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Phloem-feeding whiteflies can fool their host plants, but not their parasitoids

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Summary

1. Herbivore attack induces plants to mobilize chemical defences, including the release of volatiles that attract natural enemies of the herbivore. This commonly involves the jasmonic acid (JA) pathway. However, phloem-feeding whiteflies specifically trigger salicylic acid (SA)-signalling, thereby suppressing JA-based defences and enhancing host plant suitability.

2. Here, we show with *Arabidopsis thaliana* plants that the whitefly parasitoid *Encarsia formosa* outsmarts this apparent host plant manipulation by exploiting the SA-triggered emission of β-myrcene. Assays with various *Arabidopsis* mutants and phytohormone and gene-expression analyses reveal that the whiteflies induce the accumulation of endogenous SA, thereby enhancing the expression of SA-regulated genes, one of which encodes ocimene/myrcene synthase, which resulted in the recruitment of parasitoids under greenhouse conditions. Performance assays confirmed that whiteflies directly benefit from suppressing JA-based defences.

3. Taken together, we conclude that by activating SA-signalling whitefly feeding suppresses direct, JA-based defences, but that parasitoids can adapt to this by exploiting specific, SA-induced volatile emissions for host location.

4. Our work further confirms that herbivory contributes to selective pressure governing the evolution of inducible volatile signals as indirect plant defences.

Key-words: *Bemisia tabaci*, *Encarsia formosa*, indirect plant defence, phloem-feeding herbivore, plant–herbivore interactions, salicylic acid

Introduction

Upon herbivore attack, plants can employ a variety of defence mechanisms, some of which directly influence herbivore performance and others that attract natural enemies of the herbivore, thus defending the plant indirectly. A proposed indirect plant defence, the release of volatile compounds specifically after herbivory, is known to attract parasitoids and predators of the herbivores (Dicke *et al.* 1990; Turlings, Tumlinson & Lewis 1990; Dicke 2009). Field studies confirm the role of such signals in host and prey location (Thaler 1999; Kessler & Baldwin 2001; Poelman *et al.* 2009; Schuman, Barthel & Baldwin 2012), but their defensive function remains a topic of discussion (Janssen, Sabelis & Bruin 2002; Hare 2011; Kessler & Heil 2011). The signal-transduction pathway involved in the production of volatiles in response to tissue-damaging herbivores, such as caterpillars and mites, has been extensively investigated (Thaler *et al.* 2002; Van Poecke & Dicke 2002; Ament *et al.* 2004; Bruinsma *et al.* 2009). It has been consistently shown that the octadecanoid pathway, with the plant hormone jasmonic acid (JA) as central component, not only plays a key role in regulating direct plant defences against herbivores, but also in the release of volatiles induced by caterpillars or mites (Thaler *et al.* 2002; Schmelz *et al.* 2003; Ament *et al.* 2004; Arimura *et al.* 2008; Bruinsma *et al.* 2009). Blocking the JA signalling

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pathway results in the reduction in volatile emission (Engelberth *et al*. 2001), and consequently, attenuates the attraction of predators to herbivore-damaged plants (Thaler *et al*. 2002; Ament *et al*. 2004; Zhang *et al*. 2009; Wei *et al*. 2011).

Inducible plant volatiles have also been extensively reported as mediating the interactions between plants and the natural enemies of phloem-feeding insects (Du *et al*. 1998; Birkett *et al*. 2003; Girling *et al*. 2006; Blande, Pickett & Poppy 2007). Unlike chewing herbivores, phloem feeders do not inflict extensive tissue damage as they use their stylet to access the vascular tissue to extract phloem (Tjallingii & Hogen Esch 1993; Walling 2000) and they therefore do not activate pathways that involve JA or ethylene (ET) (Moran & Thompson 2001; Kempema *et al*. 2007; Zarate, Kempema & Walling 2007). In fact, accumulating evidence indicates that phloem-feeding insects, similar to biotrophic pathogens, by triggering salicylic acid (SA)-signalling, can suppress JA-regulated defences, leading to enhanced performance on their host plants (Zhu-Salzman *et al*. 2004; Kempema *et al*. 2007; Zarate, Kempema & Walling 2007; Pieterse *et al*. 2012). For example, in response to whitefly nymph feeding, SA-regulated genes or proteins increase locally and systemically, while JA- and ET-regulated genes or proteins remain unchanged or decline, for example in *Arabidopsis* (Van de Ven *et al*. 2000; Zarate, Kempema & Walling 2007). This has also been shown to lead to a reduction in emissions of JA-dependent volatiles in Lima bean (Zhang *et al*. 2009). How then do parasitoids of phloem feeders manage to use plant volatiles to find their hosts?

The co-evolutionary arm races between plant and herbivore should eventually lead plants to evolve ways to recognize the phloem-feeding insects through specific elicitors and to respond by launching specific defences against the sucking attackers (Felton & Tumlinson 2008; Hilker & Meiners 2010; Erb, Meldau & Howe 2012). In crop plants, however, the absence of natural selection may prevent this evolutionary route to herbivore recognition, possibly explaining their tremendous success as pests. In contrast, natural selection readily operates on parasitoids that specialize on sucking insects, and it can be expected that they have adapted to exploit volatiles that are typically induced by such insects as foraging cues. With this in mind, we hypothesized that the SA-signalling pathway plays a key role in volatile-mediated indirect defence against phloem-feeding insects. This hypothesis seems to be supported by emerging evidence that the SA-signalling pathway plays a role in regulating certain herbivore-induced plant volatile emissions (Ozawa *et al*. 2000; Van Poecke & Dicke 2002), and that the methyl ester of SA (MeSA) is a herbivore-induced attractant for predators (De Boer, Posthumus & Dicke 2004; Zhu & Park 2005).

