

A test of genotypic variation in specificity of herbivore-induced responses in *Solidago altissima* L. (Asteraceae)

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Abstract Plant-induced responses to multiple herbivores can mediate ecological interactions among herbivore species, thereby influencing herbivore community composition in nature. Several studies have indicated high specificity of induced responses to different herbivore species. In addition, there may be genetic variation for plant response specificity that can have significant ecological implications, by altering the competitive strength and hierarchical relationships among interacting herbivore species. However, few studies have examined whether plant populations harbor genetic variation for induction specificity. Using three distinct genotypes of *Solidago altissima* plants, we examined whether specialist herbivore species *Dichomeris leuconotella*, *Microrhopala vittata*, and *Trirhabda virgata* elicit specific induction responses from plants (specificity of elicitation), and whether induction differentially affects these herbivore species (specificity of effect). Results from bioassays and secondary metabolite analyses suggest that there is specificity of both elicitation and effect in the induced responses: *D. leuconotella* and *M. vittata* preferred and performed better on leaves damaged by conspecifics than heterospecifics, and induced qualitatively different secondary metabolite profiles. In contrast, *T. virgata* equally avoided but physiologically tolerated all types of damage. These patterns of specificity suggest that plant-induced responses

mediate asymmetric competitive interactions between herbivore species, which potentially intensifies inter-specific relative to intra-specific competition. Plant genotypes widely differed in overall susceptibility to the herbivores and secondary metabolite production, yet we found no genotype-by-treatment interactions in insect performance, preference and plant secondary metabolite production. This lack of genetic variation for induction specificity suggests that competitive interactions between herbivore species on *S. altissima* are homogeneous across plant genotypes.

Keywords Induced resistance · Genetic variation · Secondary metabolites · *Solidago altissima*

Introduction

Plant-induced responses to herbivory can mediate ecological interactions (e.g., competition, facilitation) among herbivore species on a shared host (Denno et al. 1995; Kaplan and Denno 2007), and thereby influence herbivore community composition in nature (Van Zandt and Agrawal 2004b; Viswanathan et al. 2005; Poelman et al. 2008, 2010). Increasing evidence suggests that plant responses—both morphological (leaf toughness, thorns, trichome) and chemical (toxic or anti-nutritive compounds; Karban and Baldwin 1997)—can be specific to herbivore species (Karbon and Baldwin 1997; Stout et al. 1998; Agrawal 2000; Messina et al. 2002; Van Zandt and Agrawal 2004a; Viswanathan et al. 2005). Plants may express specific induction responses to damage by different herbivore species (specificity of elicitation), and herbivore species may be differentially affected by induction (specificity of effect; Karban and Baldwin 1997). Such specificity in plant-induced responses suggests that plant–herbivore interactions may

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be more complex than previously thought, as the identity of initial colonizers can alter the trajectory of herbivore accumulation on a plant (Van Zandt and Agrawal 2004b; Viswanathan et al. 2005; Ando and Ohgushi 2008).

Few studies have examined whether different plant genotypes express varying degrees of induction specificity to multiple herbivore species (Bingham and Agrawal 2010). Genetic variation in specificity has important ecological implications, as it could alter relative competitive strengths among herbivore species on individual plants, and may promote co-existence of herbivore species (Smith et al. 2008; Anderson et al. 2009). A lack of genetic variation among host plants will homogenize interactions between herbivores, and lead to more predictable herbivore community composition. The few studies that have examined differences in response specificity among plant genotypes showed mixed results, with only a subset of examined plant responses exhibiting variation for specificity (e.g., Valkama et al. 2005; Bingham and Agrawal 2010).

Here, we tested the hypothesis that indirect, plant-mediated interactions between herbivores on shared host plants are affected by plant response specificity, and that the pattern of this specificity differs among plant genotypes. We measured herbivore-specific, induced responses in tall goldenrod, *Solidago altissima*, using three plant genotypes that showed a broad range of induced resistance to a model herbivore, *Spodoptera exigua* (R. Bode, personal communication). The goldenrod system provides an excellent opportunity to examine herbivore interactions via induced responses because of its diverse herbivore community (Root and Cappuccino 1992), including three specialist leaf herbivores that we examined (*Dichomeris leuconotella*, Gelechiidae, Lepidoptera; and *Microrhopala vittata* and *Trirhabda virgata*, Crysomelidae, Coleoptera). Previous research suggests that herbivore-induced responses may be an important component of defense in *S. altissima*, as damaged plants were more repellent to *T. virgata* larvae than undamaged plants (A. Kessler, unpublished). We assessed specificity of elicitation and effect in a full-factorial manner, by challenging *D. leuconotella*, *M. vittata*, and *T. virgata* with plants from each of four treatments: plants initially damaged by each of the three herbivores, and an undamaged control. This method allowed us to quantify the competitive relationships among herbivore species (Agrawal 2000).

Previous studies assessed specificity of plant responses with respect to herbivore preference (Van Zandt and Agrawal 2004b; Viswanathan et al. 2005), performance (Agrawal 2000; Messina et al. 2002), and defense-related plant traits, such as trichomes and secondary metabolites (Stout et al. 1998; Van Zandt and Agrawal 2004a; Valkama et al. 2005; Bingham and Agrawal 2010; Steinbrenner et al. 2011), suggesting an important role of plant response specificity in mediating complex interactions among herbivores.

