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LETTER

Shared signals – 'alarm calls' from plants increase apparency to herbivores and their enemies in nature

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Abstract

The attraction of natural enemies of herbivores by volatile organic compounds as an induced indirect defence has been studied in several plant systems. The evidence for their defensive function originates mainly from laboratory studies with trained parasitoids and predators; the defensive function of these emissions for plants in natural settings has been rarely demonstrated. In native populations and laboratory Y-tube choice experiments with transgenic *Nicotiana attenuata* plants unable to release particular volatiles, we demonstrate that predatory bugs use terpenoids and green leaf volatiles (GLVs) to locate their prey on herbivore-attacked plants. By attracting predators with volatile signals, this native plant reduces its herbivore load – demonstrating the defensive function of herbivore-induced volatile emissions. However, plants producing GLVs are also damaged more by flea beetles. The implications of these conflicting ecological effects for the evolution of induced volatile emissions and for the development of sustainable agricultural practices are discussed.

Keywords

cis-α-bergamotene, *Epitrix hirtipennis*, flea beetle, *Geocoris pallens*, kairomone, *Manduca sexta*, predation, tritrophic interaction.

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INTRODUCTION

Plants use both direct and indirect defences to protect themselves against herbivore attack, and the genes that mediate these defences are rapidly being elucidated in model plant systems (Gatehouse 2002). However, our understanding of whether these traits function as defences in plants' natural environments has not kept pace. A recent example is the discovery (Kappers *et al.* 2005; Schnee *et al.* 2006) of genes involved in the biosynthesis of herbivore-induced (HI) volatile organic compound (VOC) emissions that attract natural enemies to plants attacked by herbivores and thereby function as an induced indirect defence: a volatile 'alarm call' (Dicke & Sabelis 1988). Fitness benefits have been demonstrated for maize (Hoballah & Turlings 2001) and *Arabidopsis thaliana* (van Loon *et al.* 2000) plants infested by parasitized herbivores compared with plants attacked by non-parasitized herbivores, suggesting a fitness-increasing effect of a VOC-mediated attraction of parasitoids. For the majority of the studied systems, these beneficial effects of HI-VOCs remain to be demonstrated under natural conditions outside of well-controlled laboratory experiments with trained predators and parasitoids. De Moraes *et al.* (1998) demonstrated in field trials the attraction of the parasitic wasp *Cardiochiles nigriceps* by a VOC bouquet specifically elicited by *Heliothis virescens* larvae feeding on tobacco, maize and cotton plants. Phytohormone elicitation and augmentation of the volatile bouquets of wild type (WT) plants have provided further compelling evidence that these responses attract natural enemies. Methyl jasmonate treatment of tomato plants increased parasitism of *Spodoptera exigua* larvae by the wasp *Hyposoter exigua* (Thaler 1999). Similarly, in the wild tobacco plant *Nicotiana attenuata,* treatment with synthetic homologs of HI-VOCs released by the plant in response to tobacco hornworm (*Manduca sexta*) herbivory increased predation rates on *M. sexta* eggs by the generalist predator *Geocoris pallens,* which attacks a wide variety of herbivores, including hornworm eggs and larvae, mirid bugs and flea beetles, found on *N. attenuata* (Kessler & Baldwin 2001). These studies, while demonstrating the function of plant VOCs – namely, to attract natural enemies, do not control for the spatially and temporally complex

pattern of VOC release by herbivore-attacked plants. Synthetic compound or hormone applications uncouple the induced VOC release from the actual herbivore feeding and the corresponding release regulation including specific elicitation kinetics and diurnally and spatially controlled release rates (Peñuelas & Llusià 2001). Additionally, these approaches do not demonstrate whether the responses can be manipulated, e.g. by genetic engineering, to increase how efficiently a plant attracts natural enemies and subsequently benefits.

Mutants and transgenic plants with modified volatile emissions represent the most powerful means of testing the ecological function of individual HI-VOCs. In tomato, the *def-1* and *spr-2* mutations in the jasmonic acid (JA) signalling cascade, the main phytohormone signal involved in eliciting herbivore-induced plant terpene releases, reduce mono- and sesquiterpene emission and alter the profile of GLVs. In the laboratory, lepidopteran herbivore-infested *def-1* and *spr-2* mutants attract fewer predatory mites (Thaler *et al.* 2002) and receive fewer ovipositions from *M. sexta* and whitefly adults (Sanchez-Hernandez *et al.* 2006). Manipulation of GLV production in *A. thaliana* by ectopically expressing or silencing hydroperoxide lyase activity results in increased or decreased attraction of *Cotesia glomerata* wasps, respectively, and the increased attraction was correlated with a higher parasitization rate of *Pieris rapae* larvae (Shiojiri *et al.* 2006a). Again, the relevance of these results for the resistance of plants outside the laboratory remains unknown. Clear demonstrations that HI-VOC emissions reduce the herbivore loads of plants growing in their native habitats or agricultural fields is a necessary first step if these responses are to live up to predictions that they will be agriculturally useful (Dicke *et al.* 1990; Whitfield 2001; Degenhardt *et al.* 2003; Turlings & Ton 2006). In a recent study, using maize cultivars with differential emissions of the sesquiterpene caryophyllene, Rasmann *et al.* (2005) demonstrated increased parasitization of corn root worm larvae on maize genotypes releasing caryophyllene, which is highly attractive to entomopathogenic nematodes.

