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Research Article

Differential leaf resistance to insects of transgenic sweetgum (*Liquidambar styraciflua*) expressing tobacco anionic peroxidase

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Abstract. Leaves of transgenic sweetgum (*Liquidambar styraciflua*) trees that expressed tobacco anionic peroxidase were compared with leaves of *L. styraciflua* trees that did not express the tobacco enzyme. Leaves of the transgenic trees were generally more resistant to feeding by caterpillars and beetles than wild-type leaves. However, as for past studies with transgenic tobacco and tomato expressing the tobacco anionic peroxidase, the degree of relative resistance depended on the size of insect used and the maturity of the leaf. Decreased growth of gypsy moth larvae appeared mainly due to decreased consumption, and not changes in the nutritional quality of the foliage. Transgenic leaves were more susceptible to feeding by the corn earworm, *Helicoverpa zea*. Thus, it appears the tobacco anionic peroxidase can contribute to insect resistance, but its effects are more predictable when it is expressed in plant species more closely related to the original gene source.

Key words. Plant resistance; tent caterpillar; fall webworm; corn earworm; gypsy moth; fall armyworm; cigarette beetle; *Nicotiana*.

Insect feeding damage can cause significant economic losses to herbaceous and woody plants. Resistance to insects has been associated for some time with chemical factors, but increasingly biochemical factors, such as protein toxins, appear to be involved [1]. More recently, sugar metabolizing enzymes [2] have demonstrated activity against insects. However, enzymes that interact in plant growth processes have received only limited study in insect resistance. Although peroxidase activity has often been associated with resistance to plant pathogens [3], recent research has indicated that peroxidase, for a variety of reasons, may be associated with plant resistance to insects [4–6]. This concept has been validated with work on transgenic tobacco and tomato overex-

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pressing tobacco anionic peroxidase [7–9]. If a peroxidase isozyme that appears to play a defensive role against insects in one plant species is expressed in a more distantly related plant species and results in greater insect resistance, then the defensive role of such an isozyme would be further validated.

Sweet gum, *Liquidambar styraciflua*, is a large deciduous tree native to the United States, which has been introduced into other countries due to its ornamental value [10]. Its wood is used for furniture, plywood and pulp [10], and has potential for fermentative production of ethanol [11]. This species, along with *L. orientalis*, is also important as a source of storax gum, used in the perfume and pharmaceutical industries [10, 12]. However, *L. styraciflua* is fed upon by a number of different species of boring insects and caterpillars [13]. Especially important as a pest of *L. styraciflua* is the fall webworm, *Hyphantria cunea*, which defoliates the tree and decreases its aesthetic value due to the presence of large webs (ca. 30–40 cm across) that protect the caterpillars from many predators and parasites [14]. Serious leaf defoliation can also be caused by the forest tent caterpillar, *Malacosoma disstria*, which can significantly retard growth [15]. Where their ranges overlap, *L. styraciflua* is also a favoured host of the gypsy moth, *Lymantria dispar* [16], the most important defoliator of deciduous tress in eastern North America [17]. Recent successful genetic engineering has produced *L. styraciflua* trees that contain the gene for tobacco anionic peroxidase, as indicated by Southern hybridization analysis [18]. Leaves from these trees were used to determine if the tobacco anionic peroxidase would also cause increased resistance to insects in a plant species more distantly related to the enzyme source plant than those species examined previously.