To encompass important aspects of plant resistance (Underwood et al. 2002), we simultaneously measured herbivore preference and performance in bioassays, as well as the production of two classes of secondary metabolites, phenolics and diterpene acids, which are suggested as major defense-mediating compounds in *Solidago* species (LeQuesne et al. 1986; Hull-Sanders et al. 2007). For each plant response measured, we asked (1) whether there is specificity of elicitation (i.e. significant treatment effects) or effect (i.e. differential effect of treatment on herbivore species) in plant-induced responses, and (2) whether there is variation in specificity among plant genotypes (i.e. genotype-by-treatment effects). We discuss how observed patterns of induction specificity are likely to shape herbivore community structure.

Materials and methods

The tall goldenrod, *S. altissima* L. (Asteraceae), is a dominant perennial species of old-field communities in eastern North America (Werner et al. 1980) that is attacked by a diverse assemblage of herbivore species (Root and Cappuccino 1992; Root 1996). Larvae of *D. leuconotella*, *M. vittata*, and *T. virgata* are *Solidago* specialists that commonly co-occur in our study areas in Tompkins Co., New York (USA). Samples of *D. leuconotella* and *T. virgata* could potentially include low rates of their ecologically equivalent congeners (*D. flavocostella* and *D. levisella*, and *T. borealis*, respectively) because they are difficult to morphologically distinguish in the field. However, such “impurity” is likely to be minimized in our study, because we collected insects in late June, when the sample populations are known to be dominated by *D. leuconotella* (Loeffler 1994) and *T. virgata* (Messina 1982). *Dichomeris leuconotella* and *M. vittata* are shelter-feeders (leaf-rollers and miners, respectively), but they exit their old shelters and initiate new ones as their food resource diminishes (Damman 1994; Loeffler 1994). *Trirhabda virgata* is an external-feeder, which is highly mobile even during its larval stage (A. Kessler, unpublished). The two beetle species, *M. vittata* and *T. virgata*, severely damage *S. altissima* plants, and alter plant community composition during outbreak years (Meyer and Root 1993; Carson and Root 2000).

Plant materials

Sixteen clones of *S. altissima* were initially collected in 2008 from long-term (12 years) herbivore removal experimental plots, within an old field at Whipple Farm (Tompkins Co. NY). Environmental effects were controlled by clonally propagating plants for three cycles (initiated from rhizome cuttings) in a common greenhouse environment. For this

experiment, we selected three genotypes that exhibited low (genotype 11B2), medium (genotype 8A2) and high (genotype 10A4) levels of induced resistance to a model herbivore, *Spodoptera exigua* (R. Bode, personal communication).

In June 2010, we grew 120 plants (40 plants \times 3 genotypes) from rhizomes in the greenhouse. As plants reached about 20 cm in height, they were bagged with mesh sleeves and divided into 4 treatments: a control without damage, and three damage treatments, each damaged by *D. leuconotella*, *M. vittata*, or *T. virgata*. For each damage treatment, we added five insects per plant for 5 days prior to the bioassays. Additional insects were added if necessary to achieve at least 13 damaged leaves per plant. On average, each damaged leaf had 10.0, 17.7, and 12.3 % of its leaf area consumed by *D. leuconotella*, *M. vittata*, and *T. virgata*, respectively (significant difference between *D. leuconotella* and *M. vittata* consumptions, $P = 0.01$ with Tukey's HSD).

Preference and performance tests

Herbivore preference was compared among treatments for each plant genotype using a 10-cm² Petri dish choice arena. There were 10 plants per genotype-treatment combination. We sub-sampled six leaves from each plant, and created choice arenas that contained four excised leaves from each of the four treatments, while keeping genotype constrained (180 arenas in total = 3 genotypes \times 10 plants \times 6 leaves). Dishes were then evenly divided into three groups (20 arenas, per herbivore species, per genotype), where each of the three herbivore species was tested for leaf preference by scoring the leaf area (ImageJ; Schneider et al. 2012) consumed during the 3-day assay period. We used one larva of *D. leuconotella* and *T. virgata* per arena and three larvae of *M. vittata* to ensure mine initiation (the probability of successful mine initiation can be low; Damman 1994). We measured local, rather than systemic, induction responses by selecting leaves with visible chewing marks (in damage treatments). To standardize leaf age within a choice arena, leaves at similar positions on the plant were chosen. Leaves were kept fresh by wrapping the petiole with a moist paper towel. Leaves were placed in the choice arena in a random order to avoid preference biases caused by the leaf orientations in the arena.

