Non-Target Effects of Transgenic Blight-Resistant American Chestnut (Fagales: Fagaceae) on Insect Herbivores

Author(s): K. H. Post and D. Parry
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Non-Target Effects of Transgenic Blight-Resistant American Chestnut (Fagales: Fagaceae) on Insect Herbivores

K. H. POST1,2,3 AND D. PARRY1

ABSTRACT American chestnut [Castanea dentata (Marshall) Borkhausen], a canopy dominant species across wide swaths of eastern North America, was reduced to an understory shrub after introduction of the blight fungus [Cryphonectria parasitica (Murrill) Barr] in the early 1900s. Restoration of American chestnut by using biotechnology is promising, but the imprecise nature of transgenesis may inadvertently alter tree phenotype, thus potentially impacting ecologically dependent organisms. We quantified effects of genetic engineering and fungal inoculation of trees on insect herbivores by using transgenic American chestnuts expressing an oxalate oxidase gene and wild-type American and Chinese (C. mollissima Blume) chestnuts. Of three generalist folivores bioassayed, only gypsy moth [Lymantria dispar (L.)] was affected by genetic modification, exhibiting faster growth on transgenic than on wild-type chestnuts, whereas growth of polyphemus moth [Antheraea polyphemus (Cramer)] differed between wild-type species, and fall webworm [Hyphantria cunea (Drury)] performed equally on all trees. Inoculation of chestnuts with blight fungus had no effect on the growth of two herbivores assayed (polyphemus moth and fall webworm). Enhanced fitness of gypsy moth on genetically modified trees may hinder restoration efforts if this invasive herbivore’s growth is improved because of transgene expression.

KEY WORDS genetic modification, forest restoration, oxalate oxidase, gypsy moth
gesting that this protein may provide protection against blight development (Welch et al. 2007).

Because genetic engineering is essentially a random process (Chyi et al. 1986, Mullins et al. 2001), it may disrupt gene expression (Feldmann and Marks 1987, Feldmann et al. 1989), leading to changes in phenotype (Dale and McPartlan 1992). Similarly, the pleiotropic effects of transgenes also may change phenotypic expression (Dale and McPartlan 1992, Kääpeli and Auberson 1998). Thus, genetic transformation may inadvertently affect foliar chemistry and impart fitness changes on insect herbivores (e.g., Tiimonen et al. 2005, Hjältén et al. 2007).

Few studies have investigated impacts of transgenic plants with modified OxO expression on insect herbivores. In corn (Zea mays L.), three transgenic lines reduced leaf feeding and stalk tunneling by European corn borer (Ostrinia nubilalis Hübner), and this insect exhibited slower rates of growth and development when fed an artificial diet supplemented with H₂O₂ (Ramputh et al. 2002). In these corn lines, the secondary metabolite ferulic acid was found responsible in reducing herbivore fitness (Mao et al. 2007). Tobacco hornworm [Manduca sexta (L.)] and suckfly [Tupiocoris notatus (Distant)] performance and preference, respectively, increased on one of three transgenic coyote tobacco (Nicotiana attenuata Torrey ex Watson) lines with silenced germín-like gene expression, which coincided with lower levels of H₂O₂ (Lou and Baldwin 2006). These studies support the potential roles of OxO and H₂O₂ activity in plant–herbivore interactions.

The primary objective of our study was to determine if American chestnut transformed with a plasmid (pΔVspB-OxO) containing an OxO gene driven by a vascular promoter (event LP28) affects the performance of three generalist insect folivores: gypsy moth [Lymantria dispar (L.)], Lepidoptera: Lymantriidae]; polypemus moth [Antherea polyphemus (Cramer), Lepidoptera: Saturniidae]; and fall webworm [Hyphantria cunea (Drury), Lepidoptera: Arctiidae] that feed on chestnut in nature. We also assessed whether inoculation of all chestnut types with blight fungus affects polypemus moth and fall webworm growth.

Materials and Methods

Site Description and Plant Material. All experiments were conducted at the State University of New York’s College of Environmental Science and Forest-

![Image](image-url)

**Fig. 1.** Layout of the chestnut plot at Lafayette Road Experiment Station. Tree types are as follows: wild-type American (WTA), transgenic American (TA), and Chinese (Ch) chestnut. Inoculated with C. parasitica strain SG-1 on 4 June 2008. Inoculated with C. parasitica strain EP155 on 4 June 2008.

