Ten lessons from 15 years of transgenic *Populus* research

STEVEN H. STRAUSS*, AMY M. BRUNNER, VICTOR B. BUSOV[1], CAIPING MA AND RICHARD MEILAN[2]

Department of Forest Science, Oregon State University, Corvallis, OR 97331-5752, USA
[1] Present address: School of Forest Research and Environmental Science, Michigan Technological University, Houghton, MI 49931, USA
[2] Present address: Department of Forestry and Natural Resources, 715 West State Street, West Lafayette, IN 47907-2061, USA
* Corresponding author. E-mail: steve.strauss@oregonstate.edu

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Summary

Based on extensive experience with transgenic poplars in laboratory and field environments, we have found that transformation is an extremely useful tool for research in biotechnology and functional genomics. The key lessons from our experience are: (1) stable gene expression is the rule in vegetatively propagated transgenic poplars; (2) somaclonal variation is modest and manageable; (3) transformation and field tests are extraordinary functional genomics methods; (4) there are many social and technical motivations for transformation centres; (5) regulations may choke biotechnology without scientist involvement; (6) the value of transgenic traits look high, but await careful, broad evaluation; (7) public-sector scientists need to play a serious, free role in value studies; (8) gene flow is complex and needs careful consideration; (9) sterility systems can be developed via diverse means; and (10) domestication transgenes can provide new avenues to promote biosafety. In short, transformation in poplar is extremely reliable and there are diverse and promising means for improving biosafety, but considerable time, institutional commitments and public–private partnerships are required to deliver them to society.

Our system and perspective

The goal of this paper is to describe the diverse kinds of research and interactions with the private and public sectors we have had while creating and field-testing many different kinds of transgenic poplars (genus *Populus*, aspens and cottonwoods) over the last ~15 years. [We use 'transgenic' to refer to any trees produced using asexual gene transfer, regardless of the origin of the genes.] Our laboratory has generated more than 6500 independent gene-transfer events in 17 different genotypes of *Populus*, and field-tested more than 1600 of these lines. This has given us extensive experience with how transformation affects the behaviour of trees grown on a moderately large scale. It has also given us a different perspective about the reliability of transformation than seems to be common in most academic laboratories that study transgenic plants for basic research purposes.

We have benefited from excellent growing conditions for poplars in the Pacific Northwestern USA, where trees grow in the order of 3 m in height

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per year under natural or fertigated (fertilized during irrigation) conditions (Figure 1). In 1–3 years, we produce transgenic trees of a size that foresters will pay attention to. Although the large majority of funding in our laboratories has been from public sector competitive grants, we have also benefited from private-sector support through an academic–private–government consortium formerly called the Tree Genetic Engineering Research Cooperative (TGERC; now named the Tree Biosafety and Genomics Research Cooperative, TBGRC; http://wwwdata.forestry.oregonstate.edu/tgbb/). The focus of the consortium has been on genetic engineering methods to mitigate gene flow to address environmental concerns over the deployment of transgenic trees, using poplar as a model taxon almost exclusively. Despite this private sector support, our research projects and publications are under the control of Oregon State University, and none of the views we express below have been reviewed, edited, or directly influenced by any private sector companies. Moreover, none of the authors of this paper have a financial conflict of interest with respect to commercialization of transgenic poplars we have produced or studied.

Although 'biosafety traits' (e.g. modified flowering and stature) have been our area of emphasis, we have also engaged in several field studies of potential commercial traits, especially herbicide tolerance and insect resistance (Meilan *et al.*, 2000), and conducted basic research on poplar functional genomics using transformation as a tool (e.g. Busov *et al.*, 2003; Groover *et al.*,