The whitefly *Bemisia tabaci* is a typical phloem-feeding insect, which is known to cause extensive agricultural damage in temperate climates around the world. *Bemisia tabaci* is a generalist pest of *Brassica* species (McKenzie, Anderson & Villarreal 2004); therefore, studies on the related model plant *Arabidopsis* are relevant and will advance our understanding of the underlying mechanisms involved in volatile-mediated attraction of the natural enemies of whiteflies. In the present study, we investigated the role of SA pathway in triggering the release of volatiles that attract the specialist parasitoid *Encarsia formosa* (Hoddle, Van Driesche & Sanderson 1998) towards *Arabidopsis thaliana* plants infested with *B. tabaci*. We compared the response of *E. formosa* to volatiles from *B. tabaci*-infested wild-type (WT) *Arabidopsis*, and a number of mutant lines, including *dde2-2* (JA-deficient; Von Malek *et al*. 2002), *ein2-1* (ET-insensitive; Guzman & Ecker 1990), *NahG* (SA-deficient; Delaney *et al*. 1994), *npr-1* (SA-insensitive; Cao *et al*. 1997) and *cpr-6* (constitutively high SA level; Clarke *et al*. 1998). We correlated the parasitoids responses to changes in volatile emissions and in endogenous JA and SA levels in response to *B. tabaci* infestation, as well as in the transcript levels of JA- and SA-dependent genes. Finally, we conducted bioassays under greenhouse conditions to determine whether uninfested *cpr-6* plants or *B. tabaci*-infested WT plants recruited more parasitoids than SA-impaired plants. With these experiments, we provide the first evidence that parasitoids of whiteflies have evolved to exploit plant volatile emissions that are regulated by SA-signalling pathway. This was further confirmed under simulated field conditions in which plants exhibiting constitutive or inducible activation of the SA-signalling pathway were better at recruiting parasitoids.

Materials and methods

PLANTS AND INSECTS

Arabidopsis thaliana ecotype Columbia (Col-0; WT) and the mutant lines, *dde2-2*, *NahG*, *npr-1*, *ein2-1* and *cpr-6* were grown from seed in a climate room (22 ± 1 °C, R.H. 60–70%, 8L:16D) under a light intensity of *c*. 50 μmol m^{-2} s^{-1}. Two weeks later, the seedlings were transferred to plastic pots (5 cm in diameter) containing a soil mixture (Baltisches substrate, HAWITA BALTIC®, Hawita-Gruppe GmbH, Germany) that was autoclaved at 80 °C for 2 h. To ensure *cpr-6* plant had comparable rosette sizes relative to WT plant, 8–10-week-old *cpr-6* plants were used. For the WT plant and remaining mutant lines, 6–8-week-old plants were used. The mutant lines are all in the Col-0 background.

A colony of virus-free whitefly *B. tabaci* (Gennadius) MEAM1 (Hemiptera: Aleyrodidae) was maintained on *Brassica oleracea* plants (var. capitata cv. Jing-feng No. 1) in a separate greenhouse compartment (25 ± 2 °C, R.H. 50–70%, 14L:10D). The parasitoid wasp, *E. formosa*, was reared on nymphs of *B. tabaci* that were reared on *B. oleracea* plants under greenhouse conditions (25 ± 2 °C, R.H. 50–70%, 14L:10D). Because it was not possible to maintain a continuous culture of *E. formosa* on *B. tabaci* reared on *Arabidopsis*, all wasps were provided with a WT *Arabidopsis* plant heavily infested with *B. tabaci* nymphs (2nd and 3rd instars), and allowed to gain oviposition experience on the plant for 24 h before each bioassay. Various studies have shown that parasitoids acquire a strong preference for plant volatile cues that they perceived during previous successful oviposition experience (Blande, Pickett & Poppy 2007). The wasps were about 2–5 days

old when tested. Olfactory bioassay was performed between 12·00 and 15·00 h, when wasps were observed to be most active.

PLANT TREATMENTS

Plants were subjected to the following treatments:

1. Whitefly treatment: Fifty adult whiteflies were introduced onto a plant that was kept in a ventilated cage (21·0 cm high, 13·5 cm diam). Whiteflies were allowed to feed on the plant for 1, 3, 7 and 10 days, respectively.
2. Jasmonic acid treatment: Each plant was sprayed with 1 mL of 1·0 mM JA (Sigma-Aldrich, St. Louis, MO, USA) solution (containing 0·1% Tween 20). Treated plants were used for olfactory tests 24 h after JA application.
3. Salicylic acid treatment: Each plant was sprayed with 1 mL of 1·0 mM SA (Sigma-Aldrich, St. Louis, MO, USA) solution (containing 0·1% Tween 20). Treated plants were used for olfactory tests 24 h after SA application.
4. Undamaged plant treatment: Intact plants were sprayed with 1 mL of water (containing 0·1% Tween 20) and used as controls for comparison with JA- or SA-treated plants. Intact plants, that received no treatment, were used as controls for comparison with whitefly-damaged plants.