Herbivore performance was tested in a Petri dish (10 cm in diameter) by allowing an individual insect to feed on one excised leaf for 3 days, during which none of the insects consumed the entire leaf. As in preference assays, we used leaves with visible chewing marks in the case of damage treatments to measure local induction responses. From each of 10 plants per genotype-treatment combination, we sub-sampled 6 leaves, and divided them into three groups where each of the three herbivore species were tested (a total of 720 dishes). Test herbivore fresh

mass was measured before and after the bioassay to control for variation in the initial size of field collected insects (*D. leuconotella*: 16.5 ± 5.3 g, *M. vittata*: 5.7 ± 1.8 g, *T. virgata*: 22.6 ± 8.0 g). Larvae that had been parasitized were removed from the analysis because parasitoids might manipulate the behavior and growth of their hosts (Hoballah and Turlings 2001).

We chose to use excised leaves to maximize our ability to detect differences in plant-induced responses to herbivore species. Using excised leaves limits additional induction while test insects are feeding, thereby allowing us to quantify the effect of initial induction treatments. It also allows to standardize leaf ages in bioassays that could affect herbivore responses. The effect of leaf deterioration on insect preference and performance was minimized by conducting the bioassays within 3 days of leaf collection, during which no observable deterioration of the leaves was found. Preliminary data on *S. altissima* showed that the effect of damage treatments on *T. virgata* choice and growth does not differ between excised and intact leaves (K. Morrel, personal communication), suggesting that our results, based on excised leaves, are ecologically relevant.

Chemical analysis

To assess induced changes in secondary metabolites, we collected the youngest, fully expanded leaf from each plant. These leaf samples were taken independently from the herbivore performance assays. To measure local induction responses from damage treatments, we ensured that the collected leaves had visible chewing marks. Leaves were flash-frozen in liquid nitrogen and stored at -80 °C. Samples were extracted in 1 mL 90 % methanol using FastPrep[®] tissue homogenizer (MP Biomedicals[®], Solon, Ohio, USA) at 6 m/s for 60 s with 0.9 g grinding beads (Biospec[®], Zirconia/Silica 2.3 mm). Fifteen μ L of the supernatant was analyzed for secondary metabolites by high-performance liquid chromatography (HPLC) on an Agilent[®] 1100 series HPLC equipped with a Gemini C18 reverse-phase column (3 μ m, 150 \times 4.6 mm; Phenomenex, Torrance, CA, USA). We used a standard method that targets phenolic compounds (Keinanen et al. 2001), with a slight modification to simultaneously quantify both phenolics and diterpene acids. Our elution system consisting of solvents (A) 0.25 % H₃PO₄ in water (pH 2.2) and (B) acetonitrile was: 0–5 min, 0–20 % of B; 5–35 min, 20–95 % of B, and 35–45 min, 95 % of B, with a flow rate of 0.7 mL/min. Phenolic compound peaks were identified to their compound classes using UV spectra. Diterpene acids were identified by mass spectral comparisons with published spectra (Sawabe et al. 2000). Peaks were quantified at 320 and 230 nm for phenolics and diterpene acids, respectively, and the relative concentration of each compound was

expressed as peak intensity relative to fresh tissue mass of each sample.

Statistical analysis

Herbivore preference was tested using Friedman's rank correlation tests to account for the dependency of data among four choices of leaves. Prior to the analyses, we converted the continuous data for consumed leaf area into binary data, with the most preferred treatment receiving 1 and the rest of the treatment receiving 0. Using this preference distribution, we first examined whether the responses of each of the herbivores to the damage treatments differed on the three plant genotypes (genotype effect). Because a genotype effect was not detected (see "Results"), we pooled data from all three genotypes to test herbivore preferences using a Friedman rank correlation test for each herbivore species using the actual data for leaf area consumed.

Herbivore performance was analyzed separately for each test herbivore by asking whether the identity of inducers (damage treatments) influences the growth of the herbivore. Effects of damage treatment and genotype on final larval mass were examined using a linear mixed model with treatment and genotype as fixed effects, initial larval mass as a covariate, and plant identity as a random effect. The random effect was included to avoid pseudo-replication because two leaves from each plant were used to measure the growth of each herbivore species. Model selection was done following Crawley (2007) to search for the minimum appropriate model. Values of induced resistance to each insect species were extracted from the output of tests that showed residual differences between undamaged control and the three damage treatments, while controlling for the covariate and the random effect. Based on these values, we further estimated the competitiveness of each herbivore species following Agrawal (2000): "induction caused" indicates the mean reduction in growth that a specific herbivore caused on heterospecific herbivores relative to control, and "effect experienced" indicates the mean reduction in growth that the herbivore experienced due to induction by heterospecifics compared to control (Fig. 4).

To examine the composition of leaf phenolics (Ph) and diterpene acids (Dt), we initially scanned the HPLC profile for optically active peaks, and identified seven Phs and three Dts. We conducted quantitative analyses on these 10 compounds, but the inclusion of four other major peaks of unknown classes did not qualitatively change the results. MANOVA was conducted to assess whether overall composition of the blends differed between treatments and genotypes. All chemistry data were log-transformed prior to analyses to improve normality, and then tested for treatment, genotype, and the treatment \times genotype interaction using Pillai's trace. Further post hoc tests were conducted

by running multiple MANOVAs for pairs of treatments (Scheiner 1993), and significance was corrected with the Holm–Bonferroni method. Because MANOVA showed a significant treatment effect, we carried out univariate analyses to identify specific compounds whose production differs between treatments. Post hoc tests using Tukey's HSD were conducted to examine which of the damage treatments differed from the control. Inducibility of each compound was calculated as: $(C_{\text{damage}} - C_{\text{control}})/C_{\text{control}}$, where C_{damage} is the compound concentration in a damaged treatment, and C_{control} is that in an undamaged control (Fig. 3a).