Materials and methods

Plants. Transgenic *L. styraciflua* that contain the tobacco anionic peroxidase gene were used for assays. Production of these plants has been described previously [18]. Plants were originally obtained from Dr. Kim Steiner (The Pennsylvania State University, University Park, PA, USA) and were #85KS003 progeny from a plant originally obtained from New Brunswick, NJ, USA. The binary TI-plasmid tobacco anionic peroxidase overexpression vector, pML507, has been previously described [19]. It is composed of a 1256-base pair complementary DNA (cDNA) insert of the tobacco anionic peroxidase gene [which includes the 22-amino acid signal peptide and the poly(A) addition site] cloned between a CaMV 35S transcription promoter and terminator and coupled with the neomycin phosphotransferase gene of Tn5 (NPII) fused to the nopaline synthase promoter and terminator sequences and the transferred-DNA (T-DNA) left and right borders [19]. The binary TI-plasmid vector was introduced into *L. styraciflua* by cocultivation with *Agrobacterium tumifaciens* [18]. An *L. styraciflua* shoot regenerated in the presence of kanamycin and tested with Southern hybridization analysis indicated the presence of a 1.2-kb restriction fragment that hybridized with the tobacco anionic peroxidase gene probe, as would be predicted for an intact tobacco anionic peroxidase gene [18]. Genomic DNA from the representative transformant digested with Pst I also yielded unique-sized fragments that confirmed integration of the tobacco anionic peroxidase gene [18]. Vegetatively propagated plants derived from this transformant were used in bioassays as described below. Wild-type plants used in bioassays are also described below.

Peroxidase assays. Peroxidase was extracted from leaves used in *O. nubilalis* and *H. cunea* bioassays with a combination of methods published previously [20, 21]. Leaf tips of ca. 0.5 cm^2 were homogenized in a ground glass tissue grinder containing 1 ml of 0.1 M, pH 7.4 sodium phosphate buffer with 5 mM sodium bisulphite and 2% Triton-X-100. The homogenate was allowed to stand on ice for 30 min, then centrifuged at 12,000*g* for 15 min. The supernatant was used for spectrophotometric enzyme assays using guaiacol as the substrate similar to previously described methods [8], except that a 10-min assay period was used with enzyme homogenates containing ca. 0.03 mg of protein. The pellet was also analysed for peroxidase activity using a method similar to the spectrophotometric assay, except the assay was run discontinuously. Buffer was initially added, and the pellet was broken up with a spatula and then resuspended by vortex mixing for 30 s. After adding the rest of the assay reactants, the suspensions were incubated at 25 °C for 10 min. The suspensions were placed on ice for 30 min, and then centrifuged at 12,000*g* for 15 min. The supernatant was removed, and its absorbance at 470 nm was quantitated. The colour of the pellet was also rated using the '/2' column of the 5Y Munsell colour chart as described previously [6]. The protein content of the supernatant was determined using the Bio-Rad packaged assay (Bio-Rad, Richmond, CA, USA).

Insects. The *H. cunea* larvae were collected from wild cherry (*Prunus cerasus*) in Fulton County, IL, in the fall of 1994. The web containing the caterpillars was held at 4 °C until larvae were needed. The *H. cunea* larvae used in bioassays (presumably third instars) were ca. 1.5 cm long and weighed ca. 20 mg. Late (presumably fifth) instar larvae of the eastern tent caterpillar, *Malacosoma americana*, ca. 3 cm long and 200 mg, were collected from apple (*Malus sylvestris*) in Peoria County, IL, in the spring of 1995. Eggs of *L. dispar* were received from

the Canadian Department of Forestry, Insect Population Laboratory, Sault Ste. Marie, Ontario, Canada, and larvae were reared on river birch (*Betula nigra* L.) foliage in plastic boxes at 25 °C and a 15:9 light:dark photoperiod until used in assays. Foliage turgor of birch leaves was maintained by keeping freshly cut branches in floral water-picks sealed with Parafilm.

Although fall armyworms (*Spodoptera frugiperda*), corn earworms (*Helicoverpa zea*) and European corn borers (*Ostrinia nubilalis*) are not reported to feed on *L. styraciflua*, they feed on a wide variety of plants in different families [14], and so were included in bioassays for comparative purposes. The *S. frugiperda* and *H. zea* were reared on pinto bean-based diets as described previously [22]. The *O. nubilalis* were obtained from M. R. McGuire, USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL, USA. Cigarette beetles (*Lasioderma serricorne*) are capable of feeding on a wide variety of plant materials generally toxic to insects, including tobacco and aromatic spices [14]. The *L. serricorne* were reared on 45% white corn meal, 45% white corn grits and 10% brewer's yeast as described previously [23].