<table>
<thead>
<tr>
<th>Row</th>
<th>Tree 1</th>
<th>Tree 2</th>
<th>Tree 3</th>
<th>Tree 4</th>
<th>Tree 5</th>
<th>Tree 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ch</td>
<td>TA</td>
<td>WTA</td>
<td>WTA</td>
<td>Ch</td>
<td>TA</td>
</tr>
<tr>
<td>2</td>
<td>TA</td>
<td>TA</td>
<td>WTA</td>
<td>WTA</td>
<td>Ch</td>
<td>Ch</td>
</tr>
<tr>
<td>3</td>
<td>TA</td>
<td>Ch</td>
<td>Ch</td>
<td>WTA</td>
<td>TA</td>
<td>WTA</td>
</tr>
<tr>
<td>4</td>
<td>TA</td>
<td>WTA</td>
<td>TA</td>
<td>Ch</td>
<td>Ch</td>
<td>WTA</td>
</tr>
<tr>
<td>5</td>
<td>TA</td>
<td>Ch</td>
<td>Ch</td>
<td>Ch</td>
<td>WTA</td>
<td>TA</td>
</tr>
<tr>
<td>6</td>
<td>Ch</td>
<td>Ch</td>
<td>TA</td>
<td>WTA</td>
<td>WTA</td>
<td>WTA</td>
</tr>
</tbody>
</table>
Paper toweling was placed in each of 40 150-mm × 25-mm petri dishes to absorb excess humidity. Because mature leaf sizes varied considerably within and between the tree species, we tried to standardize leaf area offered to larvae by placing one large or two smaller-sized leaves in a petri dish. Moist paper toweling was wrapped around the petiole of each leaf to maintain turgor. Before experimentation, leaves were examined and any inadvertently collected insects removed. One gypsy moth caterpillar was haphazardly selected, weighed using a microbalance, and randomly assigned to a labeled dish, and then placed in an environmental chamber. A second dish using foliage from the same tree was prepared in an identical manner. Larvae were reared separately so that the growth rate of individuals could be quantified. Thus, 10, 12, and 18 dishes (subsamples) were prepared from wild-type American, transgenic American, and Chinese chestnut foliage, respectively. The caterpillars fed for 3 d at 15.5 h (24°C):8.5 h (20°C) L:D with no dishes requiring more leaves during that time. Moistened paper towels were replaced ≈24 h and 48 h into the experiment.

After 72 h, caterpillars were removed from dishes and individually placed into labeled microtubes, placed in a zip-loc bag, and frozen before being dried in an oven at 45°C for 6 d. Dried caterpillars were weighed to determine final dry weights. Initial dry weights (mg) were calculated using a fresh:dry weight conversion estimate \( y = 0.1306x - 0.00002; R^2 = 0.962 \) from a regression of a subset of larvae \( n = 21 \) from a previous study (D. P., unpublished data), where \( y \) = dry mass and \( x \) = wet mass. Relative growth rate (RGR) for each caterpillar was determined over this 72-h period (Gordon 1968) and used as the response variable.

**Polyphemus Moth Bioassay.** *A. polyphemus* are large, solitary, summer-feeding native caterpillars. We conducted a bioassay using fourth-instar polyphemus moths to investigate the effects of tree type and blight fungus inoculation on their growth rate at the end of July. Approximately half of each tree type was randomly inoculated with one of two strains of chestnut blight fungus, SG-1 or EP155, on 4 June 2008 (Fig. 1). Inoculations were made through a small wound cut on one stem with a scalpel. A plug of mycelium (the fungus on potato dextrose agar medium) was overlaid on each tree species were placed into separate dishes. In total, 14, 21, and 24 dishes were prepared from wild-type American, transgenic American, and Chinese chestnut foliage, respectively. Of these, 9, 10, and 12 dishes, respectively, were from inoculated trees. Caterpillars were oven dried for 3 d at 13 h (24°C):11 h (20°C) L:D. Foliage was added to one dish at 48 h. Larvae were removed from each dish, and numbers of living and dead larvae were recorded and placed in separate, labeled microtubes. Caterpillars were oven dried for 8 d and weighed in groups. Initial individual dry weights were determined from a fresh:dry weight conversion estimate \( y = 0.1331x - 7.2213; R^2 = 0.907 \) derived from a regression of a subset of 14 larvae excluded from this bioassay. Relative growth rate over this 72-h period was again the response variable and was determined as above for gypsy moth.