To examine which secondary metabolites affect growth of each herbivore species, we conducted multiple regression analyses using a Regression with Empirical Variable Selection (REVS) procedure (Goodenough et al. 2012). Initially, herbivore growth was converted to specific growth rate (SGR): $\text{SGR} = \ln(\text{final fresh mass}/\text{initial fresh mass})$, and averaged over two leaves taken from the same plant. We included samples across the genotypes and treatments to maximize the total variation in leaf chemistry and SGR estimates available from this experiment. The concentration of each compound was converted into unit variance prior to the analysis. REVS uses all-subset regression to quantify empirical support for each compound explaining insect SGR, and builds new sets of models by entering an increasing number of compounds in the order of empirical support. The best model is selected based on Akaike's Information Criterion (AIC) (Goodenough et al. 2012). Model selection by this method is more parsimonious and explains higher variation than full, stepwise, or all-subsets models, and is used in ecological studies where multicollinearity is often a problem (Goodenough et al. 2012). The impact of individual compounds, which were selected in the final model, on herbivore SGR was evaluated using a multiple regression analysis. This analysis tested whether the production of individual compounds, either at constitutive or induced levels, are correlated with the performance of each of the three herbivore species. Our data, however, do not allow to test the hypothesis that inducibility of secondary metabolite production per se (shown in Fig. 3a) affects herbivore performance as, for example, suggested in the "moving target hypothesis" (Adler and Karban 1994). All statistical analyses were conducted with R (R v.2.14.1; R Foundation for Statistical Computing, Vienna).

Results

Insect preference

For each herbivore species, the effect of damage treatments on each herbivore's preference did not vary among plant genotypes ($\chi^2 = 3.33$, $P = 0.77$; $\chi^2 = 6.98$, $P = 0.32$;

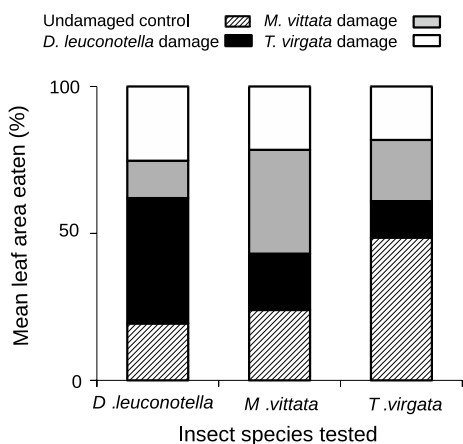


Fig. 1 Mean preferences of *Dichomeris leuconotella*, *Microrhopala vittata*, and *Trirhabda virgata* for the four treatments (hatched undamaged control, black *D. leuconotella* damage, gray *M. vittata* damage, and white *T. virgata* damage) across the three plant genotypes. *D. leuconotella* and *M. vittata* preferred leaves damaged by conspecifics ($\chi^2 = 16.2$, $P < 0.001$ and $\chi^2 = 10.9$, respectively), whereas *T. virgata* preferred undamaged control leaves ($P = 0.01$, $\chi^2 = 38.7$, $P < 0.001$)

$\chi^2 = 7.25$, $P = 0.30$ for *D. leuconotella*, *M. vittata*, and *T. virgata*, respectively), indicating no genotypic variation in induction specificity. A Friedman rank correlation test with pooled data from all three genotypes showed that *D. leuconotella* and *M. vittata* larvae preferred leaves damaged by members of their own species ($\chi^2 = 16.2$, $P < 0.001$ and $\chi^2 = 10.9$, $P = 0.01$, respectively, Fig. 1). Such specificity of elicitation was not observed in *T. virgata* preference assays: *T. virgata* larvae preferred undamaged control leaves and avoided all herbivore damage treatments ($\chi^2 = 38.7$, $P < 0.001$; Fig. 1).

Insect performance

There were no genotype \times treatment interactions for performance of each herbivore species (Table 1), indicating no genotypic variation in induction specificity. Thus, we excluded genotype \times treatment interactions in estimating the main effects of treatment and genotype. Initial larval mass positively affected final larval mass in all herbivore species. The identity of initial damagers (i.e. damage treatment) significantly influenced performances of *D. leuconotella* and *M. vittata*, but did not affect *T. virgata* performance, indicating both specificity of elicitation and specificity of effect (Table 1; Fig. 2). Contrasting each of the insect damage treatments with the undamaged controls, we found that damage by *M. vittata* and *T. virgata*, but not by *D. leuconotella*, induced resistance to *D. leuconotella* growth (respectively: $t_{108} = 2.8$, 2.5 , and 0.94 , $P = 0.0066$, 0.015 , and 0.35). Similarly, damage by *D. leuconotella*