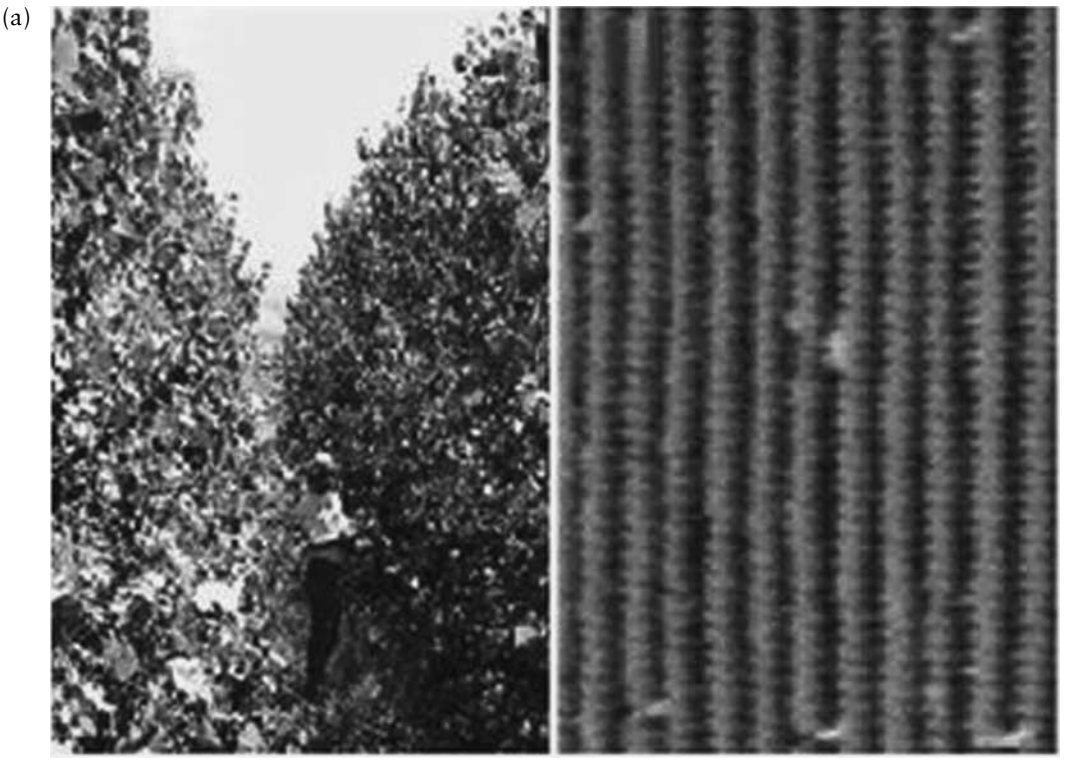

Figure 1. Field trial of transgenic poplars (*Populus deltoides* × *P. nigra*) in their second growing season. Several ramets of nine independent transgenic lines, and one non-transgenic control line of the same parent clone, are planted randomly within alternating rows; all of the trees in alternate rows contain only the non-transgenic line. (a) View between adjacent rows (the man is 1.8 m in height). (b) Infrared aerial photograph of a section of the plantation, showing uniformity. Reprinted from *Trends in Plant Science*, Vol. 9, Brunner *et al.*, 'Poplar genome sequence . . .', pp. 49–56, ©2004, with permission from Elsevier.

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2004). The transformability, clonability, rapid growth, small genome, and extensive genomic resources, including a publicly available genome sequence (reviewed in Brunner *et al*., 2004), make poplar an extraordinary system in which to investigate the potential for transgenic biotechnology, and to make real contributions to tree domestication (Bradshaw and Strauss, 2000; Strauss and Brunner, 2004).

We are motivated by a strong ethical imperative to move forward with genetic engineering (GE) research based on the expectations that: (1) we can help to improve productivity and economic value of plantations for social benefit; (2) increased productivity in established plantations provides society with the option of protecting and restoring more land for environmental purposes; (3) new methods for improving biosafety of transgenic trees, the focus of our work, can help to alleviate current environmental problems, including the spread of exotic and highly bred organisms into the wild; (4) many products of GE, such as trees with modified wood, improved bioremediation activity, and resistance to exotic pests, can provide major and direct environmental benefits; and (5) by connecting genes to traits via genomics and biotechnology, we are advancing the science of breeding, ultimately allowing trees to be bred in a more directed, predictable and safe manner.

