NRC Research Press

ARTICLE

Bt-Cry3Aa transgene expression reduces insect damage and improves growth in field-grown hybrid poplar

Amy L. Klocko, Richard Meilan, Rosalind R. James, Venkatesh Viswanath, Cathleen Ma, Peggy Payne, Lawrence Miller, Jeffrey S. Skinner, Brenda Oppert, Guy A. Cardineau, and Steven H. Strauss

Abstract: The stability and value of transgenic pest resistance for promoting tree growth are poorly understood. These data are essential for determining if such trees could be beneficial to commercial growers in the face of substantial regulatory and marketing costs. We investigated growth and insect resistance in hybrid poplar expressing the *cry3Aa* transgene in two field trials. An initial screening of 502 trees comprising 51 transgenic gene insertion events in four clonal backgrounds (*Populus trichocarpa* × *Populus deltoides*, clones 24-305, 50-197, and 198-434; and *P. deltoides* × *Populus nigra*, clone OP-367) resulted in transgenic trees with greatly reduced insect damage. A large-scale study of 402 trees from nine insertion events in clone OP-367, conducted over two growing seasons, demonstrated reduced tree damage and significantly increased volume growth (mean 14%). Quantification of Cry3Aa protein indicated high levels of expression, which continued after 14 years of annual or biannual coppice in a clone bank. With integrated management, the *cry3Aa* gene appears to be a highly effective tool for protecting against leaf beetle damage and improving yields from poplar plantations.

Résumé : La stabilité et la valeur de la résistance transgénique aux ravageurs pour favoriser la croissance des arbres ne sont pas bien connues. Ces données sont essentielles si l'on veut déterminer dans quelle mesure de tels arbres transgéniques pourraient être profitables pour des producteurs commerciaux considérant les coûts substantiels reliés à la réglementation et la mise en marché de tels arbres. Les auteurs ont étudié la croissance et la résistance aux insectes de peupliers hybrides exprimant le transgène *cry3Aa* à l'aide de deux tests au champ. Une première étude de 502 arbres après 51 événements d'insertion du gène transgénique au sein de quatre clones récepteurs (clones 24-305, 50-197 et 198-434 de *Populus trichocarpa* × *Populus deltoides* et clone OP-367 de *P. deltoides* × *Populus nigra*) a permis de produire des arbres transgéniques qui avaient subi beaucoup moins de dommages dus aux insectes. Une étude à grande échelle de 402 arbres après neuf événements d'insertion chez le clone OP-367 et réalisée pendant deux saisons de croissance a démontré que les arbres avaient subi moins de dommages et avaient une croissance en volume significativement accrue (moyenne de 14 %). La quantification de la protéine Cry3Aa a révélé de forts niveaux d'expression, qui se poursuivaient après 14 ans de recépage annuel ou biannuel dans une banque clonale. Dans un contexte de gestion intégrée, le gène *cry3Aa* apparaît être un instrument hautement efficace de protection contre les dommages foliaires causés par la chrysomèle du peuplier et d'amélioration du rendement des plantations d'arbres. [Traduit par la Rédaction]

Introduction

In the Pacific Northwest, hybrid poplars are an important source of raw material for the paper and pulp industries, as well as for the emerging biofuel industry (Stanton et al. 2008). As with other crop species, insect damage is an important concern because insect defoliation can lead to a substantial reduction in productivity and may result in tree death (Kosola et al. 2001). A major pest of poplar is the cottonwood leaf beetle (CLB, *Chrysomela scripta* Fabricus). CLB larvae and adults defoliate trees in poplar plantations, which, in turn, results in decreased tree vigor and increased susceptibility to pathogenic infections (Coyle et al. 2000).

CLB infestations can be treated by either chemical or biorational methods. While chemicals are effective in controlling CLB, these compounds are expensive and can be broadly toxic in the environment (Coyle et al. 2000). Biorational methods for controlling CLB include the use of specific toxins (proteins) isolated from the bacterium *Bacillus thuringiensis* (Bt). Bt toxins can be used either through direct sprays or by incorporation of genes encoding them into plants. Exogenous application of Bt can be problematic because the effects are transient and the toxin is only effective against younger stages of the larvae (reviewed in Sanahuja et al. 2011). In addition, the majority of Bt toxin never comes in contact with the target pest and can affect nontarget organisms. These limitations can be largely overcome by expressing specific Bt genes to target problematic pests in the host plant itself.

Received 10 July 2013. Accepted 24 October 2013.

A.L. Klocko, C. Ma, and S.H. Strauss. Department of Forest Ecosystems and Society, Oregon State University, 321 Richardson Hall, Corvallis, OR 97331, USA.
R. Meilan. Department of Forestry and Natural Resources, Pfendler Hall, Room G021E, 715 W. State Street, Purdue University, West Lafayette, IN 47907, USA.
R.R. James. USDA-ARS Pollinating Insects Research Unit, Department of Biology UMC 5310, Utah State University, Logan, UT 84333, USA.
V. Viswanath. Global Institute of Intellectual Property (GIIP) 254, Third Floor, Okhla Industrial Estate Phase III, New Delhi 110 020, India.
P. Payne. Walla Walla Public Schools, 364 South Park Street, Walla Walla, WA 99362, USA.
L. Miller. Oregon Department of Forestry, 2600 State Street, Salem, OR 97310, USA.
J.S. Skinner. Bayer Vegetable Seeds, Crop Genomics, 279 Cousteau Place, Suite 200, Davis, CA 95618, USA.
B. Oppert. USDA ARS Center for Grain and Animal Health Research, 1515 College Ave., Manhattan, KS 66502, USA.
G.A. Cardineau. Centro de Agrobiotecnología, Escuela de Biotecnologia y Alimentos, Tecnologico de Monterrey, Monterrey, N.L. 64849, Mexico.
Corresponding author: Steven H. Strauss (e-mail: Steve.Strauss@oregonstate.edu).

Published at www.nrcresearchpress.com/cjfr on 28 October 2013.

Table 1. Summary of tree genotypes used in the experiments.

	Bioassay		Initial screening field trial		Large-scale field trial		Cry3Aa initial ELISA		Cry3Aa maintenance ELISA	
Poplar clone	No. of events	No. of ramets	No. of events	No. of trees	No. of events	No. of trees	No. of events	No. of trees	No. of events	No. of trees
24-305	34	204	16	153	—	—	16	38	—	—
50-197	31	186	17	169	—	—	17	43	—	—
OP-367	17	102	9	90	9	402	9	18	3	5
189-434	18	108	9	90	—	—	9	18	—	—
Non-Tr control	NA	24	NA	40	NA	47	NA	4	NA	1

Note: Non-Tr (non-transgene) control numbers exclude rows of refuge and border trees. NA, not applicable.