Table 1 Results of LMM model describing the insect performance

Test insect	Factors	df	Likelihood ratio	P
<i>Dichomeris</i>	Initial weight	3	38.2	<0.0001
	Genotype (G)	4	4.92	0.085
	Treatment (T)	6	10.4	0.015
	G \times T	9	5.47	0.48
<i>Microrhopala</i>	Initial weight	3	224.2	<0.0001
	Genotype (G)	4	15.27	0.0005
	Treatment (T)	6	7.97	0.046
	G \times T	9	4.08	0.66
<i>Trirhabda</i>	Initial weight	3	69.1	<0.0001
	Genotype (G)	4	2.72	0.26
	Treatment (T)	6	1.59	0.66
	G \times T	9	3.77	0.70

Initial damage treatment and genotype were included as fixed effect, and initial weight as covariate. Overall significance of fixed factors was tested with likelihood ratio tests

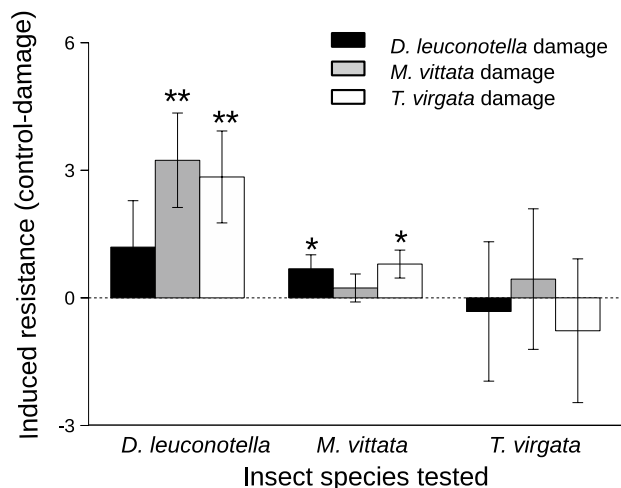


Fig. 2 Induced resistance (measured as differences in final larval mass (mg) between control and damage treatment controlling for variations due to initial larval mass in a linear mixed model) to *D. leuconotella*, *M. vittata*, and *T. virgata* in leaves initially damaged by *D. leuconotella* (black bars), *M. vittata* (gray bars) and *T. virgata* (white bars). Error bars ± 1 SE. P values for treatment effect are indicated by: * <0.05 , ** <0.01 , as determined by linear mixed model analysis

and *T. virgata*, but not by *M. vittata*, induced resistance to *M. vittata* growth (respectively: $t_{114} = 2.1$, 2.4 , and 0.7 , $P = 0.04$, 0.017 , and 0.48). No damage treatments induced resistance to *T. virgata* ($t_{114} < 1.15$, $P > 0.25$). Genotype had a strong effect on *M. vittata* growth and a marginal effect on *D. leuconotella* growth, with growth decreasing in the order of genotype 11B2 $>$ 8A2 $>$ 10A4. Genotype had no effect on *T. virgata* growth (Table 1).

Secondary metabolites

Mirroring the behavioral assays, damage from the three herbivore species resulted in differential induction of leaf secondary metabolites. Overall MANOVA on 10 compounds showed a significant effect of damage treatment ($F_{39,294} = 3.1$, $P < 0.0001$), genotype ($F_{26,194} = 269.8$, $P < 0.0001$), and genotype \times treatment ($F_{78, 606} = 1.5$, $P = 0.005$). To test whether the induction of overall compound production differs among damage by the three herbivores, we conducted a subsequent MANOVA excluding the undamaged control. We identified a treatment ($F_{26, 140} = 2.4$, $P = 0.0005$) and a genotype ($F_{26, 140} = 192.1$, $P < 0.0001$) effect, but no genotype \times treatment interaction ($F_{52, 288} = 1.2$, $P = 0.19$). Post hoc tests revealed that the control treatment was significantly different from each of the damage treatments ($F_{13,42} > 4.6$, $P_{\text{adj}} < 0.0001$), suggesting that all three herbivore species induced changes in secondary metabolite production. Moreover, the compound production in leaves damaged by *D. leuconotella* was significantly different from that of *M. vittata* ($F_{13,42} = 3.12$, $P_{\text{adj}} = 0.015$), whereas *T. virgata*-damaged leaves did not differ from the others ($F_{13,42} < 2.18$, $P_{\text{adj}} > 0.17$). Univariate comparisons showed that two phenolics, Ph11.5 and Ph12.9, were induced by all herbivores ($P_{\text{adj}} < 0.02$), and two diterpene acids, Dt29.8 and Dt31.0, were induced by *D. leuconotella* damage ($P_{\text{adj}} = 0.01$ and 0.0076 , respectively; Fig. 3a).

