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REVIEW

Genetic containment of forest plantations

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Abstract Dispersal of pollen, seeds, or vegetative propagules from intensively bred, exotic, or recombinant DNA modified forest plantations may cause detrimental or beneficial ecological impacts on wild or managed ecosystems. Insertion of genes designed to prevent or substantially reduce dispersal could reduce the risk and extent of undesired impacts. Containment measures may also be required by law or marketplace constraints, regardless of risks or benefits. We discuss: (1) the context for when genetic containment or mitigation systems may be needed; (2) technology approaches and mechanisms; (3) the state of knowledge on genes/genomics of sexual reproduction in forest trees; (4) stability of transgene expression during vegetative growth; (5) simulation studies to define the level of containment needed; and (6) needed research to deliver effective containment technologies. We illustrate progress with several examples from our research on recombinant DNA modified poplars. Our simulations show that even partial sterility can provide very substantial reductions in gene flow into wild trees. We conclude that it is impossible to define the most effective containment approaches, nor their reliability, based on current genomic knowledge and technological tools. Additional genomic and technological studies of a wide variety of options are needed. Studies in field environments are essential to provide data relevant to ecological analysis and regulatory decisions and need to be carried out in phylogenetically diverse representatives of the economically most important taxa of forest trees.

Keywords *Populus* · *Pinus* · *Eucalyptus* · Sterility · Confinement · Ablation · Excision · Genetic engineering · Genetic modification · Forest biotechnology · Gene flow · Trees · Simulation · Stability

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> *"It is essential that new molecular gene-containment strategies...be developed and introduced."* Editorial, Nature Biotechnology 20, 527 (2002)

Context for gene containment approaches

In an ideal world, industrial forest plantations would operate in harmony with, and in isolation from, natural

ecosystems. Plantations would occur within a landscape designed to maintain biodiversity and minimize ecological impacts of plantations on external ecosystems, and economic goals would be the primary consideration within plantations. However, the reality is that plantations have multiple ecological connections with other managed and wild ecosystems and operate in a social milieu where their actual and perceived impacts may or may not be tolerated. Regulations, laws, and marketplace mechanisms such as certification systems set limits on the kinds of activities that may occur within plantations and on the impacts that these activities may have outside of plantations. All of these mechanisms strongly constrain research and commercial application of genetically engineered (GE) trees[1] (reviews in Strauss and Bradshaw 2004).

Forest certification systems represent a growing mechanism for expression of social preferences in the marketplace (Cashore et al. 2003). One major forestry certification system aimed at environmental and social compliance, that of the Forest Stewardship Council, bans all forms of GE trees on certified lands. This rule is absolute; it applies regardless of the level of containment, whether the genes are from the same or different species, whether the goal is purely scientific research vs application, or whether the primary aim is the solution of substantial environmental problems rather than economic benefits (Strauss et al. 2001a,b). Such a broad ban, which covers even contained research with environmental goals, is difficult to justify on scientific grounds, especially given the long-standing scientific consensus that "product not process" should dominate risk assessment for GE organisms (Snow et al. 2005). It shows that social considerations can overwhelm technical innovations. Thus, containment systems may be required even for genes where no significant biological impact, or even a positive environmental effect, are expected to occur. By allowing effective isolation of trees produced in different ways on the landscape, containment systems should provide a mechanism whereby different social values can more easily coexist.

However, genetic mechanisms for isolation have never before been required even when highly bred or exotic species have been used in agriculture or forestry; their novelty, therefore, creates new forms of social controversy. Although genetic containment systems have long been called for by ecologists and other scientists to reduce a number of undesired effects of GE crops (NRC 2004; Snow et al. 2005), there has been strong pressure on companies and governments against use of any forms of "Terminator-like" containment technology (ETC 2006). For example, a law against the use of such technology in Brazil [Law 11,105/05, banning "...the commercialization of any form of Gene Use Restriction Technology (GURTs)]" delayed approval of a field trial of a reduced lignin, putatively sterile eucalypt (ISAAA 2006). In agriculture, these concerns primarily are about control of intellectual property and the forced repurchase of seed by farmers. But in the forestry area, there has also been activism against containment technology because of a lack of confidence that it will be fully effective, concerns about loss of biodiversity associated with modification or loss of floral tissues (Cummins and Ho 2005), and legal uncertainties and liability risks from the dispersal of patented genes. These biological concerns occur despite the intention to use such technology mainly in plantations that, due to breeding, high planting density, and short life spans, already produce few flowers and seeds compared to long-lived and open grown trees. The powerful inverse association between forest stand density and degree of tree reproduction is widely known (Daniel et al. 1979). There is also an abundance of means to avoid and mitigate such effects at gene to landscape levels (Johnson and Kirby 2004; Strauss and Brunner 2004). Government regulations against the dispersal of genes from research trials also pose very substantial barriers to field research to study the efficiency of containment mechanisms (Strauss et al. 2004; Valenzuela and Strauss 2005). Thus, genetic containment technology is, itself, difficult and highly controversial, requiring special social conditions even to carry out research.

