Of moths, mites and microbes - The role of bacteria in the life history of two arthropod herbivores

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The bacterial symbionts *Wolbachia*, *Cardinium* and *Spiroplasma* affect gene expression and survival of their spider mite host and impact distinct induced responses in plants.


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Abstract
Herbivore-associated microbial symbionts may facilitate plant utilization of their hosts directly by providing nutrients or by supporting the breakdown of food. Symbionts may also indirectly facilitate plant utilization by manipulating plant physiology to their host’s benefit. Here we investigated the role of the symbiotic prokaryotes Wolbachia (W), ‘Candidatus Cardinium’ (C) and Spiroplasma (S) in plant utilization by two strains of the spider mite Tetranychus urticae: Santpoort-2, an ‘inducer’ strain of plant defence in tomato (Solanum lycopersicum), and DeLier-1, a ‘suppressor’ strain of plant defence. We sequenced the bacterial communities of both strains and found that the inducer strain carries Cardinium and Spiroplasma (C+S+), while the suppressor strain carries Wolbachia and Spiroplasma (W+S+). After mites were treated with antibiotics to remove these symbionts, we obtained mite lines for 5 groups: W+S+, W-S+, W-S- for the suppressor and C+S+, C-S- for the inducer strain. In the suppressor strain, we found that the absence of Wolbachia correlated with lower mite survival on tomato, while the absence of Spiroplasma did not affect mite survival. In the inducer strain, food intake, survival and fecundity were increased when Cardinium and Spiroplasma were removed from the host. Using microarrays, we determined to what extent the absence of symbionts affected the mite’s transcriptome. Interestingly, many of the mite genes that were differentially regulated in mites with or without bacteria encode proteins that play a role in digestion and detoxification or are predicted to be secreted, some of them from the salivary gland. Specifically, in the suppressor strain we found a reduced expression of these genes when Wolbachia was absent, while in the inducer strain the absence of Cardinium and Spiroplasma had the opposite effect. Finally, we investigated to what extent the antibiotics-treated mites induced or suppressed plant responses compared to the non-treated control strains. Excluding Wolbachia from the suppressor mites while retaining Spiroplasma (W-S+) resulted in a dramatically reduced accumulation of several jasmonic acid (JA)-intermediates, such as 12-oxo-phytodienoic acid (OPDA), but did not affect the end product JA-Ile nor the expression of defence marker-genes downstream of JA. We did however detect an increased salicylic acid (SA) accumulation, but without a uniform effect on the downstream marker genes. Removal of both (endo)symbionts from the suppressor strain yielded an intermediate plant phenotype. Feeding by C-S- inducer mites resulted in higher levels of abscisic acid (ABA) and lower expression of SA marker genes compared to feeding by C+S+ mites, while SA levels were unaffected. Our data suggest that Wolbachia is beneficial for the suppressor mites, by possibly enhancing the ability of its host to deal with toxic secondary metabolites in its food. In contrast, Cardinium seems to negatively affect the performance of the inducer mites, possibly by inducing expression of (SA-responsive) pathogenesis-related genes in tomato leaflets, while simultaneously inhibiting detoxification processes in the mite. Spiroplasma might counteract these effects to some extent through direct interactions with Wolbachia and Cardinium. In conclusion, spider mite (endo)symbionts seem to interact directly and indirectly with their host. While Wolbachia has the characteristics of a mutualist, Cardinium has the characteristics of a parasite.
INTRODUCTION

Herbivores face the challenge to digest, absorb and utilize plant material in order to grow, develop and reproduce. In many cases, plant material is poor or unbalanced in nutrients or contains structural molecules that are hard to digest like cellulose or lignin (Schoonhoven et al., 2005; Karban & Baldwin, 2007). Moreover, herbivores have to cope with plant defences ranging from mechanical barriers, such as thorns and trichomes, to the production of poisonous substances (Schoonhoven et al., 2005; Karban & Baldwin, 2007; Mithöfer & Boland, 2012). Not only herbivores, but also microbes have evolved to consume plant material either as (biotrophic or necrotrophic) pathogens or as detritivores. In some cases, herbivores and microbes have established symbioses (i.e., the living together of dissimilar species; De Bary, 1879) that benefit both partners (mutualistic symbiosis). For instance, some herbivores provide nutrients and shelter to microbes, while in return they make use of the huge metabolic capabilities of these microbes to feed on otherwise unpalatable plants or plant parts, thereby expanding their niche space (Douglas, 2009; Feldhaar, 2011; Engel & Moran, 2013; Hansen & Moran, 2014).

Plant usage by herbivores may be facilitated either by beneficial bacterial symbionts directly, or indirectly via the host plant (Barbosa et al., 1991; Frago et al., 2012; Casteel & Hansen, 2014). For example, direct facilitation may occur via bacteria that upgrade low quality food, by producing essential amino acids or vitamins that the host diet lacks, or by the production of enzymes which enhance the digestion of refractory food sources (Douglas, 2009; Feldhaar, 2011; Engel & Moran, 2013). In contrast, indirect facilitation may occur via interaction of symbiotic bacteria with the plant to benefit their host’s fitness (Frago et al., 2012; Hansen & Moran, 2014). For example, bacteria associated with oral secretions, such as that of the Colorado potato beetle (*Leptinotarsa decemlineata*), were shown to alter plant resistance and turn the host plant into better food (Chung et al., 2013).

Not all bacterial symbionts are (always) beneficial for their hosts. In fact, with respect to the effect on host fitness, a symbiosis can span the entire range from beneficial to the microbe but harmful to the host (parasitism), via a neutral relationship (commensal symbiont) to true mutualism. Moreover, symbiosis can have mixed effects and may change over (evolutionary) time, depending on environmental conditions (Werren et al., 2008; Zug & Hammerstein, 2015). Among arthropods, the most prevalent microbial symbionts are so-called reproductive parasites such as *Wolbachia* (Rickettsiales), ‘*Candidatus Cardinium*’ (Cytophagales) and *Spiroplasma* (Entomoplasmatales) (Duron et al., 2008). Reproductive parasites commonly secure their prevalence in a host population by increasing the proportion of infected females through various mechanisms including cytoplasmic incompatibility, feminization, parthenogenesis or male killing (Werren, 1997; Duron et al., 2008; Werren et al., 2008; Engelstädter & Hurst, 2009). In most cases, these manipulations by the symbiont are not beneficial to the host, yet very effective for the persistence of the symbiont. This was demonstrated by a recent study in which
approximately 40% of all terrestrial arthropod species was estimated to be infected with *Wolbachia* (Zug & Hammerstein, 2012). However, direct beneficial effects of reproductive manipulators on host fitness are thought to mediate their spread within populations as well, especially when manipulation of host reproduction is weak (Hoffmann *et al.*, 1998; Fry *et al.*, 2004). Accordingly, evidence has accumulated that reproductive manipulators, which have long been considered parasites, can benefit their hosts or vectors in various ways (Casteel & Hansen, 2014; Sugio *et al.*, 2015; Zug & Hammerstein, 2015). For instance, reproductive manipulators have been shown to protect their host against parasitoids, predators and bacterial or viral pathogens (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Moreira *et al.*, 2009; Jaenike *et al.*, 2010a; Xie *et al.*, 2010, 2014; Walker *et al.*, 2011; Hamilton *et al.*, 2014). Beside protection, *Wolbachia* is known to function as nutritional mutualist in filarial nematodes (Foster *et al.*, 2005), while some examples also exist for arthropod hosts (Brownlie *et al.*, 2009; Hosokawa *et al.*, 2010; Unckless & Jaenike, 2012). Interestingly, infection of arthropods with *Wolbachia* has even been associated with the manipulation of plant physiology (Barr *et al.*, 2010; Kaiser *et al.*, 2010; Body *et al.*, 2013; Robert *et al.*, 2013).

The two-spotted spider mite *Tetranychus urticae* can harbour several (endo)symbiotic bacteria that are known as reproductive manipulators in mites (Breeuwer & Jacobs, 1996; Gotoh *et al.*, 2003, 2007; Enigl & Schausberger, 2007). However, the infection status was shown to vary widely among and within spider mite populations (Breeuwer & Jacobs, 1996; Gotoh *et al.*, 2007). *Tetranychus urticae* is a highly polyphagous pest species found on over 1100 plant species worldwide, including economically important crops like tomato, cucumber, strawberry, bean and cotton (Bolland *et al.*, 1998; Grbic *et al.*, 2011; Migeon *et al.*, 2011). Plants have evolved a wide array of defences, which are organized by the action of several phytohormones in which jasmonic acid (JA) and salicylic acid (SA) are the two central players (Erb *et al.*, 2012; Pieterse *et al.*, 2012). Defence against biotrophic pathogens is orchestrated by SA, while jasmonates, in particular jasmonic acid-isoleucine (JA-Ile), are crucial for defence against herbivores and pathogens with a necrotrophic lifestyle (Kessler *et al.*, 2004; Staswick & Tiryaki, 2004; Glazebrook, 2005). However, like other piercing-sucking arthropods (Walling, 2000), spider mites induce these hormones simultaneously, albeit JA-mediated defences seem to be most important for increased resistance against mites (Li *et al.*, 2002; Ament *et al.*, 2004; Kant *et al.*, 2004, 2008; Zhurov *et al.*, 2014; Alba *et al.*, 2015). Recently, we isolated mites from natural *T. urticae* populations and demonstrated that some of them suppressed JA-mediated defences of tomato to uphold a relatively high reproductive performance on this hostile plant (Alba *et al.*, 2015). One of these suppressor strains, designated as the DeLier-1 strain, was characterized in more detail and shown to significantly reduce JA- and SA-mediated defences that were elicited by the ‘normal’ inducer strain Santpoort-2 (Alba *et al.*, 2015).
Against the background that (endo)symbiotic bacteria can influence host fitness in various ways, we investigated the bacterial communities that are associated with the *T. urticae* plant defence suppressor strain DeLier-1 and the defence inducer strain Santpoort-2, hereafter referred to as the suppressor and the inducer strain, respectively. The suppressor strain was found to contain *Wolbachia* and *Spiroplasma*, while the inducer strain harboured ‘*Candidatus Cardinium*’ and *Spiroplasma*. We subsequently treated both mite strains with antibiotics to kill the bacteria and tested how their presence was correlated with the performance and transcriptome of the mites. Moreover, we investigated induced plant responses in tomato (*Solanum lycopersicum*) by analyzing phytohormone profiles and expression levels of defence-related genes after infestation of the plants with antibiotics-treated and untreated control mites of both strains that differed in their bacterial community. Together, this enabled us to assess to which extent mite endosymbionts impact mites directly or indirectly via the plant.

**MATERIAL AND METHODS**

**Plants**
Tomato (*S. lycopersicum* cv. Castlemart) and bean (*Phaseolus vulgaris* cv. Speedy) were germinated and grown in a greenhouse (25/18 °C day/night temperature, 16L:8D photoperiod, 50-60% relative humidity [RH]). Experiments involving plants were carried out in a climate room (default settings: 25 °C, 16L:8D photoperiod, 60% RH, 300 µE m\(^{-2}\) s\(^{-1}\)), to which plants were transferred seven days in advance.

**Spider mites**
We used spider mites from the *T. urticae* strains Santpoort-2 (‘inducer’) and DeLier-1 (‘suppressor’). The Santpoort-2 mites have been described before as inducers of tomato JA- and SA defences, to which they are also susceptible (Kant, 2006; Alba et al., 2015), while DeLier-1 mites suppress these defences (Alba et al., 2015). Spider mites from both strains were reared separately on detached bean leaflets in a climate room. For all plant infestation experiments and mite performance assays, we used age-equilibrated adult females.

**I. Bacterial communities of a Tetranychus urticae suppressor and inducer strain, in antibiotics-treated and non-treated mite lines**
Results from a preliminary assessment of the presence of bacteria in the two mite strains indicated that they harboured different endosymbiotic bacteria. Suppressor mites contained *Wolbachia* sp., while *Candidatus Cardinium* (referred to as *Cardinium*) was identified in inducer mites. In addition, *Spiroplasma* sp. was found in both mite strains (data not shown).

**Ia. Antibiotics treatments and nomenclature of mite lines**
We treated mites from both strains with antibiotics to remove *Wolbachia, Cardinium*
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and Spiroplasma bacteria. In short, offspring from randomly selected mated adult females (‘founder mites’) was divided over two treatments: (i) antibiotics-treated and (ii) untreated controls. For the antibiotics treatment, adult female spider mites were first kept on bean leaf discs placed on cotton wool soaked with tetracycline hydrochloride (Sigma-Aldrich, St Louis, MO, USA) for 2-3 days, after which they were transferred to new leaf discs on water-saturated cotton wool to produce eggs. Two days later, adult females were individually sampled in Eppendorf tubes and stored at -80 °C until DNA was extracted for diagnostic PCRs to establish the bacterial infection status of the mites (see below). The eggs on the leaf discs were allowed to hatch and mature in a climate room, after which the antibiotics treatment was repeated. In parallel, untreated control mites were kept on leaf discs placed on water-saturated cotton wool and after egg production mites were sampled for diagnostic PCRs as described below. Three subsequent generations of mites were treated in this way, but with increasing concentrations of tetracycline (i.e., 0.15, 0.20 and 0.30% vol/vol) to obtain mites free of Wolbachia, Cardinium and Spiroplasma as assessed via diagnostic PCR. From generation 4 onwards, all mites (antibiotics-treated or not) were reared on untreated detached bean leaflets to accommodate larger populations (from here on referred to as ‘lines’).

During the antibiotics treatment, we kept track of the individual mites and their offspring and only kept those lines (antibiotics-treated versus control) that both originated from the same ‘founder mite’, i.e., these were ‘sister lines’. This was done to minimize genetic variation between antibiotics-treated and untreated control lines. Following these criteria, we obtained four lines for the suppressor strain, designated as line 1, 2, 3 and 4. Each of the four lines had three sublines: W+S+ contained both Wolbachia and Spiroplasma; W-S+ was free of Wolbachia, but contained Spiroplasma, W-S- was free of Wolbachia and Spiroplasma. We did not manage to obtain W+S- sublines. For the inducer strain, we obtained four lines as well, which were designated as lines 5, 6, 7 and 8. Each of the four lines had two sublines: C+S+ contained Cardinium and Spiroplasma, and C-S- was free of Cardinium and Spiroplasma. Sublines with the same respective bacteria will be referred to as ‘groups’. In the suppressor strain the groups were W+S+, W-S+ and W-S-, in the inducer strain the groups were C+S+ and C-S (see Figure 5.1 and Table 5.1 for an overview of the mite lines and their bacterial communities). Mites from each strain and subline were regularly checked for their bacterial infection status by diagnostic PCR and kept on untreated detached bean leaflets for approximately 15 generations before they were used for the plant infestation assay and mite fecundity tests.