Multiple regression analyses (REVS) were conducted to identify secondary metabolites that explain herbivore performance (Fig. 3b). Growth of *D. leuconotella* was negatively correlated with Ph3.0 ($t = -3.5$, $P = 0.00067$) and positively with Ph11.5 and Dt31.0 ($t = 3.63$, $P = 0.004$; and $t = 2.96$, $P = 0.0037$, respectively). Growth of *M. vittata* was negatively correlated with Ph4.3 ($t = -4.96$, $P < 0.0001$), and positively with Ph3.0 and Ph5.0 ($t = 2.83$, $P = 0.005$; and $t = 4.9$, $P < 0.0001$, respectively). Finally, *T. virgata* growth was negatively correlated with Ph11.5 ($t = -2.81$, $P = 0.0058$) and positively with Ph3.0 ($t = 3.97$, $P = 0.0037$; Fig. 3b).

Discussion

Evidence for specificity of elicitation and effect

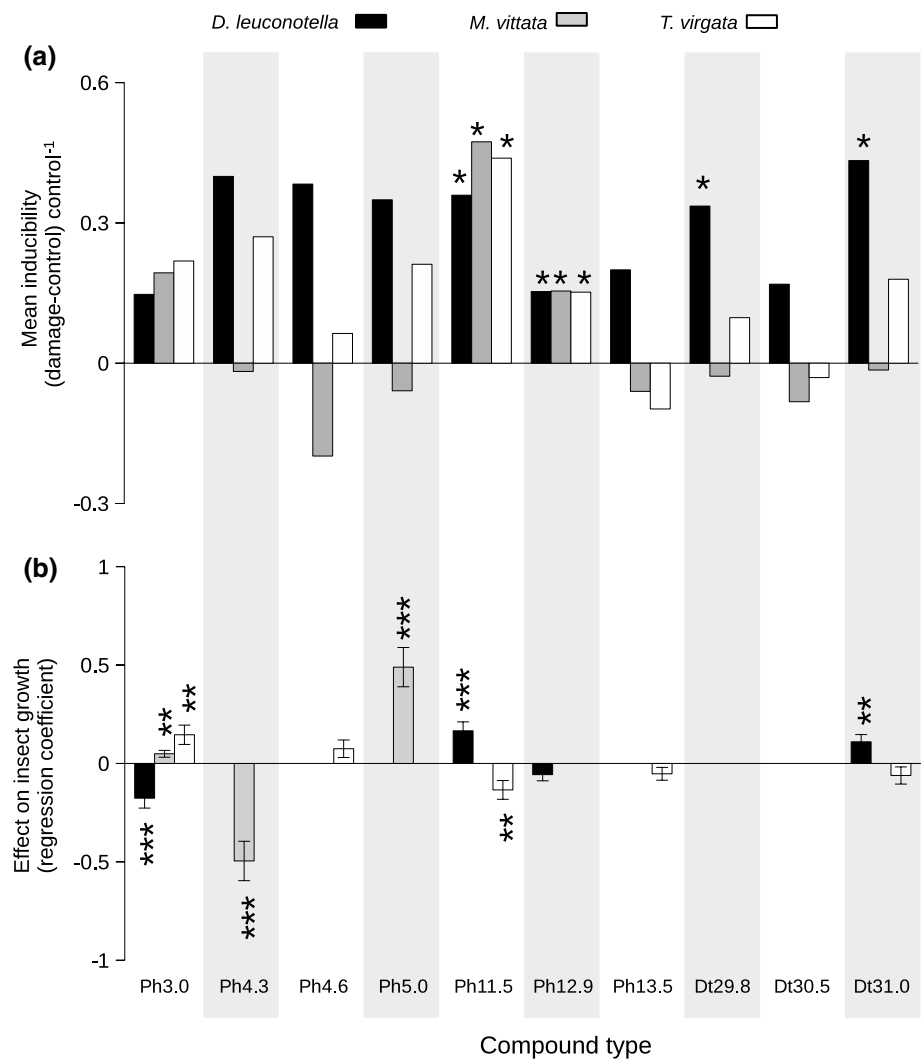
Larvae of *D. leuconotella* and *M. vittata* preferred leaves damaged by conspecifics over other leaves, while *T. virgata* avoided all damaged leaves. These preference patterns suggest that insect species elicit different plant responses, and that the specific pattern of induction differentially affects the preference of *D. leuconotella* and *M. vittata*. Interestingly, *D. leuconotella* and *M. vittata*

even preferred conspecific-damaged leaves over undamaged controls, although their performance on the damaged leaves did not exceed that on control leaves (see below). Such attraction to the conspecific-damaged leaves could be non-adaptive, or could suggest an additional benefit on these leaves, for example, by providing larvae with an enemy-free space (Bernays and Graham 1988). The predation risks for *D. leuconotella* and *M. vittata* larvae increase dramatically outside their shelters (Damman 1994; Loeffler 1994), and leaves previously rolled or mined may provide for faster shelter construction than unmanipulated leaves (Hunter 1987). In contrast, *T. virgata* larvae indiscriminately avoided all damaged leaves, although none of the damage treatments seemed to induce negative impact on their growth (Fig. 2). This behavioral avoidance of damaged leaves could allow mobile *T. virgata* larvae to escape natural enemies if plant-induced volatile production attracts enemies (Meiners et al. 2005). Further studies, including examining the effect of plant induction on tritrophic interactions, could potentially explain the observed patterns of preference specificity.