Ectopic expression of Bt genes has proven very effective as a pest control measure in herbaceous crop species, and has been credited with substantial reductions in pesticide applications and a decrease in crop losses (Qaim 2009). The potential value of transgenic pest-resistant trees has been widely discussed but little is known about their field performance (van Frankenhuyzen and Beardmore 2004). Transgenic hybrid poplar trees expressing the Bt endotoxin gene *cry1A* were developed in 1991 (McCown et al. 1991) and were successfully tested in the laboratory against a variety of insect pests (Ramachandran et al. 1993; Robinson et al. 1994). The first field test of *cry1A* transgenic trees revealed their resistance to the larvae of forest tent caterpillars and gypsy moths (Kleiner et al. 1995). However, no data were reported regarding tree growth or stability of gene expression over time — important parameters for a woody-tree crop. In China, transgenic poplars containing a modified *cry1A* gene were planted in 1994 and were authorized for commercial production in 2000 (Sedjo 2005). Trees expressing *cry3Aa*, a coleopteran-specific Bt gene, are known to be effective against poplar leaf beetle larvae (*Chrysomela tremulae*) under greenhouse conditions (Genissel et al. 2003). However, these trees were not evaluated under field conditions. Similar to our study, a codon-optimized version of the *cry3Aa* was used to enhance gene expression in plants (Adang et al. 1993).

Our goals were to extend the results of (Genissel et al. 2003) to field conditions and to elaborate on a preliminary conference report (Meilan et al. 2000). We examined whether the *cry3Aa* gene imparted resistance in hybrid poplar clones under natural insect pressure in field trials that mimicked conditions found in commercial plantations. We report significant improvement of both insect resistance and tree productivity as a consequence of transgene expression.

Materials and methods

Generation of binary vectors and plant transformation

A 3.4 kb fragment containing 35S promoter:*cry3Aaorf25* terminator (strain 6; Adang et al. 1993) was excised with ClaI from Mycogen's plasmid 48 and inserted into the ClaI site of pKH20 flanked with matrix attachment regions (MARs) as per (Han et al. 1997), with transcription directed toward the right border, to create pKH20SBT-9 (Supplementary data Fig. 1;[1] Davis et al. 2006). The codon pattern of the *cry3Aa* gene had been modified such that it represented an average dicotyledonous DNA sequence, lacking AT richness (Adang et al. 1993). This binary vector was transformed into *Agrobacterium tumefaciens* strain C58 via the freeze–thaw method (Holsters et al. 1978) and used to transform four hybrid cottonwood clones using a previously published method (Han et al. 2000). Clone OP-367 is a *Populus deltoides* × *Populus nigra* hybrid, and the three additional clones used, 24-305, 50-197, and 189-434, are *Populus trichocarpa* × *P. deltoides* crosses. Clones 24-305 and 189-434 are triploids, whereas 50-197 and OP-367 are diploids. All transgenic plants were rooted on kanamycin-containing media and, as described in the following, also subjected to laboratory bioassays with CLB to select the best events for field trials (Table 1).

ELISAs of Cry3Aa

Cry3Aa protein levels were measured using the ELISA Complete Kit for Bt-Cry3A obtained from Agdia (Elkhart, Indiana). In the initial ELISA assay, performed in 1998, leaves were collected from trees near Wallula, Washington. In the second ELISA, performed in 2012, leaves were collected in October 2011 from clone bank trees grown near Corvallis, Oregon. These trees were propagated via branch cuttings taken from the field trials planted in Corvallis in June of 2003, and coppiced every 1–2 years thereafter. Leaves were ground to a fine powder in liquid nitrogen. Samples of 100 mg were used for protein extraction, and ELISAs were performed following the manufacturer's protocol. Whole-cell protein extract concentrations for samples were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, California) to standardize protein inputs between samples. Data were normalized to subtract nonspecific background cross-reactivity from control samples.

Bioassays

Laboratory bioassays were performed to evaluate the effect of each of the transformants on CLB survival for 17 to 34 events per clone (see Table 1), following a published method (James et al. 1999). A complete randomized block experimental design was used, with date of experiment as the replicate block (six dates were studied). For each replicate and event, 10 second-instar CLB larvae were applied to a leaf disc (3.6 cm in diameter) from the transgenic event being tested and allowed to feed for 24 h, after which time they were fed whole leaves taken from nontransgenic plants of the same clone. Mortality was assessed after larvae had fed on the nontransgenic leaves for 72 h. The controls were larvae solely fed nontransgenic leaves of the relevant clonal variety. The larvae used in these feeding tests were from laboratory-reared cultures established using insects collected from an International Paper plantation in South Carolina. The beetles had been in culture for one year and reared on leaves from a variety of greenhouse-grown poplar clones. Transgenic events that did not affect larval survival were not evaluated further.

Field screening

The initial field trial, to evaluate beetle resistance and growth of the transgenic trees, was established in the spring of 1998 at a site near Wallula, Washington. Transgenic and control trees were planted at a spacing of 2.29 m (7.5 ft) within rows and 3.05 m (10 ft) between rows. The perimeter and every other inner row (a total of six rows) were planted with nontransgenic refuge trees to encourage insect development. Alternate inner rows were planted with a total of 10 trees from each transgenic event arranged randomly

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfr-2013-0270.

Table 2. Damage rating system used to evaluate field-grown trees in both the initial screening and large-scale field trials.

Score	Description
0	No visible damage
1	Some possible nibbling apparent (<0.1% leaf area eaten)
2	<5% of total leaf area eaten
3	5%–25% of total leaf area eaten
4	25%–50% of total leaf area eaten
5	50%–75% of total leaf area eaten
6	75%–100% of total leaf area eaten

with interspersed control trees (Table 1). Size measurements (stem height and stem diameter at breast height) were taken at the time of planting and at the end of the first growing season. Two damage measurements were recorded for each tree: one assessing the damage to the whole tree and the second assessing the most badly damaged leaf cluster (five to six outer leaves) on the tree (see Table 2). Tree damage was assessed by visual scoring. Trees were rated using this system during the second growing season.

Large-scale field study

A second field trial to evaluate the resistance and growth of transgenic events from clone OP-367 began in the spring of 1999 near Wallula, Washington. Trees derived from cuttings taken in the initial field trial were planted at a spacing of 2.29 m (7.5 ft) within rows and 3.05 m (10 ft) between rows. Perimeter rows and every other inner row (a total of six rows) were planted with nontransgenic nurse trees. Five inner rows were planted with 10 ramets each of every transgenic event and one nontransgenic control. Size measurements (stem height and stem diameter at breast height) were collected at the end of each growing season. Two different damage scores were made for each tree, one assessing the damage to the whole tree and the second assessing the most badly damaged leaf cluster on the tree (Table 2). Trees were rated using this system during the first growing season.

