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Direct and Indirect Plant Defenses are not Suppressed by Endosymbionts of a Specialist Root Herbivore

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Abstract Insect endosymbionts influence many important metabolic and developmental processes of their host. It has been speculated that they may also help to manipulate and suppress plant defenses to the benefit of herbivores. Recently, endosymbionts of the root herbivore *Diabrotica virgifera virgifera* have been reported to suppress the induction of defensive transcripts in maize roots, which may explain the finding of another study that once attacked plants become more susceptible to subsequent *D. v. virgifera* attack. To test this hypothesis, we cured *D. v. virgifera* from its major endosymbiont *Wolbachia* and tested whether endosymbiont-free individuals elicit different defense responses in maize roots. The presence of *Wolbachia* did not alter the induction of defense marker genes and resistance in a susceptible maize hybrid and a resistant line. Furthermore, attacked maize plants emitted the same amount of (*E*)-β-caryophyllene, a volatile signal that serves as foraging cue for both entomopathogenic nematodes and *D. v. virgifera*. Finally, the effectiveness of the entomopathogenic nematode *Heterorhabditis bacteriophora* to infest *D. v. virgifera* was not changed by curing the larvae from their endosymbionts. These results show that the defense mechanisms of maize are not affected by *Wolbachia*. Consequently, *D. v. virgifera* does not seem to derive any plant-defense mediated benefits from its major endosymbiont.

Keywords *Diabrotica virgifera* · *Zea mays* · *Wolbachia* · Plant defense · Suppression · Entomopathogenic nematodes

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Introduction

Symbiotic bacteria influence many important traits of their insect hosts (Clark et al., 2010). They can confer tolerance to heat stress (Montllor et al., 2002), increase resistance to parasitoids and pathogens (Oliver et al., 2003; Kroiss et al., 2010), and improve nutrition by supporting the digestion of plant components (Tokuda and Watanabe, 2007) and by synthesizing essential amino acids (Akman Gündüz and Douglas, 2009). Consequently, facultative endosymbionts can determine host plant specialization (Tsuchida et al., 2004) and pest status (Hosokawa et al., 2007) of certain herbivores. Some recent reports suggest that endosymbiotic bacteria and viruses also may manipulate plant metabolism by disrupting defense responses (Zhu-Salzman et al., 2004) and changing nutrient mobilization patterns (Kaiser et al., 2010). However, the effect of bacterial endosymbionts on plant defenses remains poorly understood (Stout et al., 2006; Felton and Tumlinson, 2008).

An interesting insect model species to study tripartite interactions between plants, herbivores, and their endosymbionts is the western corn rootworm *Diabrotica virgifera virgifera*. In several parts of the world, the larvae of this important insect pest feed and develop nearly exclusively on maize (*Zea mays*) roots. Maize plants respond to *D. v. virgifera* infestation by releasing volatile organic compounds that can attract entomopathogenic nematodes (Rasmann et al., 2005). Although defensive markers like proteinase inhibitors also are induced at the transcriptional level by *D. v. virgifera* attack, infested maize plants do not show any pronounced changes in secondary metabolites (Erb et al., 2012) and do not become more resistant but more susceptible to the herbivore (Robert et al., 2012a); this may in part be explained by the fact that *D. v. virgifera* is tolerant to major maize defensive compounds like benzoxazinoids (Robert et al., 2012c). It also has been found that herbivore-attacked roots have higher concentrations of free amino acids that could increase their nutritional value. Furthermore, roots that have been attacked by *D. v. virgifera* show impaired responses to future attacks, and thus, a possible decrease of their resistance potential (Robert et al., 2012b).

As a root herbivore, *D. v. virgifera* interacts with many soil microbes. Recent studies show for example that infection of maize roots with a phytopathogenic, mycotoxin-producing fungus (*Fusarium verticillioides*) reduces larval development (Kurtz et al., 2010). In turn, *D. v. virgifera* feeding influences the composition of the root microbial community (Dematheis et al., 2012b). The insect's digestive tract also contains numerous microbes, including large quantities of the genus *Wolbachia* (Dematheis et al., 2012a). *Wolbachia* are maternally inherited and infect a wide range of arthropod species (Jeyaprakash and Hoy, 2000; Hilgenboecker et al., 2008). They currently are divided into several supergroups (Bordenstein and Rosengaus, 2005). In the absence of a clear species identification system (Bordenstein and Rosengaus, 2005), we refer to the entire *Wolbachia* genus hereafter.