IIb. Illumina sequencing

To assess the presence of Wolbachia, Cardinium, Spiroplasma and other potentially present bacteria in mites from the five groups (W-S-, W-S+, W+S+, C-S- and C+S+) that were used for the plant infestation assay, we sampled five tomato-habituated mites per subline (as described above) for Illumina sequencing. DNA was extracted
from single mites using a fast Chelex method modified from Breeuwer & Jacobs (1996). To isolate the DNA, a single mite was ground and homogenized in 100 μl sterile 5% (wt/vol) Chelex (Sigma-Aldrich) with a sterile pestle, after which 2.5 μl proteinase K (20 mg/ml, Sigma-Aldrich) was added. Samples were then incubated at 56 °C for 1 h, followed by incubation at 95 °C for 8 min to complete the DNA extraction. DNA from the five mites from the same subline was pooled to form one sample. DNA concentration was adjusted to 25-35 ng/μl per sample. In total, 20 (pooled) samples were sent for sequencing, one for each subline. Amplification and sequencing of the 16S rRNA gene fragment was done by LGC Genomics (Berlin, Germany) using an Illumina MiSeq sequencer (2 x 250 bp paired-end reads; Illumina, San Diego, CA, USA) and the universal primers 341F and 785R (modified from Klindworth et al., 2012, see TABLE S5.1). Since the Chelex method does not yield highly pure DNA, the initial PCR amplification of the 16S rRNA genetic region was done on 20 times diluted DNA. Furthermore, the PCR was run with 35 instead of the usual 30 cycles.

Sequences were provided as adapter clipped FASTQ files and analysed in QIIME (quantitative insights into microbial ecology), which is a standard pipeline for microbial community analysis (Caporaso et al., 2010a). First, forward and reverse reads were joined with the join_paired_ends.py algorithm. Joined sequences were quality filtered, applying a Phred threshold of 20. Subsequently, sequences were clustered into operational taxonomic units (OTUs) with the open reference OTU picking command, applying the uclust algorithm (Edgar, 2010) and 97% similarity cut-offs. First, sequences were clustered against the reference Greengenes 16S rRNA gene database (http://greengenes.lbl.gov/; DeSantis et al., 2006). Sequences that did not cluster with the reference sequences were clustered de novo. The most abundant sequence from each OTU cluster was taken as representative sequence. Representative sequences were aligned with PyNAST, using the Greengenes core set as a template (Caporaso et al., 2010b). PyNAST-aligned sequences were checked for chimeras with Chimera Slayer (Haas et al., 2011). Identified chimeras were removed from de novo clustered sequences for downstream analysis. Taxonomy was assigned to the representative sequences using the uclust consensus taxonomy classifier (Edgar, 2010). The resulting OTU table was manually edited; global singletons and sequences identified as chloroplast and mitochondrial DNA were removed from the dataset. For the graph (FIGURE 5.1), we show OTUs that were present at > 0.5% in at least one of the sublines.

Ic. Diagnostic PCRs on mites
To verify the presence or absence of the most common endosymbionts in mites, viz. Wolbachia, Cardinium and Spiroplasma (Breeuwer & Jacobs, 1996; Gotoh et al., 200, 20073; Enigl & Schausberger, 2007), we performed diagnostic PCRs on DNA extracted from spider mites using genus-specific bacterial primers (TABLE S5.1). Adult female mites, by default not surface-sterilized, were sampled in Eppendorf
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tubes and their DNA was extracted using the fast Chelex method as described above. Samples were stored at 4 °C until they were used for PCR.

One μl of the mite-derived DNA solution (approximately 30-40 ng of DNA) was used as template in a 10.5 μl PCR reaction, further containing 2 μl 5x Phire Hot Start Buffer (Thermo Fisher Scientific, Waltham, MA, USA), 2 μl dNTPs (1 mM), 0.5 μl of each primer (10 μM each) and 0.1 μl Phire Hot Start DNA Polymerase (Thermo Fisher Scientific). PCR amplification of *T. urticae actin* was used as a positive control for DNA quality. The PCRs were run on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following cycling conditions; an initial denaturation for 30 s at 98 °C, followed by 32 cycles of 5 s denaturation at 98 °C, 5 s primer annealing at 52 °C (for *Wolbachia* and *Spiroplasma*), 57 °C (for *Cardinium*), or 58 °C (for *T. urticae actin*) and 10 s elongation at 72 °C; finalized with a 1 min elongation step at 72 °C. PCR-generated amplicons were analysed by agarose-gel electrophoresis. Primer specificity was confirmed by Sanger sequencing of the amplicons.

**II. Effects of Wolbachia, Cardinium and Spiroplasma on spider mites**

**IIa. Spider mite performance assay**

To establish whether (endo)symbionts had an effect on mite performance, we assessed spider mite fecundity and survival on wild type tomato plants. For the experiment, an ‘egg-wave’ (see Alba et al., 2015) was generated by allowing random adult females from each strain to produce eggs on the adaxial surface of detached bean leaflets, which had been put flat on wet cotton wool. After 48 h of egg production, all mites were removed from the leaflets and collected in Eppendorf tubes (25-35 mites from the same subline were pooled), flash-frozen in liquid nitrogen and stored at -80 °C, until their DNA was extracted for diagnostic PCRs. The eggs were allowed to hatch and mature in a climate room for another 9 days. The bean leaflets with mites were then transferred to leaves of 21-day-old tomato plants to habituate the mites to tomato. Three days later, the then 1 ± 1-day-old adult female mites were collected from the tomato leaves and transferred to ‘new’ 21-day-old tomato plants for the mite performance assay. Plants were infested with 5 mites per leaflet; 3 leaflets per plant; 3-6 plants per treatment. A lanolin (Sigma-Aldrich) barrier was made around the petiolule to prevent the mites from escaping. After 4 days, the number of eggs pro-duced by the mites, as well as the number of alive, dead and missing (i.e., migrated) mites, was recorded using a stereo microscope (Leica MZ6, Leica Microsystems, Wetzlar, Germany). This experiment was repeated 2-3 times for all four lines of the suppressor strain. However, for the inducer strain, populations from the C-S- subline of line 6, as well as both sublines of line 7 went (for yet unknown reasons) extinct before we could complete the performance assays. Data from the C+S+ subline of line 6 was included for analysis.

*Statistical analysis of spider mite performance assay*

To test the effect of *Wolbachia, Cardinium* and/or *Spiroplasma* on mite oviposition, we constructed one linear mixed effect models (LMM) for each mite strain.
(suppressor and inducer) in the lme4 package (Bates et al., 2013), using either ‘average number of eggs per number of females that were originally put on the leaves’ (5 mites), or ‘the average number of eggs per number of females that survived until the end of the experiment’ as response variable. To test effects of bacteria on survival of the mites, we used generalized linear mixed models in the lme4 package using a binomial distribution to analyse the proportion of mites that were dead or alive at the end of the oviposition experiment (i.e., after 4 days). In the models of oviposition and survival, we used ‘bacterial group’ as explanatory variable and ‘line’ was added as random effect. Additionally, since experiments were spread over different experimental days, and in total 6-9 plants were used per line (with three leaflets per plant), we added a nested random effect with ‘leaflet’ nested in ‘plant’, nested in ‘day’ (1 | day/plant/leaflet) to the model. Pairwise comparisons for suppressor strain were done using Tukey contrasts in the multicomp package (Hothorn et al., 2008) and applying Holm adjustments to account for multiple comparisons. All analyses were performed using the statistical software R 3.0.2 (R Core Team, 2013).

IIb. Spider mite RNA isolation and microarray analysis

To determine if the presence of Wolbachia, Cardinium and/or Spiroplasma in the mites is associated with gene expression of their host, we isolated RNA from the same mites that were used for the plant infestation assay (see below) and analysed it by means of a microarray. After 7 days of tomato infestations, mites were sampled, as described below in the ‘plant infestation assay’ section. All mites from the same plant (maximum 3 x 15 = 45; dead and alive) were pooled to form one biological replicate. Total RNA was isolated from the pooled spider mites using the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA), according to the instructions of the manufacturer. RNA integrity was checked by agarose-gel electrophoresis and a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific) was used to assess RNA purity and quantity.

For the microarray hybridizations, equal amounts of RNA (400 ng), derived from the five biological replicates of each subline, were pooled to form one sample. In total there were twelve samples for the suppressor strain (4 lines x 3 sublines) and eight samples for the inducer strain (4 lines x 2 sublines), which were hybridized separately on two arrays, i.e., one with suppressor strain samples, the other with inducer strain samples.

The RNA integrity of each sample was verified with the 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA) using the RNA ScreenTape (Agilent Technologies). The amount of RNA per µl was measured on a NanoDrop spectrophotometer (ND-2000). Per sample, 100 ng of total RNA, combined with Spike A, was amplified and labeled according to the Agilent Two-Colour Microarray-Based Gene Expression Analysis guide version 6.6 (G4140-90050, Agilent Technologies) using the Low Input Quick Amp Labeling Kit (Agilent Technolo-
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gies). For the common reference, an equimolar pool of all samples was made and
100 ng samples were amplified similarly as the test samples with the exception that
Spike B was used. Synthesized antisense RNA (aRNA) was purified with the EZNA
MicroElute RNA Clean Up Kit (Omega Bio-Tek, Norcross, GA, USA). The
NanoDrop ND-2000 was used to assess aRNA quantity and CyDye incorporation.

Each hybridization mixture was made up from 1.1 µg Test (Cy3) and 1.1 µg
Reference (Cy5) sample. Samples were dried and 1.98 µl water was added. The
hybridization cocktail was made according to the manufacturer’s instructions
(NimbleGen Arrays User’s Guide – Gene Expression Arrays version 5.0, Roche
NimbleGen, Basel, Switzerland) and 7.2 µl of this mix was added to each sample. The
samples were incubated for 5 min at 65 °C and 5 min at 42 °C prior to loading onto a
12 x 135k microarray (Roche NimbleGen), custom designed as described in the next
paragraph (Kant et al., 2004, 2008; Alba et al., 2015). Microarrays were hybridized for
20 h at 42 °C with the NimbleGen Hybridization System (Roche NimbleGen).
Afterwards, the slides were washed according to the NimbleGen Arrays User’s Guide
(Gene Expression Arrays version 6.0, Roche NimbleGen) and scanned with an Agilent
DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was
performed with NimbleScan v2.6 (Roche NimbleGen).

Microarrays were designed using normalized transcriptomes of T. urticae
strains Houten-1 (‘KOP’; Kant et al., 2004), Santpoort-1 (‘KMT’; Kant et al., 2008) and
DeLier-1 (Alba et al., 2015). Transcriptomes had been prepared from a normalized
shotgun cDNA library via random priming (Houten-1) and three normalized 3-prime
libraries cDNA (Houten-1, Santpoort-1 and DeLier-1) via 5’ sequencing of poly A-
selected fragments of 600-800 nts long via 454 Titanium sequencing (Eurofins,
Germany) delivering 1,416,647 reads in total with an average length of 330 nts. For
the microarray probe design, we assembled only the 744,649 3’ fragment reads,
because these will contain more cDNA-specific sequence information than the shotgun
reads. These fragment reads were assembled into 16,372 isotigs (with an average
length of 571 nts; N50 = 597 nts), using Newbler (using a 99% identity setting and a
minimum of 40 nts overlap), from which we could extract 14,335 unique 60-mer
probes. After assembly, 167,643 3’ reads remained as singleton (i.e., reads that could
not be assembled), from which another 120,000 unique 60-mer probes were extracted.
Together, these were printed in 12-fold on a 12 x 135k array in Nimblegen format.

Probe sequences were (re)mapped to the latest annotation of the T. urticae
genome (December 2014) using Bowtie2-2.10 with the ‘very sensitive’ preset option
(Langmead et al., 2009). Of the 134,672 probes on each array, 30,759 aligned with the
T. urticae genome (Grbic et al., 2011), representing 9,424 (51%) of the 18,414 in
silico predicted protein-coding sequences. Prior to gene expression analysis, signal
intensity data were log2-transformed and normalized (Loess and Aquantile). The
biological replication within each mite strain and its cured sublines were assessed by
a PCA-analysis, using the prcomp function in R. The first two principal components,
which explained a cumulative proportion of the total variance of 65.6 and 32.6% for
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the inducer and suppressor strain, respectively, were used to explore the data and to identify outliers. Next, a linear model of a non-connected design was fitted to the processed data, using limma (Smyth, 2004; Smyth & Altman, 2013). An empirical Bayes approach (Smyth, 2004) was applied to assess relative transcript levels and the associated P-values between different treatments. When multiple probes aligned to the same mite gene, the expression values of the individual probes were averaged. To control for false discovery rate, obtained P-values were adjusted for multiple testing according to Benjamini and Hochberg’s step-up procedure (FDR; Benjamini & Hochberg, 1995). A transcription heat map was constructed, using the relative gene-expression levels obtained in limma (bioproject website). All analyses, except for the genome alignment with Bowtie2-2.10 (Linux), were performed using the statistical software R 3.0.2 (R Core Team, 2013). Biological functions were ascribed to the DEG lists as described in Wybouw et al. (2015).

III. Effects of spider-mite associated Wolbachia, Cardinium and Spiroplasma on mite-plant interactions

IIIa. Plant infestation assay

To measure phytohormone levels and plant defence gene expression upon mite-inflicted feeding damage, tomato plants were infested with spider mites, as described before (Alba et al., 2015), with an additional habituation step (of 2 days) on tomato to minimize possible effects of the previous diet (i.e., bean) on mite behaviour, mite performance, and/or induced/suppressed tomato defences. Dietary effects are known to persist for at least 48 h, after which they diminish rapidly (Storms, 1971). For the experiment, we used age-equilibrated adult female spider mites obtained from an egg-wave. The eggs were allowed to hatch and mature in a climate room for another 12 days. The bean leaflets with mites were then taken from the cotton wool and placed upside-down on leaves of 28-day-old tomato plants to infest these (i.e., habituation step). Two days later, the 3 ± 1-day-old adult female mites were collected and transferred to 21-day-old tomato plants for the plant infestation assay, according to our standard infestation protocol (Alba et al., 2015); 15 mites per leaflet, 3 leaflets per plant. To prevent the mites from escaping, a lanolin (Sigma-Aldrich) barrier was made around the petiolule, which was also applied to uninfested control leaflets. A total of 5 plants was infested per mite subline. To verify the bacterial infection status of each strain, 5 tomato-habituated mites per strain were individually collected in Eppendorf tubes, flash-frozen in liquid nitrogen and stored at -80 °C until DNA was extracted for PCR amplification and subsequent Illumina sequencing of the 16S rRNA genetic region (see ‘Illumina sequencing’ section).