Insect bioassays. Leaves from transgenic *L. styraciflua* trees and the same cultivar of untransformed trees ca. 3 years old were used in bioassays for all insects except *L. dispar*. Mature leaves that *H. cunea* normally encounters and feeds upon were used in bioassays with this insect species. Fully expanded leaves which were 2–5 leaves below the youngest fully expanded leaf were randomly selected from three terminals from three different trees. Leaves were placed in 20 × 20 cm press-seal plastic bags along with five *H. cunea* larvae. Bags with leaves were held at 27 ± 1 °C, 40 ± 10% relative humidity and a 14:10 light:dark photoperiod. Ten leaves of each plant type were used in the assays. Leaves were examined at 3, 4 and 5 days. After 5 days, the number of dead larvae was determined, and surviving larvae were weighed. Leaf damage was determined by visually estimating the area consumed to the nearest cm^2. *M. americana* phenologically encounters and primarily feeds upon immature leaves. Thus, assays were performed in a manner similar to those with *H. cunea*, except immature leaves (6 cm from petiole to leaf attachment to leaf tip) were used. Because of the larger size of the available *M. americana* larvae, only two larvae per leaf were used. A total of 13 wild-type and 19 transgenic leaves were used for the assays. Leaves were examined daily for feeding damage until larval mortality exceeded 80%.

Newly moulted fourth instar *L. dispar* were used in assays and weighed (to the nearest 0.01 mg) immediately prior to initiation of the assay. Nine most recently matured, fully expanded leaves were taken from each of eight transgenic and eight wild-type *L. styraciflua* in late June. Wild-type *L. styraciflua* included seven trees ranging from ca. 5–25 years old planted in three different locations on the Ohio Agriculture Research and Development Center (OARDC) campus in Wooster, OH, USA, and one tree from the same cohort as the transgenic trees. Leaves were placed in Ziploc resealable bags at ca. 4 °C, and transported to the laboratory. Bioassays were set up the same day the leaves were collected. Three larvae were randomly assigned to each of 16 trees, with each larva offered three leaves from its assigned tree (a total of 24 larvae used for each tree type). The combined area (cm^2) of the three leaves was determined via digital image analysis using CI-400 Computer Image Analysis System and software (CID, Vancouver, WA) prior to being offered to each respective larva. Individual larvae with leaves were confined to petri dishes (15 cm in diameter, 2.5 cm high) containing a base of plaster of paris mixed with activated charcoal (19:1) to provide a consistent gas phase during the assays. Water added to the plaster base provided a high humidity environment which maintained the turgor of detached leaves. Assay dishes were held under the same conditions used to rear larvae. After 48 h the bioassay was terminated and the larvae were immediately weighed. Duration of the bioassay was quantified to the nearest 15-min interval. Frass and leaves were collected, oven-dried at 45 °C for 48 h and weighed. The cumulative area of leaf portions was determined prior to drying. Initial leaf dry weight was then calculated as initial dry weight = (initial leaf area × final dry weight)/final leaf area.

To determine potential mechanisms of peroxidase effects on growth and consumption by *L. dispar*, standard gravimetrical nutritional indices were estimated [24, 25]. Larval growth was estimated as growth = final weight − initial weight. The dry weight of foliage consumed (consumption) was estimated by subtracting final dry leaf weight from initial leaf weight. The percentage of consumed leaf that was subsequently digested (approximate digestibility, or AD), was calculated as (weight consumed – frass weight)/frass weight. The percentage of digested leaf subsequently converted to biomass (efficiency of conversion of digested food, or ECD) was calculated as growth/consumption.

