Stability of Herbicide Resistance over 8 Years of Coppice in Field-Grown, Genetically Engineered Poplars

Jingyi Li, Richard Meilan, Cathleen Ma, Michael Barish, and Steven H. Strauss

ABSTRACT

Herbicide resistance may be useful for reducing costs and environmental impacts, and improving yields, during weed control in poplar plantations. However, genetically engineered traits can sometimes show instability, which would compromise their commercial value. To study the stability of herbicide resistance, we analyzed resistance to the contact herbicide glufosinate in 384 transgenic plants originating from 32 gene-insertion events created within two *Populus* hybrids (*P. tremula* × *P. alba* and *P. tremula* × *P. tremuloides*). Resistance was measured at the start and end of an 8-year period during which trees were cut and allowed to resprout in the field several times. The transgenic events had widely varying levels of resistance, ranging from complete tolerance to nearly complete sensitivity. When analyzed as three classes (tolerant, intermediate, and sensitive), the levels of resistance remained stable, and there were no cases of resistance breakdown. The level of the resistance-conferring PAT protein, based on enzyme-linked immunosorbent assays, was strongly correlated with resistance class; thus, simple protein assays should enable early screening for acceptable resistance levels. Our data suggest that commercial levels of herbicide resistance and stability can be introduced into elite clones of hybrid poplar with as little as 2–3 years of transformation and field testing.

Keywords: *Populus*, genetic modification, biotechnology, transgene, glufosinate, gene silencing

A genetically engineered (GE) [1] organism is defined as one in which recombinant DNA methods were used to isolate, modify, and insert genes (Food and Agriculture Organization 2004). GE crops have had a major impact on agriculture in the last decade, occupying more than 100 million hectares in 22 different countries in 2006 (James 2007). Herbicide resistance (HR) has been a major trait adopted by US farmers. By 2005, HR soybeans accounted for 87% of total US soybean acreage, whereas HR cotton accounted for approximately 60% of total cotton acreage. The major benefits from HR have been reduction of weed-control costs; simplification of weed management, flexibility in timing of postemergence treatments; increased use of no- or low-till weed control and its attendant ecological benefits, such as carbon cycling and erosion control; and improvements in ecotoxicology of the herbicides applied (Fernandez-Cornejo and Caswell 2006). Many of these same benefits have been proposed for the use of HR genes in plantation-grown forest trees, especially those grown under short rotations (Strauss et al. 1997, Sedjo 2001).

The key ecological constraint to use of GE-HR, as well as for the several other GE traits under development (Campbell et al. 2003, Food and Agriculture Organization 2004, Frankenhuyzen and Beardmore 2004), is the propensity for long-distance spread of transgenes by pollen and seed when trees reach sexual maturity (DiFazio et al. 2004). The occurrence of off- or on-site HR "volunteers," can, for some types of resistance, create new weed management problems (Strauss et al. 1997, Meilan et al. 2002a). For this reason, it has been proposed that genes that cause very high levels of sterility be inserted along with HR transgenes, when spread of the latter would cause weed management, ecological, or legal conflicts (reviewed in Strauss et al. 1995). However, for this to be an acceptable mitigation option, the GE sterility trait must be highly efficient and highly stable over several years and under field conditions. Unfortunately, there have been many reports of trait instability associated with sexual reproduction during early stages of GE crop development in agriculture (reviewed in Brunner et al. 2007), raising concern about flowering-control transgenes as biosafety options. Compared to annual crops, much less is known about stability of GE traits in forest trees, particularly under vegetative propagation and field conditions, although results to date suggest that the extent of instability may be modest (reviewed in Hoenicka and Fladung 2006, Brunner et al. 2007).

Populus was the first GE tree produced (1986), and is by far the most commonly studied tree genus for genetic modification purposes today (Peña and Seguin 2001, Strauss et al. 2001, Brunner and Nilsson 2004). This is a result of its amenability to GE methods, clonal propagation, use in short-rotation-intensive culture, and suitability for research on gene and genome function (Brunner et al. 2004, Strauss and Martin 2004). The latter has recently been aided