From a biological viewpoint, however, there are good reasons to employ containment technologies to control some forms of highly domesticated, exotic, or GE organisms. Once genes or organisms move beyond plantation boundaries, the risks to external ecosystems are virtually impossible to control, and as with other biological introductions of mobile organisms, may be irreversible. Novel organisms of all kinds may impair the health of some wild ecosystems or create management problems for human-dominated ecosystems (James et al. 1998). If we could confidently segregate intensely domesticated trees by control of reproduction, it would avoid the need for much of the complex, imprecise, and costly ecological research that would otherwise be required to try to understand and predict impacts of spread. The costs and obstacles to conducting commercially relevant environmental research with GE trees are great and occur for a number of reasons:

1. laboratory cost of GE tree production, including production and study of many kinds of gene constructs and gene transfer events;
2. ecological complexity in space and time and high stochastic variance in gene flow and related ecological

[1] GE or genetically engineered, genetically modified, or transgenic organisms, as used in the paper, are defined as those which have been modified using recombinant DNA and asexual gene transfer methods —regardless of the source of the DNA employed.

processes, requiring many sites, environmental conditions, long time frames, and large spatial scales;
3. cost of needed patents, licenses, publication agreements, and transactions for access to genes intended for commercial use (required if results are to be directly relevant to regulatory decisions);
4. cost of record keeping and compliance with regulations, which can be very demanding and legally risky for complex programs that span many years and sites;
5. uncertainty over what data regulators will require due to vagueness in regulatory standards and political volatility creating substantial changes in regulations or their interpretations over time;
6. risk of spread into the environment during research, including costly steps to prevent any spread (e.g., premature termination of trials, bagging all flowers in test plantings, use of noncommercial but sterile genotypes, or use of geographically distant planting environments);
7. disincentives to undertaking costly and risky research, as a result of possible marketplace rejection and separation costs; other significant disincentives result from primary ownership of the genes and gene transfer methods generally being out of the hands of the tree breeders and producers that bear most of the risks and costs of field testing.

These very formidable obstacles, many of which have substantial similarities in many other crop species, have forced companies and governments to ask whether these obstacles do more harm than good by blocking economically and environmentally beneficial technologies. It has also prompted calls for regulations that would place GE organisms into risk categories that call for dramatically different levels of research and containment depending on the novelty and risk of the new traits (Bradford et al. 2005). For example, it has been suggested that "genomics guided transgenes (GGTs)," where the expression of native or functionally homologous genes are altered in a manner analogous to conventional breeding, and "domestication transgenes" that encode traits highly likely to reduce fitness in the wild, should be put into a low risk category or exempted from regulation entirely (Strauss 2003). In contrast, new types of GE plants that are more likely to produce ecologically novel traits, or produce hazardous forms of pharmaceutical or industrial compounds, would be regulated with increased stringency. The United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS), which regulates all field research in the USA, is currently undergoing a major review, with one goal being the creation of risk categories. The obstacles to field research have also called for increased emphasis on ecogenetic models, where the spread and impacts of transgenes with different properties, and under different environmental and social conditions, can be studied over decades as they spread within the containment of a computer (reviewed below).

The sense for a "mandate" to use containment technologies was also inspired by the creation of GE-based male and female sterility mechanisms during the early 1990s (Mariani et al. 1990, 1992), when the possibilities of plant biotechnology seemed limitless, public acceptance was not an issue, and regulatory hurdles appeared modest (reviewed below). It was also stimulated by the suggestion of "mitigation" genes that can both increase value in managed environments and reduce competitive ability in the wild (Gressel 1999). If gene spread creates irreversible risks and social discomfort, and technology exists to greatly reduce these risks, is it not the ethical responsibility of scientists and companies to act to minimize these risks? The incorporation of biosafety features into GE organisms during their design has been promoted as key elements of good stewardship (Doering 2004).