At 7 days post-infestation (dpi), mites and tomato leaflets were harvested separately. First, spider mites were removed from the leaflets and collected in Eppendorf tubes (all mites obtained from the same plant were pooled), flash-frozen in liquid nitrogen and stored at -80 °C until their RNA was extracted for microarray analysis (see ‘Spider mite RNA isolation and microarray analysis’ section). A
vacuum pump, sterile 1 ml pipet tip, and mite-proof gauze were used to quickly sample the mites without touching and hence mechanically damaging the leaflets. Subsequently, the mite-cleared leaflets were excised without the petiolule. The three detached leaflets obtained from the same plant, along with a scale marker, were then aligned on black paper, gently covered with a thin glass plate to flatten them out, and photographed with a Canon EOS 300D DSLR camera (Canon, Tokyo, Japan) equipped with a Canon EF-S 18-55 mm lens to enable *in silico* calculation of spider mite-inflicted feeding damage, using Adobe Photoshop CS6 Extended (Adobe Systems, San Jose, CA, USA) as described by Kant et al. (2004). Finally, the leaflets were flash-frozen in liquid nitrogen and stored at -80 °C until we extracted their phytohormones and RNA. The 3 leaflets obtained from the same plant were pooled to form 1 biological replicate. In total, it took less than 5 min per plant to complete these 3 steps and harvest the leaflets. Care was taken to not damage them. Except for removal of the mites, uninfested control leaflets were processed in the same way.

**IIIb. Isolation of phytohormones and analysis by means of LC-MS/MS**

Per sample, 200-300 mg of frozen leaf material was homogenized (Precellys 24, Bertin Technologies, Aix-en-Provence, France) in 1 ml of ethyl acetate which had been spiked with D₆-SA and D₅-JA (C/D/N Isotopes, Pointe-Claire, Quebec, Canada) as internal standards with a final concentration of 100 ng/ml. Tubes were centrifuged at 13,000 rpm (15,493 x g; Sigma 3-30KS; SIGMA Laborzentrifugen, Osterode am Harz, Germany) for 10 min at 4 °C and the supernatant (the ethyl acetate phase) was transferred to new tubes. The pellet was re-extracted with 0.5 ml of ethyl acetate (without internal standards) and centrifuged again at 13,000 rpm for 10 min at 4 °C. Both supernatants were combined and then evaporated to dryness on a vacuum concentrator (CentriVap Centrifugal Concentrator, Labconco, Kansas City, MO, USA) at 30 °C. The residue was re-suspended in 0.1 ml of 70% methanol (vol/vol), centrifuged at 14,800 rpm (20,081 x g) for 15 min at 4 °C, and the supernatants were transferred to glass vials and then analysed using a liquid chromatography tandem mass spectrometry (LC-MS/MS) system (Varian 320-MS LC/MS, Agilent Technologies). A serial dilution of pure standards of abscisic acid (ABA), traumatic acids, 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile) and salicylic acid (SA) was run separately. We injected 10 µl of each sample onto a Kinetix 5u C18 100A column (C18 phase, 5 μm particle size, 100Å pore size, 50 × 2.1 mm; Phenomenex, Torrance, CA, USA) equipped with a Phenex-RC guard cartridge (Phenomenex). The mobile phase comprised of solvent A (0.05% formic acid in LC-MS-grade water; Sigma-Aldrich) and solvent B (0.05% formic acid in LC-MS-grade methanol; Sigma-Aldrich). The program, with a constant flow rate of 0.2 ml min⁻¹, was set as follows: (i) 95% solvent A/5% solvent B for 1 min 30 s; (ii) followed by 6 min in which solvent B gradually increased till 98%; (iii) continuing with 98% solvent B for 5 min; (iv) then a rapid (in 1 min) but gradual decrease returning to 95% solvent A/5% solvent B until the end of the run. A
negative electrospray ionization mode was used for detection. LC-MS/MS parameters, e.g., analysed compounds, their parent ions, daughter ions, and collision energies used in these analyses, are listed in TABLE S5.1. FIGURE S5.1 shows a comprehensive overview of the most important compounds and their 'position' within the plant JA and SA defence response pathways induced by T. urticae. For all oxylipins and ABA, we used D5-JA to estimate the recovery rate and their in planta concentrations were subsequently quantified using the external standard series. For SA we used D6-SA to estimate the recovery rate and it was quantified using the external standard. Phytohormone amounts were expressed as ng per gram fresh mass leaf material (ng/g fresh weight). Compounds for which a pure standard was not available (i.e., 13(S)-hydroperoxyoctadecatrienoic acid (13-HPOT), 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid (OPC-8), 3-oxo-2-(2-pentenyl)-cyclopentane-1-hexanoic acid (OPC-6) and C12-derivatives of the hydroperoxide lyase (HPL) pathway) were analysed similarly, but their amounts were expressed as ‘Ion Count’ per gram fresh mass leaf material (IC/g fresh weight).

IIIc. Gene expression analysis by quantitative reverse-transcriptase PCR (qRT-PCR)

To determine the effect of mite-associated bacteria on defence gene expression, we performed qRT-PCRs on plant defence marker genes. Therefore, total RNA was isolated from the tomato leaf tissue that was used for phytohormone isolation, using the hot phenol method (Verwoerd et al., 1989). RNA integrity was checked by agarose-gel electrophoresis and a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific) was used to assess RNA purity and quantity. Per sample, 3 μg DNase (Ambion, Austin, TX, USA)-treated RNA was used as template for reverse transcription and first strand cDNA synthesis using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). For gene expression analysis, 1 μl of 10x diluted cDNA (i.e., the equivalent of 7.5 ng total RNA) served as template in a 20 μl qRT-PCR using the 5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) kit (Solis Biodyne, Tartu, Estonia) and the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), according to the instructions of the manufacturers.

We monitored expression of genes involved in JA biosynthesis: Allene Oxide Synthase 1 (AOS1) (Howe et al., 2000; Sivasankar et al., 2000) and OPDA reductase 3 (OPR3) (Strassner et al., 2002), as well as JA-defence marker genes; Jasmonate-inducible Protein-21 (JIP-21) (Lisón et al., 2006), Threonine Deaminase-2 (TD-2) (Gonzales-Vigil et al., 2011), Proteinase Inhibitor Iic (PI-Iic) (Gadea et al., 1996), SA-defence marker genes; Pathogenesis-related protein 1a (PR-1a) (Tornero et al., 1997), PR-P6 (van Kan et al., 1992), and finally putative OPDA-responsive genes; Tomato Wound-induced 1 (TWI-1) (Truesdale et al., 1996), Alcohol Dehydrogenase (ADH) (Tiemann et al., 2007), an uncharacterized Glutathione S-transferase (‘GST6’) (Solyc06g009020.2) and an uncharacterized Glutaredoxin (‘GRX’) (Solyc07g053550.1). See FIGURE S5.1 for a schematic overview of the plant defence
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response against mites, which includes the marker genes whose expression levels were analysed in this study. The amino acid sequences of established Arabidopsis thaliana OPDA-responsive genes (ORGs; (Taki et al., 2005; Mueller et al., 2008; Park et al., 2013)) were used to identify their putative tomato homologs: At2g15480 (AtUGT73B5) for SITWI-1, At1g09500 (AtCAD) for SLADH, At2g47730 (AtGST6) for SIGST6, and At1g28480 (AtGRX480) for SLGRX. Actin was used as a reference gene to normalize expression data and hence correct for variance in quantity of cDNA input. Standard dilution series of selected samples were included with each qRT-PCR run to calculate primer efficiency. PCR-generated amplicons were sequenced to verify primer specificity. Gene identifiers, primer sequences and references are listed in TABLE S5.2. The normalized expression (NE) data were calculated by the ΔCt method as described before (Alba et al., 2015): NE = (PE_{target}^{Ct_{target}})/(PE_{reference}^{Ct_{reference}}), in which PE is the primer efficiency and Ct the number of cycles to reach the cycle threshold value.

Statistical analysis of the plant infestation assay data (phytohormones, qRT-PCR, feeding damage)

To test the effect of bacteria on phytohormone levels, tomato gene expression and amount of spider mite-inflicted feeding damage, we constructed LMM using the package lme4 (Bates et al., 2013). The respective amounts of phytohormones (ng/g FW or IC/g FW), normalized gene expression (NE) or total amount of feeding damage (mm²) for three leaflets of one plant combined were used as response variable, while ‘presence of bacteria’ (i.e., W/S/C) was used as explanatory variable. To test the level of induction or suppression as compared to uninfested control plants, in the case of phytohormones and gene expression this explanatory variable also included control plants that were not infested. Since we had four lines which were present as sublines in all mite-bacteria groups, we added ‘line’ as a random factor to the model to account for variation between the lines. The response variables were transformed using log, sqrt or 1/sqrt if applicable for meeting the assumptions of homogeneity of variance and normality of residuals required for LMM. For pairwise comparisons, we used Tukey contrasts with Holm adjustment for multiple comparisons in the multcomp package (Hothorn et al., 2008). To assess how the phytohormones SA and ODPA correlated with expression levels of putative ORGs (TWI-1, GRX, GST-6, ADH and OPR3), we calculated linear correlations between phytohormone amounts and the NE level of these genes using the R package Hmisc 3.15 (Harrel et al., 2015). Further, we calculated linear correlations between SA amounts and NE levels of the SA marker genes PR-P6 and PR-1a. P-values of correlations were adjusted for multiple testing, using the Holm method. All analyses were performed using the statistical software R 3.0.2 (R Core Team, 2013).
RESULTS

I. Bacterial communities of a Tetranychus urticae suppressor and inducer strain, in antibiotics-treated and non-treated mite lines

Adult female mites, obtained from laboratory populations of either the T. urticae suppressor or T. urticae inducer strain (Alba et al., 2015), harboured different bacterial communities, as was determined by Illumina sequencing of the 16S rRNA derived PCR products (FIGURE 5.1, TABLE 5.1). Most evidently, in the suppressor strain a high percentage of the reads corresponded with the endosymbiotic bacterium Wolbachia sp. (W) (Rickettsiaceae), with an average of 30.41% (± 13.58 SD). Wolbachia was identified in the inducer strain as well, albeit at low relative levels with an average of 0.25% (± 0.32 SD). In contrast, in the inducer strain a high percentage of reads corresponded to the endosymbiotic bacterium ‘Candidatus Cardinium’ (C) (Bacteroidaceae), with an average of 29.05% (± 8.72 SD). Cardinium was also found in the suppressor strain, but at low relative levels with an average of 0.0032% (± 0.0037 SD). In addition, the same Spiroplasma sp. (S) (Spiroplasmataceae) OTU was present in similar relative amounts in both mite strains: an average of 4.03% (± 1.06 SD) in the suppressor mites and 4.42% (± 2.67 SD) in inducer mites. Therefore, the suppressor strain was classified as W+S+ and the inducer strain as C+S+.

To assess the effect(s) of the bacteria on mite performance, transcriptome and induced plant responses, mites from both strains were treated with antibiotics (tetracycline) as described above. The antibiotics treatments successfully cleared Wolbachia from the suppressor mites, as only very few (0-6) reads were detected per line in the W-S+ and W-S- groups (FIGURE 5.1, TABLE 5.1). Spiroplasma was completely removed in the latter group, but was relatively more abundant in the W-S+ group than in the W+S+ one, i.e., on average at 7.98% (± 1.71 SD). The antibiotics treatment was also successful in the inducer mites (FIGURE 5.1). Only a small fraction of the reads (< 0.04%) recovered from tetracycline-treated C-S- mites corresponded to Cardinium or Spiroplasma (TABLE 5.1). Moreover, presence of Wolbachia, Spiroplasma or Cardinium was no longer detectable in the antibiotics-treated sublines by means of PCR, using bacteria-specific primers on DNA of individual mites, which was also used for Illumina sequencing (data not shown).

Besides Wolbachia, Cardinium and Spiroplasma, bacteria from the families Enterobacteriaceae (Enterobacteriales) and Pseudomonadaceae (Pseudomonadales) were also present in varying amounts in all groups and sublines of both mite strains (FIGURE 5.1). Other bacterial families reached high relative abundances in some of the sublines, e.g., two different Oxalobacteraceae (Burkholderiales), one in line 5 subline C-S-, the other line 6 subline C+S+ and line 2 subline W+S+; Sphingobacteriaceae (Sphingobacteriales) in line 2 subline W+S+, or Nocardiaceae in line 4 subline W-S+. Taken together, this demonstrates that we cleared the mites from Wolbachia, Spiroplasma or Cardinium, but not from all bacteria residing in or on the mites, possibly because we had not surface-sterilized mites before assaying them.
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Therefore, we only focused on the consistent presence/absence of *Wolbachia*, *Spiroplasma* or *Cardinium* in all our analyses, to which we refer when we use the terms ‘bacteria’ or ‘mite-associated bacteria’.

**II. Effects of Wolbachia, Cardinium and Spiroplasma on spider mites**

**IIa. Effects of Wolbachia, Cardinium and Spiroplasma on spider mite performance**

To assess if the mite-associated bacteria were correlated with the fitness of their host, we determined the number of eggs and survival of adult female mites by performing oviposition assays on tomato. For the suppressor mites, we found an overall significant effect of the factor ‘bacterial group’ (i.e., presence/absence of *Wolbachia* and/or *Spiroplasma*) on mite survival (FIGURE 5.2a). More W+S+ mites

![Figure 5.1](image-url)

**Figure 5.1.** Bacterial community composition derived from Illumina 16S rRNA amplicon-sequencing in two strains of the spider mite *Tetranychus urticae* which had been treated with tetracycline and hence did (+) or did not (-) contain *Wolbachia* (W), *Spiroplasma* (S) and/or *Candidatus Cardinium* (C); Plant defence suppressor strain DeLier-1 with three mite groups: W+S+, W-S+ and W-S-; Plant defence inducer strain Santpoort-2 with two mite groups: C+S+ and C-S-; 4 lines per group. Bacterial operational taxonomic units (OTUs) were combined at the family level. Numbers behind family names indicate how many OTUs of one family were combined. Rare OTUs that were overall represented less than 0.5% are not shown; square brackets around taxa indicate that the taxon name is not fully established yet.
TABLE 5.1. Antibiotics treatments of two strains of the spider mite *Tetranychus urticae* resulted in the (near) complete removal of their associated bacteria *Wolbachia*, *Spiroplasma* and/or *Candidatus* Cardinium, as demonstrated by Illumina 16S rRNA amplicon-sequencing. The *T. urticae* plant defence suppressor strain DeLier-1 contained *Wolbachia* and *Spiroplasma* (*W+S*) and tetracycline treatments either removed only *Wolbachia* or both bacteria, yielding the groups *W-S* and *W-S-,* respectively. The plant defence inducer strain Santpoort-2 harboured *Cardinium* and *Spiroplasma* (*C+S*), which were both removed by the tetracycline treatments, yielding the group of *C-S* mites. Each group is represented by four independent mite lines. Note that mites from lines with the same number are ‘sister lines’ which originate from the same untreated ‘founder mite’, hence each DeLier-1 line consists of three sublines (*W+S+, W-S+, W-S-*) and each Santpoort-2 line consists of two sublines (*C+S* and *C-S-*). Shown are the overall total number of Illumina reads obtained per subline, as well as the total number of reads corresponding to all 11 *Wolbachia* operational taxonomic units (OTUs), all 3 *Spiroplasma* OTUs and the only *Cardinium* OTU identified. The 16S rRNA amplification was done with universal 341F and 785R primers, modified from Klindworth et al. (2012), see TABLE S5.2.

