

Insect Herbivory on Low-Lignin Transgenic Aspen

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ABSTRACT Ecological effects of genetically modified plants cannot always be predicted based on knowledge of the plant species or transgene. We studied the effects of transgenic aspen (*Populus tremuloides* Michaux) with reduced lignin and altered growth phenotypes on the feeding performance of gypsy moth larvae (*Lymantria dispar* L.) and forest tent caterpillars (*Malacosoma disstria* Hübner). Developmental trials were conducted using one control line and four separate transgenic lines of aspen. Gypsy moth larvae showed a significant reduction in survival on one high-lignin reduction transgenic tree line relative to all other lines, but weights of surviving larvae were similar across tree lines. Forest tent caterpillars showed similar survival and weights on all tree lines. Trials were also conducted to evaluate whether gypsy moth larvae preferred feeding on high-lignin reduction transgenic aspen lines or control trees. While gypsy moth larvae showed no significant preference between the control line and the transgenic line that caused significant reductions in larval survival during developmental trials, they did strongly prefer transgenic leaves causing no such reductions in larval survival. Because effects on feeding larvae varied among tree lines, we concluded that any potential phytochemical alterations in the transgenic lines could not be directly linked to lignin reduction. Because only one transgenic tree line had a negative effect on the herbivores, we propose that this may be an indirect consequence of transgenic manipulation resulting from the insertion point of the antisense *PtACL* gene in the genome, rather than 4CL suppression or lignin reduction.

KEY WORDS plant–insect interactions, *Populus tremuloides*, *Lymantria dispar*, *Malacosoma disstria*, transgenic lignin reduction

Separating lignin from cellulose during pulping of wood entails significant energetic and chemical expenses and significant environmental costs (Chiang 2002, Pilate et al. 2002). Lignin separation costs the paper and pulp industry ≈20 billion dollars per year (Mann and Plummer 2002). For these reasons, transgenic aspen (*Populus tremuloides* Michaux) with reduced lignin has been generated, using antisense down-regulation of a *PtACL1* (4-coumarate: CoA ligase) gene, controlled by a Cauliflower Mosaic Virus 35S constitutive promoter (Hu et al. 1999). 4CL is involved in phenylpropanoid metabolism (Fig. 1), catalyzing the activation of hydroxycinnamic acids into high-energy CoA-intermediates for lignin and flavonoid synthesis. Therefore, transgenic alterations of 4CL could impact tree metabolism, growth, and defense allocation of nonlignin phenolics in addition to reducing lignin content. Analysis of these transgenic aspen trees revealed increased cellulose and hemicellulose deposition in the xylem, substantially increased root growth, leaf size, and overall growth rates (Hu et al. 1999). Additional tests found that the transgenic

aspen stem wood contained increased wall-bound ferulic, sinapic, and 4-coumaric acids (Hu et al. 1999). Within the leaves, cell wall esterified 4-coumaric and ferulic acids were decreased (Harding et al. 2002).

Alterations in both leaf growth rates and phenolic profiles in the transgenic aspen could potentially impact ecological relationships between plants and herbivores. Increased plant growth is often associated with reduced defenses against herbivores (Hwang and Lindroth 1997). However, phenolic compounds are important and widely distributed plant allelochemicals (Schowalter 2000). They make up the majority of the defense compounds found in aspen, especially in the forms of phenolic glycosides and condensed tannins (Arteel and Lindroth 1992). Therefore, changes in either plant growth rates or phenolic profiles could impact which insects feed on lignin-reduced transgenic aspen. The digestive and assimilative capabilities, development time, and survival of feeding insects could also be altered. Changes in fundamental aspects of the plant–herbivore relationship could be either positive or negative for herbivorous insects, depending on which chemicals are altered; feeding on such plants could be easier because of lower or otherwise altered defensive compounds or more difficult because of increased or otherwise altered defensive compounds. Changes in feeding capabilities may also

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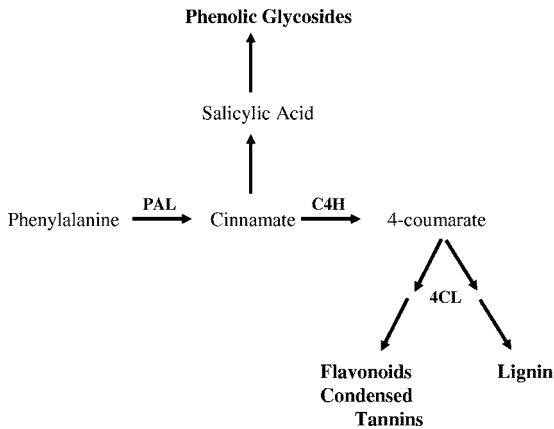


Fig. 1. Schematics of phenylpropanoid metabolism that supports phenolic glycoside, flavenoid, and lignin biosynthesis. PAL, Phe ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:coenzyme A ligase.

not be uniform across all insect species and may depend on the characteristics of individual species. Some insect species could respond positively to phenolic changes, whereas other species respond negatively to the same changes.