We comment on what we see as the 10 most important lessons about transformation, biosafety, and associated issues regarding commercial deployment. Although strongly influenced by the transformability and other facilitating aspects of poplar biotechnology research, similar results concerning the performance of transgenic trees has been observed in other tree species, including conifers (Pena and Seguin, 2001; Meilan *et al*., 2004b).

Ten lessons

Transformation is powerful and reliable

(1) *Stable gene expression is the rule*

Perhaps because most transgenic crops and model organisms are sexually propagated, and because scientists focus on exceptions to study mechanisms, there appear to have been many more papers reporting unstable transgene expression or gene silencing compared with those reporting stable transgene expression (e.g. Finnegan and McElroy, 1994; Pawlowski *et al*., 1998). Because of their high heterozygosity and the commercial preference for well-characterized, and often heterotic, clones, it is stability of vegetative propagules that is most relevant to deployment of transgenic poplars. Likewise, in contrast to studies of sexually propagated crops, we are aware of no reports of associations between transgene structure or copy number and gene silencing under vegetative propagation, even in well-studied taxa such as potato (D. Duncan, Monsanto, personal communication) and poplar (Meilan *et al*., 2004b). Instability of gene expression has been reported in studies of transgenic poplar that employ transgenes whose variation in expression is dramatically amplified by its effects on plant development (e.g. *rol*B; Kumar and Fladung, 2001). However, there is as yet no evidence that expression of transgenes under vegetative propagation is more variable than expression of most endogenes. Normal variation in expression might be dramatically amplified by linkage to potent growth regulatory protein.

We have studied stability of gene expression extensively in transgenic poplars using easily visualized marker genes (β-glucuronidase (GUS), herbicide tolerance, and/or insect resistance) and, in some cases, native poplar genes (Rottman *et al*., 2000). In more than 1000 independent events that we produced containing one or more of these genes we have yet to see a single case of obvious post-regeneration gene silencing despite many rounds of vegetative propagation, annual cycles of growth, and diverse environments to which they have been subjected (Meilan *et al*., 2002, 2004b). Similar results have been reported for other field studies of transgenic poplars that used a similar transformation method and included some of the same genotypes that we often employ (Pilate *et al*., 1997). Finally, most academic studies do not make use of a large number of gene transfer events, nor high intensities of selection, both of which are common in breeding programmes; over 95 per cent of events are typically discarded early in the screening process in molecular breeding. Although few data on

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stability are available for a biosafety trait such as sterility, we expect that once key target genes are identified, effective transgenes based on them are configured, and the most desirable events selected, they should impart highly stable sterility. The quantitative adequacy of sterility will, of course, vary widely depending on the transgene concerned, its environmental benefits and risks, and the extent of wild or non-GE flowering plantations that buffer transgene spread (DiFazio *et al*., 2004; Slavov *et al*., 2004). For example, for a lignin modification gene that is expected to have a negative or neutral effect on fitness of wild trees, and if grown where wild populations are common, even a very modest level of sterility should be adequate (i.e. if sterility is required at all).

(2) *Somaclonal variation is modest and manageable*

The large majority of poplars that we produced have shown normal growth and form. We have observed obvious morphological abnormalities that were not induced by transgene expression in only three events (~0.06 per cent) of the thousands we have studied. Interestingly, in all cases the aberrant phenotypes were not observed until after trees had gone through dormancy in the field. However, when they occurred, they affected all ramets of the transgenic events, showing that it was a transgenic event-associated phenomenon. A dwarf phenotype of a hybrid cottonwood seen after vegetative propagation and release from dormancy in all propagated ramets is shown in Figure 2. These results suggest that accelerated dormancy cycles may be an effective means to rapidly screen out aberrant events. Other laboratories appear to have observed higher levels of somaclonal variation than we have (Wang *et al*., 1996; Kumar and Fladung, 2001). However, none appear to be so high as to pose a significant constraint on commercial programmes (where events undergo intensive selection) or on functional genomics studies (where several events are studied for each experimental treatment).