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Figure 1. Schematic diagram of the T-DNA region of the plasmid vector pTTM8 used to transform the two hybrid poplar clones. OCSt, the 3′ untranslated region from the octopine synthase gene; NEO, neomycin phosphotransferase II; pNOS, the promoter from the nopaline synthase gene; pTA29, the promoter from tobacco anther-specific gene TA29; NOSt, the 3′ untranslated end of the nopaline synthase gene; pSSUARA-TP, the promoter from the *atS1A* ribulose-1,5-biphosphate carboxylase small subunit gene from *Arabidopsis thaliana*; G7t, the 3′ untranslated fragment from the TL-DNA gene 7; RB, right border; LB, left border. T-DNA regions are not drawn to scale.

by the publication of a high-quality genome sequence—only the third such sequence for any plant species (Tuskan et al. 2006). In spite of over 200 field trials of GE trees that have been reported in at least 35 countries, the only commercially deployed transgenic trees are insect-resistant *Populus nigra* and white poplar (clone 741) in China (Food and Agriculture Organization 2004), and virus-resistant papaya in Hawaii (Gonsalves and Ferreira 2003). Until the stability of GE traits, and means for reliable containment and social acceptance are established, further commercial use may be extremely limited in most parts of the world (Brunner et al. 2007).

Our goal was to study the stability of transgene expression in trees under field conditions relevant to its practical use and propagation. We selected herbicide tolerance because it is both a trait of commercial value, and because it also makes such studies highly efficient, as the effects of the expression of a single gene can be readily observed without expensive molecular analyses. We report very high levels of stability of resistance over 8 years, as well as a high degree of predictability of HR level based on a simple enzyme-linked immunosorbent assay (ELISA) for the transgene-encoded protein. These results suggest that high stability of this trait, and perhaps transgenic traits generally, may be achievable with a modest, short-term screening effort.

Materials and Methods

The plasmid vector pTTM8 was used to transform two hybrid poplar clones: INRA 353-38 (*P. tremula* × *P. tremuloides*) and 717-1B4 (*P. tremula* × *P. alba*). The transferred DNA (T-DNA) region contains two chimeric genes conferring resistance to the antibiotic kanamycin and the herbicide glufosinate-ammonium, respectively, and another chimeric *BARNASE* gene, which can impart male sterility (Figure 1). The herbicide-resistance gene was driven by the promoter of a strongly photosynthesis-associated gene, the small subunit of ribulose bisphosphate carboxylase/oxygenase, derived from *Arabidopsis*. The construct was transformed into a rifampicin-resistant derivative of *Agrobacterium* C58, under kanamycin selection. A total 19 independent transgenic events were produced for poplar clone 353-38, and 13 events were produced for clone 717-1B4. Twelve ramets for each of these 32 transgenic events, plus an equal number of nontransgenic control trees, were propagated and rooted in vitro, transplanted into soil in pots, and then planted in the field in Benton County, OR, in 1997. Two ramets of each transgenic event and control were randomly planted in each of six blocks.

Over-the-top herbicide sprays were applied in three different years (1997, 2004, and 2005). Most plants were coppiced between growing seasons to keep them short enough for spraying; however, some reached heights up to approximately 5 m before cutting. In 1997, herbicide was sprayed on four of the six plots shortly after planting. Two of these plots were sprayed with a 1× concentration (1.5 lbs · ha^{-1} glufosinate), whereas the other two plots were

Figure 2. Herbicide damage after herbicide application. Herbicide was sprayed with a hand applicator at a concentration of 3.0 lbs · ha^{-1} active ingredient (glufosinate). Leaves on the nontransgenic control plants quickly desiccated and browned, whereas leaves on the highly tolerant transgenic plants remained green and free of lesions (only shadows from bright sunlight are visible).

sprayed with a 2× concentration (3.0 lbs · ha^{-1}). In 2004 and 2005, all plots were treated with the 2× concentration. All treatments were applied using a backpack sprayer. In 1997 and 2005, the degree of herbicide resistance was scored approximately 4 weeks after herbicide application and categorized into three classes: 0, no resistance (heavy necrosis or dead plants); 0.5, intermediate resistance (moderate necrosis); 1, high resistance (no or very slight necrosis).