Because of their typically higher quality and lower levels of defensive compounds, younger leaves were used in assays with insects not adapted to feeding on *L. styraciflua*. The youngest fully expanded leaves were used in assays with *S. frugiperda* and *L. serricorne*. These leaves were obtained from the same terminals as were the fully expanded leaves used in the *H. cunea* assays. Leaf disks were cut from leaves and randomly selected for use in assays. For assays with *S. frugiperda*, a single leaf disk was placed in a 3.5-cm diameter petri dish (Falcon 1008, Baxter, McGaw Park, IL) along with a 3-cm piece of moist filter paper and either 10 newly

Table 1. Peroxidase activity of leaves used in bioassays.

Assay/source	Parameter	Relative amount	
		wild-type	transgenic
O. nubilalis assays (immature leaves)			
Supernatant	protein (mg/ml)	0.25 ± 0.02	0.30 ± 0.01*
Supernatant	peroxidase†	0.033 ± 0.010	0.047 ± 0.021
Pellet	peroxidase†	0.106 ± 0.014	0.212 ± 0.019*
Pellet	brownness‡	5.3 ± 0.1	3.2 ± 0.2*
H. cunea assays (full-size leaves)			
Supernatant	protein (mg/ml)	0.64 ± 0.04	0.78 ± 0.07
Supernatant	peroxidase†	0.188 ± 0.023	1.930 ± 0.184*
Pellet	peroxidase†	0.135 ± 0.025	0.416 ± 0.066*
Pellet	brownness‡	4.6 ± 0.1	3.3 ± 0.2*

Values listed are means $\pm$ SE. Values in rows followed by an '*' are statistically different at $P < 0.05$ by analysis of variance. †Values are changes in absorbance over the 10-min assay period. ‡Values that are lower in number are darker. A '5' value corresponds to a grayish tan and a '3' value corresponds to a dark brown.

hatched or 5 second instar larvae of *S. frugiperda*. Five plates of each leaf type were used in each experiment. Plates were examined after 1 day for second instars and after 2 days for first instars to determine feeding damage and mortality. Feeding damage was rated on an integer scale of 0 to 10, with 0 being no feeding and 10 being the total leaf disk consumed. Assays with *L. serricorne* were similarly designed. Five adults were placed with each leaf disk, and five disks of each leaf type were used in each assay. Plates were examined after 7 days for insect mortality and leaf feeding.

Assays with intact immature leaves were performed with *H. zea* and *O. nubilalis* by confining ca. 3-cm leaves (measured from petiole attachment of leaf to leaf tip) in petri dishes with tight-fitting lids (Falcon 1006) with 10 first instar larvae. Ten leaves of each type were used with each insect species. Leaf disk assays of mature leaves were performed with *H. zea* and *O. nubilalis* essentially as described for *S. frugiperda*, except the petri dishes with tight-fitting lids were used. Twenty leaf disks of each type were used with each insect species. Assays were run for 4 days.

Mortality data were analysed for significant differences using chi-square analysis. Weights and leaf damage for assays not involving *L. dispar* were analysed for significant differences using SAS [26] analysis of variance (ANOVA). More sophisticated analyses were used for *L. dispar* due to the interest in mechanisms involved in peroxidase activity. Data were analysed by ANOVA (SAS, PROC GLM; type III sum of squares) [27]. Individual trees were considered the experimental unit, with multiple observations (insects) per tree analysed using a nested model. The two larvae that failed to survive the experiment were excluded from analyses. Treatment effects on growth and consumption were tested by analysis of covariance using initial larval mass as the covariate. The assumption that data were distributed normally was tested using the Shapiro-Wilks test and by visually inspecting residuals (SAS PROC UNIVARIATE [27]). The assumption of homogeneity of variance was tested using Bartlett's test. In all cases data conformed with necessary assumptions.