**Fall Webworm Bioassay.** To further assay potential effects of tree type and pathogen inoculation on herbivore growth, we used fall webworm, a native, colonial, late-season feeder and conducted a feeding bioassay at the end of August. Larvae were collected from black cherry (*Prunus serotina* Ehrhart) in Syracuse, NY and maintained in the laboratory on cut foliage for 48 h before experimentation. For the bioassay, two large or four smaller-sized chestnut leaves were obtained from the same trees used in the Polyphemus moth bioassay, placed in individual zip-loc bags, and stored in a refrigerator overnight to minimize desiccation. For each tree, two groups of five representative caterpillars of various instars of this colonial species were placed into separate dishes. In total, 14, 21, and 24 dishes were prepared from wild-type American, transgenic American, and Chinese chestnut foliage, respectively. Of these, 9, 10, and 12 dishes, respectively, were from inoculated trees. Caterpillars were oven dried for 8 d and weighed in groups. Initial individual dry weights were determined by dividing group fresh weights at the outset of the experiment by the number of caterpillars in a group and using a fresh:dry weight conversion estimate \( y = 0.1383x + 0.434; R^2 = 0.969 \) from a subset of 23 larvae excluded from this bioassay. As with the other insects assayed, RGR was the response variable but for fall
webworm, it was the mean value for all living caterpillars in a dish at experiment termination rather than an individual.

**Statistical Analysis.** All bioassays used a completely randomized design with subsampling. Thus for each experiment, response variables were recorded from individual petri dishes (subsamples), which were nested under individual trees (experimental units or replicates). The gypsy moth bioassay was conducted before pathogen inoculation of the trees and was analyzed as a one-way analysis of variance (ANOVA) with three treatments: wild-type American, transgenic American, and Chinese chestnut. The remaining experiments were performed after trees were injected with the blight fungus and thus were analyzed as 2 by 3 factorial designs. Pathogen inoculation (noninoculated or inoculated) and tree type were the two treatment factors investigated for these assays. An additional set of eight one-way ANOVAs was performed for both the polyphemus moth and fall webworm feeding experiments on all tree types, both individually and combined, but differentiating pathogen strains, SG-1 and EP155, to ascertain whether strains should be considered separate treatments. An added or inoculated) and tree type were the two treatment factors investigated for these assays. An additional set of eight one-way ANOVAs was performed for both the polyphemus moth and fall webworm feeding experiments on all tree types, both individually and combined, but differentiating pathogen strains, SG-1 and EP155, to ascertain whether strains should be considered separate treatments. For these same two bioassays, RGR variances between noninoculated and inoculated, SG-1-inoculated, and EP155-inoculated chestnuts for all tree types, both individually and combined, were tested for homogeneity using Levene’s test (Levene 1960). This test determined whether herbivore growth response differed among single and multi-stemmed chestnuts injected with the blight fungus. An analysis of the latter two feeding trials incorporating only noninoculated trees was also conducted to exclude the pathogen inoculation treatment factor and allow comparison of tree type, as in the gypsy moth bioassay. An orthogonal set of contrasts was constructed for all ANOVAs, and where applicable, individual and main effect means were separated using Tukey’s Honestly Significant Difference (HSD) (Tukey 1949) and Scheffe’s (Scheffe 1953) tests, respectively. All statistics were computed using SAS (PROC GLM, SAS Institute 2002).