OLFACTOMETER TESTS

Behavioural responses of parasitoids to plant volatiles were investigated in a Y-tube olfactometer. The transparent glass Y-tube olfactometer had a 6 cm stem and two 6 cm arms at a 75° angle, and its internal diameter measured 0·5 cm. Two streams of purified air (filtered through activated charcoal) were each led through a 2·5 L glass container into the olfactometer arms at 100 mL min^{-1}. The base of the olfactometer was connected to a vacuum line generating a flow of 200 mL min^{-1}. The experiment started with the release of individual wasp at the base of the Y-tube. Each wasp was observed for a maximum of 10 min, and a choice for one of the two odour sources was recorded when the wasp reached the end of either arm and stayed in that arm for at least 15 s. When the wasp did not make a choice within 10 min, a 'no choice' was recorded. Each wasp was used only once. After testing five wasps, odour sources were interchanged to avoid any influence of unforeseen asymmetries in the set-up. Four plants of each treatment were used as an odour source. This experiment was repeated for 3–4 days with 10–15 wasps per day for each odour comparison.

As β-myrcene was the only component which was significantly induced by *B. tabaci* infestation, we subsequently examined the effect of β-myrcene on the attraction of parasitoids. Synthetic β-myrcene (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in hexane, and the dose of β-myrcene solution was 36 ng μL^{-1}. In a Y-tube olfactometer, β-myrcene solution emitted from a 10-μL-glass microcapillary was added downwind of the WT plants in one arm, and hexane emitted from microcapillary was added downwind of the WT plants in another arm. The release rate of β-myrcene from glass microcapillary is 360 ng h^{-1}, which is within the range of release rates that can be measured for β-myrcene emitted from whitefly-infested *Arabidopsis* plants. The behavioural response of *E. formosa* wasps towards the two odour sources was tested.

VOLATILE COLLECTION AND ANALYSIS

Headspace volatile samples were collected as described in detail by Zhang *et al.* (2013). The four plants were placed together in a 2·5-L-glass jar. After 4 h of trapping under continuous light, fresh weight of the four plants was measured. For each treatment, the volatile collection was repeated five times and each on each experimental day (replication) collections was made for each treatment in parallel.

Sample analyses were carried out with a Shimadzu GC-2010 plus GC-MS (Shimadzu, Kyoto, Japan) equipped with an Rxi-5MS (30 m–0·32 mm i.d., 0·25 μm film thickness) column in splitless mode. Column effluent was ionized by electron impact ionization (70 eV). Mass scanning was done from 33 to 300 m/z. The temperature programmes of the GC were as follows: 40 °C (4-min hold), 10 °C min^{-1} to 250 °C (5-min hold). Compounds were identified using GC-MS solution software (Shimadzu) by comparing the mass spectra with those of authentic standards or with NIST 08 spectra. Quantification of identified compounds was based on comparison with a set of authentic compounds (including (Z)-3-hexen-1-ol, linalool, methyl salicylate, and β-myrcene; Sigma-Aldrich, St. Louis, MO, USA) injected in five concentrations ranging from 2·5 ng to 40 ng μL^{-1} methanol. Response factors were linear for all reference compounds within this concentration range.

QUANTIFICATION OF ENDOGENOUS JA AND FREE SA

To determine whether or not *B. tabaci* infestation induces JA or SA in *Arabidopsis* plants, we assessed the kinetics of the JA and free SA titres in *B. tabaci*-infested or undamaged WT plants. We collected the leaf samples at 1, 3, 7, 10 days after whitefly infestation. Also, we compared the free SA titres in uninfested WT (Col-0) and *cpr-6* plants. Twelve plants were used for each treatment, and leaves collected from three individual plants were pooled as one biological replicate. Extraction and quantification of endogenous JA and free SA followed a protocol modified from that of Schulze *et al.* (2006). In brief, plant material (250–300 mg) was frozen and ground under liquid nitrogen. For quantification, [9,10-$^2H_2$]-9,10-dihydro-JA (250 ng) and [3,4,5,6-$^2H_4$] SA (500 ng) were added as internal standards. Samples were analysed with a Finnigan GCQ ion trap mass spectrometer (Thermoelectron, Bremen, Germany). The instrument was run in a CI-negative ion mode.

QUANTITATIVE REAL-TIME PCR

To further verify which defensive pathways were affected by *B. tabaci*, we measured the expression of five genes in leaves of WT plants infested with *B. tabaci* and compared it with the expression in uninfested leaves. Phytoalexin deficient4 (*PAD4*) and pathogen-related protein (*PR1*) are two genes which are known to function upstream and downstream of SA, respectively (Jirage *et al.* 2001). Lipoxygenase (*LOX2*) is a key enzyme in the biosynthesis of JA along the octadecanoid pathway (Bell, Creelman & Mullet 1995). 12-Oxophytodienoate reductase 3 (*OPR3*) is the isoenzyme involved in JA biosynthesis (Schaller *et al.* 2000). Terpene synthase 10 (*TPS10*) codes for the enzyme ocimene/myrcene synthase (Bohlmann *et al.* 2002). Nine plants were used for each treatment, and three leaves from three individual plants were pooled as one biological replicate. Total RNA extraction, purification and cDNA synthesis were done as described by Zhang *et al.* (2011). To quantify *LOX2*, *OPR3*, *PR1, PAD4* and *TPS10* transcript levels in different samples, real-time quantitative RT-PCR was performed. The real-time PCR was carried out on an ABI 7500 Real-Time PCR System with a 96-well rotor. The amplification reactions were performed in 20 μL final volume containing 10 μL of iQ™ SYBR® supermix (BioRad, Hangzhou, China), 0·8 μL of forward primer (5 μM) and reverse primer (5 μM) pairs and 2 μL cDNA first strand template. Thermal cycling conditions were 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 55–62 °C (see Table S1, Supporting Information) and 30 s at 72 °C. Subsequently, melting curve was recorded between 60 and 95 °C with the hold every 5 s. Primers used for quantitative RT-PCR are given in Table S1 (Supporting Information). All

reactions were run in duplicate, and average values were used in the analyses. Normalized gene expression was calculated using $2^{-\Delta C_t}$ method with *GAPDH* as endogenous control gene, and subsequently $\log_2$ transformed.