Results

Extracted protein was relatively uniform for the different leaves of each tree type. Soluble (supernatant) leaf peroxidase activity of immature leaves used in *O. nubilalis* assays was variable, but greater in the transgenic leaves overall (table 1). Full-sized leaves used in *H. cunea* assays had about 10-fold higher soluble peroxidase activity than did wild-type leaves. Bound (pellet) peroxidase activity from both ages of the transgenic leaves was at least twofold higher and significantly greater than bound peroxidase activity of both ages of the wild-type leaves. The pellets of the transgenic leaves were also significantly darker than the pellets of the wild-type leaves for both leaf ages. Based on the previously reported specific activity of pure tobacco anionic peroxidase towards guaiacol under the same assay conditions used in the present study [28], the quantity of tobacco anionic peroxidase expressed in the mature leaves would be approximately 9 nmol per gram of fresh leaf.

A significantly greater number of *H. cunea* larvae caged with fully expanded transgenic leaves died compared with larvae caged with wild-type leaves (table 2). Although there were no significant differences in average weights of survivors, significantly ($P < 0.05$) fewer insects fed the transgenic leaves weighed more than 25 mg compared with those fed the wild-type leaves (3.6 vs. 20.5%, respectively). The mean area of leaf damaged by *H. cunea* larvae caged on the transgenic leaves was

Table 2. Effect of wild-type and transgenic *L. styraciflua* leaves on *H. cunea* and *M. americana* larvae.

Leaf type	% Mortality	Weight (mg)	Feeding rating
H. cunea caged with mature leaves removed from trees			
Wild-type	22.0	17.7 ± 1.1	3.1 ± 0.7
Transgenic	44.0*	15.7 ± 1.0	0.1 ± 0.1*
M. americana caged with immature leaves removed from trees			
Wild-type	88.5	ND	3.2 ± 0.9
Transgenic	81.6	ND	4.3 ± 1.0

Weight and rating values are means $\pm$ SE. Feeding rating based on no. of cm^2 of leaf consumed (estimated to the nearest cm^2). Values of like studies in columns followed by an '*' are significantly different at $P < 0.05$ by chi-square analysis (mortality) or ANOVA (mean values). ND = not determined.

ca. 30 times less than the area damaged by larvae caged with the wild-type leaves (table 2). For the leaves used in *H. cunea* assays, supernatant peroxidase activity was inversely correlated with the amount of feeding by the caterpillars (R^2 of -0.60, $P = 0.005$). There were no significant differences in mortality or feeding damage when *M. americana* larvae were caged with immature leaves (table 2).

Growth of *L. dispar* larvae feeding on *L. styraciflua* foliage that expressed tobacco anionic peroxidase was significantly decreased (by 33%) relative to that of larvae feeding on wild-type foliage (table 3). Larval consumption was also significantly decreased, but not as dramatically. Larvae feeding on wild-type foliage consumed 21% more foliage than larvae feeding on foliage from transgenic trees (table 3). Expression of tobacco anionic peroxidase had no significant effect on the ability of the *L. dispar* larvae to digest leaves (AD) (table 3). However, the presence of tobacco anionic peroxidase had a noticeable (but statistically insignificant) effect on the ability of larvae to convert digested food to biomass (ECD), which was decreased 20% for larvae feeding on transgenic leaves compared with those feeding on wild-type leaves (table 3). While we cannot reject the null hypothesis of lack of treatment effect on ECD, we also cannot rule out the possibility that the presence of tobacco anionic peroxidase affected the ECD. The statistical power of the test was only 0.24, indicating that the probability of a type II error was 0.76.

Although the leaves used in *L. dispar* assays were from different trees, the amount of variability within a particular treatment (wild-type or transgenic) was of little consequence. The nested analysis of variance revealed significant variation in larval growth among trees within a treatment. However, this variation was much less than that which occurred between the two treatments ($F = 2.99$ vs. $F = 20.05$, respectively). There was also significant variation in approximate digestibility among trees within a treatment, but no significant variation among the two treatments ($F = 2.61$ and 1.10, respectively). There was no significant variation among trees within a treatment in relative consumption rate or ECD.