**Results**

**Gypsy Moth Bioassay.** Relative growth rates of fourth-instar gypsy moth were significantly affected by tree type ($F = 5.64; df = 2, 17; P < 0.014$). Planned comparisons showed no difference between tree species ($F = 2.59; df = 1, 17; P < 0.127$), whereas caterpillar growth rate was significantly higher (16.4%; $F = 8.08; df = 1, 17; P < 0.012$) on transgenic American compared with wild-type American chestnut leaves (Fig. 2). Larvae reared on Chinese chestnut foliage had significantly lower (13%; $P < 0.05$) RGRs than those on transformed American chestnut leaves but similar growth rates when compared with individuals fed nontransformed American chestnut foliage ($P > 0.05$) using two Tukey’s HSD tests.

**Polyphemus Moth Bioassay.** There was no interaction between tree type and inoculation ($F = 0.96; df = 2, 25; P = 0.297$) on fourth-instar polyphemus moth RGR, thus only main effects were considered. Growth rate of caterpillars was not significantly altered by fungal injection ($F = 0.19; df = 1, 25; P < 0.665$). However, the main effect of tree type was significant ($F = 4.49; df = 2, 25; P < 0.022$; Fig. 3A), and a planned comparison of caterpillar growth rates among species was highly significant ($F = 8.87; df = 1, 25; P < 0.007$). Specifically, larvae fed leaves from wild-type American trees had the greatest RGR among the three types, which was only 3.2% greater than its transgenic counterpart ($F = 0.26; df = 1, 25; P < 0.613$), according to
Inoculation reduced larval growth rates by significantly affected growth (\(F = 1.10; \text{df} = 2, 12; P < 0.05\)). The variance of larval RGR did not differ (\(P = 0.56; \text{df} = 2, 12; P < 0.589\); Fig. 3B). Fall webworm growth was also not affected by treatment (\(F = 1.10; \text{df} = 2, 12; P < 0.366\)).

**Discussion**

Nontarget effects of noninsecticidal transgenic plants have not been extensively examined; this is especially true for trees (but see Tiimonen et al. 2005, Halpin et al. 2007, Hjältén et al. 2007). In our study, the effects of American chestnut genetically modified for blight resistance were variable, increasing the growth rate of gypsy moth relative to nontransformed American chestnut, but having no effect on polyphemus moth and fall webworm.

The faster growth of fourth-instar gypsy moth fed transgenic American chestnut foliage presents a potential problem for restoration efforts. The North American range of gypsy moth (Sharov et al. 2002) coincides with much of the area formerly dominated by American chestnut (Gravatt 1949). Though this particular transgenic line (LP28) is not currently under consideration for forest restoration (W. A. Powell, personal communication) enhanced performance of gypsy moth, a polyphagous, exotic, outbreak species (Elkinton and Liebhold 1990), on genetically modified chestnut foliage could become a concern because chestnuts expressing OxO after transformation with a

<table>
<thead>
<tr>
<th>Herbivore and tree type</th>
<th>No pathogen (mg m(^{-2}) d(^{-1}))</th>
<th>SG-1 strain (mg m(^{-2}) d(^{-1}))</th>
<th>EP155 strain (mg m(^{-2}) d(^{-1}))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyphemus moth</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type American</td>
<td>0.35 (0.029)ab</td>
<td>0.37 (0.025)</td>
<td>0.41 (0.023)</td>
<td>&lt;0.495</td>
</tr>
<tr>
<td>Transgenic American</td>
<td>0.36 (0.015)</td>
<td>0.40 (0.022)</td>
<td>0.38 (0.020)</td>
<td>&lt;0.278</td>
</tr>
<tr>
<td>Chinese</td>
<td>0.35 (0.014)ab</td>
<td>0.36 (0.020)a</td>
<td>0.28 (0.022)b</td>
<td>&lt;0.045</td>
</tr>
<tr>
<td>All</td>
<td>0.36 (0.013)</td>
<td>0.35 (0.016)</td>
<td>0.36 (0.016)</td>
<td>&lt;0.621</td>
</tr>
<tr>
<td><strong>Fall webworm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type American</td>
<td>0.12 (0.040)</td>
<td>0.12 (0.042)</td>
<td>0.13 (0.040)</td>
<td>&lt;0.966</td>
</tr>
<tr>
<td>Transgenic American</td>
<td>0.13 (0.022)</td>
<td>0.15 (0.035)</td>
<td>0.12 (0.029)</td>
<td>&lt;0.823</td>
</tr>
<tr>
<td>Chinese</td>
<td>0.15 (0.023)</td>
<td>0.16 (0.033)</td>
<td>0.12 (0.031)</td>
<td>&lt;0.389</td>
</tr>
<tr>
<td>All</td>
<td>0.14 (0.014)</td>
<td>0.16 (0.020)</td>
<td>0.12 (0.018)</td>
<td>&lt;0.363</td>
</tr>
</tbody>
</table>