Similar to preference, we found evidence for a specificity of elicitation in *D. leuconotella* and *M. vittata* performance, with heterospecific but not conspecific damage, suppressing their growth. In contrast, *T. virgata* performance was unaffected by any of the damage treatments, which suggests that induction differentially affects each herbivore species (specificity of effect).

The chemistry data confirm that damage from each herbivore species induces qualitatively different secondary metabolite production. Damage by all three species caused significant changes in secondary metabolite production compared to control, including phenolic compounds, Ph11.5 and Ph12.9, which were induced by all herbivores. The composition of secondary metabolite blends significantly differed between leaves induced by *D. leuconotella* and *M. vittata*. Damage by *D. leuconotella*, tended to increase production of phenolics and diterpene acids, and significantly induced production of two diterpene acids, Dt29.8 and Dt31.0 (Fig. 3a). Interestingly, two compounds induced by *D. leuconotella*, Ph11.5 and Dt31.0, were positively correlated with its growth (Fig. 3b), suggesting that *D. leuconotella* induces production of compounds that improve its own growing condition. Damage by *M. vittata* induced production of few compounds (Fig. 3a). Because initial treatment by *M. vittata* caused greater damage than other species, the lack of induction of compound production did not result from lack of damage. Rather, it may indicate active suppression of compounds by which they are negatively affected. Damage by *T. virgata* larvae induced chemical profiles that were intermediate between the other two herbivores. Their performance was negatively affected by Ph11.5, a compound induced by all herbivores, and,

Fig. 3 Phenolic (*Ph*) and diterpene acid (*Dt*) compounds that were **a** induced by *D. leuconotella*, *M. vittata*, and *T. virgata* damage [expressed as inducibility: (level after damage – level in control)/level in control, averaged for the three genotypes], and **b** correlated with herbivore specific growth rate [SGR: expressed as regression coefficients (\pm 1SE) in REVS]. Different sets of samples were used to calculate (a) and (b). Closed bars indicate values for *D. leuconotella*, gray bars for *M. vittata* and open bars for *T. virgata*. Asterisks indicate significance: * <0.05 , ** <0.01 , *** <0.001 , as determined by Tukey's post hoc test for ANOVA for (a) and multiple regression analysis in (b)



although this trend did not translate into a significant effect of damage on their growth, it may explain the behavioral avoidance by *T. virgata* larvae of all damaged leaves.

We observed that *D. leuconotella* and *M. vittata* growth was negatively affected when pre-induced by heterospecifics, while not significantly affected when pre-induced by conspecifics (Fig. 2). This suggests that these two species may display a high level of tolerance to the particular changes that they induce themselves, but not necessarily to those induced by other species (Fig. 3). Along those lines, there is accumulating evidence that some herbivores, such as leaf-chewing or -mining lepidopteran larvae, are also able to attenuate induced resistance. For example, *Manduca sexta* and *Helicoverpa zea* caterpillars attenuate nicotine accumulation in tobacco plants (*Nicotiana* spp.) via compounds from their oral secretions (Kahl et al. 2000; Musser et al. 2002). However, it is unclear if this is overall more beneficial for the herbivore or for the plant, since nicotine induction could incur resource-based costs that

lower plant fitness in competition (Voelckel et al. 2001). Furthermore, *Eriocrania* leaf-miners were found not to induce an increase in total phenolics when feeding on silver birch (*Betula pendula*) (Fisher et al. 2000), or induce these to a lesser extent than do chewing herbivores (Hartley and Lawton 1987). Hence, it is tempting to speculate that *D. leuconotella* and *M. vittata* may attenuate the accumulation of key defense metabolites in *S. altissima*, while they cannot do so when these compounds are already induced by another species. Tolerance to self-induced changes and the ability to manipulate plant responses are not mutually exclusive hypotheses, and our current data cannot favor one over the other.

Although we found compelling parallels among the three measures of plant responses, there were some discrepancies between the bioassay and chemistry results. Our bioassays showed that *T. virgata* damage induced stronger resistance to both *D. leuconotella* and *M. vittata* than to conspecific damage (Fig. 2), suggesting

that *T. virgata* would induce a greater change in secondary metabolite production. Yet, *T. virgata* did not induce chemical profiles distinct from those of others, at least for the set of compounds analyzed here. The results indicate that the secondary metabolites we examined only partially explain the induced resistance observed in bioassays, and that traits not measured, such as other defense-related compounds, nutritional quality, and morphological traits, could also mediate plant resistance. For example, protease inhibitors (PIs) reduce plant digestibility and are highly inducible by herbivore attacks (Green and Ryan 1972). However, Bode et al. (2013) found that both serine- and cysteine-PIs in *S. altissima* are induced without specificity in response to two herbivores, *T. virgata* and *Spodoptera exigua*, suggesting that PIs are unlikely to contribute to the specificity found in our study.