No significant mortality was noted when first or second instar *S. frugiperda* were caged with leaf disks (table 4). Although there were no significant differences, *S. frugiperda* larvae feeding on the transgenic leaf disks generally caused less damage than those feeding on the wild-type disks. No significant mortality of *L. serricorne* adults was noted after feeding on either type of leaf disk. However, three times less transgenic leaf disk was consumed compared with the wild-type disks (table 4).

Mortality of *O. nubilalis* was significantly greater and feeding damage was significantly less for immature leaves from transgenic plants compared with those from wild-type plants, although surviving caterpillars that fed on the transgenic leaves were significantly larger (table 5). Little difference in any parameter was noted for those *O. nubilalis* larvae confined with leaf disks from mature leaves of each plant type. In contrast, the *H. zea* confined with both mature and immature leaves from transgenic trees often consumed significantly greater amounts of leaf material than those confined with wild-type tree leaves. The *H. zea* larvae were significantly larger when they fed on immature leaves of transgenic trees compared with wild-type leaves. However, weights of survivors after 4 days were not significantly different for *H. zea* larvae fed on the two types of mature leaves.

Discussion

Lignin production is a major function of peroxidases [29]. Peroxidase-mediated lignification appears to be involved in insect resistance in plants [7]. A number of other insect resistance factors may be enhanced due to higher levels of peroxidases as a result of peroxidase generation of highly reactive compounds from allelochemicals not involved in lignification [30, 31]. These reactive compounds can be toxic and inhibitory, and may be involved in further binding and cross-linking, as has been recently reviewed [7]. There is evidence that

Table 3. Comparison of performance of *L. dispar* larvae fed transgenic and wild-type mature *L. styraciflua* leaves.

Parameter	Wild-type	Transgenic	df	F value	P value
Growth (mg)	9.0 ± 0.8	6.1 ± 0.8	1,13	6.71	0.021
Consumption (mg)	82.3 ± 5.1	65.3 ± 5.1	1,13	5.50	0.034
% Leaf digested (AD)	50.6 ± 2.9	47.8 ± 3.0	1,14	0.42	0.527
% Digested foliage converted to biomass (ECD)	24.0 ± 2.5	19.1 ± 2.6	1,14	1.75	0.208

Values are least square means $\pm$ SE.

peroxidase activity contributes to insect resistance in tomato, *Lycopersicon esculentum* [4, 7, 8, 32]; tobacco, *Nicotiana tabacum* and *N. sylvestris* [7–9]; soybeans, *Glycine max* [33]; and maize, *Zea mays* [5, 6, 34–37]. Dark brown regions were noted in the transgenic compared with wild-type *L. styraciflua* leaves where insects fed, and the pellet of the transgenic leaf homogenates also turned much darker compared with that from wild-type leaves in the presence of guaiacol and peroxidase. More rapid and extreme browning has also been noted in plant tissues (over)expressing tobacco anionic peroxidase compared with wild-type plant tissues in *Nicotiana* spp. and in *L. esculentum* [7]. The degree of enhancement of total peroxidase activity noted in mature leaves in the present study is similar to that reported for leaves of transgenic *Nicotiana* spp. and *L. esculentum* (over)expressing tobacco anionic peroxidase [20, 38], and was also associated with increases in resistance to first instar *H. zea* [7–9]. However, in contrast to the simple extraction techniques used for *Nicotiana* spp. and *L. esculentum* [7–9, 20] peroxidase, the *L. styraciflua* peroxidase activity had to be extracted using detergent and is thus apparently bound to other materials, as occurs in other tree species [21].

Table 4. Effect of wild-type and transgenic *L. styraciflua* leaves on *S. frugiperda* and *L. serricorne*.

