HIGH LEVELS OF ROUNDUP" AND LEAF-BEETLE RESISTANCE IN GENETICALLY ENGINEERED HYBRID COTTONWOODS

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ABSTRACT

Uncontrolled weed and insect pests have significant impacts on survival and growth of short rotation poplar plantations. We tested genes widely used in agricultural crops that can improve the efficiency of pest control and reduce management costs. We studied herbicide tolerance using 1 10 transgenic lines (i.e., products of asexual gene transfer) of hybrid cottonwood during several years of field trials. The trees were screened for tolerance to glyphosate (the active ingredient in Roundup® herbicide) using genes developed by Monsanto. This paper describes the results of screening 70 diploid transgenic lines produced from commercial clones *(Populus trichocarpa x P. deltoides* and *P. trichocarpa x P. nigra),* which were grown at a site in eastern Oregon during 1998. We identified a number of transgenic lines that showed no foliar damage or reduction in growth rate after being sprayed at herbicide concentrations far above normal commercial rates. For insect resistance, we field-tested 51 hybrid lines *(P. trichocarpa x P. deltoides* and *P. deltoides x P. nigra)* that were transformed with a rebuilt *Cry3A Bacillus thuringiensis* toxin gene provided by Mycogen. This gene was intended to impart resistance to the primary insect pest of poplars in Oregon and Washington, the cottonwood leaf beetle *(Chrysomela scripta* Fabricius). Nearly all of the transgenic lines showed very low feeding damage under natural infestation in eastern Washington, whereas the non-transgenic lines sustained significant defoliation. In addition, the non-transgenics grew an average of 13% less than the transgenic lines. Both kinds of genes appear to hold considerable promise for aiding pest management in poplar plantations.

Keywords: Hybrid cottonwood, genetic engineering, glyphosate tolerance, insect resistance, field trials, pest management.

INTRODUCTION

Short-rotation intensive culture (SRIC) tree plantations are increasing in significance worldwide, and are likely to become more important in the next century (Sedjo and Botkin 1997). A growing demand for fiber has caused plantation area in the developing world to double between 1980 and 1995, and it is expected to double again by 2010. During that same period, per capita consumption of wood pulp has increased 50% in the developed world and 300% in the developing world (FAO 1997). New short-rotation tree crops, including hybrid poplar farms in the Pacific Northwest (PNW), are a response to fiber scarcity, and may be a critical future source for biofuels (Wright 1994) and for reducing pollution from annual crops (Tolbert and Schiller 1996).

Cottonwoods are desirable for SRIC because they have short fibers for making high-quality paper, and their rapid decomposition is important for use in facial and bathroom tissues. Their light-colored wood lowers bleaching requirements during paper manufacturing (Withrow-Robinson et al. 1995), thus reducing concentrations of undesirable by-products in mill effluent. Clones derived from interspecific crosses, such as between black cottonwood (Populus *trichocarpa)* and eastern cottonwood (*P deltoides),* often lead to the identification of genotypes that have very rapid growth (12-15 ft/year). These elite clones enable rotations as short as six years. In addition, cottonwoods flower at a young age and are easy to propagate vegetatively, allowing for facile deployment of selected hybrids.

In addition to their commercial importance, poplars provide a good model system for tree biotechnology. Because they are amenable to genetic engineering methods involving transfer of DNA during in vitro culture, they can benefit from genes isolated from other species. Poplars were the first trees to be genetically altered, and they can still be engineered more economically and effectively than most other agronomic and forest species. Thousands of transgenic poplars have been produced in laboratories around the world with traits such as modified wood properties, reproductive sterility, and insect and herbicide resistance. The Tree Genetic Engineering Research Cooperative (TGERC; http://www.fsl.orst.edu/ tgerc/index.htm) at Oregon State University has itself produced 1,700 transgenic poplars over the last few years (Strauss et al. 1998). Although the genes being studied by TGERC for herbicide tolerance and insect resistance were developed for use in soybean and potato, they are likely to be beneficial for poplar culture.