(3) *Transformation and field tests are extraordinary functional genomics methods*

Given the health of transgenic trees and stability of imparted traits, it is no surprise that transformation can be used to study gene function with high efficiency. This capability has been employed both in reverse genetics, where specific genes are chosen based on sequence and then mutants generated by altering expression of those genes, and in forward genetics, where mutants are first generated and the affected genes then identified. In gene-tagging and promoter/gene-trap methods, phenotypes are generated in a

Figure 2. Dwarf somaclonal variant first observed after release from dormancy in a field trial of fertigated transgenic poplars. The photograph was taken near the end of their first growing season after being planted as 40-cm dormant 'sticks.' A normal transgenic tree and 1.8-m man is in the background.

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manner that greatly facilitates isolation of the underlying genes. For example, we used activation tagging, where genes are identified when a strong enhancer amplifies their expression in a dominant manner, to produce a large number of tagged mutants in poplar (Busov *et al*., 2003). In this study, we found that screening for mutants in the field during one complete growing season resulted in a six-fold increase in detection frequency compared with the number observed in the laboratory. We believe that this was due to an accumulation of genotype (event) × environment interactions over time. Forty-two putatively tagged mutants were found in a field-grown population of 627 transgenic events after 1.5 years of growth.

(4) *There are many social and technical motivations for transformation centres*

We have been fortunate to have had long-term industrial, academic and grant support for our transformation centre TGERC/TBGRC. This has enabled us to manage several problems that often confound academic laboratories or small companies operating transformation programmes in long-lived species. There are many steps in transgenic research, including gene identification and modification, transformation, *in vitro* and *ex vitro* propagation, multiple-year field testing, and analysis of transgene expression, which require a diversity of personnel with diverse skills. Because transgenic organisms are regulated by government agencies, to avoid legal penalties they must be created, transported, monitored and devitalized in specific ways, regardless of the level of biological risk the transgenes may pose. As a result of the controversies surrounding biotechnology, security considerations are important both in the laboratory and the field. We have security systems in place to ensure that the location of plots is not widely disseminated and that unknown personnel cannot enter our laboratory wing after working hours. These measures are a result of vandalism committed against our transgenic field plots, and arson attacks against some of our collaborators (Service, 2001). Finally, an organized centre can readily offer services to others for specific genes or constructs of interest, as we have done on numerous occasions for academic collaborators.

Scientists need to be active in regulation and public acceptance

(5) *Regulations may choke biotechnology without scientist involvement*

The public debates over crop biotechnology have been far more acrimonious and protracted than even the most pessimistic scientists expected when transgenic plant research began in the mid-1980s. As a consequence, regulation of biotechnology continues to be a dynamic process. Nowhere in the world does a stable, satisfactory system appear to exist. Even in the USA, with its more than 60 government-authorized transgenic varieties, the core system at USDA–APHIS is now undergoing a complete re-evaluation. The nature of regulation that ensues, its costs and its stringency, will dictate both the kind of research and the kind of commercial products that will be possible. Many NGOs (non-governmental organizations) that are against biotechnology seem intent on regulating all GMOs stringently, despite strong and long-standing scientific consensus that it is 'product not process' that determines benefit and safety (references in Strauss, 2003). Scientists working in transgenic crop research, particularly those knowledgeable about plantation forestry, ecology and biosafety, need to be actively involved in advising governments and informing the public as these regulations evolve. If they do not, the costs of compliance may effectively preclude field research and, thus, release of commercial products. This appears to be the case today for transgenic tree field research in most of Europe. Trees also raise some special issues, due to their propensity for long-distance movement of pollen and seeds. This elevates concern about 'genetic pollution' from non-transgenic crops or unintended spread into wild or feral populations. Paradoxically, the very biosafety research called for to better manage GE trees, such as field-testing the efficacy of containment/sterility systems (Ellstrand, 2003), may pose the greatest risk of regulatory restriction. If, as in Europe, regulations are enacted that treat all transgene spread as hazardous or undesirable – with little regard for the benefits and safety considerations of specific genes, crops and environments – research with transgenic trees will be extremely difficult.