Evaluation of insect damage to field-grown trees

Trees were subjected to natural insect pressure, and CLB larvae were often observed feeding on leaves at the initial field site, leading to readily observable differences in leaf damage (Fig. 1). To quantify these observations, we developed a scoring system ranging from 0 (no visible damage) to 6 (75%–100% of total leaf area eaten; Table 2).

Statistical analyses

Laboratory bioassays and ELISA tests

For the laboratory bioassays, the number dead out of the total number of insects placed in each trial was modeled as a function of clone and event nested within clone using a generalized linear model assuming a binomial distribution, a logit link and overdispersion. All larvae that fed on leaves from event 125 of clone OP-367 survived; as an outlier with conspicuously low Cry3Aa expression, this clone was excluded from further analysis. Likewise, all of the controls (24-305, OP-367, 189-434, and 50-197) were omitted, as they showed either very low or no insect mortality. We tested for correlation between the insect mortality data from the bioassays and the Cry3Aa levels from the ELISA tests using regression analysis using the Statgraphics program (statgraphics). For the subset of trees that were used both for bioassays and quantification of Cry3a levels, the data were analyzed using a comparison of regression lines approach with mean proportion of larvae dead (noted as "bioassay") as the dependent variable and protein endotoxin levels measured using ELISA assays (noted as "ELISA") and clone as the independent variables. The relationship of bioassay to ELISA was allowed to vary among clones by including a clone × ELISA term in the model. Because bioassay was restricted to be between 0 and 1, a modified logit transform (log((bioassay + 0.005)/(1 – bioassay + 0.005))) was applied to satisfy normality assumptions and to assure that confidence limits would extend only within this range. A small constant was added to allow application of the log transformation when observed proportion was 0 (Wonnacott and Wonnacott 1981).

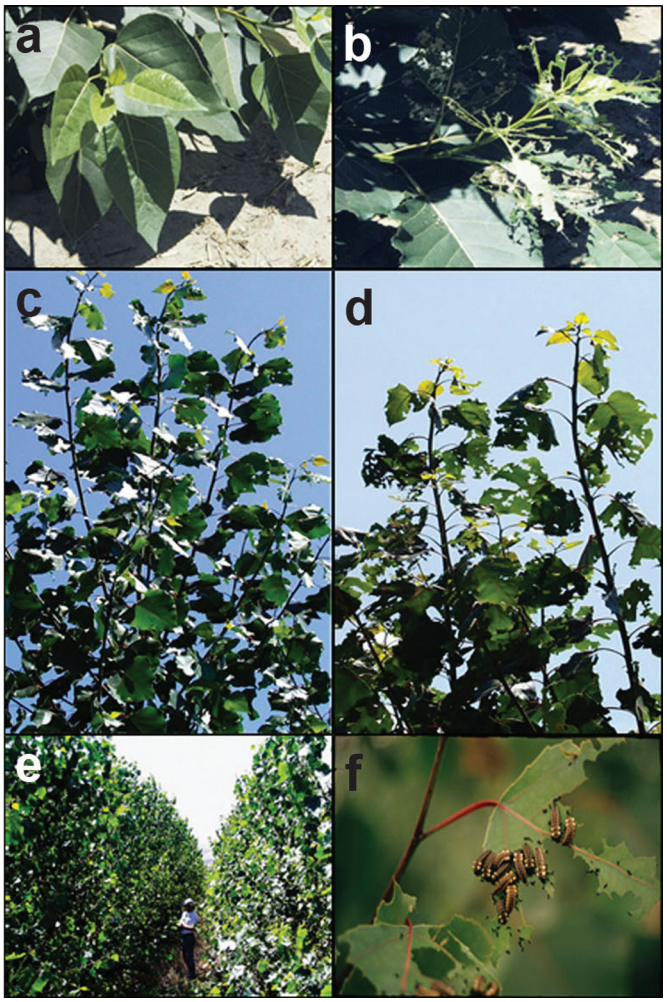


Fig. 1. Overview of tree damage seen in the initial screening and large-scale field trials. Young transgenic tree with a leaf cluster damage score of 0 (*a*) and a young control tree with a leaf cluster damage score of 6 (*b*) in the initial screening trial. Large transgenic tree with a whole-tree damage score of 1 (*c*) and large control tree with an overall tree damage score of 3 (*d*). The full damage scoring system is given in Table 2. The view down two rows of trees in the large-scale field trial (*e*) showing transgenic (left row) and control trees (right row). Cottonwood leaf beetle larvae observed feeding on leaves (*f*).

Field-screening trial

The field trial data were analyzed as a randomized block design with planting row as the blocking factor and clone and transgenic-event-within-clone as the fixed effects. Volume growth (height × diameter^2) was averaged over both ramets of each transformation event from each row in the first year and modeled as a function of clone, transgenic indicator variable, and event-nested-within-clone, using a linear mixed-effects model (PROC MIXED) in SAS (SAS Institute Inc. 2008). Type II tests of fixed effects (appropriate for nested designs) were used for hypothesis testing.

Large-scale field study

Clone OP-367 was selected for this trial as a result of its potential commercial relevance to Boise Cascade, who hosted the study. Size measurements were analyzed as a randomized block design with row as the blocking factor and clone and transgenic-event-within-clone as the fixed effects. Repeated measurements of height and diameter in the first and second years were modeled as a function of clone, an indicator of transgenic status, event-nested-within-a-clone, and year using PROC MIXED in SAS (SAS Institute Inc. 2008). The type II test of fixed effects was again used for hypothesis testing. Sources of variance accounted for were the planting row (block), ramet, and year. Stem volume from the first and second years were again modeled as a function of clone, an indicator variable of transgenic status, and event-nested-within-clone, using PROC MIXED in SAS (SAS Institute Inc. 2008) with hypotheses testing using type II tests of fixed effects. Sources of variance accounted for were the planting row (block) and ramet.