Wolbachia endosymbionts generally manipulate reproduction of their host by inducing either thelytokous parthenogenesis, cytoplasmic incompatibility upon mating, feminization, and/or male killing (Werren et al., 2008; Engelstadter and Telschow, 2009; Negri et al., 2009) that enhances their own transmission. Such manipulation may negatively affect host fitness (Stouthamer and Luck, 1993). However, some *Wolbachia* strains rapidly evolve and become beneficial for their host (Riegler et al., 2005). For instance, *Wolbachia* can act as a defensive agent by conferring increased resistance in *Drosophila melanogaster* against a range of RNA viruses (Hedges et al., 2008; Teixeira et al., 2008). The presence of *Wolbachia* also has been associated with increased fertility in *Trichogramma bourarachae* (Vavre et al., 1999), *Aedes albopictus* (Dobson et al., 2002), and *Drosophila simulans* as well as *D. melanogaster* (Weeks et al., 2007; Brownlie et al., 2009). Furthermore, in the bed bug *Cimex lectularius*, *Wolbachia* acts as a nutritional mutualist (Hosokawa et al., 2010).

In *D. v. virgifera*, *Wolbachia* are responsible for the sexual incompatibility between *D. v. virgifera* and the closely related subspecies *Diabrotica virgifera zeae* (Giordano et al., 1997). The latter is identical to *D. v. virgifera* in many aspects, including life history and pheromone communication (Krysan et al., 1980), but differs in its geographical range, color and pest status (Clark et al., 2001). The fact that *D. v. virgifera* causes much more damage in agriculture than *D. v. zeae* has led to speculation about a possible role of *Wolbachia* in the interaction between the herbivore and the plant. A recent study reported that curing *D. v. virgifera* from *Wolbachia* using the antibiotic tetracycline drastically changes the transcriptional response of maize roots (Barr et al., 2010): *D. v. virgifera* larvae without *Wolbachia* induced a stronger expression of many defense related genes than did *D. v. virgifera* larvae with *Wolbachia*. The authors concluded that the microbes may help *D. v. virgifera* to suppress plant defenses and that "a reassessment of paradigms involving plant-insect interactions [was] necessary" (Barr et al., 2010).

Based on our own observations (e.g., Erb et al., 2012; Robert et al., 2012a, b) and the study by Barr et al. (2010), we aimed to understand whether the presence of *Wolbachia* in the gut of *D. v. virgifera* is advantageous for the herbivore by either suppressing direct plant resistance or reducing the negative impact of entomopathogenic nematodes. To investigate this question, we measured growth and survival of wild type and *Wolbachia* cured *D. v. virgifera* larvae on a resistant and a susceptible maize line. Additionally, we compared the volatile blends released from maize roots infested by wild-type and *Wolbachia* cured beetle larvae. We also compared infection rates of wild type and *Wolbachia* cured *D. v. virgifera* larvae by nematodes that are attracted by feeding-induced root volatiles. Finally, we quantified the expression of several established defensive marker genes in *D. v. virgifera* roots damaged by wild type or *Wolbachia* cured *D. v. virgifera* larvae.

Methods and Materials

Diabrotica virgifera virgifera Treatment and Rearing *Wolbachia*-infected *D. v. virgifera* were obtained originally from the North Central Agricultural Research Laboratory (NCARL) in Brookings SD, USA, and maintained on freshly germinated maize in the Plant Genetics Research Laboratory at the University of Missouri, Columbia, MO, USA, for several years. *Wolbachia*-free colonies were obtained by feeding *D. v. virgifera* adults with an artificial

diet (Jackson, 1985) containing first tetracycline (Sigma-Aldrich, St. Louis, MO) and later doxycycline hyclate (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the artificial diet initially contained 0.15 % tetracycline from October to December 2008, 0.30 % tetracycline in January 2009, 0.02 % doxycycline hyclate in February 2009. Doxycycline hyclate concentration was increased by 0.01 % per month to 0.08 %. After 2 month at 0.08 %, the percentage doxycycline hyclate was reduced to 0.07 %. This concentration was maintained. All experiments were conducted using *D. v. virgifera* eggs laid by adults that were not treated by antibiotics for at least one generation (W-). *Wolbachia* infected *D. v. virgifera* (W+) were reared under the same conditions, but without any antibiotic treatment.