For protein (ELISA) analysis, the uppermost shoot tips of the plants from one of the six plots were taken in 2005, approximately 5 weeks after herbicide treatment in June and after all trees, even susceptible ones, had recovered and resumed growth. For protein extraction, approximately 50 mg of leaf tissue was ground in 400 μl of extraction buffer (50 mM $NaHPO_4$, pH 7.0; 10 mM EDTA) in a 1.5-ml microtube with a disposal pestle. The samples were centrifuged at 16,000*g* for 15 minutes at 4° C in a tabletop microcentrifuge. Approximately 200 μl of supernatant was transferred to a new 1.5-ml tube, frozen in liquid nitrogen, and stored at −80° C until it was assayed. The total protein concentration was measured by the

Bradford method (Protein Assay Kit, catalog no. 500-0001; Bio-Rad, Hercules, CA) using a microtiter plate reader (Molecular Devices, Sunnyvale, CA) following the instructions provided by the manufacturer. The relative concentration of the phosphinothricin acetyltransferase enzyme (PAT), encoded by the *BAR* gene, was quantified using the commercially available LibertyLink PAT/*bar* ELISA kit (catalog no. AP013; Envirologix, Inc., Portland, ME) following the manufacturer's instructions. Blanks and nontransgenic controls were also included in each assay plate. Duplicated wells were used for all tested samples. The optical density (OD) was read at a wavelength of 450 nm using a microtiter plate reader 20 minutes after the stop solution was added. The mean OD from the blank wells was subtracted from the samples and nontransgenic controls before data analysis.

Results and Discussion

Herbicide Resistance Levels and Stability over Time

A total of 384 transgenic poplar plants, derived from 32 independent transformation events, were grown in the field over a period of 8 years. In 1997, the herbicide treatment was applied approximately 1 month after the establishment of the plants in the field, and herbicide resistance was scored approximately 4 weeks after the herbicide application. We found that the mean responses for the two herbicide levels used were not significantly different from each other (0.7 and 0.69, respectively), and therefore, all resistance data were pooled for further expression analysis.

After averaging herbicide resistance scores over all ramets of each individual event, we redefined herbicide classes based on the distribution of the mean scores into what might be operational levels for breeding programs: no resistance (score = 0); intermediate, noncommercial resistance (score = 0.1–0.9); and high, potentially commercial resistance (score = 1) (Figure 3). All the treated, nontransgenic plants showed severe herbicide damage for both years, which indicates that both herbicide applications were sufficient to cause damage to any major sectors of transgenic plants in which gene silencing occurred (Figure 2). Of 32 transgenic events studied in 1997, 13% of the events were highly susceptible, 28% were highly resistant, and 59% were intermediate (Table 1). All the events that belonged to the highly susceptible and highly resistant groups in 1997 remained in the same groups in 2005. However, nearly 50% of the events in the intermediate group in 1997 showed high resistance in 2005. As a result, the herbicide resistance levels in 2005 appeared to be less continuous, and a majority of the events (59%) showed high resistance. The γ correlation coefficient of herbicide resistance between the two years was 0.58 and highly statistically significant ($P = 0.0003$). Events with intermediate resistance appeared to be more likely to be affected by the plant's physiological condition and the concentration and efficiency of herbicide application, which can vary widely between years. The lower mean resistance observed in 1997 was not surprising, as herbicide had been applied shortly after planting, when the plants were small and, thus, more completely treated with herbicide. In addition, they also might have had a less developed cuticle, possibly facilitating herbicide entry into cells.

Correlation of Visualized Herbicide Resistance and Measured Protein Levels

To determine the extent to which a simple measure of transgene expression could predict herbicide resistance, we performed sandwiched protein ELISA assays on 30 transgenic events and nontransgenic controls. Of the 30 events studied, 5 showed no herbicide resistance, 6 showed intermediate resistance, and the remaining events exhibited complete resistance. We used OD as an indication of relative expression level of the protein encoded by the *BAR* gene. For each event, the mean herbicide resistance and optical density over two ramets were used for statistical analysis.

The association between transgene expression and herbicide tolerance was strong. The relative protein concentration of events in the highly sensitive class was very low, close to that of nontransgenic controls, whereas those of the high resistance events showed much higher OD readings (Figure 4). The γ coefficient between herbicide