Results

Laboratory bioassays

To select events for our initial field trial, we evaluated the toxicity of plants transformed with our optimized *cry3Aa* construct (Supplementary data Fig. 1).[1] We tested the toxicity of 100 transgenic events and four controls from the four different poplar clones. Larvae that were fed control leaves exhibited an extremely low mortality rate, with no observed mortality for larvae that were fed leaves from clones OP-367, 50-197, or 189-434. Two of 60 larvae that were fed leaves from clone 24-305 died (the average percent mortality was 0.03% for this control). By contrast, transgenic events from all four clones exhibited an average of more than 30% insect mortality (Supplementary data Fig. 2).[1] We found significant differences between clonal backgrounds ($F_{3,590} = 3.33$, $p = 0.019$) and between events within clones ($F_{95,495} = 1.64$, $p = 0.0005$). Because transgenic events from all four clones resulted in significant mortality, we concluded that Cry3Aa protein was effective against CLB larvae. We selected the 51 transgenic events representing the range of insect mortality and four non-transgenic clones as controls for the initial screening field trial. We planted 10 trees from each event and each control. A few trees died from various causes resulting in a total of 542 transformed trees for this study (see Table 1).

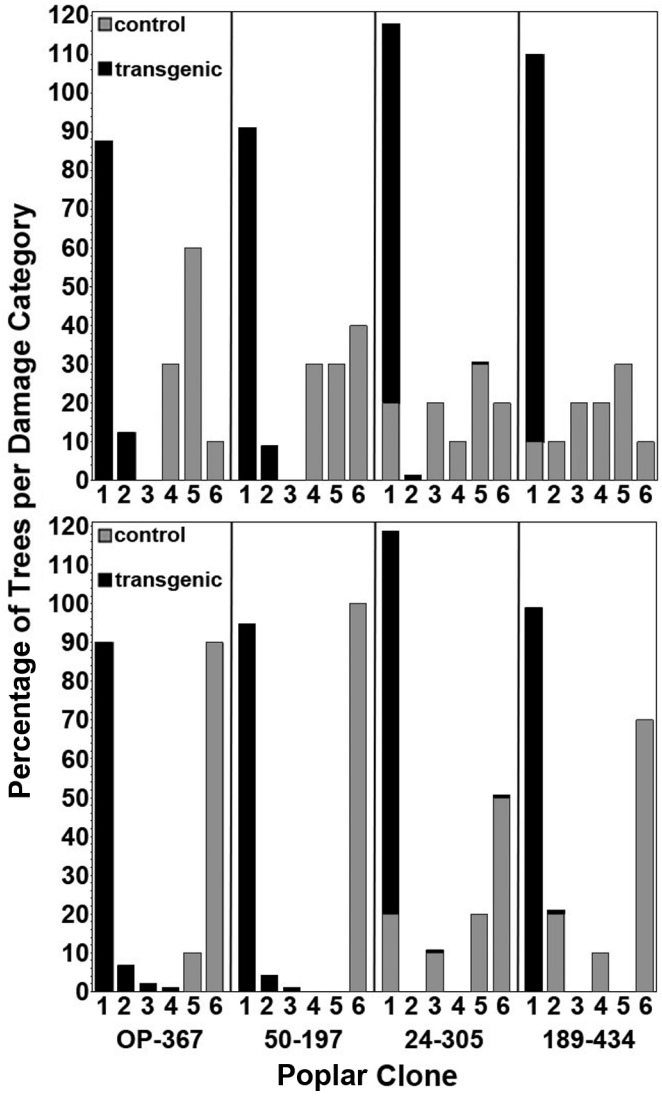
Initial screening trial: plant growth

In the first year of our field trial, we assessed overall biomass productivity using stem volume index (stem height × stem diameter^2). We calculated the volume growth of each tree by subtracting the stem volume at the time of planting from the stem volume at the end of the first growing season. We found that our four clones had significantly different volume growth from each other ($\mathbf{F}_{3,215} = 50.54$, $p < 0.0001$). Clones OP-367 and 50-197 did not differ from each other ($t_{215} = 1.1$, $p = 0.27$) but had significantly greater volume growth than the other clones (Supplementary data Fig. 3).[1] The average volume growth of transgenic events in clone 50-197 was significantly larger than their corresponding nontransgenic control ($t_{215} = -2.80$, $p = 0.006$); none of the other events were significantly different from their clonal controls (all $p > 0.30$).

Initial screening trial: damage scores year 2

Trees were rated for whole-tree and worst-cluster damage during the second growing season. All of the trees showed some level of whole-tree damage. Overall, the transgenic trees sustained much less damage than the control trees (Table 2 and Fig. 2). All transgenic trees in clones OP-367 and 50-197 had whole-tree damage scores of 2 or lower (less than 5% damage; Fig. 2, upper panel). By contrast, all control trees in these clones had scores of 4 and above, corresponding to greater than 25% whole-tree damage. Similar results were obtained for clones 24-305 and 189-434 (Fig. 2). Similarly, all leaf clusters had some level of damage (Fig. 2, lower panel). In all transgenic events, greater than 97% of leaf clusters had scores of 2 or lower, indicating less than 5% damage. By comparison, control trees from clones OP-367 and 50-197 showed greater than 50% damage for all worst-damaged leaf clusters.

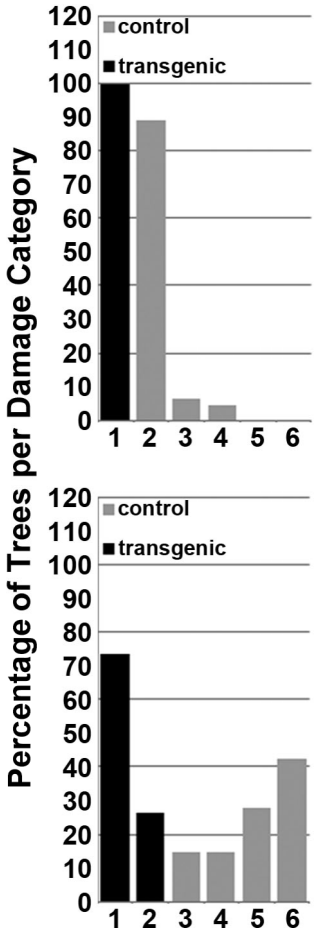
Fig. 2. Field-grown transgenic trees of all four clones sustained less insect feeding damage than control trees. Whole-tree damage scores (top panel) and worst cluster damage scores (bottom panel) recorded during the second growing season of the initial screening trial. Damage scores (horizontal axis) ranged from 1 (some possible nibbling apparent) to 6 (75%–100% of total leaf area eaten). The full damage scoring system is given in Table 2.