Unfortunately, as discussed above and in genetic detail below, applying containment technology to trees is an extremely costly and difficult endeavor. Caution is, therefore, warranted in assuming that containment systems—even the use of genes with a neutral or negative effect on fitness—present good stewardship. If genetic containment were incomplete, genes that provide a significant and evolutionarily highly stable selective advantage (should such transgenes be feasible to create and deploy), could eventually spread widely. Even neutral or deleterious genes can persist and even become fixed in wild populations in situations where transgenes numerically swamp native genes (Haygood et al. 2003). Obtaining licenses to the set of patents that cover all of the elements of the best containment technology can also be very costly or impossible. On the other hand, it is also likely that the spread of fitness-improving transgenes could, in some cases, provide ecological benefits. A gene for resistance against a serious exotic pest of trees such as the chestnut blight or Asian longhorn beetle might provide large ecological benefits by maintaining or restoring healthy ecological dominants and their dependent communities. Genes for general pest or abiotic stress resistance, including against native herbivores or pathogens, might also provide net ecological benefits by increasing the vigor of a native organism like poplar, which provides habitat for myriad dependent organisms (Whitham et al. 2006), even if some introduced herbivores or plant species were disadvantaged as a consequence. It is therefore essential that containment technology is not indiscriminately required by regulations or used when its net benefits are questionable.

The goal of the remainder of this paper is to review the state of sterility technology that might be useful for sexual

containment of trees used in clonal forestry and ornamental horticulture. We previously reviewed the many options for sex-specific sterility and inducible sterility/fertility (Strauss et al. 1995) that might be used to enable continued seed propagation. Here, we focus on complete sterility under some form of vegetative propagation. Only after a simple method for strong and bisexual sterility is shown to be effective and socially accepted, is it likely that more sophisticated methods for fertility control will be developed and deployed.

Technical approaches and their advantages/ disadvantages

Below, we discuss the main approaches to engineering containment relevant to forest trees. In addition, via electronic searches, we have scanned the recent (2000 to present) scientific and patent [United States Patent and Trademark Office (US PTO)] literature and presented representative examples of developments. Tables 1 and 2 summarize the kinds of approaches being taken, nearly all of which are relevant to one kind of tree species or another.

There are five major approaches to containment. One approach, mitigation (e.g., Al-Ahmad et al. 2004), is a directed form of plant domestication such that the fitness benefits of transgenes are effectively canceled by tight linkage to a gene that is beneficial within farms or plantations, but deleterious elsewhere. It has the advantage of being applicable to vegetative and sexual dispersal, which is useful for species like poplars that can spread vegetatively. Mitigation genes could also be combined with sterility genes to provide a second layer of containment. Genes that reduce the rate of height growth in forest trees, especially for shade-intolerant species like poplars (Daniel et al. 1979), are expected to provide a very powerful competitive disadvantage in competition with wild trees (Strauss et al. 2004). Only two patents for dwarfism genes are shown under mitigation in Table 3 (Harberd et al. 2004a,b), though there are a number of such genes now reported in both the scientific and patent literature. It is unclear, however, if such genes could be used and still maintain or improve yield and adaptability in plantation grown trees, but such studies are underway [e.g., (Strauss et al. 2004; Busov et al. 2006)].

The other forms of containment affect sexual reproduction, which is overwhelmingly the most important means for large-scale propagule spread in most tree species. There are basically four GE approaches: (1) Ablation, where floral tissues are effectively destroyed or made nonfunctional by a cytotoxin; (2) Excision, where some or all functional transgenes are removed from gametes before their release; (3) Gene suppression, where the activity of one or more genes essential for reproduction are impaired at the DNA, RNA, or protein levels; (4) Repression, where the onset of flowering is postponed by modifying the expression of genes that promote vegetative growth or repress the transition to reproductive growth.