The *Wolbachia* infection status was verified by PCR prior to experiments (Baldo et al., 2006). Reacquisition of *Wolbachia* during the experiment is unlikely in the absence of horizontal and vertical transfer vectors. Individual insects were pulverized using liquid nitrogen, and DNA was extracted using the DNeasy Blood and Tissue Kit following the manufacture's specifications (Qiagen, Valencia, CA, USA). Primers designed to amplify *cadherin*, an insect gene, and *WSP*, an outer-coat protein specific to *Wolbachia* (Table 1) were used to detect insect and *Wolbachia* DNA, respectively. The 20 μl PCR reaction consisted of 10 μl RedTaq Ready Mix (Sigma-Aldrich, St. Louis, MO), 5 μl of 10 ng μl^{-1} DNA, 1 μl each of the forward and reverse primers at a starting concentration of 25 ng μl^{-1}, and brought to volume with 3 μl of de-ionized distilled water. PCR reactions were amplified on an Eppendorf MasterCycler (Eppendorf, Hamburg, Germany) using the following protocol: 5 min at 95 °C, followed by 40 cycles at 95 °C for 1 min, a primer-specific annealing temperature for 2 min, and 72 °C for 3 min. The annealing temperature for *WSP* was 58 °C and CAD was 62 °C. The amplification protocol was completed with a final extension step of 72 °C for 5 min. Amplification products were visualized on an ethidium bromide stained 2 % agarose gel (Sigma-Aldrich, St. Louis, MO), which had been electrophoresed at 120 V for 30 min to 1 hr or until adequate band separation had been achieved.

Insect Survival and Performance The influence of the presence or absence of *Wolbachia* on *D. v. virgifera* survival and performance was evaluated by measuring the individual weight gain of W- and W+ larvae in three experiments. For the first two experiments, maize plants (*Zea mays* L. var. Delprim) were grown in plastic pots (11 cm high, 4 cm diam) using washed sand (0–4 mm, Jumbo, Switzerland) and commercial soil (Ricoter, Switzerland) in a climate chamber (24 °C, 60 % r.h., 16 L:8D, 50 000 lm/m^2). Twelve-d-old plants with three fully developed leaves were infested with 5 pre-weighed second-instar larvae for 4 day in two independent experiments (n_{W+}=6 and 9 and n_{W-}=10 and 8 respectively). After 4 day, the larvae were recovered and weighed to determine individual relative weight gain and survival. Both experimental runs were pooled for statistical analysis. In a third experiment, *D. v. virgifera* performance on the line CRW3(S1)C6 with resistance to *D. v. virgifera* larval feeding in the field (Hibbard et al., 2007) was evaluated using a seedling bioassay similar to Nowatzki et al. (2008). Each experimental unit consisted of a 33× 19 cm plastic container (5.7 L; Sterilite Corporation, Clinton, SC, USA) filled with a 2:1 mixture of autoclaved soil and ProMix potting soil (Premier Horticulture Inc., Quakertown, PA, USA), ~ 115 CRW3 maize seeds, and water. *Diabrotica v. virgifera* eggs suspended in a 0.15 % agar solution then were dispensed via a pipetter evenly across the soil surface in each container at a maximum rate of 500 eggs/container. All eggs were pre-incubated at 25 °C so that peak egg hatch would occur ~ 1 week following container setup. All containers were watered as needed to keep the soil moist and held in a climate chamber at constant 25 °C and 14 L:10D photoperiod. In addition, a subsample of eggs was dispensed onto moist filter paper in a petri dish and placed near the containers to estimate peak egg hatch. Larvae were allowed to feed and develop on maize roots in containers for 14 day following peak egg hatch, after which the above ground plant tissue was cut from the containers and the remaining contents emptied into modified Berlese funnels. Larvae were collected from Berlese funnels via attached half-pint mason jars filled with ~150 ml water. After 2 and 4 day, larvae were collected from jars and stored in 95 % ethanol. Dry weight and head capsule width were recorded for the recovered larvae. Treatments were arranged in a randomized complete block design and replicated 10 times.

Plant Responses to D. v. virgifera Infestation To evaluate the response of maize plants to *D. v. virgifera*, Delprim seedlings were grown as described above and infested with 5 sec-instar W- and W+ larvae for 24 and 48 hr. Control plants remained uninfested. Plant roots then were gently washed with tap water, flash frozen, and ground to a fine powder in liquid nitrogen (N=6). Gene expression quantification and volatile emissions analysis was performed on the same plants (48 h infestation time point).