Here we use two transgenic lines of the wild tobacco plant *N. attenuata,* both of which were transformed by antisense expression of endogenous genes involved in the elicitation of HI-VOC emissions to render them 'mute' in specific components of their herbivore-induced volatile 'vocabulary'. We used the transformed plants to compare how well two classes of plant-released VOCs, terpenoids and GLVs, attract the predatory bug *Geocoris pallens*, in both laboratory and field studies. This study builds on previous work, which demonstrated that *G. pallens* is attracted to the release of the sesquiterpene, *cis*-α-bergamotene, and the GLV (*Z*)-3-hexenol under natural conditions (Kessler & Baldwin 2001).

MATERIALS AND METHODS

Plant and insect culture

Seeds of wild type (WT, DI'92, 16× inbred generation) and the two transgenic *Nicotiana attenuata* (synonymous with *N. torreyana*) lines expressing either 13-lipoxygenase NaLOX3 [as-*lox*, A300; (Halitschke & Baldwin 2003)] or hydroperoxide lyase NaHPL [as-*hpl*, A337; (Halitschke *et al.* 2004)] in antisense direction were germinated on sterile Gamborg B5 media (Sigma, Steinheim, Germany) and grown in the glasshouse as described previously (Krügel *et al.* 2002). Silencing the plant's lipoxygenase NaLOX3 (as-*lox*) by antisense expression completely abolishes the release of herbivore-induced sesquiterpenoids but does not reduce the release of wound-induced GLVs (Halitschke & Baldwin 2003; Kessler *et al.* 2004). On the other hand, the release of GLVs is reduced in hydroperoxide lyase NaHPL-deficient (as-*hpl*) plants to 30% of the amount released by WT plants whereas herbivore-induced *cis*-α-bergamotene is released at a rate comparable with WT plants (Halitschke *et al.* 2004). For the field experiments, seedlings were transferred into 50 mm peat pellets (Jiffy Products, Shippegan, New Brunswick, Canada) after germination in Petri dishes and gradually adapted to the environmental conditions in the Great Basin desert habitat (high sun exposure and low relative humidity) over 2 weeks. Adapted seedlings of the same size were transplanted into the described field sites. Seedlings were watered every other day for 2 weeks until roots were established in the native soil.

Manduca sexta eggs were purchased from North Carolina State University (Raleigh, NC, USA). *Geocoris punctipes* (Entomos, Gainsville, FL, USA) used in the glasshouse experiments were reared on mirid bugs (*Tupiocoris notatus*) that fed on *N. attenuata* plants grown in the same glasshouse in which the choice experiments were performed.

Y-tube olfactometer experiments

For the Y-tube olfactometer experiment detached stem leaves (S1–S3) of plants of the three genotypes were used as the odour source. Each leaf was damaged by first-instar *M. sexta* larvae feeding for 48 h and larvae were kept on the leaves during the choice assay. In initial glasshouse Y-tube choice experiments, we tested a small population of *G. pallens* (24 individuals) collected at the Utah field station in 2005. Unfortunately, 75% of these predators did not move in the Y-tube experimental setup. Therefore, we used the commercially available biocontrol agent and close relative *G. punctipes* for further choice experiments in the glasshouse. A total of 34 and 39 adult *G. punctipes* were tested in the WT/as-*lox* and WT/as-*hpl* comparison, respectively. Each individual was tested immediately after

removal from the colony (satiated) and subsequently starved for 24 h. The arms of the transparent plastic Y-tube olfactometer (diameter: 6.5 mm; base: 22 cm; arms: 18 cm) were connected to 300 mL plastic chambers into which the detached leaves were placed as the odour source. A stream of ambient air was created by applying a slight vacuum to the base of the olfactometer, establishing a flow of 150 mL min^{-1} through each olfactometer arm. The *Geocoris* individual was placed at the base of the olfactometer, downwind of the odour source and constantly observed for maximally 15 min. A choice was recorded when the predator crossed a line marked 5 cm upwind of the split in the olfactometer arms within 15 min. Leaves were replaced and the positions of odour sources were exchanged after each choice recording to exclude positional effects of the Y-tube experimental setup. 'No choice' individuals were excluded from the analysis and data were analysed with Pearson χ^2-tests (SYSTAT; SPSS Inc., Chicago, IL, USA).

Whole plant cage experiments

Two transparent plastic cages (15 × 15 × 58 cm) were connected with pasteboard cylinders (9.5 × 4 cm diameter). One of the cages contained a WT plant; a plant of one of the two transformed genotypes (as-*lox* or as-*hpl*) was placed in the second cage. Freshly hatched *M. sexta* larvae were placed on each of the three oldest stem leaves (S1–S3) of each plant. After 24 h two adult *G. punctipes* individuals, which had been removed freshly from the colony (satiated) or kept without mirid bugs for 24 h (starved), were released in the middle of the cylinder connecting two cages. Predator positions were monitored at 1, 3, 9 and 24 h after the release. The experiment was repeated with new plants eight times (as-*hpl*) and 16 times (as-*lox*) with satiated predators, and 24 times with starved predators, for each of the two combinations of WT and transgenic plants. The choice of predators (0, 1, or 2) was compared with Wilcoxon signed rank tests (StatView; SAS Institute Inc., Cary, NC, USA) as the two individuals in a single cage setup do not represent independent replicates.

Field release experiments

In May 2004, plants were randomly planted into a watered field plot at the Lytle Preserve research station (Santa Clara, UT, USA) in pairs (n = 20) of WT/WT, as-*hpl*/WT, and as-*lox*/WT plants with 0.35 m between plants in a pair and 1.5 m between pairs. Herbivore damage and abundance on the plants was monitored four times (day 1, 2, 6 and 9) over a 9-day observation period and data were compared between plants of each pair by paired t-tests (StatView). Additionally, we analysed the dynamics (days 2, 6 and 9 after the transplant) of flea beetle and leaf hopper colonization using repeated measures ANOVAS with plant genotype as independent factor.

