothes and Seitz, 1981). 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 S3). We also hybridized antisense probes for Te28 (Figure S3), 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.*, in prep).

Transient overexpression 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

Table 1 Overview of the final five candidate effectors

Family number	Number of family members	Cloned candidate	Gene model ID ^a /Genbank accession no.	Mature protein size (aa)
19	11 <i>Tetranychus urticae</i>	Tu19	tetur05g09110	198
	7 <i>T. evansi</i>	Te19	KT182960	198
28	10 <i>T. urticae</i>	Tu28	tetur31g01040	266
	1 <i>T. evansi</i>	Te28	KT182959	338
84	2 <i>T. urticae</i>	Tu84	tetur01g01000	227
	2 <i>T. evansi</i>	Te84	KT182961	230
90	4 <i>T. urticae</i>	Tu90	tetur05g04560	287
128	2 <i>T. urticae</i>	Tu128	tetur01g00940	235
	1 <i>T. evansi</i>	Te128	KT182962	233

^a*T. urticae* gene models are available on the BOGAS genome portal (<http://bioinformatics.psb.ugent.be/webtools/bogas/>).

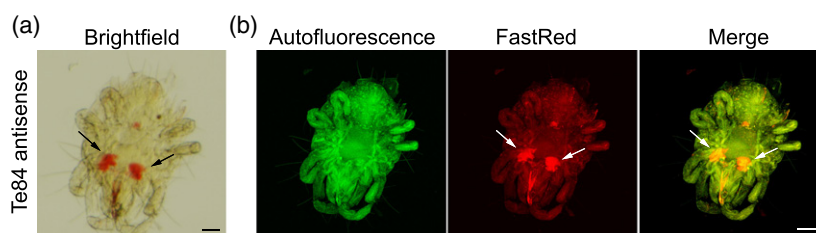


Figure 2. Whole mount *in situ* hybridization of a putative salivary protein gene belonging to family 84.

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 brightfield microscopy (a) and confocal laser-scanning microscopy (b). Scale bars indicate 50 μm .

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 phenotypes in *N. benthamiana* when expressed under control of the 35S promoter using *Agrobacterium*-mediated transient assays (Kapila *et al.*, 1997), further referred to as 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 a keep high and long-lasting transcription (Voinnet *et al.*, 2003) using the empty vector (EV i.e. the expression vector still containing the Gateway negative-selection cassette) 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 days post infiltration (DPI) (Figure 3a). The expression of the transgenes in the infiltration zone was confirmed by RT-PCR at 2 and 5 DPI (Figure 3b). Expression of candidate Te28 occasionally induced necrosis in *N. benthamiana* 4–5 DPI (Figure S4), but whether this is related to a higher expression of Te28, as the RT-PCR results suggest (Figure 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 S5) and we thus continued with the four putative effectors that did.

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 effect 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 two and five DPI.

At 2 DPI the concentration of SA was eight-fold higher in leaves agroinfiltrated with the EV than in mock-treated leaves (Figure 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 4a). In contrast, at 5 DPI the levels of SA were seven-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 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

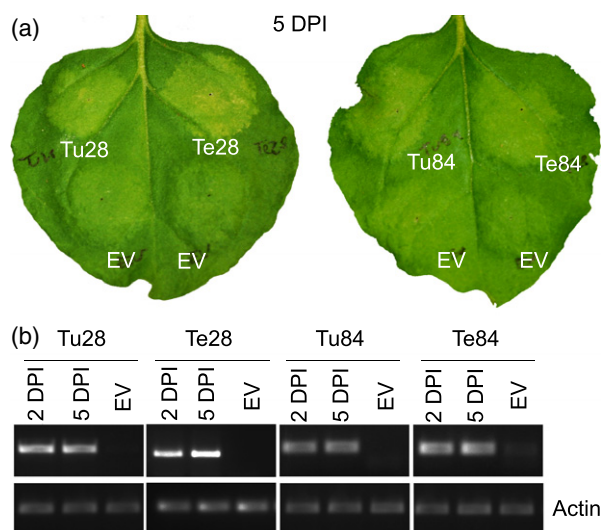


Figure 3. Four spider mite candidate effectors cause chlorosis in *Nicotiana benthamiana*.

(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 or 5 DPI. The data are representative for two experiments.

and 5 DPI respectively (Figure 4b). At 2 DPI, *PR1*-expression was six-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 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 three-fold (2 DPI) and 30-fold (5 DPI) relative to EV. However, at 2 DPI, leaves expressing its counterpart Te84, *PR1* expression was two-fold higher than in leaves with the EV (Figure 4b). Since at 5 DPI all four candidate effectors partially suppressed *PR1* induction relative to the EV control, we repeated this experiment using 35S:green fluorescent protein (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 S6).