Quantification of Defense Gene Expression Quantitative reverse transcriptase real time polymerase chain reactions (Q-RT-PCR) were conducted as previously described (Erb et al., 2010). Briefly, total RNA was purified using Qiagen RNeasy extraction kits following the manufacturer's

instructions. The quality of the extracted RNA was assessed by photometry. cDNA then was synthesized using Invitrogen Super-Script III reverse transcriptase. Q-RT-PCRs were carried out using defense gene-specific primers (Table 1). The q-RT-PCR mix consisted of 5 ul Quantace Sensimix containing Sybr Green I, 3.4 ul H2O, 100 nmol of each primer and 1ul of cDNA sample. q-PCR was achieved by incubating the samples for 10 sec at 95 °C, 20 sec at 60 °C, 15 sec at 72 °C . The final melt curve was obtained by ramping from 68 to 98 °C at a rate of 1 °C every 5 sec. Primer efficiencies and optimal quantification thresholds were determined using dilution series of cDNA mix constituted of 4 ul of every sample. Four 10-fold dilution steps were realized and the obtained standard curve was included into every q-RT-PCR run. Ct values were acquired using the automated threshold determination feature of the Rotor-Gene 6000 software and were corrected for two housekeeping genes GapC (Frey et al., 2000) and actin. In order to get average fold changes of treated plants, all Ct values were normalized to control plants levels. In an additional experiment, we quantified the expression of two differentially regulated transcripts from the study of Barr et al. (2010) (MZ00018372, MZ00036538) in the resistant line CRW3(S1)C6. Plant growth and insect infestation was performed following the protocol of Barr et al. (2010): The apical 10 mm of seminal root tips were harvested 24 h after infestation and immediately frozen in liquid nitrogen. The roots from seven individual plants were pooled to obtain three independent biological replications. RNA was extracted from 100-mg of finely ground tissue using Trizol and RNeasy MinElute Cleanup Kit with an on column DNase treatment (Qiagen, Valencia, CA, USA). First strand cDNA synthesis was completed using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. To assay cDNA synthesis efficiency, a Luciferase-derived control RNA (Promega, Madison, WI, USA) was added at a concentration of 50-pg per cDNA synthesis reaction. The resulting cDNA was subjected to real-time PCR using SYBR GreenER qPCR Mix containing the reference dye ROX (Invitrogen, Life Technologies, Carlsbad, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed on an AB7900HT (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) with the following thermocycler conditions: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min. After the completion of the qPCR, a melting curve analysis was performed ranging from 60 °C to 95 °C. Raw real-time PCR data were analyzed in a regression software package, LinRegPCR (Ramakers et al., 2003) to determine the appropriate quantification cycle (C_q) values and PCR efficiencies for each individual sample. A standard curve method was used to determine relative transcript copy numbers; the standards on the curve were pooled across each biological replication. Copy numbers were then multiplied by an RNA correction factor to correct for differences in the starting amount of RNA between biological samples.

Root Volatile Production Root volatile production was determined by SPME-GC-MS analysis as described (Robert et al., 2012a). Ground roots (0.3 g) were placed in a glass vial (Supelco, 20 ml) with a septum. A 100 μm PDMS solid

Table 1 Primer list for PCR and q-RT-PCR reactions performed in this study

Marker genes	Reference	Putative function	Forward primer (5′-3′)	Reverse primer (5′-3′)
Zm-Actin1	(Erb et al., 2009)	Actin (HKG)	ccatgaggccacgtacaact	ggtaaaaccccactgagga
Zm-B73Lox	(Ton et al., 2007b)	B73 lipoxygenase	gcgacaccatgaccatcaac	gctcggtgaagttccagctc
Zm-Bx1	(Erb et al., 2009)	DIMBOA biosynthesis gene	cccgagcacgtaaagcagat	cttcatgcccctggcatact
Zm-CysII	(Ton et al., 2007a)	Cystatin II proteinase inhibitor	tgccctgctcatactgcttg	gcgagttcctggaggtgaag
Zm-Cyst	(Ton et al., 2007a)	Cystatin proteinase inhibitor	caaggagcacaacaggcaga	ggacatgagctggcgatttt
Zm-GAPC	(Erb et al., 2009)	Glyceraldehyde phosphate dehydrogenase	gcatcaggaaccctgaggaa	catgggtgcatctttgcttg
Zm-Lox8	(Gao et al., 2008)	Jasmonic acid biosynthetic genes	atgaacgtggtggtctgcgtcagg	tacgccaccgacgggttgtt
Zm-Pal	(Erb et al., 2009)	Phenylalanine ammonia lyase	cgaggtcaactccgtgaacg	gctctgcacgtggttggtga
Zm-PR-1	(Erb et al., 2009)	Pathogenesis-related gene 1	ctgggtgtccgagaagcagt	cgggttgtagctgcagatgat
Zm-PR10	(Erb et al., 2009)	Pathogenesis-related gene 10	gtcatgccgttcagcttcat	tgttcttgcactcgacttg
Zm-PR5	(Erb et al., 2009)	Pathogenesis-related gene 5	tgcatgcatgggctagtgat	cgcacacaaatccagctacg
Zm-SerPIN	(Ton et al., 2007a)	Serine proteinase inhibitor	gacggaggaggaaggaggag	acctgatgcactgcttgcac
Zm-TPS23	(Erb et al., 2009)	Terpene synthase	tctggatgatgggagtcttctttg	gcgttgccttcctctgtgg
MZ00018372	(Barr et al., 2010)	PR-8 Class III Chitinase	cctggccagcttcaccac	cttggctcctggaatggtc
MZ00036538	(Barr et al., 2010)	Subtilisin-Chymotrypsin inhibitor	catcttcgtcgacaccgtc	acgaagcacacacatccatt
Insect cadherin	(Siegfried et al., 2005)	Cadherin (HKG)	gcagaacaggaaggtgaagc	tcgtggatccaactattctga
Wolbachia WSP	(Barr et al., 2010)	Outer-coat protein	gtccaatarstgatgargaaac	cygcaccaayagyrctrtaaa