In 2005, the WT (n = 41), as-*hpl* (n = 30), and as-*lox* (n = 26) plants were randomly transplanted in a linear transect with 3 m distance between plants into a natural population of *N. attenuata* growing in an area near Santa Clara, Utah, which was burned by a wildfire in 2004. Half of the plants of each genotype were randomly selected and received one first-instar *M. sexta* larvae on each of the first three stem leaves to elicit the release of VOCs. Additionally, each of the attacked leaves and the corresponding leaves on untreated control plants received two *M. sexta* eggs glued onto the underside of each leaf, as described by Kessler & Baldwin (2001), 24 h after the application of larvae. Larvae, eggs and the abundance of *Geocoris pallens* on each plant were monitored for 3 days. Larval mortality and egg predation were calculated for each individual plant and square root-transformed to meet ANOVA requirements. Cumulative predator abundance data were log-transformed after summing the individual observations of the three subsequent days. Transformed data were analysed with repeated measures ANOVAS (StatView) using plant genotype (for larval mortality) or genotype and elicitation treatment (for egg predation and predator abundance) as independent factors. All releases of the transformed plants were performed according to 7 CFR 340.3(c) under APHIS notification numbers 04-020-08n (2004) and 04-344-07n (2005).

Synthetic VOC application

Cis-α-bergamotene and a mixture of synthetic green leaf volatiles were tested in the Y-tube olfactometer setup described above. (*Z*)-3-hexenol (84 nmol), (*E*)-2-hexenal (2.6 nmol), (*E*)-2-hexenol (1.7 nmol), (*Z*)-3-hexenyl acetate (3.2 nmol) and (*Z*)-3-hexenyl butyrate (5.2 nmol) were applied in a 20 μL droplet of lanolin to one of the odour source chambers of the olfactometer and compared to a 20 μL droplet of pure lanolin not containing any additional compounds. A solution of *cis*-α-bergamotene (0.1 fmol in 20 μL lanolin) was similarly tested. Data were analysed as described above with Pearson χ^2-tests (SYSTAT).

In a field experiment, we added synthetic compounds to plants of the three genotypes in the linear transect in 2005. The amounts of applied compounds were based on VOC analyses of *M. sexta*-attacked field-grown plants, which demonstrated release rates of 50–200 ng h^{-1} for bergamotene and GLVs (Kessler & Baldwin 2001; Kessler *et al.* 2004). The plants were treated identically as described above; in addition, we placed a cotton swab directly beside the plant stem and applied 40 μL of lanolin containing 0.1 nmol of *cis*-α-bergamotene or a mixture of GLVs (4 nmol (*Z*)-3-hexenol, 0.4 nmol (*E*)-2-hexenal, 0.8 nmol (*E*)-2-hexenol, 0.3 nmol (*Z*)-3-hexenyl acetate, and

0.2 nmol (*Z*)-3-hexenyl butyrate). A cotton swab with 40 μL lanolin was placed close to control plants. The application was repeated every morning over the 3 days of observation. Egg predation on WT plants supplemented with synthetic VOCs was analysed with a repeated measures ANOVA after square root transformation of the data using the VOC addition as independent factor. For the genotype, comparison square root-transformed egg predation data after 3 days were recorded and analysed with an ANOVA using the complementation treatment as independent factor.

To test the activity of individual compounds, we applied equimolar amounts (4 μmol) of each of the compounds dissolved in 40 μL ethanol to artificial leaves constructed from green paper. Control paper leaves received 40 μL of pure ethanol. Ten replicate paper leaves per tested compound were attached to wooden sticks and 'planted' in the field plot at the Lytle Preserve research station in July 2005. Six *M. sexta* eggs were glued onto the underside of each paper leaf and egg predation was observed over 3 days. The application of compounds was repeated every day after the egg monitoring. Square root-transformed egg predation data were analysed with an ANOVA with the applied compound as independent factor.

RESULTS

Geocoris predatory bugs chose plants releasing complete VOC blends

First, we tested the attractiveness of the three genotypes (WT, as-*hpl*, and as-*lox*) of *M. sexta*-damaged plants in a Y-tube olfactometer using detached leaves as the odour source. Although satiated predators did not discriminate between leaves of damaged WT and as-*hpl* or as-*lox* (*P*s ≥ 0.17) plants, predators starved beforehand for 24 h preferred WT plants over GLV-deficient as-*hpl* (χ^2 = 5.143, P = 0.023) and terpenoid-deficient as-*lox* (χ^2 = 10.125, P = 0.001) plants (Table1).

In the next series of experiments, we used intact plants as the odour source to exclude the effects of leaf removal on the VOC release (Schmelz *et al.* 2001). In these cage experiments, we compared the distribution of *G. punctipes* individuals released in the middle of tubes connecting cages containing *Manduca*-attacked WT and as-*hpl* or as-*lox* plants. Individuals did not move from one cage to the other and therefore the results reflect the initial choice made by the predators upon release. Consistent with the detached-leaf assay, starved *G. punctipes* preferred WT plants damaged by feeding *M. sexta* larvae to identically treated as-*hpl* and as-*lox* plants. After 24 h, starved predators were significantly more abundant on WT than on as-*hpl* plants (WT: 21; as-*hpl*: 8; P = 0.037), while satiated individuals did not discriminate (WT: 6; as-*hpl*: 10; P = 0.36). Similarly, starved *G. punctipes* chose WT plants over as-*lox* plants (WT: 28; as-*lox*: 12; P = 0.017), whereas satiated individuals did not discriminate between genotypes (WT: 21; as-*lox*: 17; P = 0.44).