Stringent weed control is essential during the first two to three years of plantation development to enable tree establishment and rapid growth (Hansen et al. 1984), and to avoid animal damage (Strauss et al. 1997a). Cottonwoods are susceptible to many commonly used broadspectrum, post-emergent herbicides. Thus, most growers now use various combinations of pre-emergent herbicides, sheltered sprays, and tilling to control weeds, at considerable cost. Roundup®-tolerant cottonwoods may allow for better weed control near trees without damaging roots by tilling, reduce vegetation management costs, provide for low/no-till options, and promote the use of more benign herbicides. Improved weed control may also have other potential production and environmental benefits, such as reducing the need for fertilization and irrigation. If weed control is improved over that which is currently obtained in commercial plantations, there may also be a tree growth benefit. These diverse assets may encourage growers to substitute trees for annual crops, with a variety of benefits to soil, water, and wildlife (Pimentel and Krummel 1987; Hohenstein and Wright 1994; Ranney and Mann 1994).

Insect pests are often a major problem for poplar plantation managers. Two major classes of insect pest for poplars are chrysomelid beetles and lepidopteran caterpillars. Fortunately, both are susceptible to microbial pesticides derived from different strains of *Bacillus thuringiensis* (Bt). The products encoded by Bt toxin genes have been used safely as microbial pesticides in numerous crops (reviewed by Carozzi and Koziel 1997). Bt toxins are relatively selective insecticides that have very few non-target effects (reviewed by James 1997). Many different Bt strains have been identified, each affecting a select group of insects that are usually closely related phylogenetically (Thompson et al. 1995).

The use of trees genetically engineered to produce Bt toxins is preferable to spray applications for several reasons. First, vegetation, soil, and water surrounding the crop are not exposed to spray drift. Susceptible, nontarget insects in areas adjacent to the transgenic crop would not be exposed, reducing the potential for development of Bt resistance. Second, spray applications quickly degrade, persisting on leaves for, at most, only a few days (James et al. 1993; Thompson et al. 1995). Genetically engineered trees, however, can produce the biotoxin continuously, thereby avoiding sensitivities to application timing and the costs associated with multiple applications. Finally, transgenic trees produce the biotoxin within plant tissues, targeting insects harbored inside the plant, such as wood borers and leaf folders. For some of these pests, no pesticides are available that target the life stage that causes damage.

In the PNW, the cottonwood leaf beetle (CLB, *Chrysomela scripta* Fabricius) is a primary insect pest in poplar plantations. It is multivoltine and has a wide distribution; outbreaks can cause severe defoliation, particularly in young plantations (Hart et al. 1996). Significant growth loss has been shown in poplar after two years of simulated leaf beetle defoliation (Reichenbacker et al. 1996). Unlike weed control, which is only needed for the first two to three years of a crop rotation, insect control is often done repeatedly during each year of a rotation, making it costly for growers. Research done by the TGERC in collaboration with Mycogen showed a *Cry3A* Bt toxin to be highly effective against the CLB (James et al. 1999). The gene encoding this protein was used to generate the transgenics described in this paper.

METHODS

Plant Material

Three diploid hybrid clones (50-197, 195-529, both *P. trichocarpa x P. deltoides* (TxD); and 311-93, P. *trichocarpa x P. nigra)* were used to generate the herbicide-tolerant transgenic lines. The insect-resistant lines were produced in clones 24-305 and 189-434 (triploid TxD hybrids), 50-197 (diploid TxD), and OP-367 (diploid, P. *deltoides x P. nigra).*

Binary Vectors

The plant transformation vector pMON17204 was used to generate the herbicide-tolerant lines. This binary vector (provided by Monsanto Company) includes four transcriptional units within its T -DNA, two of which contain genes that impart tolerance to glyphosate (CP4 and GOX). Near the right border of the vector the Agrobacterium strain CP4 EPSPS gene (Barry et al. 1992) is fused with the chloroplast transit peptide (CTP) from *Arabidopsis thaliana EPSPS* (Klee et al. 1987). This fusion is expressed under the control of the caulimovirus figwort mosaic virus (FMV) promoter (Gowda et al. 1989; Richins et al. 1987; Sanger et al. 1990) and terminates with the polyadenylation signal from the small subunit (SSU) of RUBPcarboxylase gene of pea (E9; Coruzzi et al. 1984; Morelli et al. 1985). The second transcriptional unit contains the GUS gene (Jefferson et al. 1986), which is controlled by an enhanced version of the cauliflower mosaic virus (CaMV) 35S promoter (Kay et al. 1987) and the E9 terminator. The GOX gene follows and is expressed as a fusion with the CTP from the A. *thaliana SSU* gene (Stark et al. 1992), under the control of the FMV promoter and the nopaline synthase (NOS) terminator (Bevan et al. 1983).

