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From Induction to Suppression: How to Manipulate Plant Defenses

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Summary thesis BCJ Schimmel

From induction to suppression: how to manipulate plant defenses

Plants have evolved traits, including induced defenses, to resist herbivores. However, some plant-eating spider mites (Tetranychus spp.) have adapted to plant defenses to maintain a high reproductive performance. From natural populations of two species of mites, *T. urticae* and *T. evansi*, three lines were selected and demonstrated to suppress induced defenses of tomato plants downstream from the accumulation of the defense-regulating hormones jasmonate (JA) and salicylate (SA), and independently of JA-SA crosstalk. When sharing a leaflet, the suppression by *T. evansi* is powerful enough to co-suppress the JA and SA responses induced by *T. urticae* mites that cannot suppress defenses ('inducers'), thereby improving the reproductive performance of the latter. A subsequent analysis of the spatiotemporal dynamics of induction and suppression at the within leaflet scale revealed that these are predominantly local events. Furthermore, *T. evansi* was found to hyper-suppress defenses in response to the nearby presence of *T. urticae* competitors and this coincided with an increased reproductive performance of *T. evansi*, but not *T. urticae*. Hence, inducer mites do not always -or immediately- benefit from co-occurring suppressors. Zooming in further, inducer and suppressor *T. urticae* were shown to harbor different bacterial endosymbionts and removal of these bacteria with antibiotics affected mite performance, mite gene expression, mite feeding intensity, and tomato responses to mite feeding. Finally, genome-wide transcriptomic responses of tomato plants to feeding by suppressor and inducer mites were analyzed to identify *T. evansi*-responsive tomato promoters to use these to engineer an inducible resistance to suppressor mites.

SUMMARY

In **Chapter 1** I outline the current knowledge on the interaction between plants and phytophagous pathogens an herbivores. Plants and their attackers have evolved in close association for hundreds of millions of years. Their evolutionary trajectories were largely determined by their antagonistic interactions: while plants are equipped with numerous (defensive) traits that primarily serve to tolerate or withstand microbial and herbivorous attackers in order to not get eaten, these attackers -in turn- are equipped with traits that allow them to cope with these defenses in order to eat plants. These interactions may have resulted in co-evolutionary trajectories of specific traits and their counter-traits.

One important adaptation of pathogens and especially herbivores to plant defenses (toxic compounds) is detoxification. Another way to overcome plant defenses is by manipulating the host plant's molecular machinery such that the harmful defense responses are not properly executed and do not result in (full) resistance. Such molecular sabotage of the host is termed "defense suppression". There is ample literature to support that various plant-attacking microorganisms (e.g. bacteria, fungi) have evolved to do so. However, our knowledge on if and how herbivorous arthropods (e.g. insects, mites) suppress plant defenses is very limited. Investigating how and to which extent herbivores manipulate their host plant will help us to better understand which selective forces drive plant-herbivore interactions. Such knowledge, however, is also highly valuable for agriculture, as it can help us to improve crop resistance and hence reduce crop losses due to pests.

The research presented in this thesis is aimed at further elucidating the interaction between defense-suppressing spider mites (*Tetranychus* spp.) and their tomato (*Solanum lycopersicum*) host plant. Specifically, I have investigated how (at the transcriptional and metabolic level) a cultivated tomato plant responds to suppression of defenses by the destructive pest species *Tetranychus urticae* and *Tetranychus evansi* with the aim to genetically alter tomato plants such that defenses against these herbivores are restored.

Like humans, plants use various hormones to regulate their growth, development, reproduction, and defense (immune) responses to attackers. With respect to the latter, the two plant hormones salicylic acid (SA) and jasmonic acid (JA) are crucial for plant immunity to uphold maximal reproduction across diverse eco-physiological conditions. In general, defense responses against biotrophic pathogens and (relatively) stationary arthropods (e.g. wingless aphids, immobile larval stages such as those from whiteflies or thrips) are regulated by SA, while defense responses against necrotrophic pathogens and agile herbivores (e.g. caterpillars, mites, beetles) are mediated by JA. Both hormones mediate the transcription of defense-associated genes and thereby the production of toxic compounds and the production of defensive proteins (among others).