Table 1 *Geocoris punctipes* choice in Y-tube olfactometer experiments

	Starvation time	
	0 h	24 h
N	39	34
WT	15	20
as-*hpl*	15	8
χ^2	0	5.143
P-value (d.f. = 1)	> 0.999	0.023
N	34	34
WT	13	25
as-*lox*	21	7
χ^2	1.882	10.125
P-value (d.f. = 1)	0.17	0.001

A total of *N* satiated (0 h starvation time) or predators that had been starved for 24 h were released in a Y-tube olfactometer connected to different odour sources. The odours from individual leaves of *Nicotiana attenuata* wild type (WT) plants damaged by feeding *Manduca sexta* larvae were compared with the odours from identically treated leaves from antisense NaHPL (as-*hpl*) or NaLOX3 (as-*lox*) plants, respectively. Results of Pearson χ^2-tests are shown.

Predators are attracted by synthetic GLV and terpene compounds

We tested synthetic compounds in the Y-tube experimental setup to confirm the attractiveness of the manipulated VOCs. Consistent with the reduced attractiveness of terpenoid- or GLV-deficient plants, predators chose an odour source that released *cis*-α-bergamotene- (χ^2 = 12.665, P < 0.0001) or a GLV mix (χ^2 = 8.018, P = 0.005) over one containing pure lanolin and no additional volatile compounds (Fig. 1a). These laboratory experiments confirm the potential of both types of herbivore-induced VOCs, terpenoids and GLVs, to attract *Geocoris* predators.

To evaluate the attractiveness of individual compounds in natural populations, we applied equimolar amounts of synthetic GLVs and *cis*-α-bergamotene to artificial leaves and recorded predation rates on eggs glued to these paper substitutes. Adding individual compounds significantly increased predation rates on the eggs (Fig. 1b; ANOVA: $F_{4,45}$ = 2.59, P = 0.049). Eggs on artificial leaves treated with (*E*)-2-hexenal or *cis*-α-bergamotene received 3.3- and 3.6-fold more predation than did eggs on ethanol-treated control leaves. The application of (*Z*)-3-hexenol did not affect egg predation, and the effect of (*Z*)-3-hexenyl acetate (3.0-fold) was not statistically significant (Fig. 1b).

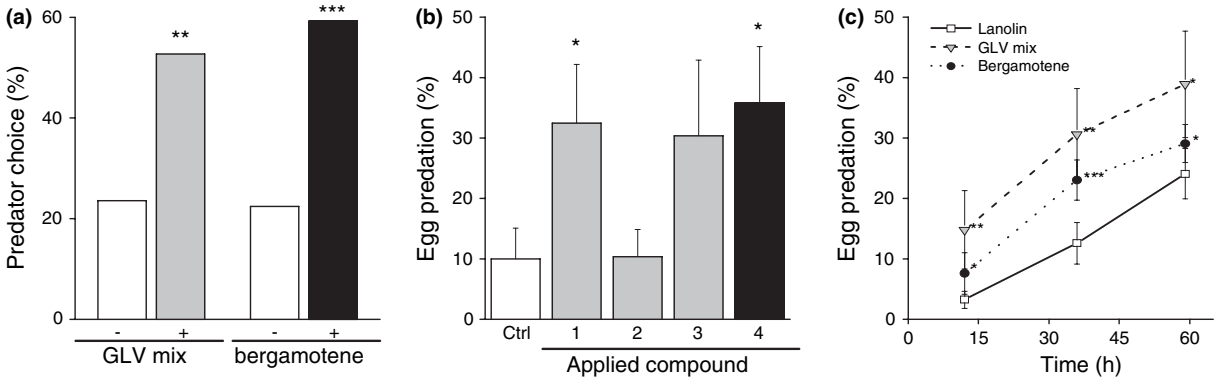

Figure 1 Response of predators to synthetic compounds. (a) *Geocoris punctipes* individuals starved for 24 h were released in a Y-tube olfactometer connected to different odour sources. A mixture of synthetic green leaf volatiles (GLV mix; $N = 55$) or pure *cis*-α-bergamotene ($N = 62$) dissolved in a 20 μl droplet of lanolin (+) was compared with pure lanolin droplets (−). (b) Solutions of individual compounds [(*E*)-2-hexenal (1); (*Z*)-3-hexenol (2); (*Z*)-3-hexenyl acetate (3); *cis*-α-bergamotene (4)] in ethanol were applied to artificial leaves made of green paper onto which 6 *Manduca sexta* eggs were glued. The paper leaves were exposed to predators at the Lytle Preserve research station in SW Utah. Solutions were re-applied every day and predation rates (mean + SE) were monitored after 3 days. Control paper leaves (Ctrl) were treated with pure ethanol. (c) Two *M. sexta* eggs were glued onto the underside of the three lowest stem leaves of field-grown *Nicotiana attenuata* wild type plants (squares, solid line), which had been damaged by first-instar *M. sexta* larvae feeding on the same leaves for 24 h. The release of terpenoid or green leaf volatiles (GLVs) was enhanced by applying *cis*-α-bergamotene or a mix of synthetic GLVs in lanolin paste to cotton swabs placed close to the plant. Egg predation (mean ± SE) was monitored over 2 days. Predator choice was compared with Pearson χ^2-tests and log-transformed predation rates by Fisher's PLSDs of ANOVAs; significant differences compared with solvent controls are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Synthetic volatile additions increase predation rates on plants in the field