Values within a row followed by no letter are not significantly different (\(P > 0.05\)).
Table 2. Variance of polyphemus moth and fall webworm relative growth rate ($mg \cdot m^{-1} \times d^{-1}$) by tree type and $C. parasitica$ treatment to determine whether stem type (single vs multi-stemmed) influenced the effect of pathogen inoculation on herbivore performance

<table>
<thead>
<tr>
<th>Herbivore and tree type</th>
<th>Control</th>
<th>Inoculated</th>
<th>SG-1 strain</th>
<th>EP155 strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphemus moth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type American</td>
<td>0.0037</td>
<td>0.0013</td>
<td>0.0019*</td>
<td>0.0005</td>
</tr>
<tr>
<td>Transgenic American</td>
<td>0.0014</td>
<td>0.0008</td>
<td>&lt;0.0001*</td>
<td>0.0014</td>
</tr>
<tr>
<td>Chinese</td>
<td>0.0018</td>
<td>0.0024</td>
<td>0.0006</td>
<td>0.0001</td>
</tr>
<tr>
<td>All</td>
<td>0.0019</td>
<td>0.0026</td>
<td>0.0009</td>
<td>0.0029</td>
</tr>
<tr>
<td>Fall webworm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type American</td>
<td>0.0027</td>
<td>0.0048</td>
<td>0.0049*</td>
<td>0.0070</td>
</tr>
<tr>
<td>Transgenic American</td>
<td>0.0024</td>
<td>0.0025</td>
<td>0.0009*</td>
<td>0.0042</td>
</tr>
<tr>
<td>Chinese</td>
<td>0.0021</td>
<td>0.0031</td>
<td>0.0019</td>
<td>0.0074</td>
</tr>
<tr>
<td>All</td>
<td>0.0024</td>
<td>0.0038</td>
<td>0.0024</td>
<td>0.0047</td>
</tr>
</tbody>
</table>

Treatment values within a row are not significantly different ($P > 0.05$) from the control.

* No test was performed due to a lack of replicates.

similar vector are now being used in field trials. Although fourth-instar gypsy moth grown on transgenic chestnut foliage were 16.4% larger than on nontransgenic foliage, similar changes have been observed for this species when comparing their performance on different nontransgenic genotypes of the same tree species (Hwang and Lindroth 1997, Osier and Lindroth 2004).

Polyphemus moth, a polyphagous, solitary mid- to late-season feeder, was unaffected by genetic modification of American chestnut for blight resistance, but analysis was complicated by fungal inoculation. Inclusion of all trees, both noninoculated and pathogen-inoculated, yielded a difference in herbivore growth rate between wild-type American and Chinese chestnuts, but neither differed from the transgenic chestnut. A similar pattern was observed in another study: in the presence or absence of phytopathogens, more adults of the spotted tentiform leafminer [Phyllonorycter blanchardella (F.)] emerged from a conventionally bred, fungal-resistant apple (Malus domestica Borkhausen) cultivar than from a susceptible variety, but neither differed from transgenic-susceptible and transgenic-resistant genotypes, though development times were comparable across all tree types (Vogler et al. 2010). However, in our study, analysis restricted to noninoculated chestnuts found no variation in larval performance among tree types. A significantly greater RGR on foliage from Chinese chestnut injected with SG-1 relative to EP155 blight fungus suggests that in future studies, pathogen strains should be considered separate treatments.