Expression of another SA-related marker *PR4*, which is known to be co-regulated by JA (Maimbo *et al.*, 2010), was six- or 27-fold higher, at 2 or 5 DPI respectively, when comparing EV to mock-treated leaves (Figure 4b). Like *PR1*, also *PR4* expression was suppressed, albeit mildly, by Te28 and Tu28 at 2 and 5 DPI, with levels being two-fold and six-fold lower than those of the EV. Candidate Tu84 significantly suppressed *PR4* induction only at 5 DPI by three-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 *Trypsin Proteinase Inhibitor (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

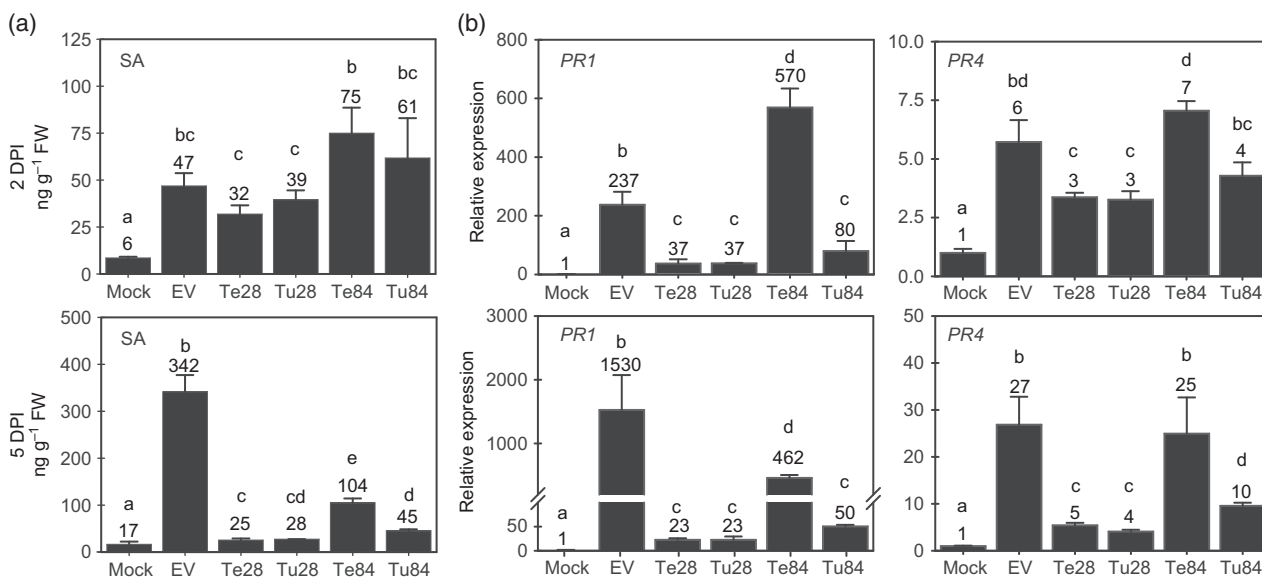


Figure 4. Spider mite candidate effectors affect the SA response induced by *Agrobacterium*.

(a) Levels of salicylic acid in ng/gFW in agroinfiltrated leaves at 2 and 5 DPI. Error bars represent standard error (SE). Data were log-transformed prior to statistical analysis. Different letters indicate statistical differences according to Fisher's LSD test ($P < 0.05$).

(b) Relative gene expression of the SA-related marker genes *PR1* and *PR4* after agroinfiltration, at 2 DPI (upper panel) or 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.

stronger for the four candidates compared to the EV and for Te28 and Tu84 down-regulation was even significant (Figure S7). 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.

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's least significant difference (LSD) test $P < 0.001$) (Figure 5). In contrast, oviposition of *T. urticae* was 25% lower on leaf discs expressing the candidate Te28, compared to the EV (Fisher's LSD test $P < 0.05$) (Figure 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 S8).

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 a defense hor-

more that constrains mite performance (Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2008; 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 to 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, within the time frame of our studies, with 3- to 4-week-old plants, the plants have a normal phenotype. After 4 days of infestation, *T. urticae* mites had deposited 10% more eggs on *nahG* plants compared to the wild type Money-maker (Figure 6) and this increase was statistically significant (Genotype effect, $P = 0.047$). This establishes that the SA defense response does have a negative effect on mite performance.