Trembling aspen has many associated insect herbivores, including gypsy moth larvae (*Lymantria dispar* L.; Lepidoptera: Lymantriidae) and forest tent caterpillars (*Malacosoma disstria* Hübner; Lepidoptera: Lasiocampidae). These polyphagous insects prefer aspen and can consume almost all available leaf material during outbreaks (Elkinton 2003, Katovich and Hanson 2002). Both gypsy moth larvae and forest tent caterpillars are sensitive to natural variations in leaf chemistry, especially genetically controlled variations in defense compounds such as phenolics (Hwang and Lindroth 1997, Hemming and Lindroth 1995, Robison et al. 1994). For example, development and survival of larvae of both species are inversely correlated with foliar phenolic levels in aspen (Hwang and Lindroth 1997).

In this study, we assessed effects of possible alterations in the leaf chemistry of low-lignin transgenic aspen on insect herbivory. We first examined the survival and growth of gypsy moth and forest tent caterpillar larvae feeding on transgenic and control leaves in developmental trials. Next, we used paired preference tests to determine whether transgenic aspen would change the host plant feeding preferences of the insects. We hypothesized that potential deviations in survival or development of larvae would be greater in larvae feeding on trees with greatly reduced lignin. However, we did not know which direction any potential deviations would take, because altered phenolic profiles could affect insect species in either a positive or negative manner. For preference testing, we hypothesized that larvae would avoid transgenic tree lines that are detrimental to larval survival or development.

Materials and Methods

Plant Materials. We included five lines of aspen in this study: a wild-type control (C), two transgenic lines (HR1 and HR2), each with a 40% lignin reduction in their stem wood compared with C, and two transgenic lines (LR1 and LR2), each with 10% lignin reduction in their stem wood compared with C (Hu et al. 1999). Each tree line contained 15–40 plants. High-lignin reduction lines will be referred to as HR, whereas low-lignin reduction lines will be referred to as LR. Descriptions of tree production and tree analyses can be found in Hu et al. (1999) and Harding et al. (2002).

To minimize environmentally caused variations in leaf chemistry (Hemming and Lindroth 1995), we maintained vegetatively propagated aspen under controlled conditions in a greenhouse at Michigan Technological University in Houghton, MI. We numbered leaves on each tree sequentially counting from the topmost, first apical unfolding leaf. To minimize age-related or microenvironmental variations in leaf chemistry, we collected leaves 5 through 8 from randomly selected trees for each feeding period. No insects received same-age leaves from the same individual tree in consecutive feeding periods.

We used only whole excised leaves during all trials (Robison et al. 1994, Hwang and Lindroth 1997). We did not cut leaf disks from whole leaves (Robison and Raffa 1994, Raffa et al. 2002) during preference trials to limit possible foliar chemistry changes caused by wounding.

Developmental Trials. Developmental trials provide a way to evaluate insect survival and growth as a measure of host leaf suitability (Kopper and Lindroth 2003, Robison et al. 1994, Hamilton and Lechowicz 1991). We used general gypsy moth and forest tent caterpillar rearing procedures (Grisdale 1985, Odell et al. 1985). Survival time was measured in days from the time when larvae hatched as neonate larvae until either pupation or death.

We performed two experiments using gypsy moth larvae, each with 15 single larva replicates reared on each tree line: C, HR1, HR2, or LR1. Because of limited tree material, the first experiment included 10 larval replicates reared on LR2, and this aspen line was not used during the second experiment. Eggs for the first gypsy moth developmental experiment came from USDA-ARS BIR. Eggs for all subsequent experiments came from the Insect Production Unit of the Great Lakes Forestry Center in Sault Ste. Marie, Ontario, Canada.

For each gypsy moth developmental experiment, we placed randomly chosen replicate neonate larvae singly in labeled plastic petri dishes with a randomly chosen leaf from the appropriate tree line and an unbleached paper towel to absorb excess water from leaf transpiration. The petiole of each leaf was inserted into a water pik containing fresh distilled water to maintain leaf turgor pressure.