<table>
<thead>
<tr>
<th>Mite strain</th>
<th><strong>Group</strong></th>
<th><strong>Suppressor strain</strong></th>
<th><strong>Inducer strain</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Group</strong></td>
<td><strong>W+S</strong></td>
<td><strong>W-S</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Line</strong></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Wolbachia</td>
<td>Total</td>
<td>3,164</td>
<td>14,303</td>
</tr>
<tr>
<td></td>
<td>Wolbachia</td>
<td>1,272</td>
<td>2,131</td>
</tr>
<tr>
<td></td>
<td>Spiroplasma</td>
<td>141</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>Cardinium</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4,975</td>
<td>20,054</td>
</tr>
<tr>
<td></td>
<td>Wolbachia</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Spiroplasma</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cardinium</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

survived until the end of the experiment (4 days) compared to both W-S+ and W-S-mites, while survival did not differ between the latter two groups, with the exception of line 2 (FIGURE S5.2a). There was no statistical difference in number of eggs between the mites from each group when we calculated the total number of eggs produced per number of females that were initially put on each leaflet (5 mites), (FIGURE 5.3a). This suggests that the fewer W-S+ and W-S- mites that did survive produced more eggs. Hence, we determined the total number of eggs produced per female that survived, and here the factor ‘bacterial group’ had an overall significant effect on number of eggs (*F*₂,₂₀₇ = 3.14, *P* = 0.045) (data not shown in graph). However, post hoc tests with Holm correction for multiple testing indicated no
significant difference between surviving W+S+ mites laying fewer eggs than W-S+ (z = -2.00, P = 0.09) and W-S- (z = -2.30, P = 0.06).

Between the two groups of the inducer strain, significantly more mites of the C-S- group generally survived until the end of the experiment (FIGURE 5.2b), but this was not consistent among the lines (FIGURE S5.2b). The number of eggs between C+S+ and C-S- mites was also significantly different: overall, C-S- mites produced more eggs than C+S+ mites (FIGURE 5.3b). This difference was significant for line 5 (F₁,29 = 14.71, P < 0.001), but not for line 8 (F₁,32 = 1.21, P = 0.27), although both lines followed the same trend (FIGURE S5.3). The oviposition calculated per surviving mite did not differ between the C+S+ and C-S- groups (F₁,76 = 3.34, P = 0.07) (data not shown in graph), possibly due to the lower survival of C+S+ mites. However, the data from the oviposition assays with the inducer mites were inconclusive, because the C-S-subline of line 6, as well as both sublines of line 7, went extinct before the performance assays were completed.

IIb. Effects of Wolbachia, Cardinium and Spiroplasma on the mite’s transcriptome

In the microarray analysis, we found significant differences in expression levels of mite genes across the five groups (W+S+, W-S+, W-S-, C+S- and C-S-). Even though the microarray represented only 9,424 (51%) of the in silico predicted protein-coding sequences (Grbic et al., 2011), we identified 201 (1.1% of the total) differentially expressed genes (DEGs; absolute fold change (FC) > 1, Ben-
jamini-Hochberg false discovery rate (FDR)-adjusted $P \leq 0.05$) across all groups. Overall, differences were relatively small, i.e., absolute FCs ranged from +1.1 to +5.2 for upregulated genes and from -1.1 to -3.4 for downregulated genes respectively, with only 23 genes displaying an absolute FC > 2 ($P \leq 0.05$, after $P$-value adjustment). The most pronounced transcriptional differences were found between W+S+ and W-S+ mites of the suppressor strain with 152 DEGs (FIGURE S5.4), 30 of which were up-regulated (see TABLE S5.3a for the top-20) and 122 were down-regulated (see TABLE S5.3b for the top-20) in W-S+ mites. Comparing the transcriptome of W+S+ and W-S- mites resulted in 50 DEGs: 14 of them were up-regulated (TABLE S5.4a), while 36 were down-regulated (see TABLE S5.4b for the top-20) in W-S- mites. Out of the 50 DEGs, 29 also differed between W+S+ and W-S+ mites (FIGURE S5.4), hence the other 21 correlated with the presence of Spiroplasma. The transcript levels of only three genes (2 up-, 1 down-regulated in W-S-) differed significantly between W-S- and W-S+ mites (TABLE S5.5, FIGURE S5.4). Based on the principal component analysis, one of the inducer C-S- samples was identified as an outlier and therefore excluded from further analysis. Using the remaining samples, we identified 33 DEGs between the two groups of the inducer strain C+S+ and C-S-, 23 were higher expressed in C-S- mites (TABLE S5.6a), while 10 were lower expressed (TABLE S5.6b). Five of the 33 DEGs were also significantly affected by Wolbachia and/or Spiroplasma in the suppressor mites (TABLE S5.6a). Many of the 201 DEGs, i.e., 36 genes (17.9%), had no homology with any of the genes characterized so far, hence their function in the
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mite physiology remains unknown. Among the DEGs in both mite strains, those encoding proteins involved in digestion, metabolic detoxification (phase I), binding/conjugation of xenobiotics (phase II detoxification), transport of xenobiotics (phase III detoxification) or encoding putative (salivary gland-derived) secreted proteins were highly abundant (TABLE 5.2). Genes related to immune-responses (Gribić et al., 2011), of which the expression is associated with the presence of Wolbachia in dipterans (Kambris et al., 2009; Moreira et al., 2009), were not differentially expressed among the mite groups (data not shown).

III. Effects of spider mite-associated Wolbachia, Cardinium and Spiroplasma on mite-plant interactions

IIIa. Effects of mite-associated Wolbachia, Cardinium and Spiroplasma on tomato induced responses

For the suppressor mite infested plants, the most consistent differences in phytohormone profiles were found between leaflets infested with W-S+ mites and those infested with W-S- and/or W+S+ mites (FIGURES 5.4 and S5.5). The (combined) presence of Wolbachia and/or Spiroplasma correlated with phytohormone levels in tomato as follows. The accumulation of the JA-precursor OPDA was correlated with mite-associated bacteria (FIGURE 5.4b). Feeding by W+S+ mites induced the accumulation of OPDA, as did feeding by W-S- mites. However, leaflets infested with W-S- mites accumulated significantly lower amounts of OPDA than W+S+ mites. Leaflets infested with the third group of mites, W-S+, accumulated significantly less OPDA than W-S- and W+S+ infested leaflets and did not accumulate more OPDA than uninfested control leaflets. This clear pattern was detected in leaflets infested with all four suppressor mite lines (FIGURE S5.5a). A similar pattern was found for several other oxylipins as well, i.e., lowest hormone levels in W-S+ compared to W+S+ and W-S- infested leaflets. First, for the JA-precursor OPC8 (FIGURE 5.4c), which is the direct downstream product of OPDA, and to a lesser extent for OPC6 (FIGURE S5.5b), but not for JA itself (FIGURE S5.5c), nor for its biologically active conjugate JA-Ile (FIGURES 5.4d and S5.5d). Second, we found the pattern for traumatic acid (FIGURE 5.4e) and 9-OH-traumatin (FIGURES 5.4f and S5.5e), which are C12-derivatives of the HPL pathway, derived from the same 13-HPOT pool as JA (FIGURE S5.1). The accumulation of 13-HPOT, one of the first intermediates in the biosynthesis process of oxylipin signalling molecules (FIGURE S5.1) (Wasternack, 2007), was not correlated with mite-associated bacteria (FIGURE 5.4a).

The levels of SA in infested leaflets followed a pattern that appeared opposite of that of OPDA: amounts of SA in leaflets that were infested with W-S+ mites were significantly higher than in leaflets infested with either W-S- or W+S+ mites, but were not higher than in uninfested control leaflets (FIGURES 5.4g and S5.5f). The accumulation of ABA was not affected by infestations with mites from any of the suppressor strain groups (FIGURE 5.4h).
### Table 5.2
Overview of differentially expressed genes (DEGs; absolute fold change > 1; \( P \leq 0.05 \), after \( P \)-value adjustment) in adult female *Tetranychus urticae* suppressor (DeLier-1) and inducer (Santpoort-2) mites after selective removal of their associated bacteria *Wolbachia* sp. (W), *Spiroplasma* sp. (S) and/or *Candidatus Cardinium* (C) and feeding from tomato (*Solanum lycopersicum*) for seven days. Shown are the DEGs encoding proteins that are involved in digestion or detoxification, or are (putatively) secreted.

▲, up-regulated in mites of the first-mentioned bacterial group; ▼, down-regulated in mites of the first-mentioned bacterial group; +, present; -, absent.

<table>
<thead>
<tr>
<th>Biological process</th>
<th>Gene family</th>
<th>Suppressor strain</th>
<th>Inducer strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>W-S+ vs W+S+</td>
<td>W-S- vs W+S+</td>
</tr>
<tr>
<td>Digestion</td>
<td>Cathepsin cysteine peptidases</td>
<td>0 / 7</td>
<td>0 / 2</td>
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<tr>
<td></td>
<td>Legumain cysteine peptidases</td>
<td>0 / 5</td>
<td>0 / 2</td>
</tr>
<tr>
<td></td>
<td>Cystatins</td>
<td>-</td>
<td>2 / 0</td>
</tr>
<tr>
<td>Detoxification</td>
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<td>0 / 3</td>
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<td></td>
<td>Carboxyl/choline esterases (CCEs)</td>
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</tr>
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<td></td>
<td>Intradiol ring-cleavage dioxygenases</td>
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<td>-</td>
</tr>
<tr>
<td>Phase II</td>
<td>Lipocalins</td>
<td>0 / 6</td>
<td>-</td>
</tr>
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<td>Glutathione S-transferrases (GSTs)</td>
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<tr>
<td></td>
<td>MFS-transporters</td>
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<td>-</td>
</tr>
<tr>
<td><strong>Unknown</strong></td>
<td>Unknown (various)</td>
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<td>2 / 6</td>
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<tr>
<td><strong>Total DEGs</strong></td>
<td></td>
<td>152</td>
<td>50</td>
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All *T. urticae* multi-gene families implicated in digestion and detoxification have been identified and/or manually annotated (Grbic et al., 2011; Santamaria et al., 2012; Dermauw et al., 2013; Ahn et al., 2014; Wybouw et al., 2015). * indicates the total number of genes (of the indicated gene family) that is represented on the microarray.

The phytohormone profiles of inducer mite (C+S+ and C-S-) infested leaflets were as follows (see Figures 5.5 and S5.6). The levels of OPDA, JA-Ile and SA were induced by C+S+ and C-S- mites compared to uninfested controls, but did not differ between the two groups. However, two of the C_{12}-derivatives of the HPL pathway, i.e., traumatic acid (Figures 5.5c and S5.6c) and 9,12-OH-(10E)-dodecenoic acid (Figure 5.5d), accumulated to higher amounts in C-S- infested than in C+S+ infested leaflets. ABA levels followed the same pattern (Figures 5.5f and S5.6d). Previously, suppression of plant defences by spider mites was shown to act downstream of phytohormones (Alba et al., 2015). We therefore augmented the phytohormone data with the expression data of downstream marker genes (Figure S5.1) with qRT-PCRs. Upon infestation with suppressor mites, the amounts of OPDA...
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in W-S+ infested leaflets differed from those in W+S+ and W-S- infested leaflets, with the latter having intermediate levels (Figure 5.4b). Transcript levels of AOS1, which encodes an enzyme involved in the biosynthesis of OPDA, were higher in suppressor mite-infested leaflets compared to uninfested controls, but there was no difference among the mite groups (Figure 5.6a). In contrast, expression of OPR3, which acts directly downstream of OPDA in the JA biosynthesis pathway, was significantly higher in W-S+ than in W+S+ or W-S- infested leaflets (Figures 5.6b and S5.7a). The same pattern was found for multiple putative OPDA-responsive genes (ORGs); TWI-1, ADH, GST6 and GRX (Figures 5.6c-f and S5.7b-e, respectively). The expression levels of these ORGs were thus negatively correlated with OPDA amounts and positively with SA amounts, while the coefficient of determination (R²) values were similar (Figure S5.8), except for expression levels of ADH, which correlated with OPDA (R² = 0.42, \(P < 0.0001\)) but not with SA (R² = 0.09, \(P = 0.22\)). Analogous to the overall induction of JA-Ile by W+S+, W-S+ and W-S- mites (Figure 5.4d), expression of the JA-defence marker genes TD-2 (Figure 5.6g) and PI-IIc (Figure 5.6h) were induced in all three groups compared to the control, but did not differ among them. However, transcripts of JIP-21 accumulated to significantly higher levels in W-S- infested leaflets than in those of W+S+ and W-S+ (Figures 5.6i and S5.7f). As for the SA-defence marker genes, PR-P6 transcript accumulation was higher in W-S+ leaflets than in W+S+ and W-S- leaflets (like SA itself) (Figure 5.6j). Here it has to be noted that this PR-P6 expression pattern was visually clear in two lines, but not in the other two (Figure S5.7g). We did not find any significant differences in the expression of PR-1a in leaflets infested with the different groups of suppressor mites, nor between infested and uninfested leaflets (Figure 5.6k). Accordingly, SA amounts showed a weak but significant correlation with expression levels of PR-P6 (Figure S5.9a) and did not correlate with PR-1a transcript levels (Figure S5.9b).