In **Chapter 2** we sampled natural spider mite populations (*T. urticae* and *T. evansi*) in the field and propagated these in the lab. For *T. urticae* we fixed the genotypic variation of hundreds of mites collected from three different host plants in the form of "strains" and tested to which extent mites from these strains; (1) induce similar defenses in tomato, and; (2) are affected -in terms of oviposition- by JA-regulated defenses of tomato. In Chapter 2 I report that these strains were markedly different for these traits. Firstly, we observed that while most strains induce JA defenses, some strains suppress them. These results show intraspecific variability for the defense-suppression trait and suggest this trait is not very rare among *T. urticae* mites. Secondly, while most strains are sensitive to JA defenses some strains appeared to be resistant (but resistance was rarer than suppression). Moreover, by using a Brazilian and Spanish haplotype of *T. evansi* we confirmed that this sister species of *T. urticae* can suppress tomato defenses strongly. Possibly in this species the suppression trait got to fixation.

In the laboratory we investigated the temporal dynamics of induction and suppression by analyzing the tomato defense response in detail over the whole course of the infestation, i.e. we monitored concentrations of JA and SA as well as transcript abundances of various defense-associated genes, from the start of the infestation until shortly before the infested leaflet died. To do

so we used the two *T. evansi* haplotypes, *T. urticae* Santpoort-2 (an inducer line) and *T. urticae* Delier-1 (a suppressor line). Although the magnitude of suppression differed, mites from both species were found to inhibit defenses downstream from JA and SA accumulation and independent from the antagonistic crosstalk that has often been observed between the signaling pathways controlled by these hormones. We further demonstrated that suppression by *T. evansi* is powerful enough to co-suppress the JA and SA responses induced by inducer *T. urticae* mites (from the Santpoort-2 strain) when these mites share the same leaflet, thereby improving the reproductive performance of the latter with as much as 45 percent. Similar suppressor/inducer co-infestation experiments with *T. urticae* DeLier-1 indicated that these are less potent suppressors of tomato defenses than *T. evansi* mites, as they only managed to co-suppress the SA-responses and had a less dramatic effect on the fecundity of *T. urticae* inducers (25 percent increase).

In **Chapter 3** we further investigated the observation that suppressor mites have a positive effect on the performance of co-occurring inducer mites via the plant and explored possible reciprocal effects of the inducer on the performance of the suppressors. To do so, we non-destructively divided tomato leaflets into three sectors perpendicular to the midvein (tip, middle and bottom) using a thin artificial barrier and infested the middle and/or tip section with either suppressor (*T. evansi*) or inducer (*T. urticae* Santpoort-2) mites. We analyzed phytohormone and gene transcript levels in each of the leaflet sectors at different time points after the infestation to determine the spatial and temporal patterns of defense responses.

Contrary to our expectations, the results indicated that induction of defenses by spider mites is very local, i.e. mostly restricted to the feeding site. As in Chapter 2, we found that defense suppression often displays itself as non-induction (i.e. not different from the control) or reduced induction (i.e. higher than the control but lower than the levels induced by *T. urticae*) of defenses, rather than suppression below control levels. However, while in Chapter 2 we observed that a 4-day period of leaflet sharing significantly improved the performance of *T. urticae*, in Chapter 3 we observed that a 2-day period of leaflet sharing did not yield this effect. Given the differences in the setup of the experiments performed in Chapters 2 and 3, we conclude that inducer *T. urticae* mites can indeed benefit from co-occurring suppressors, but this is not always or immediately the case and likely depends on the number (or ratio) of inducer and suppressor mites, the sequence of their arrival, the time they spend together on the leaf and possibly their relative position on the leaflet or plant. Moreover, we observed an intriguing and somewhat puzzling phenomenon: when *T. urticae* was introduced adjacent to an *T. evansi* feeding site, the magnitude of suppression by *T. evansi* increased. This hyper-suppression coincided with the increased expression of *T. evansi* salivary effector genes, which encode proteins secreted by mites into their host to suppress defenses, as well as an increased reproductive performance of *T. evansi*. This overcompensation response of *T. evansi* suggests that the mite responds to nearby competitors by increasing its reproductive output possibly to reinforce competitive population growth. Although I believe this overcompensation comes at a cost, i.e. at the expense of another mite activity or life history trait, we did not notice such a trade-off. In addition, the signal that elicits this response (either plant-borne or aerial since mites have practically no eye sight) remains to be identified. Based on the overcompensation response, however, I do notice that mite oviposition is a more plastic trait than previously thought.