The leaf in each petri dish was replaced with a randomly selected fresh leaf of the same tree line three

times per week. At the same time, the larvae were weighed on a scale with 0.1-mg precision, and their head capsules measured to the nearest 0.5 mm under a dissecting microscope. Head capsule width was used to identify the instar of an insect. The first experiment lasted 40 d. The second experiment lasted 43 d.

We conducted a similar experiment using forest tent caterpillars. Neonate larvae were shipped by overnight mail from the Insect Production Unit in Ontario and placed within petri dishes the day of arrival. Because of the gregarious nature of forest tent caterpillars (Robison and Raffa 1997, Katovich and Hanson 2002), each replicate petri dish contained 10 randomly chosen larvae and one randomly chosen leaf from the appropriate tree line. Each larva was monitored for survival measurements. Weight was measured for the combined larvae in each petri dish for convenience. Each tree line had three petri dishes containing a total of 30 larvae. The exception was LR2, which was limited by the number of available trees and thus had only two dishes for a total of 20 larvae. This experiment lasted 35 d.

Preference Trials. Feeding preferences between control and HR transgenic aspen leaves were tested by means of pairwise preference trials. We conducted two trials using second- and fourth-instar gypsy moth larvae. Two larval instars were used because digestive and detoxification capabilities of larvae change with age (Schultz and Lechowicz 1986). Only HR lines were tested for insect preference, because the developmental trials indicated that only one of the HR lines was significantly different from the control line. Pairwise trials were conducted because testing simultaneously for preferences between more than two choices leads to complex interpretations and requires very large sample sizes to get accurate results (Raffa et al. 2002).

To test for preference between C and HR1, 20 newly hatched larvae were placed in each of six petri dishes containing a 1-cm cube of artificial diet (high wheat germ gypsy moth diet; Bio-Serv Entomology Division, Frenchtown, NJ). The instar of each larva in each petri dish was recorded every day. On reaching the second instar, a larva was placed in a rectangular plastic box, containing two randomly chosen leaves, one from C and the other from HR1, and a paper towel. Before placement in the box, each leaf was scanned into a computer to record a measure of its area. The petiole of each leaf was placed in a water pik containing fresh distilled water and the pik was labeled. After 48 h, the leaves were removed, and the larvae were destroyed. The leaves were rescanned. Leaf images were saved as black and white bitmap files, and a histogram was produced for each file giving the number of pixels containing leaf material, using Adobe Photoshop version 7.0.1 (Adobe Systems 1990–2002). The amount consumed of each leaf was determined by comparing pre- and postherbivory pixel counts of leaf material. This procedure was repeated until a total of 30 pairs of leaves were measured. We repeated this experiment on fourth-instar larvae.

This experimental design was repeated to compare larval preferences between C and HR2, again using both second- and fourth-instar gypsy moth larvae.

Statistical Analyses. For developmental trials, differences in survival times of larvae feeding on each tree line were analyzed using Kaplan-Meier survival analysis (SPSS 2001). This method was chosen to provide an accurate representation of survival throughout each study (Dunn 2002). All pairwise comparisons of survival times of larvae feeding on each tree line were performed using Bonferroni multiple comparisons procedures (McClave and Sincich 2003). For all tests, $P < 0.05$ was chosen to indicate statistical significance.

Survival, by instar, of larvae on each tree line was examined using χ^2 tests and orthogonal contrasts; we used this method to get a more precise understanding of how tree line affected insect survival during different periods of insect development. Orthogonal contrasts examined differences in survival at each instar, comparing larvae feeding on control versus transgenic plants, HR versus LR plants, HR1 versus HR2, and LR1 versus LR2. Orthogonal contrasts were only performed when the overall χ^2 test showed significant differences to exist.

Larval weights were compared at each measurement using analysis of variance (ANOVA; SAS Institute 2002), with tree line as the factor and larval weight as the response variable. We analyzed weight at each measurement separately because of changing sample sizes resulting from larval mortality during the experiment.

Results

Developmental Trials. Preliminary analysis of the gypsy moth larvae developmental trials indicated that survival did not differ significantly between the first and second trials ($F = 1.45$, $df = 1$, $P = 0.23$); thus, we pooled the results of the two trials to analyze survival. The mean survival time differed among larvae feeding on different lines of trees (Breslow statistic = 22.64, $df = 4$, $P < 0.001$). Survival time ranged from 12.5 d on HR1 to 24.1 d on LR2 (Fig. 2). Pairwise comparisons between treatments indicated larvae feeding on HR1 had a mean survival time that was significantly shorter than for larvae feeding on all other tree lines ($P < 0.01$). No other pairwise comparisons were significant.