RECRUITMENT OF PARASITOIDS UNDER GREENHOUSE CONDITIONS

To determine whether plants exhibiting constitutive or inducible activation of SA-signalling pathway indeed recruit more parasitoids than plants without SA expression, we did a bioassay in a glass-walled greenhouse (12·1 × 6·5 m). In the first experiment, we compared parasitism on *B. tabaci*-infested WT plants that were surrounded by similarly infested plants, with parasitism on infested WT plants that were surrounded by uninfested WT plants. In the next experiment, we compared the number of parasitized nymphs of *B. tabaci* on infested WT plants surrounded by uninfested *cpr-6* (SA overproducing) plants with parasitism on infested WT plants surrounded by uninfested WT plants. The experiments were conducted from the end of March until the end of April, 2012. Prior to the bioassay, a group of whiteflies (50 adults per plant) were caged with WT plants and allowed to oviposit on these plants for 48 h in a climate room (22 ± 1 °C, R.H. 60–70%, 8L:16D); thereafter, all whitefly adults were removed. About 20 days later when most eggs had developed to 3rd instar nymphs (>100 nymphs per plant), WT plants carrying nymphs were used as oviposition target of wasps in experiments. Briefly, one WT plant carrying nymphs surrounded by five uninfested *cpr-6* plants, or by five WT plants pre-infested with 50 adults of *B. tabaci* for 3 days, was regarded as treatment groups. Another WT plant carrying nymphs surrounded by five uninfested WT plants was regarded as control group. One control group and one treatment group were oppositely placed in a ventilated cage (70 × 60 × 45 cm). The two groups were placed about 50 cm apart. At 9:00 h, 20 wasps were released in the centre of the cage between the two groups. Two days later, the wasps were carefully removed from the WT plants carrying nymphs. Thereafter, the WT plants carrying nymphs were individually placed into a ventilated cage (21·0 cm high, 13·5 cm diam) and kept in a climate room (22 ± 1 °C, R.H. 60–70%, 8L:16D). Two weeks later, the number of nymphs parasitized by wasps was recorded. Each bioassay was conducted on four or five different days to account for day-to-day variation. The position of the plant groups was randomly changed between replicates.

STATISTICAL ANALYSIS

Binomial tests were performed to analyse the Y-tube olfactometer experiments. Parasitoids that did not make a choice were excluded from the analysis. Fisher's protected least significant difference (PLSD) tests of ANOVA was used to analyse volatile and phytohormone data. The data of gene expression and the number of parasitized nymphs were statistically analysed by one-way ANOVA.

Results

CHOICES OF PARASITIC WASPS IN Y-TUBE OLFACTOMETER

In dual-choice bioassays, *E. formosa* wasps showed a significant attraction to the volatiles from WT plants 3, 7 and 10 days after *B. tabaci* infestation, when the volatiles from uninfested WT plants were offered as an alternative (binomial test; $P = 0{\cdot}008$, $P = 0{\cdot}02$, $P = 0{\cdot}01$, respectively;

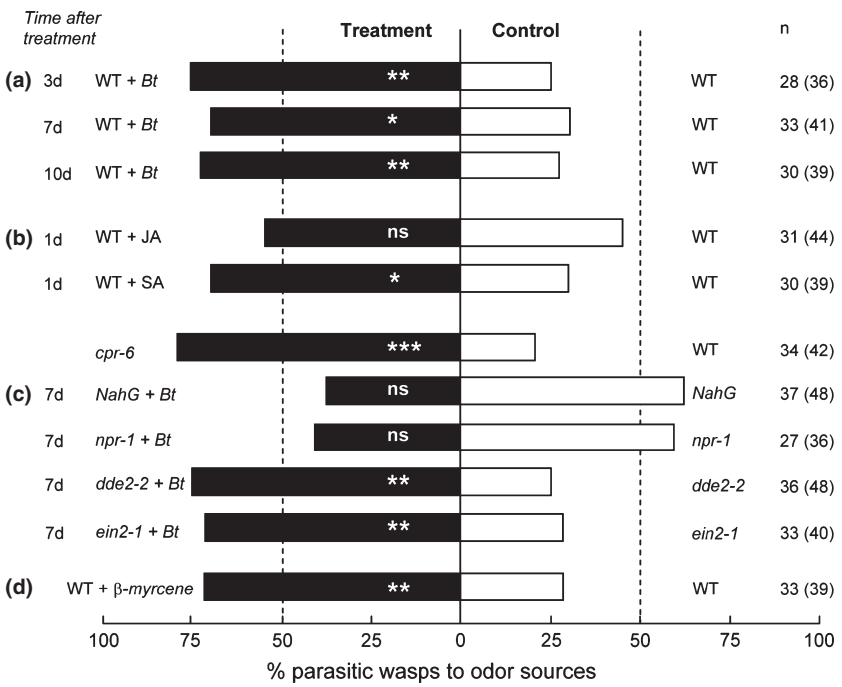

Fig. 1. Responses of *Encarsia formosa* wasps to different plants in a Y-tube olfactometer. (a) Attraction of *E. formosa* to volatiles emitted from wild-type (WT) *Arabidopsis* infested with *Bemisia tabaci* (*Bt*); (b) Attraction of *E. formosa* to volatiles emitted from WT *Arabidopsis* treated with jasmonic acid or salicylic acid; (c) Attraction of *E. formosa* to volatiles emitted from uninfested or infested *Arabidopsis* mutants. (d) Effect of spiking WT *Arabidopsis* with β-myrcene on the attraction of *E. formosa*. Bars represent the percentages of wasps choosing either of the odour sources. The numbers to the right of bars represent the number of wasps making a choice, and the total number of wasps used in olfactometer tests is indicated in parentheses. Asterisks indicate significant differences from a 50:50 distribution (binomial test; *, $P < 0{\cdot}05$, **, $P < 0{\cdot}01$; ***, $P < 0{\cdot}001$; ns, not significant).