phase micro extraction (SPME; Supelco c/o Sigma-Aldrich Chemie GmbH Buchs, Switzerland) fiber was inserted in the vial and exposed for 20 min at 35 °C. The fiber then was automatically inserted using an autosampler (Gerstel MPS2) into the injector port of a gas chromatograph (Agilent 6890 series GC system G1530A) heated at 250 °C. The sample was injected on an apolar DB-1 column under constant pressure of helium as a gas flow (18.55 psi). Following the injection, the column temperature was maintained at 60 °C for 1 min before ramping to 220 °C at a rate of 10 °C per min. The gas chromatograph was coupled to a quadrupole type mass selective detector (Agilent 5973). (*E*)-β-Caryophyllene was identified based on its retention time and mass spectrum in comparison with a pure standard. Following the SPME-Guidelines of the *Journal of Chemical Ecology*, peak areas were compared for the same compound between different treatments.

Resistance to Nematodes Entomopathogenic nematodes of the species *Heterorhabditis bacteriophora* were obtained from Andermatt Biocontrol AG (Grossdietwil, Switzerland). Twenty entomopathogenic nematodes present in 5 ml tap water were placed on the surface of a 5 cm filter paper disc in a petri dish. Ten second- to third-instar wild type or endosymbiont-cured *D. v. virgifera* larvae were added to the petri dish and initially placed along a transversal row. After 2 hr, *D. v. virgifera* larvae were recovered and placed in a new petri dish with freshly germinated maize. The infection status of the larvae was checked 7 day after contact with the parasites by dissecting the larvae and looking for the presence or absence of nematodes. The experiment was repeated twice, and data were pooled for analysis (N=6 for each replicate).

Statistical Procedures All statistical analyses were conducted using the Sigma Stat 2.0 software. Analysis of Variance (ANOVA) was performed to assess differences between treatments. Experiments that had been performed twice were pooled, and the experimental run was used as an additional factor in the model. If the factor "experiment" was not significant, it was removed from the model. Data were first analyzed using Levene and Kolmogorov–Smirnov tests to determine the heteroscedasticity of error variance and normality, respectively. If these tests showed variance homogeneity and normality, the different treatments were compared using ANOVAs. Pairwise comparisons following ANOVAs were conducted using Tukey's honestly significant difference tests. If the data did not meet the criteria of variance homogeneity and normality, nonparametric Mann–Whitney *U*-tests or Kruskal–Wallis ANOVA on ranks (H-tests) were carried out. Pairwise comparisons were conducted using Dunn's tests.

Results

Effect of Antibiotic Treatment on D. v. virgifera Endosymbionts Treatment with the antibiotics tetracycline and doxycycline resulted in the effective removal of *Wolbachia*, the predominant endosymbiont in the gut of *D. v. virgifera*. The endosymbiont-free strain did not re-acquire *Wolbachia* during the one generation without antibiotics, and all of the tested *D. v. virgifera* individuals were *Wolbachia*-free (Fig. 1).

Diabrotica v. virgifera Performance The performance of cured and non-cured larvae feeding on maize plants did not differ. Four days after the onset of feeding on Delprim seedlings, the larvae had gained similar amounts of weight and had survived with the same probability (Fig. 2a and b). Similar results were found for larvae feeding on the resistant line CRW3(S1)C6 for 2 week (Fig. 2c and d).