Finally, nearest the left-hand border is the neomycin phosphotransferase gene (NPTII) driven by the 35S promoter and terminated by the NOS 3' sequences.

The binary vector pKH20SBT 9 was used to produce the insect-resistant lines. This vector contains two transcriptional units within its T -DNA. At the right-hand border, *Cry3A* (a Bt toxin gene supplied by Mycogen) was expressed under the control of the 35S promoter and the orf25 terminator (Barker et al. 1983). Nearest the left-hand border is the NPTII gene under the control of the NOS promoter and terminator. Matrix attachment region (MAR) elements (Allen et al. 1996) are positioned between the left-hand border and the NOS promoter, and the right-hand border and the orf25 terminator.

Production and Verification of Transgenics

Sterile leaf, stem and petiole explants were co-cultivated with *Agrobacterium tumefaciens* strain ABI containing the binary vector pMON17204 or strain C58 MP90 harboring pKH20SBT 9. Transformation and regeneration were performed according to Han et al. (1999). Seventy herbicide-tolerant lines were produced in three diploid clones (15 in 50-197, 27 in 195-529, and 28 in 311-93). Fifty-one insect-resistant lines were produced in four clones (16 in 24-305, 17 in 50-197, 9 in 189-434, and 9 in OP-367).

Shoot and root production from all transformants (plants regenerated from a single cell containing the newly inserted DNA) occurred in the presence of kanamycin (25 mg/L). Leaf tissue from each glyphosate-tolerant line was stained for GUS activity in 1.0 g/L XGluc (Jefferson et al. 1986) and cleared in 95 % ethanol. Plant tissues expressing the GUS gene turn blue when stained with X-Gluc; they should also contain the glyphosate tolerance genes. All transgenic lines were also rooted in glyphosate-containing media (2.0 mg/L) to verify that the glyphosate tolerance genes were being expressed properly. The insect-resistant lines were prescreened using in *vitro* insect bioassays (data not shown) to confirm that the Bt toxin gene was being expressed.

Field Studies

Establishment

Both trials were established on xeric sites east of the Cascade Mountains. Newly planted trees were protected from desiccation with wind screens until acclimated. All trees were fertigated on a regular schedule using a previously optimized regime.

The glyphosate-tolerant lines were planted near Boardman, Oregon, on June 8-9, 1998. Rooted plantlets were placed at a 10.0 x 3.75-foot spacing (between and within plant-rows, respectively). One ramet of each of the 70 transgenic diploid lines was planted, along with untransformed controls, in each of 12 row-plots. Groups of three row-plots (zero, low, and high glyphosate spray levels) were assigned to four replicate blocks.

The insect resistance trial was installed near Wallula, Washington, on June 8, 1998. Rooted plantlets were spaced 7.5 feet apart within a row, and rows were planted 10 feet apart. One ramet of each of the 51 independently transformed lines was planted, along with untransformed controls, into each of 10 row-plots. Rows of transgenic trees were alternated with rows of four commercial clones that are equally susceptible to cottonwood leaf beetle herbivory. These "nurse rows" were intended to provide a breeding ground for the CLB in order to maintain high populations with a uniform distribution.

Herbicide Treatments

Roundup Pro™ was applied twice during the period of active growth, mid-July and mid-August. The first treatment was applied at two nominal rates: 3 and 6 qt/ ac. Low and high rates for the second treatment were 2 qt/ac. and 4 qt/ac., respectively. Treatment levels (zero, low, high) were randomly assigned to rows within each of the four replicate blocks.

Roundup® was applied from a tractor pulling a sprayer equipped with a two nozzles; one directed toward the base of the trees, the other at the ground. The spray pattern of the former provided foliar coverage to a height of 3 feet Because the trees were sprayed from both sides, the effective application rate was twice the nominal rate.

Weeds in the unsprayed plots were controlled using a rototiller between rows and hand-hoeing around the base of each tree. Not all weeds were susceptible to Roundup®, so all plots were hoed to provide comparable levels of weed control in sprayed and unsprayed plots. Differences between tree growth in Roundup® treated and control plots were, therefore, due to differential tolerance, rather than to differential weed control.