Fig. 1a). Likewise, the wasps showed a significant attraction to the volatiles from SA-treated WT plants, but not the volatiles from JA-treated WT plants, when respective control plants were offered as an alternative (binomial test, $P = 0{\cdot}03$, $P = 0{\cdot}55$, respectively; Fig. 1b). To further assess the potential roles of JA, ET and SA-signalling pathways in mediating the attraction of *E. formosa*, the response of wasps to the volatiles from mutants, including *dde2-2* (JA-deficient), *ein2-1* (ET-insensitive), *NahG* (SA-deficient), *npr-1* (SA-insensitive) and *cpr-6* (constitutively high SA level), was tested. Wasps preferred the volatiles from uninfested *cpr-6* plants over uninfested WT plants (binomial test, $P < 0{\cdot}001$; Fig. 1c). Wasps did not prefer the volatiles from *B. tabaci*-infested *NahG* or *npr-1* plants over their respective uninfested plants (binomial test; $P = 0{\cdot}14$, $P = 0{\cdot}34$, respectively); in contrast, the wasps significantly preferred the volatiles from *B. tabaci*-infested *dde2-2* and *ein2-1* plants, over their respective uninfested plants (binomial test, $P = 0{\cdot}004$, $P = 0{\cdot}009$, respectively; Fig. 1c).

In a dual-choice bioassay, the wasps preferred the odour blend from WT plants supplemented with additional β-myrcene over the blend from WT plants alone (binomial test, $P = 0{\cdot}009$; Fig. 1d), which indicated that the enhanced emission of β-myrcene from *B. tabaci*-infested WT plants plays an important role in attracting *E. formosa*.

VOLATILE BLENDS FROM *ARABIDOPSIS* PLANTS

Gas chromatography-mass spectrometry (GC-MS) analysis showed that 14 major compounds were consistently released from uninfested and *B. tabaci*-infested WT plants (Fig. 2). Quantitative analysis further revealed that the amount of the monoterpene β-myrcene (4·6% of total emission) from plants infested with *B. tabaci* was significantly increased as compared to the amount (1·9% of total emission) emitted from uninfested plants (ANOVA, $P = 0{\cdot}005$; Fig. 2).

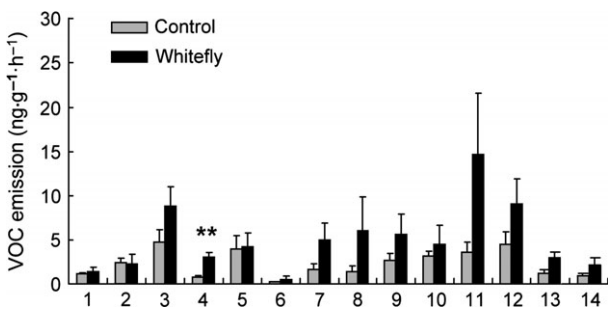

Fig. 2. Volatile compounds identified in the headspace of wild-type *Arabidopsis* plants. Comparison of mean (±SE) ($n = 5$) emission rate of volatiles from undamaged plants (Control) and plants infested with *Bemisia tabaci* (Whitefly) for 3 days. Compound numbers represent: 1, hexanal; 2, (Z)-3-hexen-1-ol; 3, octanal; 4, β-myrcene; 5, limonene; 6, linalool; 7, α-farnesene; 8, methyl salicylate; 9, (*E*)-β-ocimene; 10, dodecanal; 11, unknown; 12, (*E*, *E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT); 13, nonanal; 14, decanal. Asterisks represent significant differences (**, $P < 0{\cdot}01$) from undamaged plants as determined by Fisher's protected least significant difference test of ANOVA.

WHITEFLY PERFORMANCE

The nymphal development whitefly on the different genotypes fits with the key finding by Zarate, Kempema & Walling (2007) that by suppressing JA activation, they render the plants more suitable for themselves and their offspring (see Appendix S1, Supporting Information). Our data showed that nymphal development was significantly accelerated on JA-deficient mutant *dde2-2* (80·4% fourth instars; $P = 0{\cdot}001$) compared to WT (Col-0) plants (31·2% fourth instars; see Fig. S1, Supporting Information). Similarly, insects on *ein2-1* mutant, which does not perceive ET, showed accelerated development trends (61·3% fourth instars; $P = 0{\cdot}001$; see Fig. S1, Supporting Information). In contrast, insects on SA-defective *NahG* and *npr-1* mutants showed delayed development trends (18·1% and 19·5% fourth instars, respectively; $P = 0{\cdot}002$, $P = 0{\cdot}01$, respectively; see Fig. S1, Supporting Information). Taken together, these data suggested that reduced JA or ET responses compromised *Arabidopsis* direct defence against the whiteflies, whereas reduced SA responses enhanced *Arabidopsis* direct defence against the whiteflies.

QUANTIFICATION OF ENDOGENOUS JA AND FREE SA

The amount of free SA in leaves infested with *B. tabaci* did not differ from the amount in uninfested leaves at 1 and 3 days after infestation (Fig. 3a). At 7 and 10 days after infestation, the amount of free SA significantly increased in *B. tabaci*-infested leaves compared to uninfested leaves (ANOVA, $P = 0{\cdot}02$, $P = 0{\cdot}03$, respectively; Fig. 3a).