Plant genotypes do not differ in the pattern of induction specificity

The three genotypes of *S. altissima* tested in this study were highly variable in their susceptibility to *M. vittata* and their secondary metabolite production, yet we found no evidence of genetic variation for the specificity of induction. Performance and preference showed no genotype \times treatment effect, indicating that the plant genotypes respond to each herbivore species in a similar way. Moreover, while the chemistry data revealed an interaction effect when the control treatment was included (in the MANOVA analysis), this interaction disappeared when the control was excluded. These results suggest that genotypes differ in overall inducibility of the compounds, but nevertheless responded similarly to damage by each herbivore species. So far, only a limited number of studies have examined variation in induction specificity among plant genotypes (Valkama et al. 2005; Bingham and Agrawal 2010). Bingham and Agrawal (2010) explicitly tested for genetic variation in the specificity of latex and cardenolide elicitation by two specialist caterpillars using 20 full-sib families of milkweed (*Asclepias syriaca*). While they found evidence for genetic variation in specific latex elicitation (but not cardenolides), they did not measure insect performance and/or preference in bioassays, making it unclear whether the specific elicitation is actually ‘experienced’ by the herbivores. Valkama et al. (2005) found no variation in induction specificity among five clones of silver birch, which similarly induced formation of glandular trichomes and secondary metabolites in response to different types of mechanical defoliation treatments. However, this study did not examine plant responses to different herbivore species, and potentially missed any responses to actual herbivory induced by chemical elicitors unique to each herbivore species (Yoshinaga et al. 2010; Erb et al. 2012).

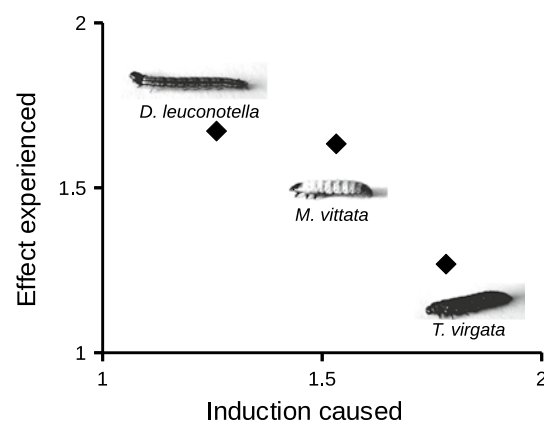


Fig. 4 Competitiveness of herbivore species measured as “induction caused” (the average reduction in growth each herbivore species caused on heterospecifics relative to control) and “effect experienced” (the average reduction in growth the herbivore experienced due to induction by heterospecifics compared to control)

Effect of specificity on interspecific competition and herbivore communities

On average, *D. leuconotella* experienced a severe performance reduction from changes induced by other insects (high effect experienced), and had little effect on the other insects (low induction caused; Fig. 4). *Trirhabda virgata* was on the opposite end of the spectrum, exhibiting high tolerance to induction by others (low effect experienced), and significantly suppressing the growth of others (high induction caused). We speculate that competition among the three herbivores should be asymmetric, with rank competitive order: *T. virgata* > *M. vittata* > *D. leuconotella*. Our measurement of local induction may possibly overestimate induction effects (Karban 2011), but studies in other systems have found that systemic responses of induction can be as strong or stronger than local induction (Kessler and Baldwin 2004). Induced resistance may also attenuate with time (Underwood 1998), and thus change the strength of competition. While the temporal dynamics of induced responses are not known in our system, other studies have shown prolonged influence of initial damage on subsequent colonizers in the field (Van Zandt and Agrawal 2004a; Ando and Ohgushi 2008; Poelman et al. 2010).

Asymmetrical competitive interactions are commonly observed among herbivorous insect species, especially for plant-mediated interactions (Kaplan and Denno 2007), with some species inducing stronger resistance and/or tolerating induced resistance more than the others (Agrawal 2000; Messina et al. 2002; Van Zandt and Agrawal 2004a; Viswanathan et al. 2005). We further observed that *D. leuconotella* and *M. vittata* induce changes that are favored by (or do not affect) conspecifics but negatively affect

heterospecifics, suggesting that plant-induced responses may mediate and intensify inter-specific competition relative to intra-specific competition. Such asymmetry and specificity of induction effect could lead to plant-mediated competitive exclusion (Anderson et al. 2009). One possible mechanism maintaining the coexistence of herbivores within a population may be that competitive hierarchy varies among plant genotypes, allowing different species to become more competitive on different plant genotypes (Smith et al. 2008). Such competitive ability tradeoffs between different plant genotypes appear unlikely on *S. altissima*, as distinct genotypes in our experiments responded similarly to different herbivore species. Our results suggest that *T. virgata* is the superior competitor independent of plant genotype.

Weaker competitors may escape competition from *T. virgata* by avoiding plant genotypes on which they are likely to encounter *T. virgata* (Amarasekare 2003). Maddox and Root (1987) found that *S. altissima* genotypes significantly differed in attractiveness to *M. vittata* and *T. virgata* (they did not examine attractiveness to *D. leuconotella*), and the trait was highly heritable. Preferences of *T. virgata* for particular plant genotypes may thus create spatial refuges for inferior competitors. Given our finding that the herbivores indirectly compete via plant-induced resistance, and competitive interactions are asymmetric and consistent across plant genotypes, we hypothesize that inter-specific competition via induced resistance may reinforce niche partitioning among herbivore species on plant genotypes.

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