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(6) *Value of transgenic traits look high, but await careful, broad evaluation*

Benefit evaluations for agricultural biotechnologies were originally focused on company and farmer profits, rather than on a broader social/environmental context. Thus, when society has asked what the benefits really are, compared with the risks, there was limited credible data on which to make decisions. This has changed in recent years, with a growing focus on environmental and health benefits (e.g. Qaim and Zilberman, 2003; Toenniessen *et al*., 2003). Because of the long time frames, and the diverse germplasm, environments and companies involved with plantation forestry, obtaining high quality data will require complex public–private partnerships, and the participation of interdisciplinary teams, over long time intervals. This will look very much like traditional tree breeding and silviculture research, rather than the development of Roundup-Ready® soybeans. Scientists engaged in biotechnology, however, can be the catalysts for such studies. The limited work of this kind that has been done suggests that the economic and environmental benefits for both quality and agronomic transgenic traits can be very substantial (Pilate *et al*., 2002; Meilan *et al*., 2000); however, these studies have been short term and have not considered the full diversity of transgenes, environments, genotypes and environmental considerations that are desirable.

(7) *Public-sector scientists need to play a serious, free role*

The 'Monsanto model' exemplified the first phase of biotechnology development, where the private sector evaluated benefits for transgenic varieties internally, or dictated the terms for evaluation. Moreover, the focus has been on benefits for the company and farmer, rather than broad social and environmental values. Thus, it has been easy to demonize corporate, patent-dominated biotechnology as solely profit-driven. For this to change, much broader public sector participation is needed, both from a technical and ethical viewpoint. As the Nuffield Foundation has shown best, there are strong ethical cases to be made for crop biotechnology (http://www.nuffieldbioethics.org/gmcrops/index.asp); however, for reasons of credibility, corporations should not be the ones doing it. Because the intellectual property constraints on transgenic products are being relaxed as patents expire, there may be increased public-sector release of regionally valuable transgenic varieties. This would also help to reduce the perception that biotechnology products only benefit corporations, and could allow many low-risk tree products to be released that would help to inform the public about the long-term efficacy of biosafety traits (e.g. sterility and dwarfism).

(8) *Gene flow is complex and needs careful consideration*

Poplars and trees present great advantages and difficulties when considering transgene dispersal into the environment, either via seeds, pollen or asexual propagation. The key advantage is that, with the limited planting of transgenics contemplated for the foreseeable future, gene flow from wild and planted non-transgenic trees are certain to astronomically swamp levels of gene flow from plantations, especially if low fertility hybrids or incompletely sterile transgenics are employed (discussed in DiFazio *et al*., 2004). However, poplars also show the ability for vegetative propagation. In nature, this is likely to mainly affect local spread; however, it also facilitates legal and illegal movement by humans. Poplars also have a high propensity for long-distance pollen movement by wind, and seed can be spread via wind and water. This means that unless there is complete sterility, some level of very long-distance migration is likely – this can raise social or legal concerns even if near-astronomical dilution renders it of extremely low biological consequence. Spread is also constrained by a number of very complex factors, including habitat suitability (poplars generally require highly disturbed, moist sites that are free of plant competition), frequency of disturbance, rotation age relative to onset of flowering, and fitness benefit/detriment from transgenes. DiFazio *et al*. (2004) have developed a simulation model called STEVE (http://www.fsl.orst.edu/tgerc/dif_thesis/difaz_thesis.pdf) which they used to estimate levels of gene flow from transgenic poplar plantations over a 50–100-year period. However, far less sophisticated methods could be used to develop order-of-magnitude gene flow estimates based on relative areas, ages and proximities of transgenic to non-transgenic tree populations.