**Figure 5.4.** Phytohormone amounts in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with a plant defence suppressor strain DeLier-1 of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain the bacteria *Wolbachia* (W) and *Spiroplasma* (S). Control plants were not infested. Phytohormones for which we assayed include (a) 13(S)-hydroperoxyoctadecatrienoic acid (13-HPOT), (b) 12-oxo-phytodienoic acid (OPDA), (c) 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid (OPC8), (d) jasmonic acid-isoleucine (JA-Ile), (e) traumatic acid, (f) 9-OH-traumatin, (g) free salicylic acid (SA), (h) abscisic acid (ABA); Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR (interquartile range), dots represent data points outside this range. Different letters above the boxes indicate significant differences at a level of \(P \leq 0.05\), after applying a linear mixed model followed by Tukey multiple comparisons with Holm adjustment. Phytohormone amounts are presented as nanogram (ng) per gram fresh leaf weight (g FW). Compounds for which we did not have a pure standard are presented as ion counts (IC / g FW). n.s. = not significant.
FIGURE 5.5. Phytohormone amounts in tomato (Solanum lycopersicum) leaflets after 7 days of infestation with a plant defence inducer strain Santpoort-2 of the spider mite Tetranychus urticae which did (+) or did not (-) contain the bacteria Candidatus Cardinium (C) and Spiroplasma (S). Control plants were not infested. Phytohormones for which we assayed include (a) 12-oxo-phytodienoic acid (OPDA), (b) jasmonic acid-isoleucine (JA-Ile), (c) traumatic acid, (d) 9,12-OH-(10E)-dodecenoic acid, (e) free salicylic acid (SA) and (f) abscisic acid (ABA) (f); Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of $P \leq 0.05$, after applying a linear mixed model followed by Tukey multiple comparisons with Holm adjustment. Phytohormone amounts are presented as nanogram (ng) per gram fresh leaf weight (g FW). Compounds for which we did not have a pure standard are presented as ion counts (IC / g FW). n.s. = not significant
FIGURE 5.6. Normalized expression (NE) of plant defence related genes obtained via qRT-PCR in tomato (*Solanum lycopersicum*) leaflets after infestation (7 days) with a plant defence suppressor strain DeLier-1 of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain the bacteria *Wolbachia* (W) and *Spiroplasma* (S). (a) Allene oxide synthase 1 (*AOS1*), (b) 12-oxophytodienoate Reductase 3 (*OPR3*), (c) Tomato wound-induced 1 (*TWI-1*), (d) Alcohol dehydrogenase (*ADH*), (e) Glutathione S-transferase 6 (*GST6*), (f) Glutaredoxin (*GRX*), (g) Threonine Deaminase-2 (*TD-2*), (h) Proteinase Inhibitor IIC (*PI-IIc*), (i) Jasmonate-inducible protein-21 (*JIP-21*), (j) Pathogenesis-related protein 6 (*PR-P6*), (k) Pathogenesis-related protein 1A (*PR-1a*); Gene expression levels were normalized to the levels of tomato actin (TABLE S5.2). Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR* (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of *P* ≤ 0.05, after applying a linear mixed model followed by Tukey multiple comparisons with Holm adjustment. n.s. = not significant.
In the leaflets infested with the inducer mites, none of the JA-defence marker genes (TD-2, PI-IIC and JIP-21) were differentially expressed (P < 0.05) between C+S+ and C-S- infested leaflets (FIGURE 5.7a-c). Interestingly, transcripts of both SA-defence marker genes, PR-P6 and PR-1a, were more abundant in C+S+ infested leaflets than in C-S- infested ones (FIGURES 5.7d-e and S5.10a,b).

IIIb. Effects of Wolbachia, Cardinium and Spiroplasma on the amount of feeding damage inflicted by the spider mites

A possible explanation for the observed differences in phytohormone and gene expression levels, which were correlated with the presence of mite-associated bacteria might be of behavioural nature, i.e., mites that feed more cause more damage to the plant and therefore might elicit stronger induced responses. To test if the magnitude of induced responses was correlated with the amount of feeding of the mites, we quantified the amount of spider mite-inflicted feeding damage (recognizable as chlorotic spots) on the same leaflets that were used for phytohormone extractions and

**FIGURE 5.7.** Normalized expression (NE) of plant defence related genes obtained via qRT-PCR in tomato (*Solanum lycopersicum*) leaflets after infestation (7 days) with a plant defence inducer strain Santpoort-2 of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain the bacteria *Candidatus Cardinium* (C) and *Spiroplasma* (S). (a) Threonine Deaminase-2 (TD-2), (b) Proteinase Inhibitor IIc (PI-IIc), (c) Jasmonate-inducible protein-21 (JIP-21), (d) Pathogenesis-related protein 6 (PR-P6), (e) Pathogenesis-related protein 1a (PR-1a). Gene expression levels were normalized to the levels of tomato actin (TABLE S5.2). Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR* (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of P ≤ 0.05, after applying a linear mixed models followed by Tukey multiple comparisons with Holm adjustment. n.s. = not significant

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tomato RNA isolation for qRT-PCRs. Overall, there was no significant difference in feeding damage between the three groups (W+S+, W-S+, W-S-) of suppressor mites (Figure 5.8a). In contrast, there was a clear difference in the amount of feeding damage caused by the two groups of inducer mites and in the type of damage these inflicted. Not only did C-S- mites feed significantly more than the C+S+ mites (Figure 5.8b), feeding by C+S+ mites resulted in rusty red/brown ‘scars’ on the leaflets (Figure 5.8c), while those infested with C-S- mites had clear white scars (Figure 5.8d). This feeding damage phenotype was evident for three out of four lines, it was less clear for line 7 (Figure S5.11).

**Figure 5.8.** Feeding damage (mm\(^2\)) on tomato (*Solanum lycopersicum*) leaflets inflicted by two strains of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain the bacteria *Wolbachia* (W), *Spiroplasma* (S) and/or *Candidatus Cardinium* (C). (a) Plant defence suppressor strain DeLier-1 with three mite groups: W+S+, W-S+ and W-S-. (b) Plant defence inducer strain Santpoort-2 with two mite groups: C+S+ and C-S-. Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR* (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of *P* ≤ 0.05, n.s. = not significant after applying a linear mixed model. (c) Typical rusty red/brown scars inflicted by feeding of C+S+ mites of the inducer strain and (d) typical white scars inflicted by feeding of C-S- mites of the inducer strain.
DISCUSSION
In this study we showed that *T. urticae* females of the plant suppressor strain DeLier-1, contained *Wolbachia* (W) and *Spiroplasma* (S) bacteria, while those from the plant defence inducer strain Santpoort-2, harboured *Candidatus Cardinium* (C) and also *Spiroplasma* (i.e., the same OTU as in suppressor mites). We determined the effects of these well-known reproductive parasites on (a) the spider mites and (b) on induced plant responses, after removing them via antibiotic treatment. In the spider mites, we showed I) that the presence of the (endo)symbionts correlated positively with the survival, but not oviposition of the suppressor mites, while it correlated negatively with both survival and oviposition of the inducer mites. II) We showed that a subset of spider mite genes that are predicted to be predominantly involved in digestion and detoxification processes, as well as genes encoding putative secreted (salivary gland) proteins, were differentially expressed in mites, with opposite trends occurring in the suppressor versus the inducer mites. III) Plant responses to mite infestations differed between the mite groups that did or did not harbour *Wolbachia, Cardinium and/or Spiroplasma*. With the suppressor strain the most prominent result was that the amount of OPDA was lowest in W-S+ infested compared to W-S- and W+S+ leaflets, while SA followed an opposite pattern (highest amount in W-S+ infested leaflets). With the inducer strain the most interesting result was that SA-marker genes were expressed at lower levels in C-S- inducer mites, than in C+S+ infested leaflets. Moreover, the antibiotics-treated inducer mites (C-S-) consumed significantly more plant material and caused visually different feeding scars compared to the non-treated (C+S+) mites. Remarkably, well-known upstream-downstream relationships between phytohormones and expression of their marker genes were in some cases not found in our study, as discussed below. Finally, we showed IV) the combined presence of *Wolbachia* and *Spiroplasma* has consequences for mite gene expression and induced plant responses. Together, our results indicate that different (endo)symbiotic bacteria may have distinct consequences for their host, as is summarized in Figure 5.9 and discussed below.

Importantly, it has to be noted that although the antibiotic treatments resulted in the (near-) complete removal of *Wolbachia, Cardinium and/or Spiroplasma* from the mites and we found their absence/presence to correlate well with certain parameters (e.g., mite performance, mite transcriptomic responses and induced plant responses), various other bacteria were (randomly) present in/on antibiotics-treated and non-treated mites as well. These bacterial strains were not restricted to a certain group of

**Figure 5.9.** Schematic and simplified overview of the most important findings of this study. Adult female spider mites (*Tetranychus urticae*) from the plant defence suppressor strain DeLier-1 and the defence inducer strain Santpoort-2 were treated with antibiotics to remove their associated bacteria *Wolbachia* sp. (W), *Spiroplasma* sp. (S) and/or *Candidatus Cardinium* (C), after which the indicated mite and plant (tomato; *Solanum lycopersicum*) parameters were assayed. For a more detailed description we refer to the Results and Discussion sections. med, intermediate; +, bacteria present; -, bacteria absent.
mites and are thus not likely to be responsible for the effects that we found between the mite groups. In addition, tetracycline treatments may have had effects on the mites other than the removal of bacteria. Direct toxic effects of tetracycline, such as inhibition of mitochondrial functioning, are unlikely to play a role in our study, because we started experiments at least 15 generations after the antibiotics treatments. Selective effects, in which tetracycline treatment has selected e.g., for more toxin resistant mites, cannot be ruled out. However, subjectively we did not notice higher mortality in the tetracycline-treated lines compared to non-treated lines.

I. Effects of Wolbachia, Spiroplasma and Cardinium on spider mite performance

When we investigated the performance of suppressor mites in a four days trial, survival was highest in mites containing Wolbachia and Spiroplasma. The presence or absence of Spiroplasma did not affect mite survival. The Wolbachia-mediated effect on suppressor mite survival could have resulted from direct effects on host gene transcript levels, from indirect effects via induced or suppressed plant defences, or a combination hereof, as discussed below. Surprisingly, we did not find that mite fecundity was higher in the presence of Wolbachia than in its absence, indicating that the Wolbachia-containing survivors may have laid eggs at a lower frequency than mites without Wolbachia. Future experiments should focus on the lifetime production of eggs by mites with and without symbionts, since differences in survival are expected to have a significant effect on reproductive performance in the long run.

The performance of inducer mites was negatively correlated with the presence of Cardinium and Spiroplasma. Both mite survival and fecundity were higher for C-S- mites than for the C+S+ mites. Unfortunately, the performance assay for the inducer strain could not be completed, because the C-S- subline of line 6, as well as both sublines of line 7, went extinct before the assays started. However, the results of the plant infestation assay and mite transcriptome analysis, which were performed with all lines, were all in line with our hypothesis that Cardinium and/or Spiroplasma have a negative influence on the performance of the inducer mites: expression of SA-marker genes in C-S- infested leaflets was reduced compared to the C+S+ infested leaflets, even though C-S- mites inflicted almost twice as much damage. Furthermore, on the microarray the expression of digestion and detoxification genes was higher in C-S- mites than in C+S+ ones.

Previous studies indicate that fitness effects of reproductive parasites may strongly depend on the genotype of symbiont and host and whether symbionts optimize their prevalence in a population by reproductive manipulation and/or by positively affecting host fitness (Bordenstein & Werren, 2000; Fry et al., 2004; Zug & Hammerstein, 2015). Accordingly, the reported effects of Cardinium, Wolbachia and Spiroplasma on host fitness are diverse. Whereas Cardinium and/or Spiroplasma negatively affected the fitness of mites in our experiments, an earlier study with a different T. urticae strain found that Cardinium did not alter survival or
egg hatchability of its host, nor did it manipulate its reproduction (Gotoh et al., 2007). Studies of other arthropods indicate that infection with Cardinium can have all possible effects on fitness parameters of its host (Weeks & Stouthamer, 2004; Ros & Breeuwer, 2009; White et al., 2011; Stefanini & Duron, 2012). The same is true for Wolbachia infection, which seemed to positively affect T. urticae fitness in our study. In T. urticae, Wolbachia was previously shown to have positive, negative or no effect on fitness parameters of this mite species (Vala et al., 2000; Perrot-Minnot et al., 2002; Xie et al., 2011). Such varying effects of Wolbachia are also known in Drosophila melanogaster (Hoffmann et al., 1994; Fry et al., 2002). Effects of Spiroplasma on animal host fitness parameters were previously found to be negative or neutral (Ebbert, 1991; Fukatsu et al., 2001; Montenegro et al., 2006; Anbutsu & Fukatsu, 2011).

II. Effects of Wolbachia, Spiroplasma and Cardinium on the mite’s transcriptome

Previous studies have established that (endo)symbiotic bacteria can affect gene expression of their host. Some data has been published on Cardinium (Nakamura et al., 2011) and Spiroplasma (Hutchence et al., 2011), but most research has focused on Wolbachia (Kambris et al., 2009; Moreira et al., 2009; Hussain et al., 2011; Zhang et al., 2013; Mayoral et al., 2014).

In T. urticae, Wolbachia has been shown to impact gene expression of its host: sex-specific differences in transcript levels of up to 251 protein-coding genes (Zhang et al., 2015), as well as up to 91 miRNAs (Rong et al., 2014) were found when comparing Wolbachia-infected and uninfected female mites. In our microarray analysis with antibiotics-treated and non-treated adult female suppressor and inducer mites, we identified 201 differentially regulated spider mite genes among the mites from all groups (i.e., W+S+, W-S+, W-S- and C-S-, C+S+), i.e., roughly 1% of the 18,414 in silico predicted protein-coding sequences (Grbic et al., 2011) of which about 50% were present on our array. Consistent with the data of Zhang et al. (2015), none of these mite genes putatively involved in immunity (Grbic et al., 2011) were differentially regulated. This is in agreement with previous findings that the immune system is not activated in response to Wolbachia in arthropod host species that are naturally infected by this bacterium, but can be activated in species that are naturally Wolbachia-free and are experimentally infected, as was shown in Aedes aegypti (Rances et al., 2012; Zug & Hammerstein, 2015).

Among the DEGs, there was a high abundance of genes that implicated the mite-associated bacteria in direct or indirect regulation of digestive and detoxification processes of their host. This is again similar to the findings of Zhang et al. (2015). However, when comparing the gene IDs of the DEGs of our study and that of Zhang et al. (2015) we found only five genes with an identical ID. Moreover, according to the data of Zhang et al. (2015), two of these five genes were differentially regulated in males but not females, while the remaining three genes were inversely regulated on our arrays. Notably, Zhang et al. (2015) worked with a
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Chinese mite strain that was sampled from bean, whereas we worked with Dutch mite lines that were sampled from tomato, while both data sets were mapped onto the *T. urticae* London genome (Grbic et al., 2011) for identification of DEGs. Possibly this is not accurate enough for identifying the homologous loci, but only for identifying homologous gene families. Additionally, *Wolbachia* strains may have differed between the two studies or *Wolbachia* may interact differently with mites on bean than with mites on tomato.