Measuring Herbicide Tolerance

Heights and basal diameters were taken on all trees immediately after planting and at the end of the growing season. Data were adjusted for variation in size at planting and between blocks by using the least-square mean (SAS 1990) of the logarithm of net growth. Trees

were evaluated for damage due to glyphosate four weeks after treatment according to the damage rating system in Table 1.

Table 1.-Rating system used to visually estimate the level of foliar chlorosis after glyphosate treatment.

Score	Description
0	No visible damage
1a	1–5% of total leaf area chlorotic
1b	6–20% of total leaf area chlorotic
2	21–40% of total leaf area chlorotic
3	41–60% of total leaf area chlorotic
4	61–80% of total leaf area chlorotic
5	81–100% of total leaf area chlorotic
6	Dead

Measuring Insect Resistance

Heights and basal diameters were taken on all trees immediately after planting, and at the same time as damage ratings were taken. Trees were evaluated for damage seven weeks after planting and again at the end of the growing season (damage rating system in Table 2).

Table 2.-The rating system used to evaluate relative degree of resistance to defoliation in the insect resistance trial.

Score	Description
1	No damage from beetle herbivory any where on tree
2	Undecided'
3	Clear evidence of beetle herbivory some where on tree

At the time the trees were rated for defoliation (15 Oct. 1998), the leaves were very brittle and sustained some damage due to cold temperatures and high winds. When this abiotic leaf damage could not clearly be distinguished from insect herbivory, trees were assigned a damage score of 2.

RESULTS AND DISCUSSION

Glyphosate Tolerance

In previous herbicide tolerance trials, we identified numerous triploid lines with complete tolerance to Roundup® (Strauss et al. 1997b), but in response to much lower application rates than were applied in the present experiment. The purpose of the current trial was to identify, within a single growing season, a few lines within each clone that exhibited very high tolerance to glyphosate and unimpaired growth. Thus, the Roundup® concentrations used for this study were considerably higher than those used operationally. The mean net growth for the three best lines within each clone is shown in Figure 1. In nearly every case, the lower concentration of Roundup® did not significantly reduce growth rate, whereas the higher application did result in less growth. In one case (line 210, clone 311-93), even the highest concentration of Roundup® did not appear to impair growth.

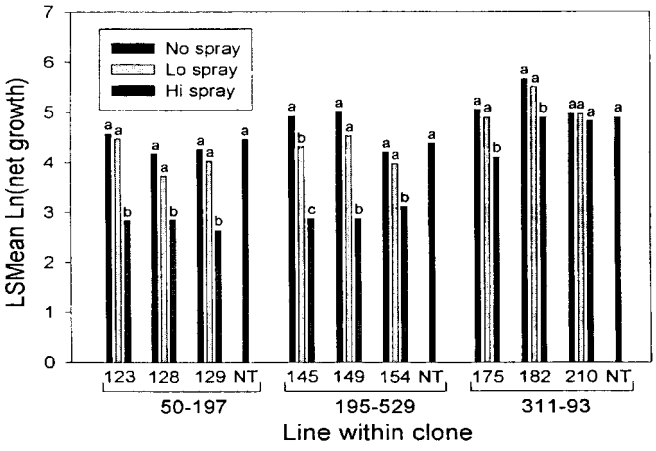

Figure 1.-The mean logarithm of net growth, by treatment, for the three most glyphosate-tolerant lines within each clone (n=4 ramets/line). Net growth is defined as the difference between tree volumes at the beginning and end of the growing season. Tree volumes were calculated as: [(basal diameter)2 x (height)]. Within each cluster, bars labeled with the same letter are not significantly different. The single bar within each clonal group represents the growth of unsprayed, non-transgenic controls (NT).

The mean damage ratings are shown in Figure 2. In general, there was no significant difference in the amount of damage caused by the low vs. the high herbicide rate. The transgenic lines produced in clone 311-93 had less damage than those from the other two clones. In both lines 182 and 210 the low rate of Roundup® did not result in any chlorosis; this was even true for the high concentration in line 210. Based on our previous experience, it is likely that if lower application rates had been used, many more lines would have exhibited no damage. However, even at the highest concentration of Roundup®, the best performing transgenic lines exhibited little or no damage, and their growth was not significantly less than that of the unsprayed transgenic or non-transgenic controls for the same clone. Figure 3 shows the range of responses seen following two Roundup® treatments at the highest rate.