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These are likely to be critical for complying with regulations regarding adventitious (unintended) presence that are common throughout the world.

Biosafety technology is promising but slow

(9) *Sterility systems can be developed via diverse means*

For transgenes whose uncontrolled dispersal could create management problems, violate management conditions mandated by certification systems (Strauss *et al*., 2001a) or pose ecological uncertainties that are hard to quantify, gene containment via engineered sterility has long been an important goal (Strauss *et al*., 1995). This confinement is likely to be essential for genes such as those used for pest/herbicide resistance and bioremediation, especially when the genes employed are of an exotic origin and not required to meet a pressing environmental or social problem. However, social considerations might dictate that containment be required for all transgenes, including those that would not increase fitness, such as wood-modification genes (Doering, 2004). If reproductive tissues are completely destroyed, mitigation measures such as interplanting with fully fertile trees may be needed to reduce undesirable impacts on fauna that are dependent on flowers and fruit (Johnson and Kirby, 2004). For transgenes that could sustainably promote fitness in interfertile wild-tree populations, long-term demonstrations of complete, stable sterility (if this is achievable) might be required before any commercial use is allowable or advisable. Alternatively, such transgenics might be banned entirely, either regionally, nationally or globally. We do not believe that single *Bacillus thuringiensis* toxin genes should necessarily require absolute confinement; insects are expected to readily evolve to overcome their effects, greatly diminishing their selective value and spread, over modest evolutionary time frames (Strauss *et al*., 2001b). The final conclusion about deployment of such genes will rest on the impact of the target pest, the environmental risks and costs of alternative control measures, including chemicals and irreversibly released biocontrol organisms, and the costs of doing nothing at all. For the foreseeable future, sterility systems are likely to need redundancy to ensure a high level of confinement, thus research on diverse mechanisms is desirable. We have focused on floral homeotic and meristem-identity genes from poplar, homologues to the *Arabidopsis* genes *AGAMOUS*, *APETALA1*, *APETALA3* and *LEAFY* (reviewed in Zik and Irish, 2003), as tools. We are studying several ways to use these genes to induce sterility, including their suppression (singly and together via RNA interference), directing a cell toxin to destroy floral tissues (ablation), and production of mutant protein forms that interfere with normal protein function (dominant negative mutants, DNMs). These studies are in various stages, ranging from identification of novel DNMs in *Arabidopsis* to evaluation of transgenic trees in the field. We are also studying DNA elements such as matrix attachment regions (Han *et al*., 1997) as potential means to increase reliability of transgene function over multiple-year time frames. The long delay until flowering in poplars (3–6 years in our region) and the difficulties of measuring sterility in large flowering trees, pose considerable logistical hurdles to the speed and accuracy of this work. We have developed the means for transformation and flower induction in one female poplar clone (*P. alba*, clone 6K10; Meilan *et al*., 2004a), with which we hope to screen constructs and, thus reduce the size of field trials. Efforts to induce the production of precocious, fertile flowers and seed via transgenes have thus far proven unsuccessful (Rottmann *et al*., 2000; Table 1). Finally, the need for isolation of trees to reduce spread of transgenic pollen and seeds during field testing can impose significant difficulties, depending on regulatory requirements. We have a permit to test transgenic aspen (section *Leuce*) and relatives in a region dominated by sexually incompatible cottonwoods (section *Tacamahaca*). Our consortium, grants for discovery of new genes, and a compatible regulatory environment in the USA have been essential for developing sterility technology in poplar.