Absence of only *Wolbachia* from suppressor mites (W-S+) correlated with reduced transcript accumulation of 35 out of 36 DEGs, which belong to 9 gene families involved in digestion and in detoxification of xenobiotics. This result indicates that *Wolbachia* may have a direct effect on mites and may impact their capability to digest food and their ability to catabolize secondary metabolites. It has been shown before for various strains of *Wolbachia* that they can metabolically assist their host, in particular in filarial nematodes in which *Wolbachia* represents an obligate nutritional mutualist, for instance by providing its host with the energy source ATP (Darby et al., 2012) or essential coenzymes, the cofactor heme and nucleotides, in return for amino acids (Foster et al., 2005). With respect to arthropod hosts, *Wolbachia* is indispensable for the bedbug *Cimex lectularius*, because it provides essential B vitamins (Hosokawa et al., 2010). Furthermore, in the parasitic wasp *Asobara tabida* and the fruitfly *D. melanogaster*, *Wolbachia* influences iron (and hence redox) homeostasis, which benefits its host in perturbed iron environments (Brownlie et al., 2009; Kremer et al., 2009). Hence, in suppressor mites, *Wolbachia* possibly performs early catabolic steps of (plant) nutrients or secondary metabolites and delivers intermediates which the mite can further process using the genes listed in TABLE 5.2. If so, this may explain why Zhang et al. (2015) observed similar functional processes but different loci in mites in correlation with the absence or presence of *Wolbachia*. In this scenario, *Wolbachia* may act as a nutritional mutualist when mites feed from tomato, which might explain the higher survival of suppressor mites that contained *Wolbachia*.

Strikingly, our data reveals fewer DEGs (roughly 65% less) when comparing W+S+ mites with W-S- (instead of W-S+) mites, while transcriptomic differences between W-S+ and W-S- mites were virtually absent. This suggests a direct antagonistic interaction between *Wolbachia* and *Spiroplasma*. Unfortunately we did not obtain a W+S- line to substantiate this. Possibly, part of the *Wolbachia*-associated transcriptional response of the mite is a direct consequence of the presence of *Spiroplasma*. It has been suggested before that *Spiroplasma* negatively affects the abundance of *Wolbachia* within the same host (Goto et al., 2006). If so, *Wolbachia* may chemically combat and/or constrain *Spiroplasma* and vice versa, leaving the mite to clean up ‘the waste’ using the genes listed in TABLE 5.2.

The transcriptome of *Cardinium*- and *Spiroplasma*-free inducer mites also differed in the expression of genes involved in digestion, detoxification and transport of xenobiotics as compared to the mites that still harboured both bacteria. However,
we found fewer DEGs and the directionality of transcriptional change was largely opposite from what we found for the suppressor strain. Expression of cysteine peptidase and lipocalin encoding genes for instance, which responded strongest to the absence of *Wolbachia*, was unaffected in C-S- inducer mites. In fact, the only overlap with suppressor mites was the increased transcript abundance of two cystatin encoding genes in C-S- versus C+S+ mites. When compared to W+S+ mites, these genes were also induced in W-S- mites, but not in W-S+ ones. Together, this suggests that these cystatin genes respond to the presence of *Spiroplasma*. Other DEGs (encoding two CYPs, a CCE, an UGT, an ABC- and two MFS-transporters) seem to respond to the presence of *Cardinium* in inducer mites, mainly because they followed a trend opposite to when *Wolbachia* was present in the suppressor strain: all but one MFS-transporter were up-regulated in C-S- mites compared to C+S+ ones. We did not manage to obtain inducer strain lines with only *Spiroplasma* or only *Cardinium*, therefore it remains difficult to pinpoint the effects of each bacterial strain more precisely. All in all, the transcriptome data suggest *Cardinium* to have a negative effect on the mite, possibly by reducing the mite’s capability to detoxify and transport harmful secondary metabolites. This is consistent with our results for mite performance, in which C+S+ mites preformed worse than C-S- mites.

Among the 201 DEGs, the ones encoding (putative) secreted proteins were also highly abundant. Again this is in agreement with the data from Zhang *et al.* (2015). Four of our identified DEGs were predicted to encode proteins that are secreted from the mite salivary gland and are thus thought to indeed directly interact with the plant. Moreover, *Wolbachia* was found to be localized in the gnathosoma (mouth and feeding parts) of both male and female *T. urticae* (Zhao *et al.*, 2013), from where the proteins could also be secreted to the plant. Mite salivary gland-secreted proteins might be recognized by the plant and induce anti-herbivore defences (i.e., act as elicitors) or they may act as effectors and interfere with plant defences (Villarroel *in prep*). Pathogens (Dou & Zhou, 2012; Lo Presti *et al.*, 2015) and nematodes (Goverse & Smant, 2014) are well-known for their use of effector molecules to subvert plant immunity and while it is likely that many arthropods have evolved them as well, empirical evidence is still scarce (Kant *et al.*, 2015). In some cases defence suppression and the secretion of effectors by arthropods was reported to depend on associated microbes. For instance, the leafhopper *Macrosteles quadrilineatus* vectors phytoplasma bacteria, that after being transmitted to the host plant, produce and secrete molecules that suppress JA-defences, which is beneficial for the leafhopper (Sugio *et al.*, 2011). Recently, the saliva of the whitefly *Bemisia tabaci* was shown to contain a small (< 3 kDa) non-proteinaceous compound(s) responsible for the effective suppression of JA-defences via manipulation of JA-SA crosstalk (Su *et al.*, 2015). Defence suppression was only detected with saliva from whiteflies that harbour the endosymbiont *Hamiltonella defensa*, substantiating a relationship between the presence of the bacterium and the production of the effector(s) (Su *et al.*, 2015). Our microarray data indicates that mite-associated
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bacteria can affect the host’s transcript accumulation of genes encoding secreted (salivary gland) proteins and may in this way affect plant defence responses.

Together, the transcriptional differences that we observed between mites with *Wolbachia*, *Cardinium* and/or *Spiroplasma* bacteria or without them, may have several, not mutually exclusive, causes. First of all, they may reflect processes in the host associated with suitability for bacterial colonization, as was shown in *A. aegyti* (Hussain et al., 2011) and this could be a direct consequence of bacterial manipulation of host transcripts e.g., via RNA-interference (RNAi). Secondly, it may reflect responses of the host to bacterial metabolites, either plant-derived or not, eligible for further metabolization or catabolization. Such metabolites may be nutritional, may be plant toxins or be generated by multiple bacterial strains competing for space or nutrients. Thirdly, they may reflect a response of the mite to differences in plant tissue quality, e.g., due to differentially induced plant responses (discussed in the next section), directly or indirectly caused by differences in the host’s bacterial composition and possibly mediated by alterations in the host’s secretome.

### III. Effects of Wolbachia, Cardinium and Spiroplasma on tomato induced responses

When we investigated the effect of *Wolbachia* and *Spiroplasma* infection of the suppressor strain on tomato induced responses, the most striking finding was that the JA-precursor OPDA did not significantly accumulate in W-S+ infested leaflets compared to uninfested leaflets and W+S+ infested leaflets, while OPDA levels were intermediate in leaflets infested with W-S- mites. Thus, the presence of *Wolbachia* in suppressor mites is correlated with enhanced OPDA accumulation, while the presence of *Spiroplasma* in these mites is correlated with suppression of OPDA accumulation. Note that the OPDA (or any other plant response) phenotype cannot be explained by the amount of damage inflicted due to mite feeding, as this was equal among all four groups. Expression of *OPR3* (Strassner et al., 2002) was highest in W-S+ infested leaflets, thus an increased conversion rate of OPDA might explain the reduced OPDA accumulation in the respective leaflets. However, the immediate downstream product of OPDA, OPC8 showed the same pattern as OPDA. Surprisingly, the end product of the oxylipin pathway, JA-Ile, which is considered the main biologically active molecule (Fonseca et al., 2009; Wasternack & Hause, 2013), did not show any pattern that was correlated with bacterial presence, neither did JA-responsive defence marker genes. In addition, the accumulation profiles of two C12-derivatives of the hydroperoxide lyase (HPL)-pathway, traumatic acid and 9-OH-traumatin, closely resembled that of OPDA across the treatments. This is notable, because both the JA and the HPL pathway represent branches of the LOX pathway that use the same substrate, i.e., 13-HPOT (Figure S5.1; Wasternack, 2007). Moreover, levels of the substrate 13-HPOT as well as transcript levels of *AOS1*, which encodes the first of two enzymes
responsible for turnover of 13-HPOT into OPDA (Howe et al., 2000; Sivasankar et al., 2000), were not correlated with the presence of bacteria. Although gene transcript levels do not necessarily reflect protein levels and/or enzyme activity (Sullivan & Green, 1993), it remains unknown how accumulation of some wound hormones is affected by spider mite-associated bacteria, while other oxylipin pathway intermediates/products are not. One explanation for the altered OPDA amounts might be that *Wolbachia* and *Spiroplasma* differentially affect conjugation of OPDA with other cellular compounds. In Arabidopsis (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*) for example, OPDA is known to form conjugates with glutathione (GSH) (Davoine et al., 2006; Mueller et al., 2008). Although we did not detect OPDA-GSH in tomato leaflets, it is possible that other conjugates (that we did not search for) were formed.

Unexpectedly, expression levels of putative OPDA-responsive genes (ORGs) in suppressor mite infested leaflets negatively correlated with OPDA amounts. While OPDA induces expression of the ORGs *UGT73B5*, *CAD*, *GST6* and *GRX480* in Arabidopsis (Taki et al., 2005; Mueller et al., 2008; Park et al., 2013), the expression of their respective putative tomato homologs *TWI-1*, *ADH*, *GST6* and *GRX* all followed exactly the opposite pattern in our experiments. The fact that all four tomato genes had identical expression profiles (we found this for more putative ORGs, data not shown) and that their sequences and gene ontologies were conserved between Arabidopsis and tomato (52-62% identical, 69-77% similar at the amino acid level), strongly suggests these genes are indeed functional homologs. An explanation for the inverse correlation of ORG transcript levels and OPDA amounts might be that these genes are regulated by one or more signals other than OPDA. This might for instance be attributed to SA, since we found significantly higher levels of SA in W-S+ than in W+S+ and W-S- infested leaflets and thus SA amounts and ORG transcript levels were positively correlated. Interestingly, some Arabidopsis ORGs have been shown to be SA responsive (Uquillas et al., 2004; Langlois-Meurinæ et al., 2005; Ndamukong et al., 2007). In tomato, the ORG *TWI-1* is also responsive to SA (O’Donnell et al., 1998). This suggests that SA might (co-)regulate the expression of the putative tomato ORGs.

SA amounts correlated only weakly with expression levels of *PR-P6* and did not correlate with *PR-1a* expression levels at all. Interestingly, *PR-1a* was not induced compared to the control in leaflets infested with suppressor mites, while it was induced in leaflets infested with inducer mites. This weak or lack of correlation with PR gene expression might thus be indicative of defence suppression by suppressor mites, which was demonstrated to occur downstream of phytohormone accumulation, e.g., at the *PR-1a* transcript level (Alba et al., 2015).

Remarkably, plant response patterns (in which W-S+ differed from W+S+ and W-S-) were not reflected in mite performance (mite survival was lower for W-S+ and W-S- than for W+S+ mites), possibly because *Wolbachia* and *Spiroplasma* do not seem to have a significant effect on the JA defence responses to which the mites are susceptible.
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(Alba et al., 2015). However, differences in OPDA amounts might have a significant impact on the fitness of mites independently from JA, because accumulation of OPDA itself is sufficient to confer resistance to several herbivores and pathogens as has been demonstrated in various studies (Stintzi et al., 2001; Stotz et al., 2011; Park et al., 2013; Bosch et al., 2014; Scalschi et al., 2015; Guo et al., 2014). We can therefore not exclude, that plant responses, that were altered in correlation to bacteria, affect fitness of the suppressor strain in other traits than we have measured.

For the inducer strain, the results form a clearer picture. Leaflets infested with C-S- mites contained higher amounts of ABA, traumatic acid and 9,12-OH-(10E)-dodecenoic acid, while transcript levels of SA-responsive PR-P6 and PR-1a were reduced. Consistent with these results, earlier studies found that feeding by arthropods with bacterial symbionts was associated with increased amounts of SA and higher expression levels of PR genes in host plants (Chung et al., 2013; Su et al., 2015). ABA appears to negatively regulate SA defences in tomatto, in particular by inhibiting expression of PR-1a (Audenaert et al., 2002). The reduced expression of PR genes in C-S- samples compared to the C+S+ samples might thus be explained by the negative action of ABA. Since these results were paralleled by different feeding intensities of mites with or without these bacteria, Cardinium and/or Spiroplasma may negatively affect mite fitness via induction of plant defences.

IV. The combined presence of Wolbachia and Spiroplasma bacteria has consequences for mite gene expression and induced plant responses

Within a host, the various symbionts that (can) co-occur possibly interact, which may affect host and bacterial fitness in various ways. Our results suggest that a single or double infection status of suppressor mites differentially affects host gene expression
as well as induced plant responses, with *Wolbachia* demonstrating characteristics of a mutualist, while the effect of *Spiroplasma* on the mite was less clear, but its presence (partially) antagonized *Wolbachia*-associated responses. This could have resulted from competition between the two symbionts, for instance for space and/or resources. Microbial competition for space (i.e., the ovaries) within the mosquito hosts *Anopheles stephensi* and *Aedes aegypti* was reported to occur between *Wolbachia* and an unrelated *Asaia* bacterium (Hughes et al., 2014; Rossi et al., 2015). It is thus possible that *Wolbachia* spatially displaces *Spiroplasma* or vice versa, i.e., away from organs where they may affect induced plant responses or mite digestive processes, like the salivary glands or gut epithelial cells. It would therefore be interesting to determine the spatial localization of both bacteria in single and double infected mites using *in situ* hybridizations. Beside the proper localization, symbiont densities are another important parameter for fidelity of their vertical transmission as well as their effect on host fitness (Rousset et al., 1999; Goto et al., 2006; Oliver et al., 2006; Unckless et al., 2009). Our Illumina MiSeq analysis showed that the relative abundance (as well as the total number of reads) of *Spiroplasma* in the W-S+ lines was higher than in the W+S+ lines, suggesting that *Wolbachia* may negatively affect the abundance of *Spiroplasma* in suppressor mites. However, since 16S amplicon sequencing is only a semi-quantitative method, bacterial abundance should be assessed by means of qRT-PCRs to test this hypothesis.