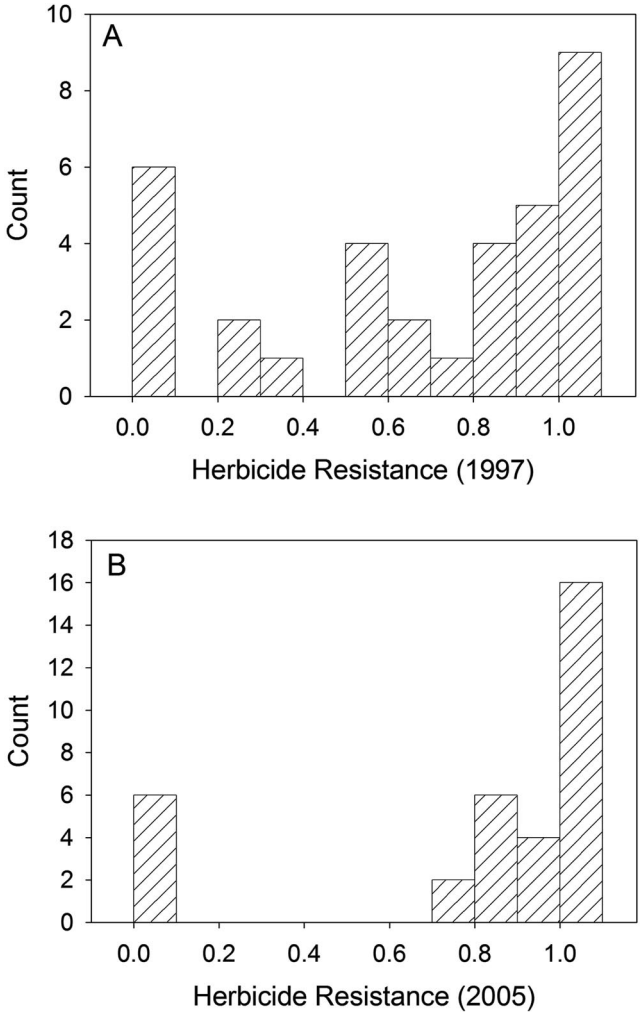

Figure 3. Distribution of mean herbicide resistance of 32 transgenic events in 1997 (A) and in 2005 (B). Herbicide resistance was scored using three classes: 0, no resistance; 0.5, intermediate resistance; 1.0, high resistance. Mean scores averaged over all ramets per event were used to create the histogram.

Table 1. Percentage of transgenic events in each of three herbicide resistance classes in 1997 and 2005.

Year	Herbicide resistance class		
	None (0)	Intermediate (0.1–0.9)	High (1.0)
1997	13%	59%	28%
2005	13%	28%	59%
Percent retention in 2005[a]	100%	47%	100%

[a] Percentage of events in each class that were originally categorized in 1997 and remained in the same class in 2005.

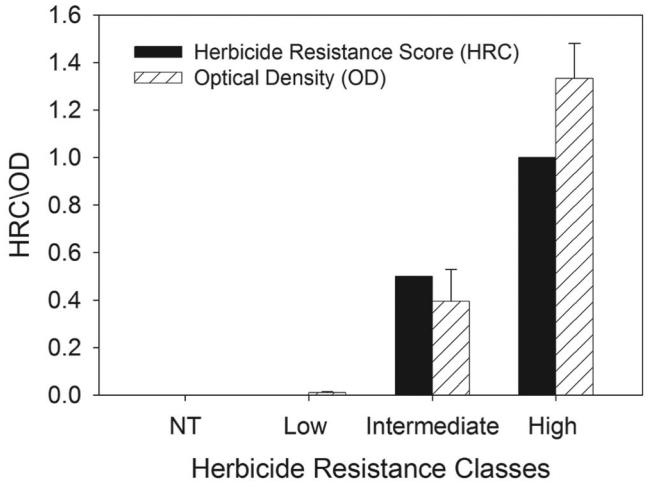

Figure 4. Association of herbicide resistance class with expressed protein level determined by ELISA. For each pair of bars, the left bar represents the mean score of herbicide resistance, and the right bar represents the mean OD reading. NT, nontransgenic controls.

Table 2. Prediction of herbicide resistance classes based on optical density (OD) percentiles. Data are percentage of events within an OD percentile for resistant classes.

	Herbicide resistance class		
OD percentile	None (0)	Intermediate (0.1–0.9)	High (1.0)
Lowest 10%	100%		
Lowest 20%	71%	29%	
Lowest 30%	56%	44%	
Lowest 40%	33%	53%	13%
Lowest 50%	31%	50%	19%
Highest 50%			100%

resistance and OD was 0.98 and highly statistically significant ($P <$ 0.0,001); thus, herbicide resistance could be predicted on the basis of measured OD (Table 2). All events in the lowest 10% of OD values were highly susceptible, and all events in the highest 50% of OD values were highly resistant. These results suggest that a simple measurement of expressed protein levels could be used in the greenhouse or during early field testing to identify the top-performing events, reducing field trial costs and/or increasing experimental precision.