To determine whether these components of the HI-VOC emission function as an indirect defence and thus play an ecologically significant role in the interaction of *N. attenuata* plants with their natural environment, we designed a series of experiments to manipulate the release rate of individual compound classes in field-grown *N. attenuata* plants. To increase the headspace concentration of plants attacked by *M. sexta* larvae, we added synthetic VOCs to cotton swabs positioned close to the attacked plant and recorded predation rates on *M. sexta* eggs. Consistent with results from the paper leaf experiment (Fig. 1b), adding synthetic GLVs or *cis*-α-bergamotene significantly increased predation on eggs on leaves of herbivore-damaged plants compared to eggs on leaves of caterpillar-damaged plants receiving only lanolin as a solvent control (repeated measures ANOVA: $F_{\text{treatment},2,79} = 10.064$, $P = 0.0001$; $F_{\text{time},2,158} = 35.898$, $P < 0.0001$; $F_{\text{treatment} \times \text{time}} = 1.203$, $P = 0.312$). Predation was enhanced in the first 12 h and increased 2.4- and 1.8-fold in response to the addition of GLVs and bergamotene, respectively, during day 2 (Fig. 1c).

Augmenting the HI-VOC release by adding synthetic compounds does not fully mimic the herbivore-induced release emanating from the plant. Consequently, we directly tested the ecological relevance of the HI-VOC release using plants that are muted in specific components of their volatile vocabulary.

Silencing GLV or terpene emissions debilitates the plant's indirect defence

In the field release experiment, plant genotype had a significant effect on the abundance of *G. pallens* on the plant (repeated measures ANOVA: $F_{\text{genotype},2,91} = 5.381$, $P = 0.0062$) and strongly influenced the induced response in *Manduca*-attacked plants ($F_{\text{treatment},1,91} = 0.102$, $P = 0.75$; $F_{\text{genotype} \times \text{treatment}} = 18.354$, $P < 0.0001$). Consistent with the results of the Y-tube choice assays, the abundance of *G. pallens* increased dramatically only on herbivore-damaged WT plants, but not on herbivore-attacked GLV- or terpenoid-deficient as-*hpl* or as-*lox* plants (Fig. 2a).

The reduced predator abundance correlated with reduced egg predation rates on volatile-deficient plants. Plant genotype (repeated measures ANOVA: $F_{\text{genotype}} = 10.32$, $P < 0.0001$) and type of elicitation treatment ($F_{\text{treatment}} = 10.02$, $P = 0.0016$) strongly affected egg predation. After 56 h, the percentage of predated eggs on undamaged WT plants (9.5 ± 2.5%) was similar to as-*hpl* (13.5 ± 4.6%) and as-*lox* (6.3 ± 4.4%) plants. Elicitation by feeding *M. sexta* larvae caused a 3.3-fold increase in the rate of egg predation on WT plants, meaning that 31.7 ± 6.0%

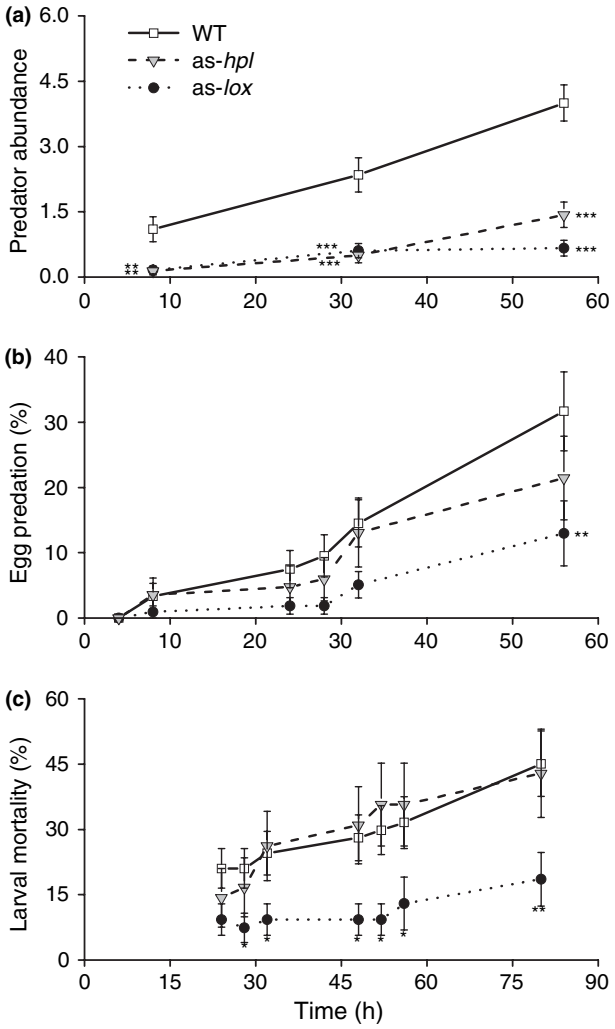

Figure 2 Predator abundance and predation rates in nature are influenced by a plant's ability to release VOCs. (a) *Geocoris pallens* individuals were counted on field-grown *Nicotiana attenuata* wild type (WT; squares, solid line) plants whose GLV emissions had been 'muted' by antisense NaHPL (as-*hpl*; triangles, dashed line) or in sesquiterpenoid emissions by antisense expression of NaLOX3 (as-*lox*; circles, dotted line) plants which had been damaged by first-instar *Manduca sexta* larvae feeding on the first three stem leaves of each plant; numbers were summed over consecutive days. (b) Two *M. sexta* eggs were glued onto the underside of each of the damaged leaves and egg predation was monitored over 3 days. (c) Mortality of *M. sexta* larvae feeding on plants of the three genotypes. Predator abundance and predation rate data (mean ± SE are shown) were square root- and log-transformed, respectively; significant differences (Fisher's PLSD of ANOVAs) compared with the WT are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

of the eggs were predated (Fig. 2b). This increase in egg predation was less pronounced on *Manduca*-damaged as-*hpl* plants (21.4 ± 6.4%) and strongly reduced on as-*lox* plants