Ablation approaches

Genetic ablation methods employ promoters active in specific cells to control the expression of a deleterious gene, usually encoding a cytotoxin (e.g., Burgess et al. 2002). However, many kinds of deleterious genes may be employed, as demonstrated by the patent applications of Dellaporta and Moreno (2004) and Spena et al. (2002), which cite in addition to the widely used RNases and protein synthesis inhibitors (Table 1), DNases, proteases, glucanases, and lipases. Höfig et al. (2006) recently reported that targeted expression of stilbene synthase, which interferes with pollen function, gave a high rate of male sterility. For engineering reproductive sterility, a floral predominant promoter has been used to control the expression of a cytotoxin such as the ribonuclease *barnase* (Mariani et al. 1990). Ideally, cytotoxin expression will be confined to floral cells; however, it appears that many floral promoters are not expressed exclusively in floral tissues (e.g., Brunner et al. 2000; Rottmann et al. 2000), and even low levels of unintended cytotoxin expression may impair tree growth (Skinner et al. 2000). Thus, great care is needed in selection of promoters and cytotoxins. Skinner et al. (2003) showed how the promoter of the poplar floral homeotic gene *PTD*, used to drive the cytotoxin *DTA*, gave rise to high levels of sterility in tobacco and *Arabidopsis* and did not impair vegetative growth in a greenhouse trial. The tapetal specific promoter *TA29* from tobacco, when fused to barnase, caused very high levels of male sterility in field-grown poplars (Figs. 1 and 2). However, Wei et al. (2006), studying poplar, and Lemmetyinen et al. (2004a) and Lännenpää et al. (2005), studying birch, found that many transgenic events with floral homeotic promoter:: barnase fusions showed abnormal growth or morphology in the greenhouse. In an attempt to avoid deleterious effects on growth seen with the poplar *LEAFY (PTLF)* promoter driving barnase, we coexpressed *barstar*, a specific inhibitor of barnase, in transgenic poplars using various promoters. We found that gene insertion events with low ratios of barstar to barnase activity had abnormal growth and morphology (Fig. 3), and that even among plants with normal growth and morphology in the greenhouse, those events with barnase grew slower in the field than events with only barstar or that lacked both genes (Wei et al. 2006). We found that we were unable to regenerate any transgenic poplars containing an intact *pAPETALA1::DTA* transgene, a likely result of leaky expression (root and leaf)

Table 1 Selected literature on genetic engineering of sterility published from 2000 onward

Phenotype	Mechanism	Promoter	Active gene	Plant species	Reference
Delayed flowering					
Late flowering	Overexpression of *FLM*	35S CaMV	*Flowering Locus M*	*Arabidopsis*	Scortecci et al. 2001
	AGL20/shoot apical meristem	35S CaMV	*AGAMOUS LIKE 20*	*Arabidopsis*	Borner et al. 2000
Cell ablation					
Male sterility	Altered pollen development	Endosperm specific promoter, *AGP2*	Fission yeast *cdc25*	Wheat	Chrimes et al. 2005
	Pollen sterility	Rice tapetum promoter (*TAP*)	Barnase/rice tapetum gene *rts*	Creeping bentgrass	Luo et al. 2005
	Alteration in tapetal cells	*Tapetum A9* promoter	Chimeric gene in transgenic plant	*Arabidopsis*	Guerineau et al. 2003
	Abnormal pollen	*BcA9*	*DTx-A*	*Brassica*	Lee et al. 2003
	Tapetal dysfunction	*TA29* promoter	*RIP*	Tobacco	Cho et al. 2001
	Reduced pollen viability	Pollen specific promoter G9	Chimeric genes *G9 uidA* and *G9-RNase*	Tobacco	Bern-d-Souza et al. 2000
Male and female sterility	Floral organ ablation with otherwise normal growth	*PopulusPTD*	*DTA*	Tobacco, poplar, *Arabidopsis*	Skinner et al. 2003
Recoverable block of function (RBF)	Inducible fertility	Sulfhydryl endopeptidase, heat-shock promoter	*Barnase* (the blocking construct) and *barstar* (recovering construct)	Tobacco	Kuvshinov et al. 2001
Gene suppression					
Male sterility	Distorted pollen morphology	Various	*AtMYB32 AtMYB4*	*Arabidopsis*	Preston et al. 2004
	Temperature sensitive male sterility due to silencing choline biosynthesis	*S*-adenosyl-L-methionine	Phosphoethanolamine *N*-methyltransferase (PEAMT)	*Arabidopsis*	Mou et al. 2002
	Mitochondrial dysfunction	Tapetum specific promoter	Antisense pyruvate dehydrogenase E1α subunit	Sugar beet	Yui et al. 2003
	Abnormal pollen	*Nin88* promoter	Antisense *Nin88*	Tobacco	Goetz et al. 2001
	Abnormal pollen	Glutenin subunit gene promoter	Antisense sucrose non-fermenting-1-related (SnRK1) protein kinase	Barley	Zhang et al. 2001
Restoration of fertility	Glucanase gene suppression	*pA9*	Sense and antisense PR glucanase	Tobacco	Hird et al. 2000