Initial screening trial: ELISAs of Cry3Aa

After the initial field trial was established, we used ELISAs to quantify the amount of Cry3Aa protein present in the different events. We collected leaves from each event and nontransgenic control trees of each clone. Control samples had a low level of reactivity, likely because of nonspecific antibody binding, so the data were normalized to subtract this background. Transformed events had levels of Cry3Aa ranging from 45.3 to 133.6 ng/μg total leaf protein (Supplementary data Fig. 4).[1] One event in clone 24-305 had 5.6 ng Cry3Aa / μg total protein; a level close to that of the controls. Nearly all larvae fed a diet of control leaves survived, indicating that the leaves were not toxic. By comparison, the transgenic trees had higher levels of Cry3Aa protein and higher

Fig. 3. Transgenic clone OP-367 trees sustained less insect feeding damage than control trees. Whole-tree damage scores (top panel) and worst cluster damage scores (bottom panel) recorded during the first growing season of the large-scale field trial. Damage scores (horizontal axis) ranged from 1 (some possible nibbling apparent) to 6 (75%–100% of total leaf area eaten).



levels of insect mortality. When evaluating the full data set of Cry3Aa levels and insect mortality, there was strong evidence that mortality was related to levels of Cry3Aa ($F_{1,47} = 72.24$, $p < 0.0001$), but there was no evidence that the relationship differed among clones ($F_{3,47} = 1.16$, $p = 0.34$). When the controls and nonexpressing event were excluded from the analysis, there was no significant relationship between Cry3Aa levels and insect mortality. However, when the clones were examined individually, it appeared that clone 24-305 had a weak and nominally significant relationship among the variables ($F_{14} = 4.91$, one-tailed $p = 0.022$, $r^2 = 27.4\%$); the three other clones showed no evidence of a relationship between the variables (Supplementary data Fig. 5).[1]

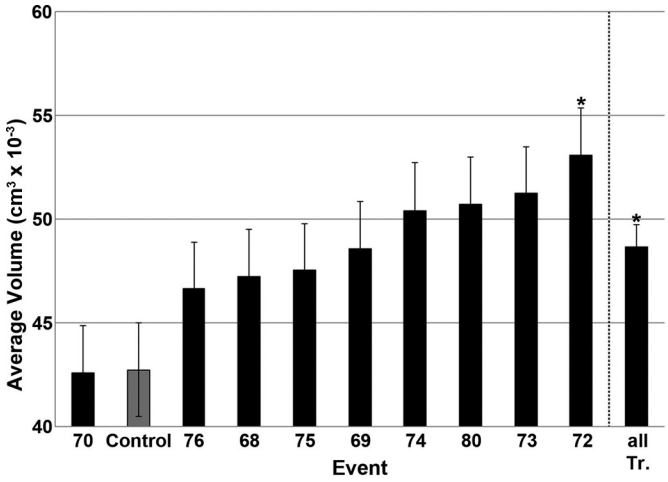
Large-scale field study

The nine transgenic events from clone OP-367 that were tested in the initial field trial were selected for further field evaluation (Table 1). Fifty control trees and 50 trees from each event were planted for this trial. A few trees died, resulting in a total of 419 transgenic and 47 control trees available for evaluation.

Large-scale field study: tree damage scores year 1

Trees were rated for whole-tree and worst-cluster damage during the first growing season. As in our initial screening trial, all trees had some degree of insect damage. All 419 of the transgenic trees had whole-tree damage scores of 1 (less than 5% damage; black bars, Fig. 3, upper panel), whereas the control trees received whole-tree scores of 2–4 (5%–50% damage; grey bars, Fig. 3, upper panel). With regard to leaf clusters with the worst damage (Fig. 3, lower panel), all of the transgenic trees had scores of 1 or 2, (less than 5% damage), whereas the control trees all scored 3 or above (greater than 5% damage), with 43% of the control trees scoring a 6 (70%–100% damage).

Fig. 4. Transgenic clone OP-367 trees grew well in a two year large-scale field trial. Average volume index of each event in the OP-367 clone after the second year of growth in the large-scale field study. The "all Tr." bar shows the average volume of all transgenic trees. Asterisks indicate significant differences between events and control trees ($p < 0.05$). Bars show standard error of the mean based on trees within an event.



Large-scale field study: net volume growth

As an index of stem volume growth, we compared the height × diameter^2 attained by each tree at the end of the second growing season. We found that our transgenic trees grew as well, or significantly better, than the control trees in this second large-scale field trial (Fig. 4). Compared with the controls, the transgenic trees grew in stem volume index an average of 13.9% larger after two growing seasons in the field, and the best growing event, No. 72, grew 24.2% larger. Analysis of the net growth of each event from season one to season two showed the same pattern as the total growth, with the transgenic trees having an average of 8% more net growth than the control trees.

Maintenance of Cry3Aa expression over time

Because trees are usually grown for many years prior to harvest, we were interested to determine if Cry3Aa expression would be maintained across growth cycles. Therefore, we tested for the presence of Cry3Aa in trees a subset of the same events as our large-scale field trial, but that were planted in a different location, and underwent eight growth cycles at that location with regular coppicing most years. For trees from these three events that were maintained in the field for 14 years, we found high levels of Cry3Aa still being produced in leaves in the 14th year. Concentrations of Cry3Aa were within the same range as our initial assay (see Supplementary data Fig. 4)[1] and ranged from 94.1 to 129.9 ng Cry3Aa / μg total leaf protein, whereas no Cry3Aa was detected in the control trees (Supplementary data Fig. 7).[1]

Discussion

Adoption of insect-resistant herbaceous crops, such as cotton and maize, has led to decreased insecticide use and crop losses (Qaim 2009); based on our results, it appears that similar benefits could accrue from transgenic trees. Unlike annual crop species,

trees are maintained in the field for many cycles of growth and, therefore, are at risk of repeated insect damage over their lifespan. If trees can be developed that are insect-resistant and grow well, they could be beneficial to commercial growers.

Two previous field studies of hybrid poplar in China, one with *cry1Ac* and the second a double transformant of *cry3Aa* and *oryzacystatin I*, reported a reduction in leaf damage and a decrease in insect infestation, showing that Bt proteins are effective under field conditions (Hu et al. 2001; Zhang et al. 2011). However, neither study evaluated tree growth. The Cry3Aa toxin used in our study is effective against *C. tremulae* and *C. scripta* under lab or greenhouse conditions (Genissel et al. 2003; Hjalten et al. 2012). However, the field performance of trees expressing the *cry3Aa* gene had not previously been reported. We produced several transgenic events in four different clones and evaluated these events in both the lab and the field. Our results showed insecticidal effects of Cry3Aa in every poplar clone tested.