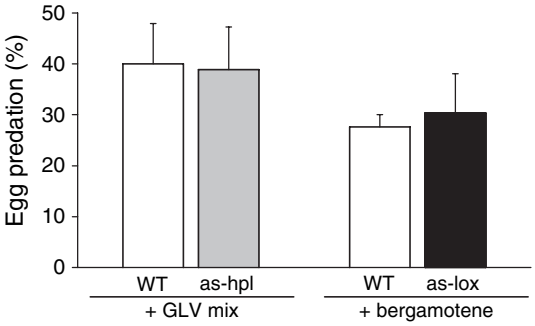

Figure 3 Recovery of silenced volatile emissions. Two *Manduca sexta* eggs were glued onto the underside of the three lowest stem leaves of field-grown *Nicotiana attenuata* wild type, antisense NaHPL (as-*hpl*), and antisense NaLOX3 (as-*lox*) plants which had been damaged by first-instar *M. sexta* larvae feeding on the same leaves for 24 h. The reduced release of terpenoid or green leaf volatiles (GLVs) in the transgenic lines was complemented by applying *cis*-α-bergamotene or a mix of synthetic GLVs in lanolin paste to cotton swabs placed close to the plant. The egg predation rate (mean + SE) was recorded after 2 days, square root-transformed and compared with predation rates on identically treated WT plants by Fisher's PLSDs of ANOVAs.

(13.0 ± 5.0). Complementing the volatile-deficient as-*hpl* and as-*lox* plants with a mixture of GLVs or *cis*-α-bergamotene as described above restored the egg predation rates to those of identically treated WT plants (Fig. 3; ANOVA; $F_{3,36} = 0.42$, $P = 0.7399$).

Similar to the egg predation rate, the mortality of first-instar *M. sexta* larvae was reduced on as-*lox* plants but only slightly on as-*hpl* plants (Fig. 2c; repeated measures ANOVA: $F_{\text{genotype}} = 3.512$, $P < 0.0381$).

Silenced-GLV production reduces the plant's apparency to herbivores

To more closely examine the consequences of decreased GLV production, we monitored colonization and damage on pairs of WT/WT, as-*hpl*/WT and as-*lox*/WT plants in a plantation at the Lytle Preserve research station (Santa Clara, UT, USA). As previously reported for as-*lox* plants with strongly suppressed defence capabilities (Kessler *et al.* 2004), as-*lox* plants received significantly more herbivore damage by *Empoasca* spp. leafhoppers (repeated measures ANOVA: $F_{\text{genotype},2,57} = 27.55$, $P < 0.0001$) than did WT and as-*hpl* plants during the 9-day observation period (Fig. 4). Interestingly, a pronounced difference in the distribution of flea beetles (*Epitrix hirtipennis*) on the as-*hpl*/WT plant pairs was observed. Plant genotype had a significant effect on the colonization by flea beetles over the 9-day observation period (data not shown; repeated measures ANOVA: $F_{\text{genotype},1,38} = 4.722$, $P = 0.0361$) resulting in a highly

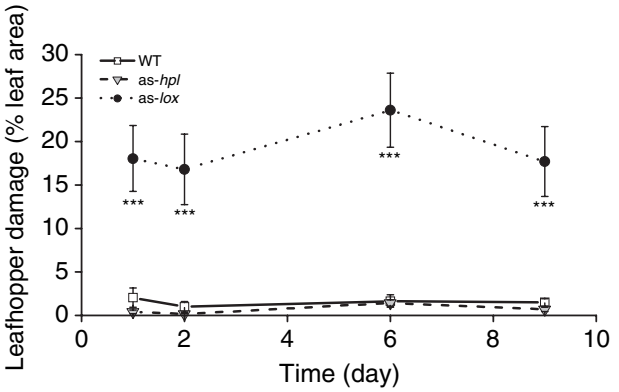

Figure 4 Leafhopper damage on *Nicotiana attenuata* plants. Wild type (WT; open squares), antisense NaHPL (as-*hpl*; gray triangles), and NaLOX3 (as-*lox*; black circles) plants were planted in a field plot in pairs with untransformed WT plants. Leafhopper (*Empoasca* spp.) damage, as a percentage of total leaf area (mean ± SE), on the plants was recorded over 9 days after the removal of the insect screen. Significant differences in leafhopper damage compared with the WT are indicated by asterisks (Fisher's PLSD of ANOVAs; $***P < 0.0001$).

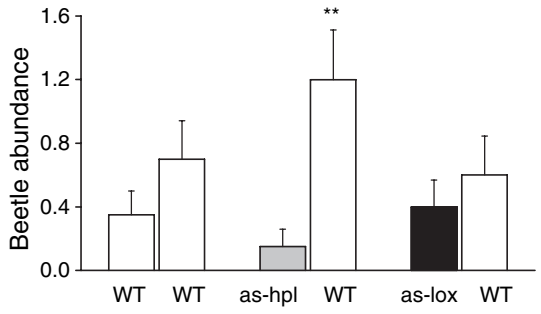

Figure 5 Flea beetle abundance on *Nicotiana attenuata* plants. Wild type (WT; open bar), antisense NaHPL (as-*hpl*; gray bar), and NaLOX3 (as-*lox*; black bar) plants were planted in a field plot in pairs with untransformed WT plants. (a) The distribution of flea beetles (*Epitrix hirtipennis*) in the plant pairs was recorded on day 9 after the removal of the insect screen. Significant differences in flea beetle abundance (mean + SE) are indicated by asterisks (paired *t*-test; $**P < 0.01$).

skewed distribution of flea beetles in the as-*hpl*/WT pairs towards the WT member (Fig. 5; paired *t*-test; $P = 0.0032$). No differences were detected in beetle abundance between plants in WT/WT and as-*lox*/WT pairs (*P*s > *0.05*).