We did not detect JA in uninfested and *B. tabaci*-infested leaves at any time points. However, it is possible that the JA level exhibits a transient burst at a time point that was missed in our analyses (Engelberth *et al.* 2001) or that the amount of JA in the leaves was below the detection limit. By contrast, the amount of free SA in uninfested *cpr-6* plants was significantly higher than that in uninfested Col-0 plants (ANOVA, $P = 0{\cdot}003$; Fig. 3b), which is consistent with previous findings (Clarke *et al.* 1998).

GENE-EXPRESSION CHANGES IN RESPONSE TO *B. TABACI* INFESTATION

PAD4 transcript level was significantly induced by *B. tabaci* at 3 days after infestation (ANOVA, $P = 0{\cdot}027$), but not at 1, 7 and 10 days after infestation (Fig. 4). Infestation by *B. tabaci* after 1 day did not affect *PR1* transcript level, but after 3, 7 and 10 days of infestation *PR1* transcript levels had significantly increased (ANOVA, $P < 0{\cdot}001$, $P < 0{\cdot}001$, $P < 0{\cdot}001$, respectively; Fig. 4). *Bemisia tabaci* caused a significant reduction in *LOX2* and *OPR3* transcript levels at 1 and 3 days after infestation (ANOVA,

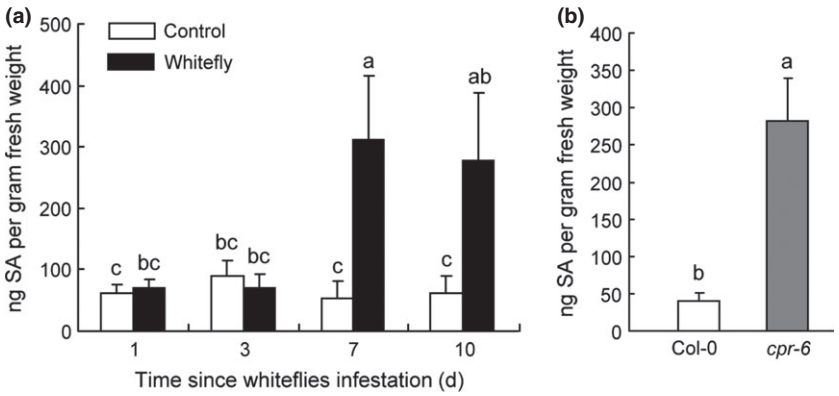

Fig. 3. Quantification of free salicylic acid (SA) levels. (a) Mean (±SE, $n = 4$) concentrations of free SA detected in undamaged (Control) and *Bemisia tabaci*-infested (Whitefly) leaves of wild-type (WT) *Arabidopsis* plants at different time points. (b) Mean (±SE, $n = 5$) concentrations of free SA detected in undamaged leaves of WT (Col-0) and *cpr-6* plants. Different letters above bars indicate significant differences in the quantities between control and treatment (Fisher's protected least significant difference test of ANOVA, $P < 0{\cdot}05$).

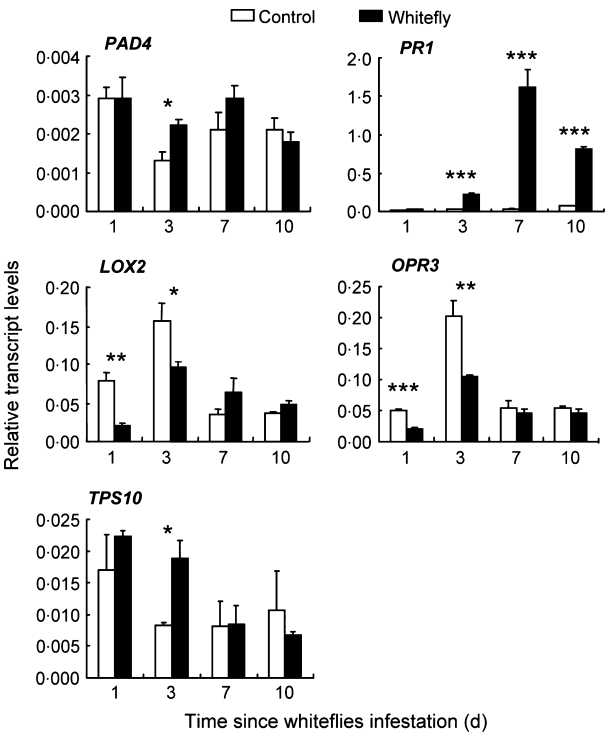

Fig. 4. Gene expressions in response to *Bemisia tabaci* infestation. Time-related changes in transcript levels of *PAD4*, *PR1*, *LOX2*, *OPR3* and *TPS10* in undamaged (Control) and *B. tabaci*-infested (Whitefly) leaves of wild-type *Arabidopsis* plants. The relative gene expression was calculated as $2^{-\Delta C_t}$ method and subsequently $\log_2$-transformed. Values are shown as the mean (±SE) of three biological replicates. Asterisks represent significant differences from control plants as determined by one-way ANOVA (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$).

LOX2: $P = 0{\cdot}004$, $P = 0{\cdot}05$, respectively; *OPR3*: $P < 0{\cdot}001$, $P = 0{\cdot}009$, respectively), but not at 7 and 10 days after infestation (Fig. 4). *Bemisia tabaci* significantly induced *TPS10* transcript levels 3 days after infestation (ANOVA, $P = 0{\cdot}02$), but not 1, 7 and 10 days after infestation (Fig. 4).