Conclusions

In vegetatively propagated tree species, maintenance of transgene expression is required over many cycles of propagation and over many years during which biotic and abiotic stresses will vary widely. Although stability of expression has been studied extensively in annual, sexually propagated crops, there is little information regarding stability of expression for traits of economic importance in field-grown trees (Brunner et al. 2007). The 8 years of our study can easily exceed a commercial rotation for poplars grown as pulp or biomass crops. The repeated coppicing simulates the standard methods used for propagation or regeneration of planted or wild stands in many areas (Zobel 1992, Rackham 2001). In contrast to reports with annual crops, we found a very high degree of stability for herbicide resistance. All events from two different hybrid poplar clones, which involved three different parent species that showed high resistance during the 1st year and remained highly resistant in the 8th year. In addition, we did not observe any cases of gene silencing (i.e., trait breakdown resulting from loss of gene expression). In another study, using more than 2,000 transgenic poplar trees, HR was also highly stable over 3 years in the greenhouse and field (Li 2006). Based on these two studies and related work (e.g., Pilate et al. 1997, Meilan et al. 2000, Meilan et al. 2002b, Hawkins et al. 2003), it appears that transgene silencing in vegetatively propagated poplars is rare, if it occurs at all at levels relevant to commercial needs (reviewed in Brunner et al. 2007). For trees such as poplars, which are vegetatively propagated and grown under short rotations, and for traits such as herbicide resistance, which can be observed following a broad range of expression levels from a single transgene and for which there is continued positive selection via application of herbicide, instability of gene expression does not appear to be major concern for commercial deployment.

The high stability we found suggests that transgenic approaches to containment of sexual propagules hold substantial promise. There are many mechanisms for control of flowering using native and exogenous genes, which may be used singly or in combination (reviewed in Brunner et al. 2007). However, the factors that might trigger instability could vary widely, depending on the transgenic mechanisms involved. For example, the level of gene silencing of an endogenous *CAD* (cinnamyl alcohol dehydrogenase) gene was dependent on the expression level of the construct triggering this effect in *Pinus radiata* (Wagner et al. 2005), suggesting that a gene that requires a very high level of expression for imparting sterility—such as some forms of dominant negative proteins—might be more prone to instability. However, there was no evidence for instability among the most herbicide-resistant trees in this study, although they resulted from the most highly expressed *BAR* transgenes. RNA interference (RNAi) can be used to create loss of function phenotypes in floral genes, leading to sterility. Li (2006) found that RNAi suppression of a *BAR* transgene remained stable during seasonal development and 2 years in the field. In *Arabidopsis*, the high degree of RNAi-induced silencing of the endogenous Δ12-desaturase gene (*FAD2*) in one transgenic event was stable over five sexually propagated generations; no reversion or reduction of gene silencing was observed (Stoutjesdijk et al. 2002). Although those results are encouraging, before a high level of sterility can be assumed it will be important to verify stability of endogens suppression for a variety of genes, tissues, and environments.

Endnotes

[1] We use the terms transgenic and transformed to refer to GE organisms—produced using recombinant DNA and asexual gene transfer, regardless of the source of the transgene(s).

Literature Cited

BRUNNER, A.M., AND O. NILSSON. 2004. Revisiting tree maturation and floral initiation in the poplar functional genomics era. *New Phytol.* 164:43–51.

BRUNNER, A.M., V.B. BUSOV, AND S.H. STRAUSS. 2004. Poplar genome sequence: Functional genomics in an ecologically dominant plant species. *Trends Plant Sci.* 9:49–56.

BRUNNER, A.M., J. LI, S.P. DIFAZIO, O. SHEVCHENKO, B.E. MONTGOMERY, R. MOHAMED, H. WEI, C. MA, A. ELIAS, K.V. WORMER, AND S.H. STRAUSS. 2007. Genetic containment of forest plantations. *Tree Genetics Genomics*, 3:75–100.