How co-infecting symbionts affect each other’s densities depends on both the species and genotype of host and symbiont (Bordenstein & Werren, 2000; Kondo et al., 2005). For instance, in double or triple infections of wasps, fruitflies or moths with different *Wolbachia* genotypes, none of the tested *Wolbachia* strains influenced densities of the other *Wolbachia* strain(s) (Rousset et al., 1999; Ikeda et al., 2003; Mouton et al., 2003). In contrast, in the beetle *Callosobruchus chinensis* one *Wolbachia* strain suppressed a second one (Kondo et al., 2005). In *D. melanogaster* infected with *Wolbachia* and *Spiroplasma*, the latter negatively affected *Wolbachia* densities, but *Wolbachia* did not affect *Spiroplasma* densities (Goto et al., 2006).

In addition, fitness effects of mixed infections with different bacterial species on hosts are diverse. For instance, female *Bryobia sarothamni* mites that were doubly infected with *Wolbachia* and *Cardinium* had a higher fecundity than singly or uninfected females (Ros & Breeuwer, 2009). In contrast, pea aphids that were doubly infected with *H. defensa* and *Serratia symbiotica* had a lower fecundity than singly or uninfected aphids (Oliver et al., 2006). Infection of *D. melanogaster* with *Wolbachia, Spiroplasma* or both did not affect fitness parameters of the flies (Montenegro et al., 2006). A mutualistic interaction has been proposed for *Wolbachia* and *Spiroplasma* that co-infect the fruitfly *D. neotestaceae* (Jaenike et al., 2010b). The frequency of co-infected fruitflies in sampled populations and estimated evolutionary age of the two bacteria that had co-infected the flies, were seen as a possible basis for the evolution of a mutualism between the bacteria (Jaenike et al., 2010b). The fact that *Wolbachia* and *Spiroplasma* bacteria in the
supressor strain seemed to differentially influence mite survival, transcriptomic responses and induced plant responses, emphasizes the importance of considering interaction effects of multiple bacteria in/on one host.

Unfortunately, we did not obtain all bacterial combinations in *T. urticae* to disentangle the exact effects of single bacterial strains and their combined effects on mite fitness, induced plant responses and mite gene expression. For instance, we did not test mites that were infected with only *Wolbachia*, hence we can only indirectly infer the role of *Wolbachia* in our experiments from comparisons between W+S+ and W+S− mites. Moreover, for the inducer strain the roles of *Spiroplasma* and *Cardinium* remain difficult to interpret because we did not have C-S+ or C+S− mites. The same *Spiroplasma* OTU occurred in both the inducer and suppressor mites. If we assume that *Spiroplasma* in inducer mites had the same effect on host fitness parameters as in suppressor mites, we could attribute the negative effects of bacteria in inducer mites to *Cardinium*. However, *Spiroplasma* may interact with *Cardinium* in the mites. Furthermore, its effects on host fitness may depend on mite genotype (Schmid-Hempel, 2011).

**Conclusions**

We assessed the influence of the (endo)symbiotic bacteria *Wolbachia*, *Cardinium* and *Spiroplasma* on the performance and transcriptome of their respective hosts, the *T. urticae* suppressor (W+S+) and inducer (C+S+) strains. Furthermore, we tested how these spider mite-associated bacteria affect the induced responses of tomato plants triggered by feeding mites. Our data indicate that the different (endo)symbiotic bacteria may have distinct consequences for their host (see Figure 5.9):

1. The presence of *Wolbachia* correlates positively with survival of suppressor mites, possibly via manipulation of OPDA and SA-related responses. We did not observe changes in JA- and SA marker gene expression levels. Further, the presence of *Wolbachia* correlates positively with transcript levels of suppressor mite genes involved in digestion and detoxification of xenobiotics. This might reflect responses of the mite to symbiont-generated or -modified metabolites, either plant-derived (transcriptome profiles of the suppressor strain resemble the OPDA phenotype) or not, that are suitable for further processing by the mite.

2. The presence of *Cardinium* and *Spiroplasma* correlates negatively with the feeding activity, survival and fecundity of inducer mites, while it correlates positively with the induction of SA-marker-gene expression as well as with the frequently observed rusty scars of infested leaves. This indicates a distinct and clearly visible (in)direct impact of the presence of *Cardinium* and *Spiroplasma* on the mite’s host plant while simultaneously their presence correlates with inhibition of digestion and detoxification processes in the mite.

3. There is no clear correlation between the absence or presence of *Spiroplasma* and survival of suppressor mites, while the induced plant responses of leaflets fed on by mites with or without *Spiroplasma* are ambiguous. The data suggests
a complex interaction between Wolbachia and Spiroplasma in which the latter may suppress OPDA-related responses that are induced in the presence of Wolbachia. In addition, some mite digestion and detoxification genes that respond positively to the presence of Wolbachia seem to be slightly suppressed when only Spiroplasma is present. Unfortunately, we did not obtain W+S- lines to disentangle this interaction.

4. The same Spiroplasma OTU was present in suppressor and inducer mites. Hence, although we did not obtain singly-infected inducer lines, a comparison of the results between the mite strains suggests that the presence of Spiroplasma in C+S+ inducer mites is not related with the observed negative effects on its host (e.g., induction of SA defences, impaired expression of detoxification genes and reduced feeding). However, we cannot exclude that Spiroplasma affects the suppressor mites differently than the inducer mites.

In conclusion, according to our data, Wolbachia has the characteristics of a mutualist, while Cardinium has the characteristics of a parasite. The role of Spiroplasma remains unclear, but it does seem to interact – directly or indirectly – with Wolbachia at the level of mite gene expression and induced plant responses (note that these two responses might be causally linked).

REFERENCES
Mite-associated bacteria


Mite-associated bacteria


Tornero, P., Gadea, J., Conejero, V. & Vera, P. 1997. Two PR-1 genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. Mol Plant Microbe Interact 10: 624-634.


Mite-associated bacteria


Mite-associated bacteria

SUPPORTING INFORMATION

**FIGURE S5.1.** Schematic and simplified overview of tomato (*Solanum lycopersicum*) defence responses typically induced by spider mite (*Tetranychus urticae*) feeding and which were monitored in this study. Infestation with inducer *T. urticae* Santpoort-2 mites (inset) results in the simultaneous, enhanced accumulation of the phytohormones jasmonic acid (JA) and salicylic acid (SA) as well as transcript levels of their downstream marker genes. JA biosynthesis originates from linolenic acid, which is converted to 13-HPOT by LOX. This compound then serves either as substrate for the enzyme HPL to generate various C₁₂ traumatic acids, or for AOS to ultimately yield JA-Ile. Arrows indicate biosynthetic steps carried out by a single enzyme, some of them are shown in red letters. Note that SA can be synthesized via different pathways and how this is done upon mite infestation is still unclear. Dashed arrows symbolize transcriptional activation of marker genes (which are in italic face). Amounts of linolenic acid and HPL transcripts were not assayed in this study.

(a) Cumulative percentage of alive, migrated & dead females:

- **dead**
- **migrated**
- **alive**
FIGURE S5.2. Survival, migration and mortality in two strains of the spider mite *Tetranychus urticae* which did (+) or did not (-) contain *Wolbachia*, *Spiroplasma* and/or *Candidatus Cardinium* after feeding on tomato (*Solanum lycopersicum*) for 4 days. (a) Four lines of the plant defence suppressor strain DeLier-1 were tested (1, 2, 3 and 4). Each line was subdivided into three mite groups: W+S+, W-S+ and W-S-; (b) Two lines (5 and 8) were tested for the plant defence inducer strain Santpoort-2 with two mite groups: C+S+ and C-S-. Additionally we tested C+S+ of line 6. C-S- of line 6 as well as both groups of line 7 went extinct before the experiment.

FIGURE S5.3. Reproductive performance (number of eggs produced per female in four days) of two lines (5, and 8) of a plant defence inducer strain Santpoort-2 of the spider mite *Tetranychus urticae*. Each of the lines was subdivided into two groups which did (+) or did not (-) contain the bacteria *Candidatus Cardinium* and *Spiroplasma*: C+S+ (grey) and C-S- (white). Additionally we tested C+S+ of line 6; the C-S- groups of line 6 as well as both groups of line 7 went extinct before the experiment. Boxes span the 25-75 percentiles, horizontal lines in the boxes represent medians, whiskers span 1.5*IQR* (interquartile range), dots represent data points outside of this range. Different letters above the boxes indicate significant differences at a level of *P* ≤ 0.05 (tested per line) after applying a linear mixed model.
**FIGURE S5.4.** Transcription heatmap of differentially expressed genes in the *T. urticae* suppressor strain DeLier-1 after removal of *Wolbachia* (W-S+) and *Wolbachia* and *Spiroplasma* (W-S-) as compared to transcription levels of the original strain (W+S+) that contained both bacteria. A total of 173 differentially expressed genes were detected after *Wolbachia* (W-S+) and *Wolbachia*-Spiroplasma removal (W-S-) and between the two treatments, mutually (FDR-corrected \( P \leq 0.05 \)). Using Euclidean distance metrics, genes were clustered based on their relative transcript levels in the W-S+ vs W+S+ and W-S- vs W+S+ comparisons. In the left sidebar, orange and green rectangles indicate genes that code for enzymes involved in the xenobiotic and digestive pathways of *T. urticae*, respectively.
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**FIGURE S5.5.** Phytohormone amounts in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with four lines (1, 2, 3 and 4) of a plant defence suppressor strain DeLier-1 of the spider mite *Tetranychus urticae*. Each of the lines was subdivided into three groups which did (+) or did not (-) contain the bacteria *Wolbachia* and *Spiroplasma*: W+S+ (dark grey), W-S+ (light grey) and W-S- (white). Control plants were not infested (darkest grey). Tested phytohormones included (a) 12-oxo-phytodienoic acid (OPDA), (b) 3-oxo-2-(2-pentenyl)-cyclopentane-1-hexanoic acid (OPC6), (c) jasmonic acid (JA) (d) jasmonic acid-isoleucine (JA-Ile), (e) 9-OH-traumatnin, (f) free salicylic acid (SA); Circles represent individual data points, horizontal lines indicate the medians. Phytohormone amounts are presented as nanogram (ng) per gram fresh leaf weight (g FW). Compounds for which we did not have a pure standard are presented as ion counts (IC/g FW).

**FIGURE S5.6.** Phytohormone amounts in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with four lines (5, 6, 7 and 8) of a plant defence inducer strain Santpoort-2 of the spider mite *Tetranychus urticae*. Each of the lines was subdivided into two groups which did (+) or did not (-) contain the bacteria *Candidatus Cardinium and Spiroplasma*: C+S+ (grey), C-S- (white). Control plants were not infested (dark grey). Tested phytomhorones included (a) jasmonic acid-isoleucine (JA-Ile), (b) free salicylic acid (SA) (c) traumatic acid, (d) abscisic acid (ABA) (f); Circles represent individual data points, horizontal lines indicate the medians. Phytomhorone amounts are presented as nanogram (ng) per gram fresh leaf weight (g FW). Compounds for which we did not have a pure standard are presented as ion counts (IC/g FW).
FIGURE S5.7. Normalized expression (NE) of plant defence related genes (qRT-PCR) in tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with four lines (1, 2, 3 and 4) of a plant defence suppressor strain DeLier-1 of the spider mite *Tetranychus urticae*. Each of the lines was subdivided into three groups which did (+) or did not (-) contain the bacteria *Wolbachia* and *Spiroplasma*: W+S+ (dark grey), W-S+ (light grey) and W-S- (white). Control plants were not infested (darkest grey). (a) 12-oxophytodienoate Reductase 3 (*OPR3*), (b) Tomato wound-induced 1 (*TWI-1*), (c) Alcohol dehydrogenase (*ADH*), (d) Glutathione S-transferase 6 (*GST6*), (e) Glutaredoxin (*GRX*), (f) Jasmonate-inducible protein-21 (*JIP-21*), (g) Pathogenesis-related protein 6 (*PR-P6*); Circles represent individual data points, horizontal lines indicate the medians.
FIGURE S5.8. Correlations between phytohormone amounts of salicylic acid (SA) and 12-oxophytodienoic acid (OPDA) and the normalized expression levels of putative OPDA responsive genes (TWI-1, GRX, GST-6, ADH and OPR3) measured in tomato leaflets after 7 days of infestations with the plant defence suppressor strain DeLier-1 of the spider mite Tetranychus urticae. TWI-1: Tomato wound-induced 1, GRX: Glutaredoxin, ADH: Alcohol dehydrogenase, GST6: Glutathione S-transferase 6; P-values were adjusted for multiple comparisons with the Holm method.
FIGURE S5.9. Correlations between phytohormone amounts of salicylic acid (SA) and the normalized expression levels of the two SA marker genes PR-P6 and PR-1a, measured in tomato leaflets after 7 days of infestations with the plant defence suppressor strain DeLier-1 of the spider mite Tetranychus urticae. PR-P6: Pathogenesis-related protein 6, PR-1a: Pathogenesis-related protein 1a; P-values were adjusted for multiple comparisons with the holm method.

FIGURE S5.10. Normalized expression (NE) of plant defence related genes (qRT-PCR) in tomato (Solanum lycopersicum) leaflets after 7 days of infestation with four lines (5, 6, 7 and 8) of a plant defence inducer strain Santpoort-2 of the spider mite Tetranychus urticae. Each of the lines was subdivided into two groups which did (+) or did not (-) contain the bacteria Candidatus Cardinium and Spiroplasma: C+S+ (grey) and C-S- (white). Control plants were not infested (dark grey). (a) Pathogenesis-related protein 6 (PR-P6), (b) Pathogenesis-related protein 1a (PR-1a); Circles represent individual data points, horizontal lines indicate the medians.
Figure S5.11. Feeding damage (mm²) on tomato (*Solanum lycopersicum*) leaflets after 7 days of infestation with four lines (5, 6, 7 and 8) of a plant defence inducer strain Santpoort-2 of the spider mite (*Tetanychus urticae*). Each of the lines was subdivided into two groups which did (+) or did not (-) contain the bacteria *Candidatus Cardinium* and *Spiroplasma*: C+S+ (grey) and C-S− (white). Circles represent individual data points, horizontal lines indicate the medians.