Leaf type	% Mortality	Feeding rating
2nd instar *S. frugiperda*		
Wild-type	0.0	6.6 ± 0.7
Transgenic	0.0	4.8 ± 0.4
1st instar *S. frugiperda*		
Wild-type	11.8	4.8 ± 0.9
Transgenic	3.7	3.2 ± 0.7
Adult *L. serricorne*		
Wild-type	0.0	3.4 ± 0.4
Transgenic	0.0	1.0 ± 0.3*

Feeding rating values are means $\pm$ SE. Values in columns of like assays followed by an '*' are significantly different at $P < 0.05$ by ANOVA. ND = not determined.

Leaf maturity can influence the relative resistance to insects produced by altering tobacco anionic peroxidase expression transgenically in different tobacco species and varieties [7, 9]. Relatively immature leaves from tobacco lines overexpressing tobacco anionic peroxidase were more resistant to *H. zea* compared with wild-type plants, while little difference in *H. zea* resistance between the two different plant types was noted for mature leaves [7–9]. The same effect was noted in the present study with *O. nubilalis*. The size of the insect can also influence the degree of resistance noted for transgenic plants overexpressing tobacco anionic peroxidase. Leaves of *N. sylvestris* tobacco plants overexpressing tobacco anionic peroxidase were equally susceptible to feeding by third instar *H. zea* compared with leaves from wild-type plants [7, 9]. However, leaves from overexpressing plants were more resistant to feeding by first instar *H. zea* compared with those from wild-type plants [7, 8]. Tomato fruit from transgenic tomato expressing tobacco anionic peroxidase was more resistant to first instar *H. zea*, but not third instars, compared with fruit from wild-type plants [7, 8]. The lack of resistance of the transgenic sweetgum to *M. americana* noted in the present study may be a result of the relatively large size larvae used, although differences in peroxidase-mediated resistance mechanisms in young vs. mature leaves may also be involved. Tobacco and tomato stems (over)expressing tobacco anionic peroxidase were more resistant to feeding by the dusky sap beetle (*Carpophilus lugubris*) than wild-type stems [7, 8]. We also noted resistance of the transgenic *L. styraciflua* leaves to a different beetle species (*L. serricorne*) relative to wild-type leaves in the present study.

We were somewhat surprised that the transgenic *L. styraciflua* leaves were more susceptible to feeding by *H. zea* compared with the wild-type plants, based on our prior studies with tobacco and tomato and the results with the other caterpillar species in the present study. However, polymerized catechin (which may be promoted by peroxidases or polyphenol oxidases) was less toxic to aphids than was catechin itself [39]. Possibly, different allelochemicals are responsible for resistance to different insect species in *L. styraciflua*. Apparently,

Table 5. Effect of immature and mature wild-type and transgenic *L. styraciflua* leaves on *O. nubilalis* and *H. zea*.

Leaf type	% Mortality†	Feeding rating‡		Weight (mg)
	day 2	day 2	day 4	day 4
O. nubilalis				
Wild-type – immature	12.3	44.5 ± 6.2	2.8 ± 0.4	0.11 ± 0.01
Transgenic – immature	38.8*	28.1 ± 3.9*	2.0 ± 0.4	0.18 ± 0.02*
Wild-type – mature	18.4	53.6 ± 2.7	2.2 ± 0.2	0.09 ± 0.01
Transgenic – mature	25.7	54.4 ± 3.2	2.5 ± 0.2	0.09 ± 0.01
H. zea				
Wild-type – immature	20.0	22.8 ± 2.7	3.9 ± 0.8	0.38 ± 0.04
Transgenic – immature	8.4*	47.7 ± 3.6*	7.6 ± 0.5*	1.04 ± 0.10*
Wild-type – mature	4.9	2.0 ± 0.1	4.6 ± 0.4	0.22 ± 0.01
Transgenic – mature	7.4	3.5 ± 0.3*	5.5 ± 0.5	0.24 ± 0.01