DISCUSSION

Previously we showed that the phytophagous mites *T. urticae* and *T. evansi*, two agronomical-relevant pest species, suppress JA and SA defenses in plant to their own benefit via an unknown mechanism (Kant *et al.*, 2008; Sarmiento *et al.*, 2011; Alba *et al.*, 2015). 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 4). Furthermore, we showed that *in planta* expression of these proteins i.e. Tu28 and Tu84 and its homologue Te84, promoted the reproductive performance of *T. urticae* (Figure 5) similar to when it expressed the *nahG* gene (Figure 6). Thus these

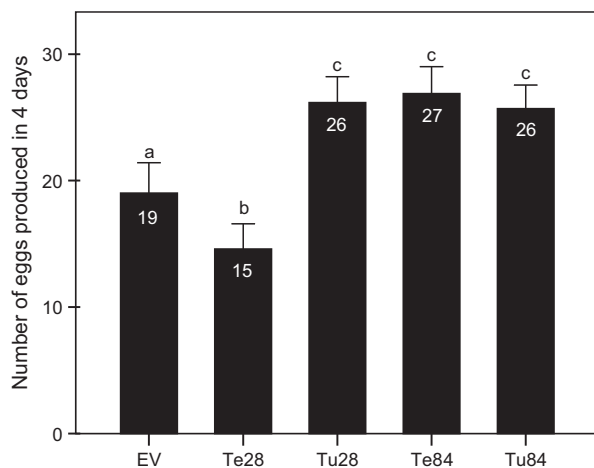


Figure 5. Three candidate effectors improve *Tetranychus urticae* performance on *Nicotiana benthamiana*.

The figure shows the average number of eggs laid by one female adult spider mite after feeding for 4 days on *N. benthamiana* leaf discs expressing the candidate effectors or the EV control. Error bars represent standard error (SE). Different letters indicate statistical differences according to Fisher's LSD test ($P < 0.05$). This experiment was repeated twice with similar results.

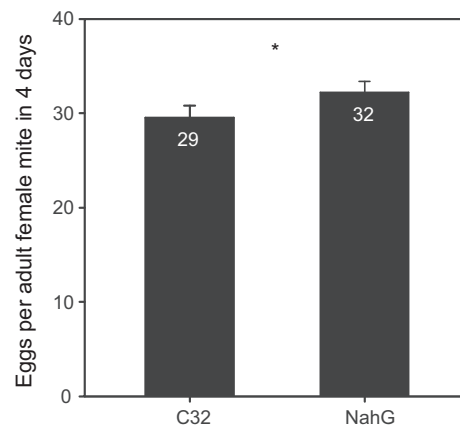


Figure 6. Spider mite performance is higher on SA-deficient tomato *NahG* plants than on wild type plants.

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 Money-maker. The experiment was performed four 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 ($P < 0.05$).

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 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 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 (Zhang *et al.*, 2012; Kiba *et al.*, 2014). 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 (Kant *et al.*, 2004, 2008; Li *et al.*, 2004; Glas *et al.*, 2014) and suppress (Sarmiento *et al.*, 2011; Alba *et al.*, 2015) 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 S7). 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 (Kant *et al.*, 2004; Glas *et al.*, 2014), and insect herbivores such as aphids (Moran and Thompson, 2001), whiteflies (Zarate *et al.*, 2007) and by the larvae of some lepidopteran species (Musser *et al.*, 2002; Diezel *et al.*, 2009). 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 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 S11). 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 S8). 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 homologues 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 S9), 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-bio-

synthesis mutant *def-1* (Kant *et al.*, 2008; Alba *et al.*, 2015) 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, as 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: 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 (Kaloshian, 2004; Jones and Dangl, 2006). 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 *T. evansi*-specific secreted proteins from group II (gene families with two 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 (Sarmiento *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 *T. evansi* and generalists like *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 *T. urticae* strains induce plant defenses (Zhurov *et al.*, 2014; Alba *et al.*, 2015; Martel *et al.*, 2015) 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 (Kant *et al.*, 2008; Alba *et al.*, 2015). 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 effec-

tors, herbivore performance assays may provide the only read-out with biological relevance.

EXPERIMENTAL PROCEDURES

High-throughput sequencing and *de novo* assembly

T. 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 five or more reads were used to predict their coding regions and protein sequences using OrfPredictor (Min *et al.*, 2005). Details on the sequencing and assembly can be found in Methods S1.

In silico prediction of the spider mite secretome

For *Tetranychus urticae* secretome prediction the predicted proteins from *T. urticae* London genome (Grbic *et al.*, 2011) were used. The signal peptide prediction was done using SignalP 4.0 (stand alone version; Petersen *et al.*, 2011) and Phobius (Kall *et al.*, 2004). Transmembrane domains were predicted using THMM (standalone version; Krogh *et al.*, 2001) and Phobius. Subcellular localization was predicted by TargetP (stand alone version; Emanuelsson *et al.*, 2000) and WolfPsort (stand alone version; Horton *et al.*, 2007). Default settings were used for all software parameters.

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 88616 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).