Interaction with Natural Enemies The presence of endosymbionts did not alter the interaction of *D. v. virgifera* with one of its main natural enemies: Infection rates by the entomopathogenic nematode *Heterorhabditis steinernema* were similar for cured and non-cured individuals (Fig. 3a). We found a clear induction of the nematode-attractant (*E*)-β-caryophyllene 24 hr and 48 hr after the onset of *D. v. virgifera* attack. However, maize plants attacked by *D. v. virgifera* emitted the same amounts of (*E*)-β-caryophyllene, irrespective of the presence of endosymbionts (Fig. 3b).

Defense Gene Expression We found a strong induction of PR-genes and genes encoding proteinase inhibitors and the (*E*)-β-caryophyllene synthase *Zm*-tps23 (Köllner et al.,

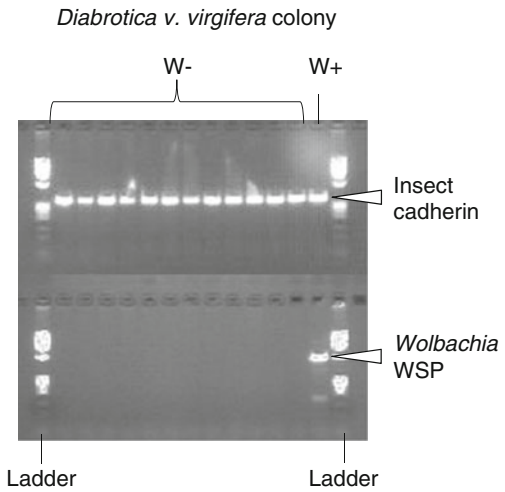

Fig. 1 Diagnostic PCRs of endosymbiont cured (W-) *Diabrotica v. virgifera* individuals. Top arrow: Insect cadherin gene as positive control for the presence of insect DNA. Bottom arrow: *WSP* gene, an outer-coat protein specific to *Wolbachia*. A non-cured individual (W+) was included as positive control

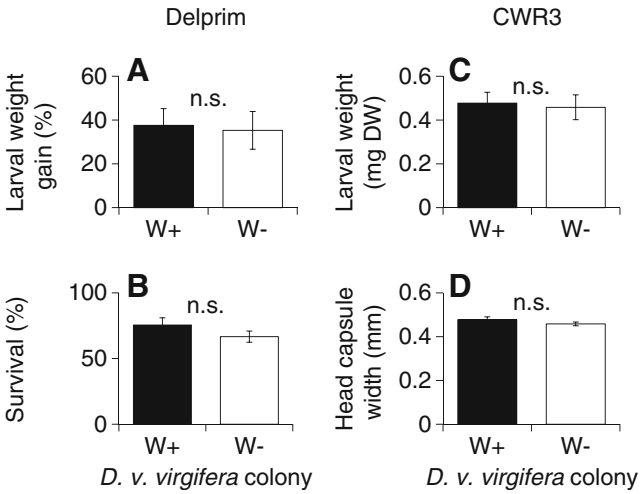

Fig. 2 Performance of wildtype (W+) and endosymbiont-cured (W-) *Diabrotica v. virgifera* larvae on the hybrid maize variety Delprim (**a**, **b**) and the resistant line CRW3(S1)C6 (CRW3, C,D). Means ± SE are shown. N.S. denotes no significant difference between colonies ($P>0.05$)

2008) in maize roots damaged by *D. v. virgifera* larvae (Fig. 4a). In accordance with previous findings, we found no strong induction of genes involved in the biosynthesis of jasmonates or secondary metabolites by the root herbivore, nor was there induction of genes involved in the biosynthesis of phenolic compounds (pal) and benzoxazinoids (bx1). A slight induction of lipoxygenase genes was observed.

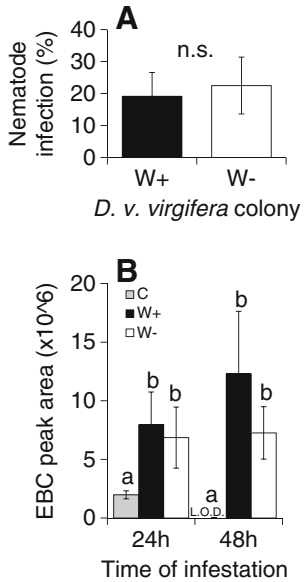

Fig. 3 Influence of *Diabrotica v. virgifera* endosymbionts on natural enemies and indirect plant defense. **a** susceptibility of widtype (W+) and endosymbiont cured (W-) *D. v. virgifera* larvae to the entomopathogenic nematode *Heterorhabditis bacteriophora*. **b** Emission of the attractive volatile (*E*)-β-caryophyllene in control (**c**) and *D. v. virgifera* attacked maize plants (W) 24 hr and 48 hr after the onset of attack. Letters indicate significant differences between treatments within time points ($P<0.05$). L.O.D. Limit of detection