In **Chapter 4** we analyzed the bacterial community associated with the *T. urticae* DeLier-1 and *T. urticae* Santpoort-2 to investigate if the absence/presence of these bacteria correlates with suppression and induction of defenses respectively. We found that these two mite strains harbor different bacterial endosymbionts: DeLier-1 mites contained *Wolbachia* bacteria, while Santpoort-2 mites contained *Cardinium*. In addition, *Spiroplasma* bacteria were associated wih mites from both strains. Subsequently we treated mites with antibiotics to remove these bacterial endosymbionts in order to assess their influence on mite performance, on the spider mite transcriptome and on the induction/suppression of defense responses in tomato plants. For DeLier-1 the antibiotics treatments resulted in strains that no longer carried *Wolbachia* nor *Spiroplasma* (W-S-), and strains that no

longer contained *Wolbachia* but that had retained *Spiroplasma* (W-S+). For Santpoort-2 the antibiotics treatments yielded strains that no longer carried *Cardinium* nor *Spiroplasma* (C-S-).

For the suppressor strain DeLier-1, removal of *Wolbachia* decreased mite survival and decreased transcript levels of mite genes associated with digestion and detoxification. Tomato leaflets infested with non-treated suppressor mites harboring *Wolbachia* as well as *Spiroplasma* (W+S+) accumulated higher concentrations of the JA precursor 12-oxo-phytodienoic acid (OPDA) and lower concentrations of SA than leaflets infested with mites that harbored only *Spiroplasma*. The concentrations of OPDA and SA were intermediate in leaflets that were infested with DeLier-1 mites that did not contain any of the endosymbionts. No consistent differences were found for JA levels. Aligning phytohormone and marker gene expression data resulted in a confusing picture that often did not follow trends known from literature. Transcript levels of one SA-defense marker gene followed the pattern of SA accumulation, but those of a second marker gene did not. Similarly, transcript levels of one JA-regulated defense-associated marker gene were highest in leaflets infested with endosymbiont-free mites, while those of two other JA-marker genes did not differ among treatments. Even more confusingly, the expression patterns of five putative OPDA-regulated genes were opposite of the accumulation pattern of OPDA itself. Overall, suppression by DeLier-1 could not be attributed to the absence/presence of bacterial endosymbionts.

For the inducer Santpoort-2, non-treated mites carrying both *Cardinium* as well as *Spiroplasma* (C+S+) had a lower survival, fecundity and feeding intensity than mites without these bacteria. Interestingly, feeding by *Cardinium* plus *Spiroplasma*-containing mites resulted in rusty red/brown feeding scars, whereas feeding by endosymbiont-free Santpoort-2 mites resulted in clear white scars. Also for *T. kanzawai* strains exist that produce either red or white scars and here the red scars are attributed to a single dominant mite gene locus. Hence, we speculate that there may be an interaction between such a gene and *Cardinium* and/or *Spiroplasma* in producing red scars and it does suggest a direct effect of these bacteria on this (unknown) plant response. No significant differences were found in the concentrations of OPDA, JA or SA in leaflets after infestation with Santpoort-2 with or without endosymbionts. Yet, concentrations of another (abiotic stress-regulating) hormone, abscisic acid (ABA), were higher after infestation with mites without *Cardinium* and *Spiroplasma* than after infestation with mites with these endosymbionts. Furthermore, transcript levels of two SA-defense marker genes were inversely correlated with ABA concentrations, i.e. they were highest in tomato leaflets after an infestation with *Cardinium* and *Spiroplasma*-containing mites. Overall these data suggest that *Cardinium* and *Spiroplasma* may contribute to the induced SA-response of Santpoort-2 mites.