(10) *Domestication of transgenes can provide new avenues to promote biosafety*

Sterility is not the only means to promote biosafety when transgene flow presents a risk. If trees were modified sufficiently so that the chances for successful spread in the wild was low, the dispersal of sexual as well as vegetative

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Table 1: Flowering genes tested in regenerated transgenic poplar whose over-expression had accelerated onset of flowering in *Arabidopsis* or other annual plant species

Gene (plasmid construct)	Poplar genotype*	No. PCR-verified events	References	Result
35S-PTLF (p104S)	353	16	Rottmann *et al.* (2000)	Very rare early floral onset, some morphological disturbance
35S-LFY (pDW151)	353 717 184–402 189–434 24–305 17–50	20 13 7 2 2 5	Weigel and Nilsson (1995), Rottmann *et al.* (2000), Skinner *et al.* (2003)	Early flowering but genotype specific, single flowers (not catkins), most effective for males, flowers infertile, highly branched/dwarf vegetative form
35S-AP1 (pAM563)	353 717 184–402	3 10 3	Mandel and Yanofsky (1995)	No flowers, normal vegetative form
35S-OsMADS1 (pGA1209)	353	6	Chung *et al.* (1994)	No flowers, normal
35S-CONSTANS (pART27/CO)	353 717 184–402 17–50 19–53	10 10 10 1 2	Putterill *et al.* (1995)	No flowers, normal vegetative form
35S-AGL20 (pSK231)	353 717 184–402 17–50	10 10 10 1	Rounsley *et al.* (1995)	No flowers, normal vegetative form

Transgenic poplars were produced in 1994–96, planted in pots in the autumn of 1996 and 1997, and monitored in the glasshouse for 3–7 years.
PCR (polymerase chain reaction) was used to verify presence of the target floral transgene in transformed plants.
*The parental genotypes were: INRA 353–53 (male (M), *Populus tremula* × *P. tremuloides*); INRA 717–1B4 (female (F), *P. tremula* × *P. alba*); 184–402 (F, *P. trichocarpa* × *P. deltoides* (TD)); 189–434 (unknown, TD); 24–305 (M, TD); 19–53 (F, TD) and 17–50 (F, TD). All TD hybrids listed are triploids.

propagules would be of little concern (Gressel, 1999; Bradshaw and Strauss, 2000). Unfortunately, long breeding cycles make tree 'domestication' via conventional means unlikely for the foreseeable future. However, transgenes that reduce competitiveness while maintaining or improving yield of plantations could have this effect. These genes would need to be tightly linked to, and preferably flank, transgenes of concern. A variety of genes could be employed for this purpose, including genes that substantially modify wood in a manner that improves industrial properties under short rotations but that would reduce fitness in long-lived trees (e.g. reduced lignin; Hu *et al.*, 1999). We have been studying 'green revolution' genes that could be used to impart semi-dwarfism to determine if they would affect, and even improve, biomass and fibre yield from plantation-grown trees. Trees engineered to be shorter than wild trees would likely be poor competitors in wild or feral forests. The genes we are testing interfere with metabolism of or signalling by

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gibberellic acids, key regulators of height growth in plants. We have tested three kinds of genes (*GAI*, *RGL1*, *GA 2-oxidase*), and found that each is effective at inducing dwarfism but has distinct efficiencies and morphological effects on plant form and root development. The degree of dwarfism also varies widely among independent transgenic events (Figure 3). Field tests are underway to see how these genes affect growth and fibre properties.

Conclusions

Regulations and social views toward transgenic plants are still in a state of rapid flux throughout the world. This gives forest biotechnology researchers the time they need to adequately study and develop GE tree crops that deliver value and environmental benefit, and may ultimately prove socially acceptable. Given the cold reception to the first generation of GE