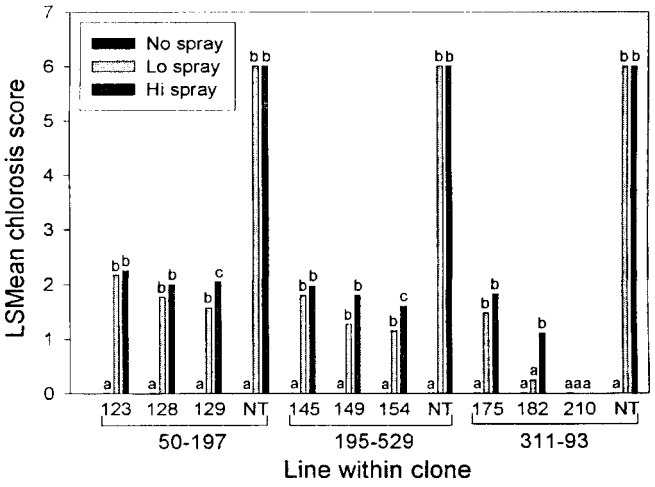

Figure 2.-Mean chlorosis, by treatment, for the three most glyphosate-tolerant lines within each clone (n=4). The herbicide damage rating system is defined in Table 1. Within each cluster, the first bar (no spray) is not visible (score of zero) but is labelled with an "a." Bars labeled with the same letter are not significantly different. The final bar within each clonal group is the nontransformed control (NT).

Figure 3.-Herbicide tolerance trial approximately two weeks after the second treatment (Sept. 2, 1998). The tree in the center is line 210 (clone 311-93); it and the others in its irrigation row received the high rate of Roundup®. The small dead tree to its left is a non-transgenic control; the yellowing (light colored leaves) on the transgenic tree to its right is chlorosis resulting from herbicide treatment.

Insect Resistance

We relied on immigration of beetles from the surrounding stands to test insect resistance. Although only representative lines are shown in Figure 4, nearly all of the Bt transgenics showed very low feeding damage, whereas the non-transgenic lines sustained significantly higher levels of defoliation. Figure 5 shows typical damage levels for transgenic and non-transgenic trees. In most cases, the mean growth for transgenic lines was greater than that for the non-transgenic controls within each clone (Figure 6).

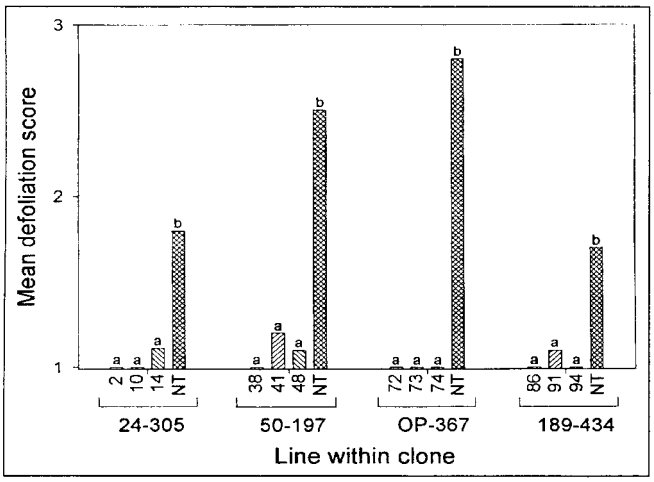

Figure 4.-Mean defoliation for representative lines within each clone (n=10 ramets/line). The defoliation scoring system is defined in Table 2. Within each cluster, bars labeled with the same letter are not significantly different. The final bar within each clonal group is the nontransgenic control (NT).