None of the defensive markers were differentially induced by cured or non-cured *D. v. virgifera* larvae 48 hr after the onset of attack (Fig. 4). A similar result was found for the two genes tested in CRW3(S1)C6 24 hr after infestation (Fig. 4b).

Discussion

Endosymbionts influence a broad range of metabolic processes in their host. Interestingly, a previous study had suggested that endosymbionts of *D. v. virgifera* may help the insect to suppress plant defenses. Evidence for this phenomenon came from a microarray study showing that cured *D. v. virgifera* larvae induced an entirely different set of defense-related genes, and that many transcripts, including pathogenesis-related (PR) and proteinase-like genes were more strongly induced by cured than by non-cured larvae (Barr et al., 2010).

Following up on these results, we hypothesized that the suppression of the host-defense response by *Wolbachia* should facilitate the growth of *D. v. virgifera* on maize roots. In an earlier study, we had found that roots attacked by wildtype *D. v. virgifera* larvae become more susceptible to the herbivore (Robert et al., 2012b), and we speculated that this phenomenon may be due to an endosymbiont-mediated suppressive effect. However, in the current study we could not find any effects of the presence or absence of *Wolbachia* and other endosymbionts on the performance of *D. v. virgifera* larvae (Fig. 2). We used both the maize hybrid Delprim, as well as the *D. v. virgifera* resistant line CRW3(S1)C6 as food plants and obtained similar results. Thus, *D. v. virgifera* does not benefit from the presence of *Wolbachia* in its gut by altering host plant resistance.

While insect-derived viruses have been shown to alter plant defenses (Mauck et al., 2010), and at least in the case of the whitefly *Bemisia tabaci* to be beneficial to their insect host (Liu et al., 2010), we are not aware of any studies that demonstrate a positive influence of insect bacterial endosymbionts on herbivore fitness via endosymbiont-mediated suppressive effects on plant defense. In vertebrate systems, surface proteins of *Wolbachia* endosymbionts of filarial nematode parasites have been shown to elicit immune responses by the vertebrate host (Brattig et al., 2004). Clearly, further research is required to understand the impact of insect-associated bacteria on plant defense and resistance.

In this study, we also investigated whether *D. v. virgifera* endosymbionts affect the attraction of entomopathogenic nematodes and/or the infection of larvae with this parasite. Neither the resistance of the larvae to nematode infection nor the induction of the attractive volatile, (*E*)-β-caryophyllene, in maize roots was altered by curing *D. v. virgifera* from