Table 2 Selected patents on genetic engineering of sterility published from 2000 onward

Phenotype	Mechanism	Promoter	Active Gene/Protein	Species	Reference
Time of flowering					
Altered floral development	Expression of floral meristem identity protein	Modified native promoter	*CAULIFLOWER* (*CAL*), *APETELA 1* (*AP1*), *LEAFY* (*LFY*)	Angiosperm or gymnosperm	Yanofsky 2000
Cytotoxin ablation					
Suicide gene to ablate gamete	Any of several cytotoxic genes expressed in gametes	Male- or female-specific promoter expressed in gamete	Various "suicide" genes (*barnase*, *tasselseed2*, *diphtheria toxin A*)	Rice	Dellaporta and Moreno 2004
Female sterility	Enhance fruit development or induce sterility	*DefH9* promoter	DNases, RNases, proteases, glucanases, lipases, toxins, etc.	Many	Spena et al. 2002
Gene suppression					
Male Sterility	Calcium/calmodulin-dependent protein kinase (CCaMK) expression	Developmental stage-specific anther promoter	Antisense RNA	Tobacco	Poovaiah et al. 2002
Reversible male sterility	Biosynthesis of amino acids inhibited in male reproductive organs, reversible by application of those amino acids	Male organ-specific promoter	Antisense RNA	*Arabidopsis*, tobacco	Dirks et al. 2001
Male sterility	Supression of *ATH1* gene to control flowering time	35S CaMV	Antisense *ATH1*	*Arabidopsis*	Smeekens et al. 2005
Delayed flowering time	Loss of function of *SIN1* by RNAi	35S CaMV	Short integuments 1 protein	Unspecified	Ray and Golden 2004
	RNAi construct	Constitutive, inducible, or tissue-specific promoter	Sequence similar to transgene or endogenous gene	Unspecified	Waterhouse and Wang 2002
Floral promoters					
Male sterility	Anther development-specific genes and promoters	Tapetum, pollen	Antisense RNA or any gene that compromises pollen viability	*Brassica*, *Arabidopsis*, tobacco	Knox et al. 2004
Female sterility	Regulatory region of corn silk/pistil genes	*C3* promoter	Silk-specific gene, *C3*	Maize	Ouellet et al. 2003
Restoration of fertility to cytoplasmic male sterile plants	Wild-type *atp6*	*AP3* promoter	Wild-type *atp6* gene fused to a mitochondrial transit peptide	*Brassica*	Brown 2002
Conditional male sterility	Upon application of acetylated toxin	Stamen-selective promoters	Deacetylase	Wheat	Quandt et al. 2002
Male and female sterility	Poplar floral homeotic genes and promoters	Native promoters	*PTLF*, *PTD*, *PTAG-1*, *PTAG-2*	Poplar	Strauss et al. 2002
Male sterility	Recessive mutant causes sterility	*Ms41*-A promoter	*Ms41*-A	*Arabidopsis*, maize,	Baudot et al. 2001
Male sterility	Absence of a functional callase enzyme	*MsMOS* promoter	*msMOS*	Soy	Davis 2000
Protein Interference					
Reversible male sterility	Dominant negative genes under anther-promoter reversed by expression of a repressor	Anther-specific promoter and *lexA* operator	Any cytotoxic methylase or growth-inhibiting gene	Maize	Cigan and Albertsen 2002
Cytoplasmic male sterility	ATP synthesis in mitochondria	Ubiquitin promoter	Unedited *Nad* 9 gene	Rice, wheat, corn,	Patell et al. 2003

Table 2 (continued)