Although differing in biological and ecological attributes, all three folivores assayed are free-feeders and thus members of the same functional guild. Other types of phytophagous insects may respond differently to these tree types. In particular, phloem-feeders may exhibit greater fitness effects from LP28 chestnuts because a vascular promoter drives the OxO gene. Changes in herbivore preference or performance on genetically modified plants could result from transgene expression, either through the primary (Vaeck et al. 1987) or secondary (pleiotropic) functions (Puterka et al. 2002) of the novel protein. Alternatively, effects on phytophagous insects may be caused by unintended alterations in plant phenotype from the transformation process (Kappeli and Auberson 1998, Conner and Jacobs 1999, Latham et al. 2006), such as somaclonal variation (Larkin and Scowcroft 1981), insertion of multiple intact or rearranged transgene copies (Jones et al. 1987, Jorgensen et al. 1987), insertional mutagenesis (Feldmann and Marks 1987, Feldmann et al. 1989), epistatic interactions between the transgene and an endogenous gene (Napoli et al. 1990), (trans)gene silencing (Matzke and Matzke 1995), or a position effect (Jones et al. 1985, Nagy et al. 1985). Instances in which herbivore fitness varies across multiple transgenic lines, especially when response to only one event deviates from wild-type plants, would be more indicative of a transgenic engineering side effect rather than the expression product itself (Titimonen et al. 2005, Brodeur-Campbell et al. 2006). As more chestnut lines become available, we will be able to more conclusively evaluate whether the effects on insect performance are indicative of transgenic OxO-expressing chestnuts or are specific to the single transformation event LP28.

Blight resistance levels determined from introduction of $C. parasitica$ to the three chestnut types contrasted with growth patterns exhibited by the three herbivores. Transgenic American chestnut line LP28 demonstrated enhanced pathogen resistance over wild-type trees, as indicated by postinoculation survival (W. A. Powell, unpublished data). When restricting analysis to noninoculated chestnuts, lack of correlation between fungal resistance levels and larval growth rates among trees types for each herbivore indicates that pathogen resistance alone is a poor predictor of folivore fitness. Unintended phenotypic effects resulting from tree transformation (Dale and McPartlan 1992, Kappeli and Auberson 1998) the inclusion of a different species to serve as a positive control, or both (Powell et al. 2007) may obscure relationships between resistance level and herbivore performance.

Foliage selection for the polyphemus moth and fall webworm bioassays was done independently of fungal injection site on multi-stemmed plants, thus potentially confounding our analyses of insect growth rate. Because inoculated stems contained greater concentrations of the blight fungus and because plants respond to pathogen infection both locally and systemically (Fodor et al. 1997, Bonello and Blodgett 2003), we assessed whether there was greater variation in herbivore growth on leaves from inoculated, multi-stemmed trees relative to single-stemmed trees (e.g., Rostás et al. 2003). However, across all tree types, there were no differences in the variances of RGR for polyphemus moth and fall webworm, respectively, suggesting that the effects of fungal inoculation and stem type were negligible.

Traditional breeding, specifically backcrossing (Burnham 1981), is an alternative to biotechnology for chestnut restoration and as with genetic engineering, produces phenotypes that vary in suitability for non-
target herbivores. In one study, gypsy moths grew faster on Chinese-American hybrid seedlings, whereas consumption did not differ from control wild-type seedlings (Rieske et al. 2003). In a subsequent study, Kellogg et al. (2005) showed that fall webworm consumption and gypsy moth development were similar on foliage from mature, blight-inoculated American, hybrid, and first generation backcross chestnuts, but gypsy moth growth, consumption, and final larval weights varied with tree type. These results and ours suggest that conventionally bred and transgenic blight-resistant trees have a similar range of effects on herbivores.

Our study showed that gypsy moth fitness is affected by genetically modified American chestnuts expressing OxO to enhance blight resistance. The inclusion of only one transgenic line prevents the determination of whether transgene expression or the inexact gene insertion process is responsible for observed differences. Though LP28 chestnuts are not being considered further for forest restoration, it is critical to determine whether this effect is driven by the transformation event, or if it is an outcome of product expression. Future studies need to incorporate greater replication, multiple transgenic lines, more feeding guilds, feeding bioassays spanning larval development, and chemical and molecular analyses of foliar tissue to confirm or refute these results, determine whether other non-target insect herbivores could be affected by transgenic blight-resistant chestnuts, and elucidate the underlying mechanism(s) involved in this system before considering genetically modified, OxO-expressing American chestnuts for forest restoration.

Acknowledgments

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