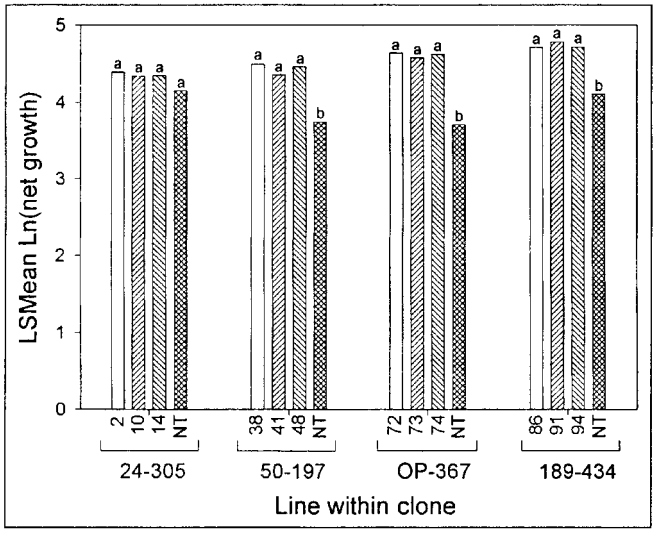

Figure 6.-Effect of insect feeding on tree growth. The mean logarithm of net growth for three typical transgenic lines, and their corresponding non-transgenic controls, are shown for each clone (n=10 ramets/line). Within each cluster, bars labeled with the same letter are not significantly different. The final bar within each clonal group is the non-transgenic control (NT). In three of the four clones, insect feeding on non-transgenic lines resulted in a significant reduction in growth.

Figure 5.-Characteristic levels of defoliation seen early in the 1998 growing season on transgenic (A) and non-transgenic (B) lines in the insect resistance trial at Wallula, WA.

Future Research

Both resistance gene classes are promising for pest management in poplar plantations, but additional work is needed to demonstrate their commercial value. This spring the TGERC will initiate a series of large-scale, long-term management trials in which we will try to determine the value of herbicide tolerance to growers. In these studies, we will compare the effects of various conventional weed-control regimes to those that fully utilize the introduced trait.

We have also begun to assess the stability of transgene expression following vegetative propagation and multiple dormancy cycles, and the effect of our transformation system on the genetic integrity of the starting material. Hardwood cuttings were taken from glyphosatetolerant triploid lines screened in a previous, two-year trial were outplanted during summer 1998 and will be challenged repeatedly with Roundup® during 1999. They will then be rated for herbicide damage and evaluated for unwanted genetic variation in growth rate arising from *in vitro* culture. In addition, we have initiated another insect resistance trial using hardwood cuttings taken from the 1998 Wallula trial. In this study we will assess the stability of insect resistance, the relationship between herbivory intensity and growth, and whether transformation has impaired growth compared to nontransgenic clones.

Hurdles to Commercialization

The potential for insect pests to develop resistance to genetically engineered crops is a major issue in pest control and a drawback to this strategy, which has few other weaknesses (DiCosty and Whalon 1997; James 1997; Roush and Shelton 1997). Before insect-resistant transgenics can be commercialized, a resistance management plan must be developed. Many management strategies have been proposed based on prior experiences with pesticide resistance (e.g., Luttrell and Caprio 1996; Roush 1997; Gould 1998; McGaughey et al. 1998). The TGERC is conducting a detailed genetic analysis of Bt resistance in the CLB to determine the number of genes involved in resistance and their mode of inheritance. To obtain regulatory approval from EPA, studies are also needed of beetle dispersal, the extent to which natural refugia are effective buffers for preventing the development of resistance, and the level of toxicity afforded by transgenics, among other things (Matten 1998).

Combining resistance genes (pyramiding or stacking) is recommended as a way to prevent or delay the development of insect resistance to transgene products (Nwanze et al. 1995; Maredia and Mihm 1997; Roush 1997). This approach is an effective strategy for resistance management with the cotton bollworm *(Helicoverpa armigera)* (Zhao et al. 1997). The TGERC is now experimenting with another Bt toxin that may operate by different mode of action. If the CLB is found to be susceptible, the gene of this second toxin will be combined with *Cry3A* in a single vector to produce additional transgenic lines.

Before genetically engineered poplars can be commercialized, federal regulators will probably require a strategy for minimizing the risk of transgene escape into wild populations. One way to reduce this risk is to engineer reproductive sterility (Strauss et al. 1995). We have been actively working in this area for the past five years, and are experimenting with a variety of approaches (review by Skinner et al. 1999). The approach that proves most effective will be stacked with other traits of interest in a final commercial product.

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