χ^2 analysis of the gypsy moth larvae developmental trials also indicated that tree line caused differential survival among gypsy moth larvae during each instar. Significant differences in survival were apparent for the first, second, third, and fifth instars (χ^2 statistics = 13.61, 29.68, 11.52, 10.79; $df = 4$; $P = 0.009$, < 0.001 , 0.021, 0.029 respectively). Orthogonal contrasts indicated that larvae feeding on HR1 had significantly higher mortality than larvae feeding on HR2 during the first, second, and third instars (χ^2 statistics = 8.53, 20.38, 4.28; $df = 1$; $P = 0.003$, < 0.001 , 0.038 respectively). Second-instar larvae feeding on LR1 had significantly higher mortality than larvae feeding on LR2 (χ^2 statistic = 5.76, $df = 1$, $P = 0.016$). Third-instar larvae feeding on HR lines had significantly higher

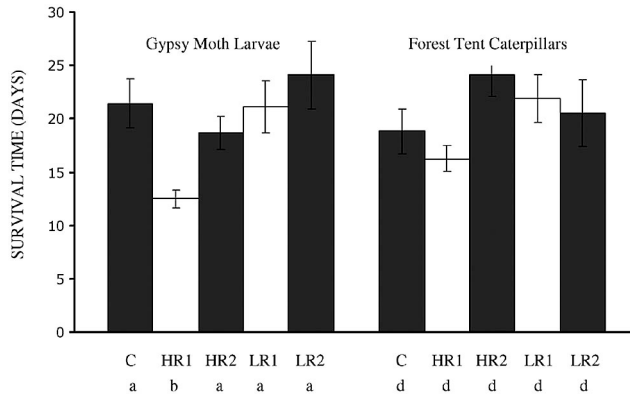


Fig. 2. Average survival times (d) for larvae. The results of the gypsy moth larvae developmental trials are pooled. Error bars represent SE. Letters beneath the tree line indicate significant differences in survival time.

mortality than larvae feeding on LR lines (χ^2 statistic = 4.04, $df = 1$, $P = 0.044$), and fifth-instar larvae feeding on transgenic lines had significantly higher mortality than larvae feeding on the control line (χ^2 statistic = 9.29, $df = 1$, $P = 0.002$). No other orthogonal contrasts were significant.

Analysis of larval weights for gypsy moth larvae indicated that there were no significant differences between larvae feeding on different tree lines.

Forest tent caterpillars feeding on different tree lines did not have significantly different survival times (Breslow statistic = 7.20, $df = 4$, $P = 0.13$; Fig. 2). Survival times ranged from 16.2 d on HR1 to 24.1 d on HR2.

χ^2 analysis on forest tent caterpillar developmental trials, by instar, indicated that tree line caused differential survival times for fourth-instar, fifth-instar, and pupating larvae (χ^2 statistics = 12.97, 20.64, 18.99; $df = 4$; $P = 0.011$, <0.001, <0.001 respectively). Orthogonal contrasts found that fourth-instar, fifth-instar, and pupating larvae feeding on HR1 had significantly higher mortality than larvae feeding on HR2 (χ^2 statistics = 10.42, 12.00, 12.00; $df = 1$; $P = 0.001$, <0.001, <0.001 respectively). In addition, fifth-instar and pupating larvae feeding on LR2 had significantly higher mortality than larvae feeding on LR1 (χ^2 statistics = 6.60, 5.61; $df = 1$; $P = 0.010$, 0.018). No other significant orthogonal contrasts were found.

Weight analysis for forest tent caterpillars indicated there were no significant differences between larvae feeding on different tree lines.

Preference Trials. Preference trials were analyzed by comparing leaf consumed between control and transgenic leaves using a paired *t*-test. Neither second-instar nor fourth-instar gypsy moth larvae showed a significant preference between C and HR1 leaves (*t*-statistics = -1.895, -0.173; $df = 29$; $P = 0.068$, 0.864, respectively), although the difference between C and HR1 leaf consumption is close to being significant for second-instar larvae. An average of 0.50 cm² of control leaves were eaten by second-instar larvae compared with 1.24 cm² of transgenic leaves. Fourth-instar larvae ate an average of 1.91 cm² of C and an average of 2.08 cm² of HR1.

Both second- and fourth-instar gypsy moth larvae showed a statistically significant preference for HR2 over C (*t*-statistics = -3.805, -3.325; $df = 29$; $P = 0.001$, 0.002, respectively). An average of 0.52 cm² of control leaves were eaten by second-instar larvae compared with 2.37 cm² of transgenic leaves. Fourth-instar larvae ate an average of 8.26 cm² of C and an average of 20.92 cm² of HR2.