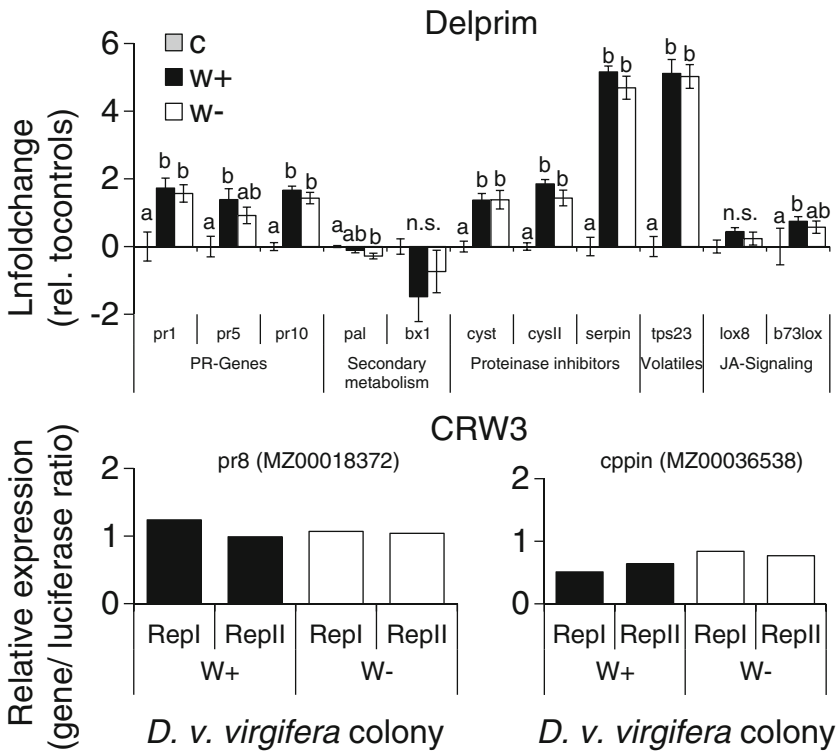

Fig. 4 Induction of root defensive marker genes by *Diabrotica v. virgifera*. **a** Ln-fold changes of defensive markers in the maize hybrid Delprim relative to control levels are shown. Maize plants were attacked by wildtype (W+) or endosymbiont-cured (W-) *D. v. virgifera* larvae for 48 hr. Letters indicate significant differences between treatments. **b** Expression levels of two defensive marker genes 24 hr after *D. v. virgifera* attack in the resistant line CRW3(S1)C6 (CRW3). Results of two independent replicates (7 pooled plants each) are shown

endosymbionts (Fig. 3). The quantity of (*E*)-β-caryophyllene emitted from maize roots has a direct influence on their attractiveness to certain entomopathogenic nematodes (Rasmann and Turlings, 2007). As the emission of (*E*)-β-caryophyllene from maize root attacked by wildtype and endosymbiont-cured larvae was similar, the tritrophic system of maize, *D. v. virgifera* and entomopathogenic nematodes is likely to remain unaltered by the endosymbionts of the herbivore. In *D. melanogaster*, it has been found that *Wolbachia* induces resistance against RNA viruses (Hedges et al., 2008; Teixeira et al., 2008) and the pathogenic fungus *Beauveria bassiana* (Panteleev et al., 2007); it has been speculated that this effect may be the result of an activation of innate immunity by the endosymbiont (but see Rancès et al., 2012). Other reports show no positive effect of the presence of *Wolbachia* on host immunity.

In *Drosophila simulans*, the endosymbiont even reduced immune responses to *B. bassiana* and resistance against *Leptopilina heterotoma*, a parasitoid wasp, and in *Spodoptera exempta*, *Wolbachia* increases susceptibility to a nucleopolydrovirus (Graham et al., 2012). In this context, it has been proposed that *Wolbachia* broadly stimulates immunity in the early association phase only and that this effect disappears over evolutionary time, as host and endosymbiont adapt to each other (Cook and McGraw, 2009). Our results are in tune with this hypothesis, as *D. v. virgifera* is likely to have already acquired *Wolbachia* during the separation from *D. v. zeae* (Giordano et al., 1997).

To verify the observations by Barr et al. (2010) that i) there is little overlap between the transcript signature of maize roots after attack by wildtype and cured *D. v. virgifera* larvae and that ii) defensive marker genes are less induced in the presence of endosymbionts, we profiled the expression of an established set of defensive marker genes in maize following *D. v. virgifera* attack. The set comprised PR and proteinase inhibitor genes as well as genes involved in the production of volatile and non-volatile secondary metabolites and the defensive hormone jasmonic acid. All these markers have been used before as indicators of defensive induction in maize (Erb et al., 2009; Robert et al., 2012b). We found most of the markers to be strongly induced by *D. v. virgifera* attack. However, in accordance with the performance and volatile measurements, we did not detect any differences in induction between the wild type and endosymbiont-cured strain (Fig. 4). To evaluate whether the lack of suppression is due to the fact that we used a different plant variety, we also measured the expression of two defenses genes, a PR-8 Class III chitinase and a chymotrypsin inhibitor homologue, which also were measured by Barr et al. (2010) in the maize line CRW3(S1)C6, using a similar growth and infection protocol. Again, we did not observe any suppression in roots damaged by *Wolbachia*-infected larvae.

There are a number of reasons that could have contributed to the striking discrepancies in gene expression between the study of Barr et al. (2010) and the results presented here. First, it is possible that the *D. v. virgifera* strains treated with antibiotics and used for our experiments may have lost or regained symbionts other than *Wolbachia* compared to the

untreated wildtype strain; these changes would not have been detected by our *Wolbachia*-specific PCR approach. Second, our gene expression profiling only encompassed a number of selected genes and time points, and it is possible that we may have missed differentially regulated transcripts that would have been picked up in a transcriptome-wide microarray approach with high temporal resolution. However, given the striking absence of any difference in direct and indirect defenses, it seems unlikely that the presence of *Wolbachia* in the digestive tract of *D. v. virgifera* has any significant influence on the defensive capacity of maize plants against this pest.

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