CAMPBELL, M., A.M. BRUNNER, M.J. HELEN, AND S.H. STRAUSS. 2003. Forestry's fertile crescent: The application of biotechnology to forest trees. *Plant Biotechnol. J.* 1:141–154.

DIFAZIO, S.P., G.T. SLAVOV, B. JAROSLAW, L. STEFANO, AND S.H. STRAUSS. 2004. Gene flow from tree plantations and implications for transgenic risk assessment. P. 405–422 in *Plantation forest biotechnology for the 21st century,* Walter, C., and M. Carson (eds.). Research Signpost, Kerala, India.

FOOD AND AGRICULTURE ORGANIZATION. 2004. *Preliminary review of biotechnology in forestry, including genetic modification.* Forest Genetic Resources Working Paper FGR/59E. Forest Resources Development Service, Forest Resources Division, Rome, Italy. 118 pp.

FERNANDEZ-CORNEJO, J., AND M. CASWELL. 2006. The first decade of genetically engineering crops in the United States. USDA, Economic Research Service, Economic Information Bulletin no. 11.

FRANKENHUYZEN, K., AND T. BEARDMORE. 2004. Current status and environmental impact of transgenic forest trees. *Can. J. For. Res.* 34(6):1163–1180.

GONSALVES, D., AND S. FERREIRA. 2003. Transgenic papaya: A case for managing risks of *Papaya ringspot virus* in Hawaii. Available online at www.plantmanagementnetwork.org/pub/php/review/2003/papaya; last accessed Apr. 6, 2007.

HAWKINS, S., J. LEPLÉ, D. CORNU, L. JOUANIN, AND G. PILATE. 2003. Stability of transgene expression in poplar: A model forest tree species. *Ann. For. Sci.* 60:427–438.

HOENICKA, H., AND M. FLADUNG. 2006. Genome instability in woody plants derived from genetic engineering. P. 301–321 in *Tree transgenesis: Recent developments*, Fladung, M., and D. Ewald (eds.). Springer, Berlin.

JAMES, C. 2007. *Global status of commercialized biotech/GM crops: 2006.* Available online at www.isaaa.org/resources/publications/briefs/35/executivesummary/default.html; last accessed Mar. 6, 2007.

LI, J. 2006. *Stability of reporter gene expression and RNAi in transgenic poplars over multiple years in the field under vegetative propagation.* PhD dissertation, Oregon State Univ., Corvallis, OR. 212 p. Available online at www.fsl.orst.edu/tgerc/strauss/LiJ_thesis.pdf; last accessed Mar. 4, 2007.

MEILAN, R., C. MA, S. CHENG, J.A. EATON, L.K. MILLER, R.P. CROCKETT, S.P. DIFAZIO, AND S.H. STRAUSS. 2000. High levels of Roundup and leaf-beetle resistance in genetically engineered hybrid cottonwoods. P. 29–38 in *Hybrid poplars in the Pacific Northwest: Culture, commerce and capability*, Blatner, K.A., J.D. Johnson, and D.M. Baumgartner (eds.). Washington State University Cooperative Extension Bulletin MISC0272, Pullman, WA. 220 pp.

MEILAN, R., K.-H. HAN, C. MA, S.P. DIFAZIO, J.A. EATON, E.A. HOIEN, B.J. STANTON, R.P. CROCKETT, M.L. TAYLOR, R.R. JAMES, J.S. SKINNER, L. JOUANIN, G. PILATE, AND S.H. STRAUSS. 2002a. The *CP4* transgene provides high levels of tolerance to Roundup herbicide in field-grown hybrid poplars. *Can. J. For. Res.* 32(6):967–976.

MEILAN, R., D.J. AUERBACH, C. MA, S.P. DIFAZIO, AND S.H. STRAUSS. 2002b. Stability of herbicide resistance and GUS expression in transgenic hybrid poplars (*Populus* sp.) during several years of field trials and vegetative propagation. *HortScience* 37(2):277–280.

PEÑA, L., AND A. SEGUIN. 2001. Recent advances in the genetic transformation of trees. *Trends Biotechnol.* 19:500–506.

PILATE, G., D. ELLIS, AND S. HAWKINS. 1997. Transgene expression in field-grown poplar. P. 84–89 in *Micropropagation, genetic engineering, and molecular biology of* Populus*,* Klopfstein, N.B., Y.W. Chun, M.S. Kim, and M.R. Ahuia (eds.). USDA For. Serv. Gen. Tech. Rep. RM-GRT-297. 326 pp.