Discussion

In terms of overall survival, the results of the gypsy moth larvae developmental trials suggest that line HR1 caused the strongest and most consistent negative effects on feeding larvae and that surviving larvae were generally similar in survival and weight. Aspen line HR1 may contain some phytochemical differences that make it less acceptable as a host tree for gypsy moth larvae. Early, rapid, and sustained mortality can be an indication of toxicity or deterrence, which most likely results from altered chemicals within the leaves of that tree line (Robison and Raffa 1994). The majority of the gypsy moth larvae mortality on line HR1 occurred within the first two instars. This is the period when larvae are most vulnerable to plant defensive chemicals because of low detoxification ability (Hamilton and Lechowicz 1991) and when they normally disperse from poor-quality hosts (Robison and Raffa 1994).

The strong survival impacts of HR1 on gypsy moth larvae may indicate a phytochemistry alteration unique to line HR1. These alterations may be caused by accumulation of lignin pathway precursors or increased metabolic allocation to other parts of the phenolic pathways, caused by antisense down-regulation of 4CL. Somaclonal variation, i.e., random but naturally occurring phenotypic variation within plants regenerated from tissue cultures, could also explain the variation in phytochemistry in line HR1. Alternatively, because HR1 was the only tree line that had a negative effect on the herbivores we propose that this may be an indirect consequence of transgenic manipulation resulting from the insertion point of the antisense *PtACL* gene in the genome, rather than 4CL suppres-

sion or lignin reduction. Despite well-established transformation techniques, exactly where the transgene is inserted into the genome cannot be controlled precisely, because transformation techniques insert the transgene into a random location in the genome (Andow 2003). This transgene insertion point varies among independent transformation events, may affect gene expression, and makes gene expression harder to predict (Han et al. 1996, Andow 2003). Further chemical analysis of the leaves or sequencing analysis of the HR1 transgenic genome could determine the exact components and mechanisms of the alteration in phytochemistry.

The forest tent caterpillar developmental trials indicated that survival rates for this species were less strongly affected by possible phytochemical differences between tree lines. No overall differences in survival rates among tree lines were noted, although specific differences in survival rates were noted between pairs of tree lines later during caterpillar development. While there may be a chemical difference between the tree lines, it may be a difference for which forest tent caterpillars are better adapted. Because forest tent caterpillars are native to the United States, in contrast to the introduced gypsy moth, forest tent caterpillars may be more tolerant of variable chemistry in *P. tremuloides* than gypsy moth larvae. These observations are supported by studies examining naturally occurring phytochemistry variations among aspen trees, in which both second and fourth instar gypsy moth larvae were more severely and negatively affected than forest tent caterpillars (Hemming and Lindroth 1995, Hwang and Lindroth 1997). The forest tent caterpillar larvae were better at detoxifying higher levels of that particular defensive compound than were the gypsy moth larvae (Hemming and Lindroth 1995, Hwang and Lindroth 1997).

Although the potential pest resistance implied in line HR1 could be considered a positive result from a commercial tree production perspective, selective pressure from several generations of insects feeding on these trees could potentially yield tolerant insects (Alstad and Andow 1995, Tabashnik 1997). It is unknown what effects this selective pressure may have on insect herbivore populations. Preference trials indicated that gypsy moth larvae do not seem to display any avoidance of HR1, despite its apparent toxicity. They also seem to prefer the transgenic leaves of HR2 to control leaves. Nonavoidance or positive preference for transgenic leaves would have to be taken into account when planning for commercial plantations, because such plantations may be more vulnerable to insect pests such as gypsy moth.

Given these results, some transgenic lines with reduced lignin may be suitable for consideration in field trials for commercial applications. Because differences in insect survival did not seem to be linked to particular amounts of lignin reduction, it seems that no direct link can be made between larval performance and the down-regulation of the *Pt4CL1* gene in aspen, despite the altered phenotype observed in the transgenic plants. However, further laboratory and field-

based evaluations, including detailed phytochemical analyses, are needed to test for interactions between transgenic aspen phytochemistry and other herbivores, such as beneficial insects, specialist insects feeding only or mostly on aspen, and vertebrate herbivores. Testing for effects on nonherbivorous insects such as predators and parasites would also be useful in looking at trophic interactions involving transgenic plants. Recommendations for transgenic plants must be based on many repeated studies involving many different organisms before conclusions can be reached about the possible effects of the plants in a natural environment. Such evaluations can be used to guide selection of transgenic lines in such a way that the transgenic plants will have minimal unintended impacts on the environment.

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