DISCUSSION

Our results demonstrate that *N. attenuata* plants use the same chemical signals to attract natural enemies to feeding herbivores for defensive purposes that are also involved in the identification of host plants and the initiation of feeding by at least two of the herbivores attacking *N. attenuata*. The 'double-edged sword' of HI-VOCs demonstrated in this report – the ploy and counterploy – vividly illustrates an example of the complex signalling that mediates interactions in natural environments and highlights the need to characterize the function of defence traits in an organism's natural environment.

Specificity of predator attraction

In contrast to the highly specific attraction of experienced parasitoids by herbivore-specific plant volatile bouquets (De Moraes *et al.* 1998; Röse *et al.* 1998; Fatouros *et al.* 2005; Hoballah & Turlings 2005), the generalist predator *G. pallens* is attracted by individual components of the VOC bouquet of *N. attenuata* plants. Kessler & Baldwin (2001) reported increased predation rates in response to the augmentation of WT plants with (*Z*)-3-hexenol, linalool and *cis*-α-bergamotene, but no increase when ocimene and methyl salicylate (MeSA) were added. In additional field trapping experiments, methyl salicylate- and (*E*)-2-hexenal-laced baits attracted *G. pallens*, whereas (*Z*)-3-hexenol, linalool, and (*Z*)-3-hexenyl acetate had no effect (James 2003, 2005). Our Y-tube and field results (Fig. 1) confirm the relatively unspecific response of *G. pallens* to individual components of the volatile blend released by herbivore-attacked *N. attenuata* plants. By individually silencing the release of GLVs or terpenoids, we demonstrate the function of both compound classes as indirect defence by attracting the predators to infested plants and reducing the herbivore load on the plant (Table 1, Fig. 2). Nevertheless, individual components of the VOC bouquet, while generally attractive for the predator, might differentially elicit specific search behaviours such as local search patterns or arrestment on the plant. These differences could explain why the effects of reduced GLV emissions from as-*hpl* plants on actual predation rates were relatively mild despite the strongly reduced predator abundance.

Interestingly, we found dramatic differences in choices of starved and satiated predators (Table 1), which could be explained by changes in sensitivity or detection thresholds in the odour perception or a change in the general prey search strategy of the predator. Starvation influences search behaviour in the predatory bug *Deraeocoris lutescens*, which changes from intensive (local) search mode in satiated to an extensive (longer distance) search mode in starved individuals (Lamine *et al.* 2005). This starvation-dependent switch is species specific (Hénaut *et al.* 2002) and additional experiments are required to determine which mechanism mediates the altered choice responses of *Geocoris* predators.

Additionally, the fact that prey might be able to sequester and use plant defences for their own defence against natural

enemies could influence the predators' behaviour and performance (Campbell & Duffey 1979). *Manduca sexta* is thought to sequester sufficient quantities of direct defences (nicotine, phenolic compounds) while feeding on cultivated tobacco to hamper top-down control by the parasitoid wasp *Cotesia congregata* (Barbosa *et al.* 1991; Bentz & Barbosa 1992). While showing toxic effects of the alkaloid on the parasitoid, these experiments do not demonstrate a reduction in the effectiveness of indirect defences in plants using the alkaloid as a direct defence. In fact, locally adapted parasitoids were not affected by the toxin and even utilize the alkaloid as a search stimulant (Kester & Barbosa 1994). In contrast to the more specialized host–parasitoid interaction, the highly generalist and opportunistic predator of our study system is less likely to be affected by the chemical composition of one of its prey. Despite the reduced accumulation of induced direct defence compounds (nicotine and TPIs) in the as-*lox* plants (Halitschke & Baldwin 2003), the predators showed no preference for larvae feeding on these plants, suggesting that the strong top-down control mediated by the indirect defence is not negatively influenced by the plant's induced direct defences. Moreover, field experiments did not reveal any differences in behaviour or performance of *G. pallens* attacking hornworm larvae on *N. attenuata* plants with genetically and pharmacologically manipulated nicotine concentrations (A. Steppuhn and I. T. Baldwin, unpublished data). Therefore, the coordinated activation of direct and indirect defence in *N. attenuata* functions synergistically as predicted by the slow growth/high mortality hypothesis.

VOC-mediated direct effects on *Manduca sexta* herbivory

Kessler & Baldwin (2001) estimated that VOC-mediated plant responses reduced herbivore loads by 90%. In addition to the indirect defence effect of egg and larvae predation/parasitation, plant-released volatiles are effective oviposition repellents for a series of lepidopteran herbivores (De Moraes *et al.* 2001; Kessler & Baldwin 2001; Sanchez-Hernandez *et al.* 2006). Although we did not observe significant oviposition by *M. sexta* or *M. quinquemaculata* moths on the transgenic plants in the release experiments, the oviposition-repellent effects of plant-derived VOCs are likely to further increase herbivore susceptibility of the GLV- and terpenoid-deficient transgenic plants.