GENE-EXPRESSION CHANGES IN RESPONSE TO EXOGENOUS SA TREATMENT

Transcript level of *TPS10* was not affected 2 h after SA application, but after 6 and 24 h of application *TPS10* transcript levels were significantly higher, as compared to untreated plants (ANOVA, $P = 0{\cdot}02$, $P < 0{\cdot}001$, respectively; Fig. 5). Similarly, SA treatment significantly induced the expression of *PR1* after 2, 6 and 24 h of application (ANOVA, $P < 0{\cdot}001$, $P < 0{\cdot}001$, $P < 0{\cdot}001$, respectively; Fig. 5). In contrast, SA treatment significantly reduced the transcript levels of *LOX2* after 24 h (ANOVA, $P < 0{\cdot}001$), but not 2 and 6 h after application (Fig. 5). These data imply that the induction of *TPS10* is SA-regulated.

RECRUITMENT OF PARASITOIDS UNDER GREENHOUSE CONDITIONS

The number of parasitized nymphs recorded in the *B. tabaci*-infested WT group was significantly higher than that recorded for the uninfested WT group (ANOVA, $P = 0{\cdot}02$; Fig. 6b). Similarly, the number of parasitized nymphs recorded in *cpr-6* group was significantly higher than that recorded for the neighbouring WT group (ANOVA, $P = 0{\cdot}01$; Fig. 6a). These data support the notion that upon activation of SA-signalling pathway the plants attract more parasitoids and thereby may indirectly protect themselves against herbivory.

Discussion

Our data confirm that whitefly infestation induces SA-signalling in plants without any direct negative impact on the herbivore; SA activation even enhanced whitefly performance by suppressing JA-mediated defences (Fig. S2, Supporting Information; Zarate, Kempema & Walling 2007). However, SA-signalling was found to induce the release of specific volatiles that can protect the plant indirectly. The parasitic wasp *E. formosa* was attracted to *B.*

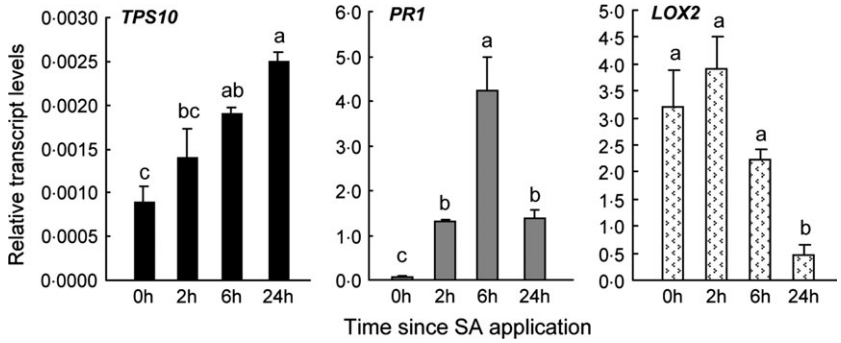

Fig. 5. Gene expressions in response to salicylic acid (SA) application. Time-related changes in transcript levels of *TPS10*, *PR1* and *LOX2* in leaves of wild-type *Arabidopsis* plants treated with SA (1·0 mM) solution. The relative gene expression was calculated as $2^{-\Delta C_t}$ method and subsequently $\log_2$-transformed. Values are the mean (±SE) of three biological replicates. Different letters above bars indicate significant difference in transcript levels between treatments (Fisher's protected least significant difference test of ANOVA, $P < 0{\cdot}05$).

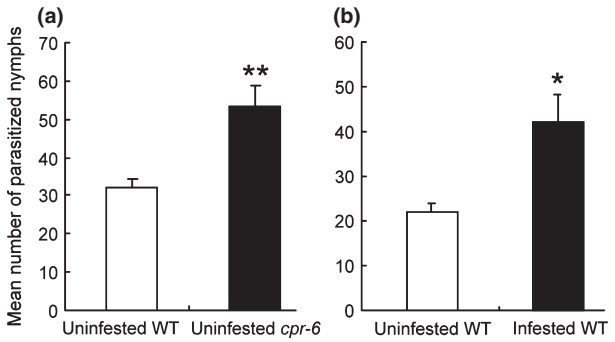

Fig. 6. Parasitized nymphs of *Bemisia tabaci*. Mean number (±SE, $n = 4$) of parasitized nymphs of *B. tabaci* under greenhouse condition. The numbers of parasitized nymphs recorded in uninfested *cpr-6* vs. uninfested wild-type (WT) (Col-0) groups (a) and in *B. tabaci*-infested WT vs. uninfested WT groups (b) were analysed by one-way ANOVA (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$).

tabaci-infested WT *Arabidopsis* plants and this attraction is likely due to the enhanced β-myrcene emission, but may also involve other SA-dependent volatiles. This is in line with Birkett *et al.* (2003) who found that *E. formosa* is attracted to bean plants 2 or 3 days after whitefly infestation. We further found that exogenous application of SA, but not of JA, resulted in the attraction of *E. formosa*. Attraction of the parasitoid was also observed for uninfested *cpr-6* (constitutively high SA level), infested *dde2-2* (JA-deficient) and *ein2-1* (ET-insensitive) *Arabidopsis* plants, whereas infested *NahG* (SA-deficient) or *npr-1* (SA-insensitive) mutants were not attractive to the parasitoid. The results for these latter two mutants even showed a tendency for the plants to become repellent upon infestation (Fig. 1c), indicating that in the absence of the proper attractants that are specifically induced by whitefly attack, other, possibly less specific volatiles may possibly repel the parasitoid. Further evidence for the role of the SA-signalling pathway in these interactions was obtained by measuring levels of gene-expression and phytohormone (Figs 3–5). Taken together, the data fully support our hypothesis that the SA-signalling pathway plays a crucial role in mediating the attraction of *E. formosa* to *B. tabaci*-infested plants.