Our initial screening data indicated that most transgenic trees performed similarly to the nontransgenic control trees (Supplementary data Fig. 3).[1] This finding was not surprising given the large environmental variation present during the initial year after establishment, and the small sample sizes in these trials. The main goal was to assess resistance to defoliation, which was found to be a characteristic of nearly all the transgenic trees in the trial (Fig. 2). Previous analysis of the wood chemistry of some of the same transgenic event trees showed no detectible differences in the composition of the control and transgenic trees (Davis et al. 2006). Therefore, it appears that expression of Cry3Aa was not detrimental to overall tree growth or wood formation. A few events, such as an event in clone 50-197 (Supplementary data Fig. 3, left-hand side),[1] grew poorly. This difference may be due to insertion-specific or somaclonal effects. When selecting trees for commercial deployment, poorly growing events such as this one would be readily identified and removed prior to large-scale planting. However, despite the presence of the poorly growing event in 50-197, on average the transgenic trees grew significantly faster than the controls in this clone.

We performed a second field trial to test events in clone OP-367, the clone of most commercial interest to east-side growers at the time of the study. This drought-tolerant clone is well-suited to the dry climate of eastern Oregon, a region of the state where its largest commercial poplar plantations are grown. In the large-scale field study, we found that transgenic events grew as well or better than control trees (Fig. 4). We saw less insect damage in this second field trial, as compared with the initial trial (compare Figs. 2 and 3, top panels). However, even with this modest natural insect challenge, the transgenic trees still grew better than the controls. It is likely that the growth benefits would be more substantial during years with higher insect pressure. In large-scale plantings in China, Cry1A-expressing trees provided substantial cross-protection for nontransgenic trees growing nearby (Hu et al. 2001), possibly by reducing the overall size of the local insect population. If this was also true for our *cry3Aa*-transformed trees, then the benefits of Cry3Aa expression for tree growth might be much larger than the estimates from this study.

During the screening trial, it was easy to classify every transgenic and control tree based on the extent of herbivory. The task was not so simple during the large-scale trial, as heavily damaged clusters of leaves were hard to locate on many trees. The reason for the lower feeding intensity in the large-scale trial is unclear, but it appears to have been associated with lower CLB populations in the vicinity at that time (P. Payne (Walla Walla Public Schools, Walla Walla, Washington) and L. Miller (Oregon Department of Forestry, Salem, Oregon), unpublished observations). It is also possible that the Bt trees had a suppressive effect on local beetle populations or perhaps repelled beetles from the plantation, as has been observed with Cry1 Bt maize (Hutchison et al. 2010) and Cry1 Bt poplar (Wang et al. 2004). In either case, as discussed previously, it is likely that the growth benefits of Bt gene expression in this study are underestimates.

If Cry3Aa-producing trees are to be utilized commercially, then it would be useful to screen for toxin levels prior to field testing. High levels of Cry3Aa expression would be desirable from a resistance-management perspective (Huang et al. 2011), and should be most effective against the target insect. A comparison of Cry3Aa expression versus whole-tree damage scores showed that events in all four clones had very little damage, even those with relatively low Cry3Aa expression (Supplementary data Fig. 6).[1] Thus, it appears that even moderate levels of Cry3Aa expression would be effective against targeted insect pests. We found that most of the observed variation in Cry3Aa levels was due to the transformation events themselves, as would be expected for the generation of independent transgenic events. The clonal background had a small but detectible effect, but events in all four clones had high amounts of Cry3Aa expression. We expect other transformed hybrid poplar clones, including those most commonly grown in commercial cultivation, would have similar expression levels. The Bt protein levels we observed (5.6%–13.3% of total soluble protein) were comparable in magnitude to those observed in other crops in which the Bt gene was under the control of a constitutive promoter, such as potato (up to 1.08%; Canedo et al. 1997–1998) and tobacco (up to 5%; McBride et al. 1995), but were higher than prior studies of transgenic poplars that showed Cry3Aa levels of up to 0.05% and 0.03% of total soluble protein (Genissel et al. 2003; Zhang et al. 2011). The MAR elements in our construct may have been at least partly responsible for high expression levels (Han et al. 1997); however, their effect seems to vary widely among genes and promoters (Li et al. 2013).

Because poplar is grown for many seasons, maintenance of transgene expression over growth cycles is essential. Our data show that Cry3Aa expression is stable over time in field-grown plants (Supplementary data Fig. 7).[1] Therefore, trees should maintain their insect-resistant phenotype. Yield gains would be compounded over multiple growth cycles under insect pressure, leading to a higher yield in the final harvested trees. Stable Cry3Aa expression may have additional benefits, such as decreasing the fluctuation in insect damage from year to year, leading to less variation in growth increment. Analysis of Bt corn revealed a lower degree of annual variation in crop yield, which was attributed to the resistance gene (Shi et al. 2013).

We have shown that the benefits from expression of a codon-optimized *cry3Aa* gene in poplar that were observed in greenhouse and controlled-feeding experiments (Genissel et al. 2003) can be extended to the field. Moreover, we have also shown that expression of Cry3Aa was stable for more than a decade and that the benefits of protecting against beetles were striking and stable over years in the field and in two different locations. Stability of gene expression in pest-resistant transgenic trees has long been a concern, but has been the subject of few studies (Ye et al. 2011). Presumably, the growth benefit resulting from Bt-gene expression is an underestimate. Cry3Aa targets several coleopteran insects and, thus, it will very likely reduce defoliation caused by other poplar beetle pests as well. It appears that, if used as part of an integrated pest-management system (James et al. 1998), the *cry3Aa* gene could provide important pest and yield protection in poplar.

Apart from modest commercial plantings in China (Ye et al. 2011), there has been an absence of the adoption of Bt technology for forest trees. There are several possible explanations, including market (Strauss et al. 2001; Costanza and McCord 2013) and regulatory obstacles (Strauss et al. 2010; Meilan et al. 2012; Haggman et al. 2013). These obstacles appear to reflect a general social and ethical unease about the use of transgenic crops and trees. Although it is reasonable to expect the introgression of Bt genes into wild poplars could have net environmental benefits (e.g., if trees are more resilient under climate-associated insect pressure), current regulations and market restrictions presume harm from the

gene-insertion process or that a departure from the "natural" is inherently undesirable. This broadly negative ethical embrace of the technology is likely to result from a broad array of factors (Ipsos 2013), all of which could change over time. Potential social changes that could result in greater acceptance include an increased sense that genetic engineering, in general, is an essential tool in the face of growing population, climate, and economic pressures on forests and crops; proven evidence of the ability of transgenic methods to provide direct ecological benefits in forestry, such as through forest restoration (Zimmer 2013); and proven evidence of tangible benefits that are identified through open scientific research as presented here. The strongly positive results we have documented suggest that additional ecological and mitigation research needed to responsibly use this technology — such as on the effectiveness and impact of sterility genes for containment (e.g., Brunner et al. 2007; DiFazio et al. 2012; and Zhang et al. 2012) — is a worthwhile investment to make.