The larval mortality data (Fig. 2c) highlighted an additional role that these VOCs play in nature. Larval mortality, in contrast to predator abundance and egg predation, integrates the direct effects of plant phenotype on the feeding herbivore with the effects of the plant's tritrophic interactions with natural enemies. *Spodoptera frugiperda* larvae are attracted to volatiles released from damaged corn leaves and this apparently 'maladaptive' preference for plants with an activated induced response is likely to represent a trade-off between the detection of a more apparent host and optimal nutritional food quality and predation/parasitation risk (Carroll *et al.* 2006). Under laboratory conditions, GLVs stimulate feeding in *M. sexta* larvae (Halitschke *et al.* 2004). Similarly, the nocturnal feeding behaviour of *Mythimna separata* larvae is regulated by the diurnal emission of plant volatiles (Shiojiri *et al.* 2006b). Although diurnal patterns of VOC emissions have been demonstrated in several plant species, the compounds stimulating the observed behaviour have not been identified. In *M. sexta* the lack of GLVs as feeding stimulant in as-*hpl* plants slows larval growth and thereby increases the larvae's vulnerability to predation by *G. pallens,* which attack larvae mainly in the first two instars (Kessler & Baldwin 2001). This increased vulnerability likely contributes to the larvae's poor performance and, ultimately, increased mortality (Fig. 2c).

Additional VOC-mediated effects on the *N. attenuata* herbivore community

In addition to functioning as oviposition repellents, VOCs also serve as host-location cues for herbivores. Whereas the release of HI-VOCs has an repellent effect on adult Lepidoptera and aphids (Turlings & Wäckers 2004), feeding by lepidopteran larvae is stimulated by the production of GLVs (Halitschke *et al.* 2004; Carroll *et al.* 2006). Furthermore, leaf beetles (Coleoptera) are generally attracted to infested plants emitting VOCs (Turlings & Wäckers 2004). Plant VOCs, especially GLVs, can directly attract coleopteran herbivores to host plants or synergize insect-derived sex or aggregation pheromones (Loughrin *et al.* 1995; Reddy & Guerrero 2004). The utilization of as-*lox* plants by *Empoasca* leafhoppers is likely to be mediated by JA-induced responses in the tissue probed by this opportunistically feeding leafhopper (Kessler *et al.* 2004) and does not necessarily involve the detection of terpenoid VOCs. However, the preference of flea beetles for GLV-producing WT over GLV-deficient as-*hpl* plants is likely mediated directly by GLVs, as the altered GLV production in as-*hpl* plants is not accompanied by a strong reduction in induced defence responses; this is the case for JA-deficient as-*lox* plants (Halitschke *et al.* 2004). Although the demonstrated function of GLVs as aggregation pheromone suggests a direct response of the beetle to the plant-derived GLVs, additional unknown responses elicited by GLV signalling in the plant could be perceived by the beetles and used as host location cues.

The integrated effect of a broad range of VOC-mediated interactions

The multiple functions of plant HI-VOCs, as host location cues, feeding stimulants, oviposition repellent and indirect

defences has several implications for the underlying evolutionary mechanisms of this induced response, particularly with regard to the maintenance of genetic diversity and ecological function of secondary metabolite production. A biased focus on the beneficial effects of HI-VOCs as indirect defences may lead to the conclusion that selection should favour plant phenotypes that constitutively emit large amounts of VOCs to attract natural enemies of their herbivores. However, considering the herbivore-attracting function of HI-VOCs, one may predict that plants not producing these compounds and thereby reducing their apparency to a fraction of their herbivore community would be favoured by natural selection. In addition, other ecological and physiological functions ranging from plant–plant signalling (Dicke *et al.* 2003; Baldwin *et al.* 2006) to tissue protection have been proposed. GLVs possess antimicrobial activity and are involved in defences against pathogen infection (Croft *et al.* 1993; Bate & Rothstein 1998). Furthermore, the wound- and herbivore-induced production of GLVs and their ability to increase transcript accumulation of a series of wound response genes suggests a function of GLVs as phytohormones involved in intraplant signalling (Sivasankar *et al.* 2000). This hormonal and antimicrobial function together with the demonstrated attraction of natural enemies should strongly favour selection for plants releasing volatiles even though these increase a plant's apparency to herbivores. However, HI-VOCs may also influence the apparency of attacked plants to neighbouring plants (Dicke *et al.* 2003; Baldwin *et al.* 2006). A plant perceiving VOC signals from damaged neighbours may gain a benefit by readying its defences (Engelberth *et al.* 2004) and the sender may incur a competitive disadvantage, which should be considered as a potential fitness cost of induced VOC emission. Interestingly, in *N. attenuata* the silencing of GLV production in the as-*hpl* plants results in an HI- VOC bouquet which elicits a large transcriptional change in neighbouring undamaged plants, a response significantly larger than that to the VOCs released by WT plants (Paschold *et al.* 2006). This example suggests that even the lack of certain signalling compounds can impose fitness costs through increased apparency to neighbouring plants. The enormous range of plant responses involving the production and release of volatiles highlights the adaptive function of these compounds and strongly negates the theory that the released VOCs are merely by-products of biosynthetic pathways (Peñuelas & Llusià 2004).

Our study emphasizes the importance of an integrated characterization of ecologically relevant traits under natural conditions to allow a complete functional evaluation without the bias of the experimental setup targeting individual responses. Moreover, the potential of indirect defences to protect agricultural crops will strongly depend on the ability to control these opposing functions and exclude compromising factors, such as the unintentional attraction of additional herbivores (Turlings & Ton 2006). Despite these potential negative effects of plant VOC emission, our study demonstrates a clear, indirect defensive function of HI-VOC emission, a defence that could be readily engineered into crops (Kappers *et al.* 2005; Schnee *et al.* 2006) to sustainably mediate top-down control of pest insects.

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