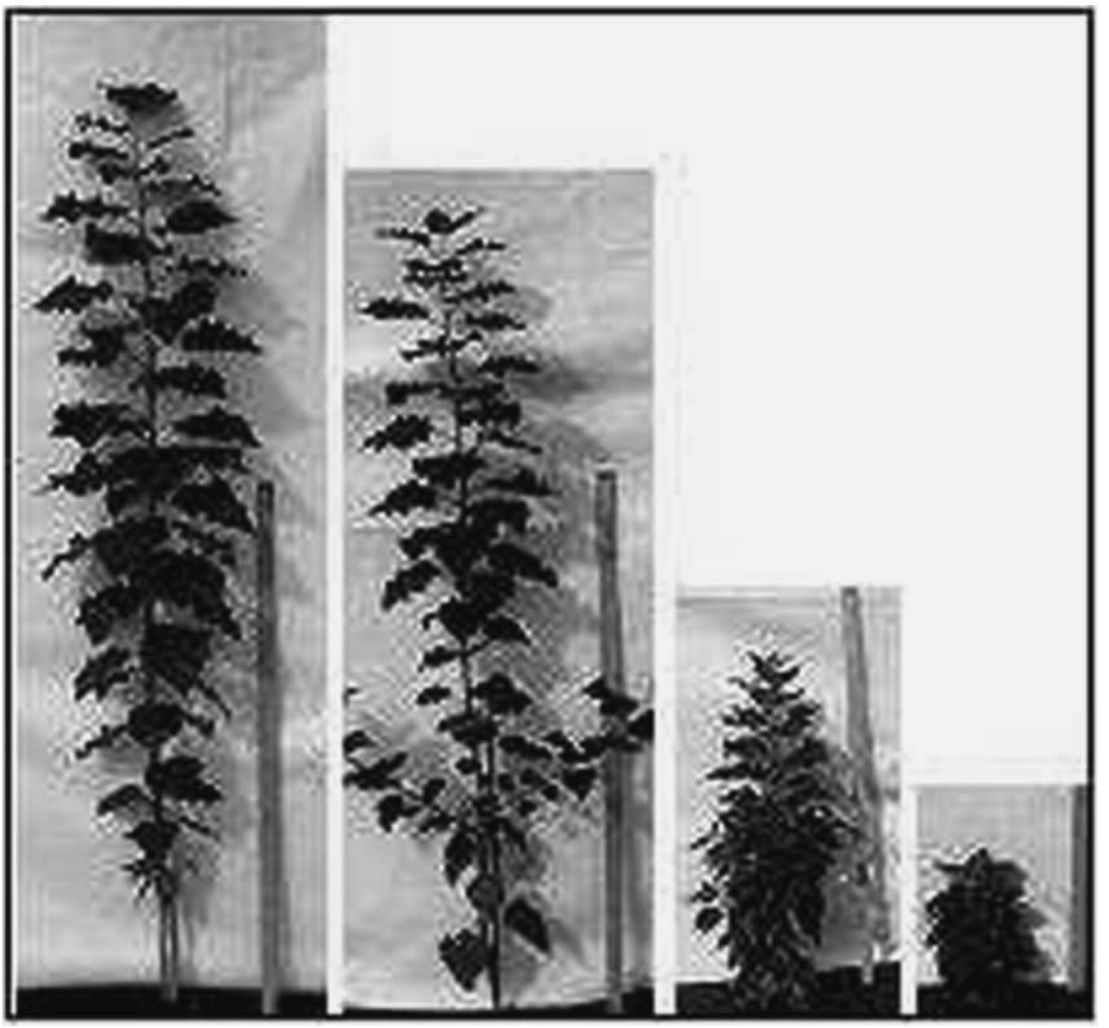

Figure 3. A range of dwarfism in poplars (*Populus tremula* × *P. alba*) from different transgenic events expressing *Arabidopsis* mutant *gai* or *rgl1* genes. The photograph was taken ~4 months after planting in a field trial. A metre-stick is shown; the plant furthest to the left is a non-transgenic control.

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agricultural crops in many parts of the world, a substantial record of efficacy and safety is likely to be needed for acceptance of GE forest trees. The many attributes of poplars, including their intensive management, short rotations and tractable biology, make it a logical candidate for developing new technologies and providing commercial demonstrations that may pave the way for broader adoption in forestry. Our experience with transgenic poplars over the last 15 years shows that GE is highly reliable, and the genomic science underlying it is powerful and growing rapidly. Progress may hinge on the willingness of public and private institutions to move cooperatively forward amidst a complex and controversial milieu.

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