Taken together, from the data of Chapter 4 we conclude that endosymbionts can affect mite performance, the expression of distinct groups of mite genes and possibly the SA-mediated defense responses induced by mite feeding. Overall, *Wolbachia* seems to behave more like a beneficial symbiont (positive effect on mite fitness), while the combination of *Cardinium* and *Spiroplasma* behaves more like a parasite (negative effect on mite fitness). Since *Spiroplasma* partially counteracted the effects of *Wolbachia*, these bacteria may interact with each other in the mite, but it is not known why, nor what the exact effect of *Spiroplasma* on its host is.

In **Chapter 5** I searched for *T. evansi*-responsive tomato promotors to use these for creating transgenic tomato plants with promotor::defense-gene fusions to bypass defense suppression. Therefore, I first compared the genome-wide transcriptomic responses of tomato plants to feeding by suppressor and inducer mites using microarrays and whole transcriptome-sequencing (RNA-seq) to identify tomato genes *induced* by *T. evansi*. Feeding by *T. urticae*, either DeLier-1 or Santpoort-2, resulted in the largest groups op up- and down-regulated tomato genes, with Santpoort-2 upregulating three times more tomato genes than DeLier-1. *T. evansi*-infested plants were most similar to uninfested control plants as *T. evansi* up-regulated six times less tomato genes than Santpoort-2. Only very few of these genes were *T. evansi* specific. The identity of the *T. evansi*-specific genes did not give clear hints for a mechanistic explanation of the defense suppression phenotype. I selected the tomato genes that are most rapidly and significantly induced by

(suppressor) mites, but are not expressed under uninfested control conditions. I subsequently isolated and cloned the promoter region (i.e. the regulatory DNA fragment that precedes the protein-coding region) of some of these genes and fused them upstream of either the protein-coding region of tomato Prosystemin to engineer an mite-inducible JA-defense or the protein coding region of the fungal avirulence protein Avr4 to engineer an inducible hypersensitive response (i.e. a defense response characterized by local cell death and the production of defensive proteins). Tomato plants were then genetically transformed with one of the generated constructs with the aim to restore defense responses against suppressor mites. My results suggest that some of the generated promoter::defense-gene constructs indeed do respond to mite-feeding and, hence, possibly can be used to re-engineer inducible defenses.

Finally, in **Chapter 6** I highlight and discuss my findings and add some preliminary data on the plant's primary metabolism (soluble sugar content and photosynthesis) in response to infestation with either inducer or suppressor mites to address the following questions: (Q1) Can we pinpoint on which part (or parts) of the defense signaling cascade the mite's suppression acts? (Q2) What might be the source (or sources) of suppression? (Q3) To which extent can inducer mites benefit from defense suppressor mites? (Q4) Is promotor-swapping a feasible approach for resistance engineering?

With regard to Q1, I conclude that suppression operates downstream of JA and SA production and independent of the JA-SA crosstalk. Furthermore, suppression affects the plant's primary as well as secondary metabolism and possibly affects resource reallocation. With respect to Q2, I conclude that salivary effectors are the prime source of suppression, possibly by targeting general regulatory systems like the proteasome. Mite-associated microbes may also influence plant responses to mite feeding, but perhaps more by inducing them than suppressing them. With respect to Q3, I conclude that inducer mites may benefit from defense suppression since it improves their diet, but that not every experimental permutation will allow for this effect to occur. In turn, suppressor mites can respond to the presence of inducer mites by initiating hyper-suppression and by overcompensation of their oviposition rate. Finally, with regard to Q4, I conclude that suppressor-mite inducible promotors are promising tools for engineering plants that display defense responses to inducer as well as suppressor mites.