Acknowledgements

We thank Manuela Huso of Oregon State University (OSU) for her statistical analysis of the bioassay, tree growth, and tree damage data, as well as her detailed review of the manuscript. We also thank Ariel Muldoon of OSU for her help with the statistical analysis of the ELISA and bioassay data; Mycogen Corporation (now Dow AgroSciences, Indianapolis, Indiana) for providing their *cry3Aa* plasmid; the industrial members of the Tree Biosafety and Genomics Research Cooperative at OSU for financial support; and Boise Cascade for providing the field site, including irrigation and management thereof. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

References

Adang, M.J., Brody, M.S., Cardineau, G., Eagan, N., Roush, R.T., Shewmaker, C.K., Jones, A., Oakes, J.V., and McBride, K.E. 1993. The reconstruction and expression of a *Bacillus thuringiensis cryIIIA* gene in protoplasts and potato plants. Plant Mol. Biol. **21**(6): 1131–1145. doi:10.1007/BF00023609. PMID:8490132.

Brunner, A.M., DiFazio, S.P., and Groover, A.T. 2007. Forest genomics grows up and branches out. New Phytologist. **174**: 710–713.

Canedo, V., Benavides, J., Golmirzaie, A., Cisneros, F., Ghislain, M., and Lagnaoui, A. 1997–1998. Assessing Bt-Transformed potatoes for potato tuber moth, *Phthorimaea operculella* (Zeller), management. CIP Program Rep. 161. Lima, Peru.

Costanza, A., and McCord, S. 2013. Regulation, certification, and use of biotech trees. Institute of Forest Biotechnology.

Coyle, D.R., McMillin, J.D., Krause, S.C., and Hart, E.R. 2000. Laboratory and field evaluations of two *Bacillus thuringiensis* formulations, Novodor and Raven, for control of cottonwood leaf beetle (Coleoptera: Chrysomelidae). J. Econ. Entomol. **93**(3): 713–720. doi:10.1603/0022-0493-93.3.713. PMID:10902320.

Coyle, D.R., McMillin, J.D., Hall, R.B., and Hart, E.R. 2003. Effects of cottonwood leaf beetle (Coleoptera: Chrysomelidae) larval defoliation, clone, and season on *Populus* foliar phagostimulants. Environ. Entomol. **32**: 452–462. doi:10.1603/0046-225X-32.3.452.

Davis, M.F., Tuskan, G.A., Payne, P., Tschaplinski, T.J., and Meilan, R. 2006. Assessment of *Populus* wood chemistry following the introduction of a Bt toxin gene. Tree Physiol. **26**(5): 557–564. doi:10.1093/treephys/26.5.557. PMID:16452069.

DiFazio, S.P., Leonardi, S., Slavov, G.T., Garman, S.L., Adams, W.T., and Strauss, S.H. 2012. Gene flow and simulation of transgene dispersal from hybrid poplar plantations. New Phytologist. **193**: 903–915.

Genissel, A., Leple, J.C., Millet, N., Augustin, S., Jouanin, L., and Pilate, G. 2003. High tolerance against *Chrysomela tremulae* of transgenic poplar plants expressing a synthetic *cry3Aa* gene from *Bacillus thuringiensis* ssp. *tenebrionis*. Mol. Breed. **11**(2): 103–110. doi:10.1023/A:1022453220496.

Häggman, H., Raybould, A., Borem, A., Fox, T., Handley, L., Hertzberg, M., Lu, M.Z., Macdonald, P., Oguchi, T., Pasquali, G., Pearson, L., Peter, G., Quemada, H., Séguin, A., Tattersall, K., Ulian, E., Walter, C., and McLean, M. 2013. Genetically engineered trees for plantation forests: key considerations for environmental risk assessment. Plant Biotechnol. J. **11**(7): 785–798. doi:10.1111/pbi.12100.

Han, K.H., Ma, C., and Strauss, S.H. 1997. Matrix attachment regions (MARs) enhance transformation frequency and transgene expression in poplar. Transgenic Res. **6**(6): 415–420. doi:10.1023/A:1018439518649.

Han, K.H., Meilan, R., Ma, C., and Strauss, S.H. 2000. An *Agrobacterium tumefaciens* transformation protocol effective on a variety of cottonwood hybrids (genus *Populus*). Plant Cell Rep. **19**(3): 315–320. doi:10.1007/s002990050019.

Hjalten, J., Axelsson, E.P., Whitham, T.G., LeRoy, C.J., Julkunen-Tiitto, R., Wennstrom, A., and Pilate, G. 2012. Increased resistance of Bt aspens to *Phratora vitellinae* (Coleoptera) leads to increased plant growth under experimental conditions. Plos One, **7**(1): e30640. doi:10.1371/journal.pone.0030640. PMID:22292004.

Holsters, M., Dewaele, D., Depicker, A., Messens, E., Vanmontagu, M., and Schell, J. 1978. Transfection and transformation of *Agrobacterium tumefaciens*. Mol. Gen. Genet. **163**(2): 181–187. doi:10.1007/BF00267408. PMID:355847.

Hu, J.J., Tian, Y.C., Han, Y.F., Li, L., and Zhang, B.E. 2001. Field evaluation of insect-resistant transgenic *Populus nigra* trees. Euphytica, **121**(2): 123–127. doi:10.1023/A:1012015709363.

Huang, F.N., Andow, D.A., and Buschman, L.L. 2011. Success of the high-dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entomol. Exp. Appl. **140**(1): 1–16. doi:10.1111/j.1570-7458.2011.01138.x.

Hutchison, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E., Hellmich, R.L., Kaster, L.V., Hunt, T.E., Wright, R.J., Pecinovsky, K., Rabaey, T.L., Flood, B.R., and Raun, E.S. 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science, **330**(6001): 222–225. doi:10.1126/science.1190242. PMID:20929774.

Ipsos Social Research Institute. 2013. Community attitudes towards emerging technology issues - biotechnology. Report prepared for the Department of Industry, Innovation, Science, Research and Tertiary Education. ISRI Project 12-025766-01. 1–135.

James, R.R., DiFazio, S.P., Brunner, A.M., and Strauss, S.H. 1998. Environmental effects of genetically engineered woody biomass crops. Biomass Bioenergy, **14**(4): 403–414. doi:10.1016/S0961-9534(97)10077-0.

James, R.R., Croft, B.A., and Strauss, S.H. 1999. Susceptibility of the cottonwood leaf beetle (Coleoptera: Chrysomelidae) to different strains and transgenic toxins of *Bacillus thuringiensis*. Environ. Entomol. **28**(1): 108–115.