RACKHAM, O. 2001. *Trees and woodland in the British landscape: The complete history of Britain's trees, woods, and hedgerows.* Phoenix Press, London. 254 pp.

SEDJO, R. 2001. *Biotechnology's potential contribution to global wood supply and forest conservation.* Resources for the Future, Washington, DC.

STOUTJESDIJK, P.A., S.P. SINGH, Q. LIU, C.J. HURLSTONE, P.A. WATERHOUSE, AND A.G. GREEN. 2002. hpRNA-mediated targeting of the Arabidopsis FAD2 gene gives highly efficient and stable silencing. *Plant Physiol.* 129:1723–1731.

STRAUSS, S.H., W.H. ROTTMANN, A.M. BRUNNER, AND L.A. SHEPPARD. 1995. Genetic engineering of reproductive sterility in forest trees. *Mol. Breed.* 1:5–26.

STRAUSS, S.H., S.A. KNOWE, AND J. JENKINS. 1997. Benefits and risk of transgenic, Roundup Ready cottonwoods. *J. For.* 95(5):12–19.

STRAUSS, S.H., S.P. DIFAZIO, AND R. MEILAN. 2001. Genetically modified poplars in context. *For. Chron.* 77(2):271–279.

STRAUSS, S.H., AND F.M. MARTIN. 2004. Poplar genomics comes of age. *New Phytol.* 164:1–4.

TUSKAN, G.A., S. DIFAZIO, S. JANSSON, J. BOHLMANN, I. GRIGORIEV, U. HELLSTEN, N. PUTNAM, S. RALPH, S. ROMBAUTS, A. SALAMOV, J. SCHEIN, L. STERCK, A. AERTS, R.R. BHALERAO, R.P. BHALERAO, D. BLAUDEZ, W. BOERJAN, A. BRUN, A. BRUNNER, V. BUSOV, M. CAMPBELL, J. CARLSON, M. CHALOT, J. CHAPMAN, G.L. CHEN, D. COOPER, P.M. COUTINHO, J. COUTURIER, S. COVERT, Q. CRONK, R. CUNNINGHAM, J. DAVIS, S. DEGROEVE, A. DEJARDIN, C. DEPAMPHILIS, J. DETTER, B. DIRKS, I. DUBCHAK, S. DUPLESSIS, J. EHLTING, B. ELLIS, K. GENDLER, D. GOODSTEIN, M. GRIBSKOV, J. GRIMWOOD, A. GROOVER, L. GUNTER, B. HAMBERGER, B. HEINZE, Y. HELARIUTTA, B. HENRISSAT, D. HOLLIGAN, R. HOLT, W. HUANG, N. ISLAM-FARIDI, S. JONES, M. JONES-RHOADES, R. JORGENSEN, C. JOSHI, J. KANGASJARVI, J. KARLSSON, C. KELLEHER, R. KIRKPATRICK, M. KIRST, A. KOHLER, U. KALLURI, F. LARIMER, J. LEEBENS-MACK, J.C. LEPLE, P. LOCASCIO, Y. LOU, S. LUCAS, F. MARTIN, B. MONTANINI, C. NAPOLI, D.R. NELSON, C. NELSON, K. NIEMINEN, O. NILSSON, V. PEREDA, G. PETER, R. PHILIPPE, G. PILATE, A. POLIAKOV, J. RAZUMOVSKAYA, P. RICHARDSON, C. RINALDI, K. RITLAND, P. ROUZE, D. RYABOY, J. SCHMUTZ, J. SCHRADER, B. SEGERMAN, H. SHIN, A. SIDDIQUI, F. STERKY, A. TERRY, C.J. TSAI, E. UBERBACHER, P. UNNEBERG, J. VAHALA, K. WALL, S. WESSLER, G. YANG, T. YIN, C. DOUGLAS, M. MARRA, G. SANDBERG, Y. VAN DE PEER, AND D. ROKHSAR. 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313:1596–1604.

WAGNER, A., L. PHILLIPS, R.D. NARAYAN, J.M. MOODY, AND B. GEDDES. 2005. Gene silencing studies in the gymnosperm species *Pinus radiata. Plant Cell Rep.* 24:95–102.

ZOBEL, B. 1992. Vegetative propagation in production forestry. *J. For.* 90:29–33.