Mortality values are based on found insects and do not include insects potentially consumed during cannibalism. Feeding rating values are means ± SE. Values in columns followed by an '*' in like assays are significantly different at $P < 0.05$ by chi-square analysis (mortality) or ANOVA (feeding rating and weights).
†Mortality reported for *O. nubilalis* and *H. zea* is after two days. Beyond this time *O. nubilalis* were difficult to determine due to extensive webbing, and *H. zea* underwent extensive cannibalism.
‡Feeding ratings are based on an estimated 0.25 mm^2 consumed after 2 days and an overall 0–10 rating for the entire leaf after 4 days (and 2 days for mature leaves fed *H. zea*).

the enhanced peroxidase activity detoxifies whatever allelochemical(s) are important for resistance to *H. zea*. Potential allelochemicals found in *L. styraciflua* that could be involved in this type of reaction include terpenoids [40, 41], cinnamic acid derivatives [42], other phenolic acids [43, 44], catechin [44], and quercetin [43]. Many of these allelochemicals contain moieties that make them substrates for peroxidases [45] or contain groups susceptible to attack by reactive compounds generated by peroxidases. Enhanced rates of lignification [46] and greater amounts of lignin [38] occur in tobacco plants overexpressing tobacco anionic peroxidase. However, a structural barrier such as lignin would likely affect insect species similarly and thus is unlikely to explain the enhanced feeding on the transgenic *L. styraciflua* leaves by *H. zea*. Results with different species of *Nicotiana* overexpressing tobacco anionic peroxidase, where the secondary chemistry and peroxidase interactions are well known, also support increased toxicity as being a major result of increased peroxidase activity [8].

Our work with *L. dispar* in the present study also supports the hypothesis that increasing total peroxidase activity in *L. styraciflua* through expression of tobacco anionic peroxidase retards growth and damage primarily due to a toxic interaction. We found no evidence to suggest that decreased growth caused by elevated peroxidase expression was due to effects on the nutritive quality of the foliage. We observed no effect of tobacco anionic peroxidase expression on the ability of larvae to digest foliage (AD), suggesting that reduced growth rates were not due to formation of indigestible complexes as a result of covalent binding of essential nutrients with oxidized compounds. The naturally oxidizing environment of the gypsy moth midgut [47, 48] may have been sufficient to oxidize available prooxidants capable of binding with nutrients, thereby rendering superfluous the increased oxidation capacity generated by elevated peroxidase. Decreased larval growth did result from decreased consumption and possibly from decreased ability to convert digested leaves to biomass (ECD). Postingestive toxic effects of peroxidase on growth and/or preingestive deterrent effects on consumption could produce such effects [49]. Toxic effects of highly reactive peroxidase products (including reactive oxygen species) could decrease growth rates and subsequently lead to decreased consumption. Decreased ECD, if indeed it does occur due to elevated peroxidase activity, would be consistent with toxicity, as has been observed previously [49, 50] and in the present study, where growth is decreased to a greater degree than consumption. However, we cannot rule out that feeding deterrence is also involved to some degree, although feeding deterrents are often also toxic [51].

Our results suggest that tobacco anionic peroxidase may be involved in a general defence mechanism against insects. Like certain tissues of tobacco and tomato expressing high levels [7–9], the immature and mature leaves of *L. styraciflua* expressing tobacco anionic peroxidase were often more resistant to different species of insects. Significant increases in resistance were noted for the sweetgum pests *H. cunea* and *L. dispar*. Provided the secondary chemistry of the plant is appropriate and expression levels are sufficient, expression of tobacco

anionic peroxidase may prove to be a useful means of increasing plant resistance to insect pests, depending on the species and size of the pest. However the present study also indicates that expression of the peroxidase in a distantly related plant may not give the same result as for the source plant, as was seen for *H. zea*. As many of peroxidase-associated factors appear to promote resistance to both disease and insects [6, 7], expression of the tobacco anionic peroxidase may also be useful in reducing susceptibility to some diseases.

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