Kleiner, K.W., Ellis, D.D., McCown, B.H., and Raffa, K.F. 1995. Field evaluation of transgenic poplar expressing a *Bacillus thuringiensis cry1a(a) d*-endotoxin gene against forest tent caterpillar (Lepidoptera: Lasiocampidae) and gypsy moth (Lepidoptera: Lymantriidae) following winter dormancy. Environ. Entomol. **24**(5): 1358–1364.

Kosola, K.R., Dickmann, D.I., Paul, E.A., and Parry, D. 2001. Repeated insect defoliation effects on growth, nitrogen acquisition, carbohydrates, and root demography of poplars. Oecologia, **129**(1): 65–74. doi:10.1007/s004420100694.

Li, Q., Dong, W., Wang, T., Liu, Z., Wang, F., Wang, X., Zhao, C., Zhang, J., and Wang, L. 2013. Effect of betaglobin MAR characteristic elements on transgene expression. Mol. Med. Rep. **7**(6): 1871–1874. doi:10.3892/mmr.2013.1424. PMID:23589234.

McBride, K.E., Svab, Z., Schaaf, D.J., Hogan, P.S., Stalker, D.M., and Maliga, P. 1995. Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Biotechnology (N.Y.), **13**(4): 362–365. doi:10.1038/nbt0495-362. PMID:9634777.

McCown, B.H., McCabe, D.E., Russell, D.R., Robison, D.J., Barton, K.A., and Raffa, K.F. 1991. Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Rep. **9**(10): 590–594. doi:10.1007/BF00232339.

Meilan, R., Ma, C., Cheng, S., Eaton, J., Miller, L.K., Crocket, R.P., DiFazio, S.P., and Strauss, S.H. 2000. High levels of Roundup and leaf-beetle resistance in genetically engineered hybrid cottonwoods. *In* Hybrid Poplars in the Pacific Northwest: Culture, Commerce and Capability. *Edited by* K.A. Blatner, J.D. Johnson, and D.M. Baumgartner. Washington State University, Pullman, Wash. Washington State UniversityCooperative Extension Bulletin MISC0272. pp. 29–38.

Meilan, R., Harfouche, A., and Nehra, N. 2012. Risk assessment of biotech trees: a path forward. J. For. **110**(3): 171–172.

Qaim, M. 2009. The economics of genetically modified crops. Annu. Rev. Resour. Econ. **1**: 665–693. doi:10.1146/annurev.resource.050708.144203.

Ramachandran, R., Raffa, K.F., Miller, M.J., Ellis, D.D., and McCown, B.H. 1993. Behavioral responses and sublethal effects of spruce budworm (Lepidoptera: Tortricidae) and fall webworm (Lepidoptera: Arctiidae) larvae to *Bacillus thuringiensis* Cry1A(a) toxin in diet. Environ. Entomol. **22**: 197–211.

Robinson, D.J., McCown, B.H., and Raffa, K.F. 1994. Responses of gypsy moth (Lepidoptera: Lymantriidae) and forest tent caterpillar (Lepidoptera: Lasiocampidae) to transgenic poplar, *Populus* spp., containing *a Bacillus thuringiensis d*-endotoxin gene. Environ. Entomol. **23**(4): 1030–1041.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., and Christou, P. 2011. *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnol. J. **9**(3): 283–300. doi:10.1111/j.1467-7652.2011.00595.x. PMID:21375687.

SAS Institute Inc. 2008. SAS component language 9.2: reference. SAS Institute Inc., Cary, N.C.

Sedjo, R. 2005. Will developing countries be the early adopters of genetically engineered forests? AgBioForum, **8**(4): 205–212.

Shi, G., Chavas, J.P., and Lauer, J. 2013. Commercialized transgenic traits, maize productivity and yield risk. Nat. Biotechnol. **31**(2): 111–114. doi:10.1038/nbt.2496. PMID:23392505.

Stanton, B., Johnson, J., and Neale, D.B. 2008. Genetic improvement of hybrid poplar for the renewable fuels industry: a Pacific Northwest perspective. *In* Proceedings of the Short Rotation Crops International Conference, Bloomington, Minnesota.

statgraphics. Statgraphics SIGMA express: Getting started. Available from http://www.statgraphics.com/statgraphics_express.htm.

Strauss, S.H., Campbell, M.M., Pryor, S.N., Coventry, P., and Burley, J. 2001. Plantation certification and genetic engineering: FSC's ban on research is counterproductive. J. For. **99**: 4–7.

Strauss, S.H., Kershen, D.L., Bouton, J.H., Redick, T.P., Tan, H.M., and Sedjo, R.A. 2010. Far-reaching deleterious impacts of regulations on research and environmental studies of recombinant DNA-modified perennial biofuel crops in the United States. BioScience, **60**(9): 729–741. doi:10.1525/bio.2010.60.9.10.

van Frankenhuyzen, K., and Beardmore, T. 2004. Current status and environmental impacts of transgenic forest trees. Can. J. For. Res. **34**(6): 1163–1180. doi:10.1139/x04-024.

Wang, L., Yifan, H., and Jianjun, H. 2004. Transgenic forest trees for insect resistance. *In* Molecular genetics and breeding of forest trees. *Edited by* S. Kumar and M. Fladung. Food Products Press, New York. pp. 243–262.

Wonnacott, T.H. and Wonnacott, R.J. 1981. Regression: A second course in statistics. John Wiley & Sons Inc., New York.

Ye, X., Busov, V., Zhao, N., Meilan, R., McDonnell, L.M., Coleman, H.D., Mansfield, S.D., Chen, F., Li, Y., and Cheng, Z.M. 2011. Transgenic *Populus* trees for forest products, bioenergy, and functional genomics. Crit. Rev. Plant Sci. **30**(5): 415–434. doi:10.1080/07352689.2011.605737.

Zhang, B.Y., Chen, M., Zhang, X.F., Luan, H.H., Diao, S., Tian, Y.C., and Su, X.H. 2011. Laboratory and field evaluation of the transgenic *Populus alba* × *Populus glandulosa* expressing double coleopteran-resistance genes. Tree Physiol. **31**(5): 567–573. doi:10.1093/treephys/tpr032. PMID:21561923.

Zhang, C., Norris-Caneda, K.H., Rottmann, W.H., Gulledge, J.E., Chang, S., Kwan, B.Y., Thomas, A.M., Mandel, L.C., Kothera, R.T., Victor, A.D., Pearson, L., and Hinchee, M.A. 2012. Control of pollen-mediated gene flow in transgenic trees. Plant Physiol. **159**: 1319–1334.

Zimmer, C. 2013. Resurrecting a forest. The Loom.