Previous studies that have explored the signal-transduction pathways involved in the induction of volatile-mediated parasitoid attraction by phloem-feeding insects have implicated JA signalling, in particular in aphid-plant interaction (Bruce *et al.* 2008; Girling *et al.* 2008). For instance, exogenous *cis*-jasmone, which is biosynthetically related to JA, strongly induces the transcript levels of two cytochrome P450 genes *CYP81D11* and *CYP72A13* in *Arabidopsis*, among which *CYP81D11* is responsible for the induced emission of (*Z*)-3-hexen-1-ol that attracts the aphid parasitoid *Aphidius ervi* (Bruce *et al.* 2008). The aphid parasitoid *Diaeretiella rapae* shows an attraction to *Arabidopsis Cev1* mutants in which JA signalling pathway is constitutively activated; in contrast, *D. rapae* is not attracted to aphid-infested *coi1*-16 plants in which JA signalling pathway is blocked (Girling *et al.* 2008). The current data imply that volatile-mediated attraction of parasitoids induced by phloem-feeding whitefly is differently controlled than for aphid parasitoids. It would be worth to check if SA-induced volatiles are also attractive to aphid parasitoids or if, indeed, there is such a clear-cut difference in foraging strategies between aphid and whitefly parasitoids.

It is important to note that, as the developmental rates of *B. tabaci* feeding on different *Arabidopsis* mutants may differ (Zarate, Kempema & Walling 2007), the infestation intensity inflicted by *B. tabaci* could be different among mutants. Such difference in infestation intensity is likely to affect the amount of volatile emission induced by whitefly infestation (Du *et al.* 1998). However, we observed that whiteflies laid comparable numbers of eggs on WT *Arabidopsis* plant and mutants (see Fig. S2, Supporting Information). Hence, the infestation intensities by *B. tabaci* were similar among the genotypes and, therefore, the attraction of parasitoids to infested *dde2-2* and *ein2-1*, and the non-attraction of parasitoids to infested *NahG* and *npr-1* were not caused by the enhanced or reduced infestation, respectively.

Bemisia tabaci is known to induce the SA-dependent responses locally and systemically in *Arabidopsis*, while they suppress the JA- and ET-dependent responses (Van de Ven *et al*. 2000; Zarate, Kempema & Walling 2007). From the perspective of whiteflies this suppression is considered beneficial, as the JA signalling pathway can trigger an effective defence against *B. tabaci* (Zarate, Kempema & Walling 2007). Indeed, we demonstrated that whiteflies showed accelerated development on JA-deficient *dde2-2* mutant plants, whereas they showed delayed development on SA-defective *NahG* and *npr-1* mutant plants (Fig. S2, Supporting Information). Apparently whiteflies have found an effective way, possibly through eliciting SA-signalling, to manipulate plant direct defence responses (Walling 2008). Our results imply that the co-evolved parasitoid *E. formosa* has adapted to outwit the whitefly's strategy to suppress the production of JA-related volatiles by exploiting volatiles that are specific for SA induction. For the plant, this offers an alternative volatile-mediated indirect plant defence against *B. tabaci*.

We reason that (i) *B. tabaci* infestation induces the expression of two genes (*PAD4* and *PR1*) that function upstream and downstream of SA (Jirage *et al*. 2001), as well as an increase in endogenous SA levels; (ii) *B. tabaci* upregulation of SA induces the expression of *TPS10* gene coding for the enzyme β-myrcene synthase (Bohlmann *et al*. 2002); and (iii) the enhanced emission of β-myrcene is essential for the attraction of whitefly parasitoids, although the role of other changes in the volatile blend cannot be excluded (Rasmann & Turlings 2007). This proposed importance of the SA-signalling pathway for parasitoid attraction was confirmed under greenhouse conditions with plants that exhibited constitutive or inducible activation (Fig. 6).

In other systems, a key role of JA signalling pathway in regulating indirect plant defence, that is, the volatile-mediated attraction of parasitoids or predators, has been well demonstrated (Thaler *et al*. 2002; Ament *et al*. 2004; Bruce *et al*. 2008; Girling *et al*. 2008; Bruinsma *et al*. 2009; Wei *et al*. 2011) and a few studies have shown that JA-dependent indirect plant defence offers benefits under field conditions (Thaler 1999; Heil *et al*. 2001). As yet, the role of other signalling pathways in attracting natural enemies of herbivores was not known, although it has been indicated that the SA-signalling plays a role in regulating certain herbivore-induced plant volatile emissions (Ozawa *et al*. 2000; Van Poecke & Dicke 2002). Hence, to our knowledge, this is the first case where SA-signalling pathway is found to mediate the attraction of a whitefly parasitoid and one of the rare examples showing that this attraction indeed results in enhanced parasitism. These results further support the notion that herbivory contributes to selective pressure governing the evolution of inducible volatile signals as indirect plant defences (Janssen, Sabelis & Bruin 2002; Hilker & Meiners 2010; Hare 2011; Kessler & Heil 2011). It can be concluded that whitefly parasitoids have found a way to outsmart their host's ability to suppress JA-based signalling.

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Supporting Information

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

Appendix S1. Additional details of plant treatments, statistical analysis and results.

Fig. S1. Nymphal development of *B. tabaci* on *Arabidopsis* plants.

Fig. S2. Mean oviposition rate of *B. tabaci* females on *Arabidopsis* plants.

Table S1. Lists of primers used for qRT-PCR.