Table S5.1. Parameters used for detection of phytohormones and related compounds by LC-MS/MS

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<th>Compound</th>
<th>Q1&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Q2&lt;sup&gt;*&lt;/sup&gt;</th>
<th>CID&lt;sup&gt;**&lt;/sup&gt;</th>
<th>CE&lt;sup&gt;***&lt;/sup&gt;</th>
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<td>9,12-OH-(10E)-dodecanoic acid</td>
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<td>266</td>
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<td>JA-Ile / JA-Leu</td>
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<td>12.0</td>
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<td>Wang et al., 2007</td>
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<td>JA-glucose</td>
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<td>209</td>
<td>-35</td>
<td>18.0</td>
<td>no</td>
<td>VanDoorn et al., 2011</td>
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</table>

<sup>*</sup> Q<sub>1</sub> mass = Molecular Weight − 1 = [M − H]+ (m/z); <sup>**</sup> CID Q<sub>2</sub> mass (of daughter with 100% intensity) (m/z); <sup>***</sup> Capacillary Collision-Induced Dissociation (V); <sup>****</sup> Collision Energy (V); <sup>*****</sup> personal communication.
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<th>Name</th>
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<th>Forward primer 5'→3'</th>
<th>Reverse primer 5'→3'</th>
<th>References</th>
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<td>16S rRNA</td>
<td><em>Actin</em></td>
<td>Solyc03g078400.2</td>
<td>ACTCTTTACTTTAAGTCAGCT</td>
<td>ACTCTTTACCTTTAAGTCAGCT</td>
<td>Tomato Genome Consortium, 2012</td>
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<td><em>Actin</em></td>
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<td>ACTCTTTACCTTTAAGTCAGCT</td>
<td>Tomato Genome Consortium, 2012</td>
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<td><em>Actin</em></td>
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<td>Tomato Genome Consortium, 2012</td>
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<tr>
<td><em>Bacteria (general)</em></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Tetranychus urticae</em></td>
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<td><em>Actin</em></td>
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<td>Tomato Genome Consortium, 2012</td>
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<td><em>Solanum lycopersicum</em></td>
<td>AOSI</td>
<td><em>Actin</em></td>
<td>Solyc03g078400.2</td>
<td>ACTCTTTACTTTAAGTCAGCT</td>
<td>ACTCTTTACCTTTAAGTCAGCT</td>
<td>Tomato Genome Consortium, 2012</td>
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<tr>
<td>Mite-associated bacteria</td>
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</table>
### Table S5.3a. List of top-20 genes significantly up-regulated in adult female *Tetranychus urticae* suppressor (DeLier-1) W-S- mites as compared to W+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days (*P* ≤ 0.05, after *P*-value adjustment).

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<th><em>P</em></th>
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<tr>
<td>1</td>
<td>tetur06g06585</td>
<td>Hypothetical protein (snosnR61, small nucleolar RNA)</td>
<td>1.81</td>
<td>0.0036</td>
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<td>2</td>
<td>tetur18g02170</td>
<td>Hypothetical protein (no hits found)</td>
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<td>0.0031</td>
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<tr>
<td>3</td>
<td>tetur24g01690</td>
<td>Hypothetical protein (similar to sorting nexin 13)</td>
<td>1.49</td>
<td>0.0261</td>
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<tr>
<td>4</td>
<td>tetur05g04420</td>
<td>Hypothetical protein (choline/ethanolaminephosphotransferase 1)</td>
<td>1.45</td>
<td>0.0344</td>
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<tr>
<td>5</td>
<td>tetur31g00280d</td>
<td>CSPCA1: CUB domain-containing Secreted Protein Conserved in Arthropods</td>
<td>1.44</td>
<td>0.0185</td>
</tr>
<tr>
<td>6</td>
<td>tetur39g00780</td>
<td>Hypothetical protein (vitellogenin1)</td>
<td>1.35</td>
<td>0.0449</td>
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<tr>
<td>7</td>
<td>tetur04g06140d</td>
<td>Hypothetical protein (lisW_ISCW012949)</td>
<td>1.34</td>
<td>0.0140</td>
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<tr>
<td>8</td>
<td>tetur05g02950</td>
<td>CYP389C2: Cytochrome P450</td>
<td>1.31</td>
<td>0.0127</td>
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<tr>
<td>9</td>
<td>tetur23g01800</td>
<td>Hypothetical protein (Rho GTPase-activating protein 29)</td>
<td>1.30</td>
<td>0.0295</td>
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<tr>
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<td>tetur09g04980</td>
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<td>tetur10g04770</td>
<td>Hypothetical protein (leukocyte elastase inhibitor)</td>
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<tr>
<td>12</td>
<td>tetur60g00070</td>
<td>Hypothetical protein (TcasGA2_TC006277)</td>
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<td>13</td>
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<td>Hypothetical protein (IscW_ISCW012949)</td>
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<td>0.0143</td>
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<td>14</td>
<td>tetur06g03890</td>
<td>Hypothetical protein (trichohyalin, putative)</td>
<td>1.24</td>
<td>0.0483</td>
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<tr>
<td>15</td>
<td>tetur05g08770d</td>
<td>Hypothetical protein (intracellular protein transport)</td>
<td>1.23</td>
<td>0.0014</td>
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<tr>
<td>16</td>
<td>tetur05g03440</td>
<td>Hypothetical protein (membrane and coiled-coil domains protein 2-like)</td>
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<td>Hypothetical protein (M-phase inducer phosphatase)</td>
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<td>18</td>
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<td>Hypothetical protein (tyrosine kinase, putative)</td>
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<td>20</td>
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<td>Hypothetical protein (mRNA splicing protein SMN)</td>
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<td>0.0344</td>
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</table>

These genes were significantly up-regulated in *T. urticae* suppressor W-S- mites as compared to W+S+ mites.

### Table S5.3b. List of top-20 genes significantly down-regulated in adult female *Tetranychus urticae* suppressor (DeLier-1) W-S- mites as compared to W+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days (*P* ≤ 0.05, after *P*-value adjustment).

<table>
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<td>Hypothetical protein (similar to WT Secreted Protein 12)</td>
<td>-3.44</td>
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<tr>
<td>2</td>
<td>tetur16g03680d</td>
<td>TuPap-31: Cathepsin L</td>
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<td>tetur16g03740d</td>
<td>WTSP12: WT Secreted Protein 12</td>
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<td>Hypothetical protein (Legumain)</td>
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<td>tetur44g00311</td>
<td>Hypothetical protein (transposon)</td>
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<td>7</td>
<td>tetur32g02297</td>
<td>SSPF13: Small Secreted Protein, Family F</td>
<td>-2.56</td>
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<td>tetur16g03670</td>
<td>TuLeg-13: Legumain</td>
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<tr>
<td>9</td>
<td>tetur16g03610</td>
<td>WTSP4: WT Secreted Protein, pseudogene</td>
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<td>10</td>
<td>tetur31g00810</td>
<td>Hypothetical protein (similar to methyl-accepting chemotaxis sensory transducer, extracellular)</td>
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<td>TuPap-44: Cathepsin L</td>
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<td>Hypothetical protein (Serpin 3 inhibitory serine protease inhibitor, putative secreted salivary gland protein)</td>
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<td>Hypothetical protein (methyl-accepting chemotaxis sensory transducer, extracellular)</td>
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Mite-associated bacteria

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<td>tetur03g02710</td>
<td>Hypothetical protein (phospholipid scramblase 2-like)</td>
<td>-1.89</td>
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</table>

* these genes were significantly down-regulated in *T. urticae* suppressor W-S- mites as compared to W+S+ mites.

**TABLE S5.4a.** List of all genes significantly up-regulated in adult female *Tetanychus urticae* suppressor (DeLier-1) W-S- mites as compared to W+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days ($P \leq 0.05$, after $P$-value adjustment).

W, *Wolbachia* sp.; S, *Spiroplasma* sp.; +, present; -, absent

<table>
<thead>
<tr>
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<th>Locus identifier</th>
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<th>P</th>
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<td>tetur06g01060</td>
<td>TuCPI-22: Cystatin (cystatin precursor)</td>
<td>2.03</td>
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<tr>
<td>3</td>
<td>tetur02g15167</td>
<td>SP68: Serine Protease (Peptidase S1)</td>
<td>1.63</td>
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<td>4</td>
<td>tetur15g00310</td>
<td>CSP1: Cell Surface Protein with GTPLASR(K,R,S) repeats</td>
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<td>Hypothetical protein (tribbles homolog 2, serine/threonine-protein kinase)</td>
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<td>tetur15g0320</td>
<td>CSPCA2: Cell Surface Protein with GTPLASR(K,R,S) repeats</td>
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<tr>
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<td>tetur04g06140</td>
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<tr>
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<td>TuGR62: Chemosensory Receptor, Gustatory receptor family</td>
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<td>tetur14g02440</td>
<td>Hypothetical protein (Ant09g06400, <em>Aspergillus niger</em> chitinase)</td>
<td>1.15</td>
<td>0.0369</td>
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</tbody>
</table>

* these genes were significantly up-regulated in *T. urticae* suppressor W-S- mites as compared to W-S+ mites. * these genes were significantly up-regulated in suppressor W-S+ mites as compared to W+S+ mites. * these genes were significantly up-regulated in *T. urticae* inducer (Santpoort-2) C-S- mites as compared to C+S+ mites. C, *Candidatus Cardinium*

**TABLE S5.4b.** List of top-20 genes significantly down-regulated in adult female *Tetanychus urticae* suppressor (DeLier-1) W-S- mites as compared to W+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days ($P \leq 0.05$, after $P$-value adjustment).

W, *Wolbachia* sp.; S, *Spiroplasma* sp.; +, present; -, absent

<table>
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<th>P</th>
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<td>1</td>
<td>tetur03g08800</td>
<td>SSPB2: Small secreted protein, family B</td>
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<td>2</td>
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<td>CYP392D8: Cytochrome P450</td>
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<td>3</td>
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<td>tetur03g09961</td>
<td>CYP392D7: Cytochrome P450</td>
<td>-2.34</td>
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TABLE S5.5. List of all genes significantly up- and down-regulated respectively, in adult female *Tetranychus urticae* suppressor (DeLier-1) W-S- mites as compared to W-S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days (P ≤ 0.05, after P-value adjustment).

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<th>P</th>
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<td>1.84</td>
<td>0.0187</td>
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<tr>
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<td>tetur17g02060 w</td>
<td>Hypothetical protein (secreted salivary gland peptide, putative)</td>
<td>1.39</td>
<td>0.0187</td>
</tr>
<tr>
<td>3</td>
<td>tetur03g08800 x</td>
<td>SSPB2: Small secreted protein, family B</td>
<td>-1.96</td>
<td>0.0235</td>
</tr>
</tbody>
</table>

* These genes were significantly up-regulated in *T. urticae* suppressor W-S- mites as compared to W+S+ mites and in inducer (Santpoort-2) C-S- mites as compared to C+S+ mites. * This gene was significantly down-regulated in both suppressor W-S- and W-S+ mites as compared to W+S+ mites. C, *Candidatus Cardinium*

TABLE S5.5a. List of all genes significantly up-regulated in adult female *Tetranychus urticae* inducer (Santpoort-2) C-S- mites as compared to C+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days (P ≤ 0.05, after P-value adjustment).

<table>
<thead>
<tr>
<th>#</th>
<th>Locus identifier</th>
<th>Annotation (BOGAS)</th>
<th>Fold change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>tetur11g05780 w</td>
<td>Hypothetical protein (no hits found)</td>
<td>5.17</td>
<td>0.0046</td>
</tr>
<tr>
<td>2</td>
<td>tetur06g06650 w</td>
<td>TuCPI-19: Cystatin (onchocystatin)</td>
<td>5.03</td>
<td>0.0001</td>
</tr>
<tr>
<td>3</td>
<td>tetur06g01060 w</td>
<td>TuCPI-22: Cystatin (cystatin precursor)</td>
<td>3.02</td>
<td>0.0001</td>
</tr>
<tr>
<td>4</td>
<td>tetur11g05760 w</td>
<td>TuCCE-33: Carboxyl/choline esterase (putative)</td>
<td>2.84</td>
<td>0.0288</td>
</tr>
<tr>
<td>5</td>
<td>tetur37g00580 x</td>
<td>Hypothetical protein (4-nitrophenylphosphatase)</td>
<td>2.32</td>
<td>0.0008</td>
</tr>
<tr>
<td>6</td>
<td>tetur08g08060 x</td>
<td>Hypothetical protein (secreted salivary gland peptide, putative)</td>
<td>2.11</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

* These genes were significantly up-regulated in *T. urticae* induced C-S- mites as compared to C+S+ mites.
Mite-associated bacteria

<table>
<thead>
<tr>
<th>Locus identifier</th>
<th>Annotation (BOGAS)</th>
<th>Fold change</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetur11g05550</td>
<td>Hypothetical protein (MFS-transporter)</td>
<td>-1.59</td>
<td>0.0219</td>
</tr>
<tr>
<td>tetur18g02050</td>
<td>Hypothetical protein (no hits found)</td>
<td>-1.55</td>
<td>0.0369</td>
</tr>
<tr>
<td>tetur11g05720</td>
<td>PLAT10: Lipase/lipoxygenase</td>
<td>-1.47</td>
<td>0.0066</td>
</tr>
<tr>
<td>tetur02g13460</td>
<td>Hypothetical protein (very acidic salivary protein)</td>
<td>-1.46</td>
<td>0.0337</td>
</tr>
<tr>
<td>tetur01g15560</td>
<td>ZN207: Zinc Finger protein 207</td>
<td>-1.37</td>
<td>0.0436</td>
</tr>
<tr>
<td>tetur28g01570</td>
<td>Hypothetical protein (deoxyhypusine synthase)</td>
<td>-1.36</td>
<td>0.0185</td>
</tr>
<tr>
<td>tetur11g06310</td>
<td>Hypothetical protein (galactose-binding domain-like)</td>
<td>-1.36</td>
<td>0.0079</td>
</tr>
<tr>
<td>tetur05g03510</td>
<td>Hypothetical protein (ribosome production factor 2 homolog)</td>
<td>-1.35</td>
<td>0.0143</td>
</tr>
<tr>
<td>tetur15g00160</td>
<td>Hypothetical protein (no hits found)</td>
<td>-1.30</td>
<td>0.0329</td>
</tr>
<tr>
<td>tetur27g02556</td>
<td>SLCP1: secreted low complexity protein, putative</td>
<td>-1.29</td>
<td>0.0313</td>
</tr>
</tbody>
</table>

\( ^{a} \) this gene was significantly up-regulated in *T. urticae* suppressor (DeLier-1) W-S- mites as compared to W+S+ mites. \( ^{b} \) these genes were significantly down-regulated in suppressor W-S+ mites as compared to W+S+ mites. \( ^{c} \) these genes were significantly up-regulated in suppressor W-S- mites as compared to both W+S+ and W-S+ mites. W, *Wolbachia* sp.

### Table S5.6b.

List of all genes significantly down-regulated in adult female Tetanychus urticae inducer (Santpoort-2) C-S- mites as compared to C+S+ mites, after feeding from tomato (*Solanum lycopersicum*) for seven days (\( P \leq 0.05 \), after \( P \)-value adjustment).

### Supplementary References


Mite-associated bacteria