RNA isolation and RT-qPCR

T. 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. In short, after grinding the material, total RNA was isolated using the Qiagen RNAeasy mini kit (Valencia, CA, USA). 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 and S3. Statistical differences of transcript abundances shown in Figure 4 and in Figures S6 and S7 were calculated by using a general linear model in spss 20 (SPSS Inc., Chicago, IL, USA). Statistical differences shown in Figure S1 were calculated using Student's *t*-test in MS Excel® (Microsoft).

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, Carlsbad, CA, USA) (Table S1). 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 1). The final destination vectors were introduced into *A. tumefaciens* GV3101 cells by electroporation.

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% humidity). All agroinfiltrations were performed on plants 4–5 weeks old. Tomato *Lycopersicon 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 (\pm 2) days old via a method described in Kant *et al.* (2004). Four days after infestation, leaflets were detached and the number of eggs were counted using a stereomicroscope. The experiment was repeated four times on 10 plants per tomato genotype: per plant, three leaflets were infested. The results presented in Figure 6 represent the mean number of eggs per mite per day. Effect on performance was analyzed using a GLM in SPSS 20 (SPSS Inc.) including plant genotype as main factor and experiment as random factor.

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, EV (pSOL2092), or 35s: p19, were grown from single colonies for 16 h in 2 ml LB medium with the appropriate antibiotics. An aliquot of each pre-culture was then inoculated into 5–10 ml LB with 10 mM MES and 20 μ M acetosyringone, using the same antibiotics and grown until an OD between 1.0 and 1.5 was reached. After centrifugation the bacteria pellets were resuspended in MMAi (2% sucrose, 10 mM MES, 0.2 mM acetosyringone) to a final OD of 0.6 and then incubated for at least 1 h at room temperature. Bacterial suspensions were infiltrated into the abaxial side of the third-youngest fully expanded *N. benthamiana* leaf using a needleless syringe.

Phytohormone extraction and LC-MS

Phytohormone analysis was performed as described in Alba *et al.* (2015) and its details can be found in Methods S3. Statistical differences in the amounts of phytohormones among samples were calculated using log-transformed values by Fisher's LSD test after analysis of variance (ANOVA) (SPSS 20, SPSS Inc.).

Spider mite performance assays

Agroinfiltrated *N. benthamiana* leaflets were detached at 2 DPI. From these detached leaves glandular trichomes were gently removed using filter paper soaked in water (Figure S10). 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 stereomicroscope. Leaf discs with either a dead female or a female that had drowned in the border of the wet cotton were discarded 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, SPSS Inc.). To evaluate performance on wild type and *nahG* tomato five adult female mites were placed on a single leaflet and for each plant three leaflets were infested in total. Leaflets were detached after 4 days and eggs were counted using a stereomicroscope. The experiment was repeated four times using 10 plants per tomato genotype each time. The data was analyzed using a general linear model in SPSS 20 SPSS Inc. using 'plant genotype' as main factor and 'experiment' as random factor.

In situ hybridization

Tissue-specific expression of Te84 and Te28 was obtained via whole mount *in situ* mRNA localization with DIG-labeled 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 S4.

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. *T. evansi* RNA-seq data can be found under the SRA accession number SRR2127882.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Screening for potential *T. urticae* salivary-gland specific genes.

Figure S2. Amino acid sequence alignment of Te28 and Tu28.

Figure S3. Detection of Te28 and Te84 gene expression in salivary glands of *T. evansi* using whole mount *in situ* hybridization.

Figure S4. Candidate effector Te28 causes tissue necrosis after agroinfiltration.

Figure S5. Agroinfiltration of candidate effectors from families 19, 90, or 128 does not induce chlorosis in *N. benthamiana*.

Figure S6. Relative gene expression of the SA-marker gene PR1 after agroinfiltration of four candidate effectors or 35s:GFP as control.

Figure S7. Relative gene expression of the JA-marker gene *TPI* after agroinfiltration of four candidate effectors.

Figure S8. Leaf discs expressing candidate Te28 show intense chlorotic symptoms.

Figure S9. Amino acid sequence alignment of Te84 and Tu84.

Figure S10. Agroinfiltrated *N. benthamiana* leaves after leaf-surface washing.

Figure S11. Exponential growth of two hypothetical populations growing on the SA-impaired *nahG* tomato plants or Moneymaker (MM) tomato plants.

Table S1. Primers sequences used for gateway cloning and *in situ* hybridization.

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

Table S3. *Tetranychus urticae* and *Tetranychus evansi* primer sequences used for RT-PCR and/or qPCR in this study.

Data S1. *Tetranychus evansi* and *T. urticae* clustered secretomes.

Methods S1. High-throughput sequencing and *de novo* assembly.

Methods S2. RNA isolation and RT-qPCR.

Methods S3. Phytohormone extraction and LC-MS.

Methods S4. *In situ* hybridization and confocal laser-scanning microscopy.

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