Phenotype	Mechanism	Promoter	Active Gene/Protein	Species	Reference
	inhibited			soybean	
Male sterility	Biotin-binding polypeptide ablates male gamete tissue, fertility can be restored	Promoter regulated by the *LexA* operon expressed in anther	Botin-binding polypeptides and inhibitory proteins	*Arabdidopsis* and tobacco	Albertsen and Huffman 2002
Male sterility	Repressor protein under male promoter repressed by antisense RNA	Male flower specific promoter	Repressor protein	Multiple	Bridges et al. 2001
Male sterility	Protein that disturbs metabolism, development, and gene for reversibility	Stamen-specific promoter	A sterility RNA, protein, or polypeptide	*Brassica*, maize, rice	Michiels et al. 2000
Mitigation					
Male sterile and dwarf	Unknown	Native promoter	*dfl1* gene	Safflower	Weisker 1995
Dwarf plants	GA insensitive	Native promoter	Mutant of *GAI*	*Arabidopsis*	Harberd et al. 2004a
Dwarf plants	*Rht* mutant dominant allele causes GA-insensitivity	Native promoter	Mutant of *Rht* (D8)	Rice	Harberd et al. 2004b

seen with this promoter in transgenic poplars with *pAPETALA1::GUS* fusion genes (data not shown). Thus, ablation-based systems need to be carefully engineered in trees via judicious choice of promoters, cytotoxins, and vectors, and then, carefully field tested.

Gene excision approaches

There have been considerable efforts to develop more precise means for manipulation of transgenes and their genomic locations via the use of site-specific recombinase systems such as *cre/lox* from bacteriophage P1 (reviewed in Gilbertson 2003). Although the primary goals have been the removal of selectable marker genes and the targeting of transgenes to defined locations, a more recent application has been to use them to selectively remove transgenes before the release of seeds and pollen. By flanking transgenes with recombinase recognition sites and placing the recombinase under the control of a floral predominant promoter, it appears that very high levels of transgene excision can be obtained. Mlynárová et al. (2006) used the microspore-predominant *NTM19* promoter to control expression of an intron-containing *cre* gene to successfully excise GUS encoding transgenes from tobacco pollen at a rate above 99.98%. No excision activity was detected other than in target tissues. Li and Pei (2006 and personal communication) used the promoter of the bisexually expressed *PAB5* gene (Belostotsky and Meagher 1996) to drive either or both the *cre* or *FLP* recombinase genes, targeting *loxP–FRT* fusion recognition sites. Based on GUS activity examined in more than 25,000 T_1 progeny per transgenic event, they reported a 100% rate of transgene removal from both male and female gametes of tobacco in 18 of 45 events studied. Although this is a promising system for transgene containment in vegetatively propagated plants, its effectiveness in the long-term under field conditions is unknown, and predicting and verifying that gametes will lack transgenes in large trees when they begin flowering will be difficult. It is also distinct from the other approaches in that it does not impair fertility, and thus, would provide containment of only the excised transgenes —not of exotic or highly domesticated organisms. However, reproductive transgene excision could be used in combination with a sterility transgene to provide a more robust containment system.

Gene suppression approaches

The activity of genes essential for fertility can be suppressed by transcriptional gene suppression, posttranscriptional gene suppression, blocking the activity of the encoded protein, or by directed mutation or deletion. As shown in Tables 2 and 3, there have been a great variety of

Table 3 Summary of studies on stability of transgene expression in plants

Taxa	Gene	Number of events (unstable)*	Environment	Propagation	Generations or years	Associated factors	Nonassociated factors	Sources
Chyrsanthemum	*35S-gus*	17 (0)	Greenhouse	V	1 gr			Pavingerová et al. 1994
Citrus	*35S-uidA*, *NOS-nptII*	70 (0)	Screenhouse	V	4–5 yrs	Copy number	T-DNA rearrangements	Cervera et al. 2000
Poplar	*FMV-cp4*, *FMV-gox*	40 (1)	Field	V	4 yrs			Meilan et al. 2002
Poplar	*35S-rolC*	6–22 (2–6)	In vitro, greenhouse, field	V	5–6 yrs	T-DNA repeat formation, flanking AT-rich sequence		Kumar and Fladung 2001
Poplar	*35S-uidA*, *EuCAD-uidA*	44 (0)	In vitro, greenhouse, field	V	6 yrs		Copy number, extra vector sequence	Hawkins et al. 2003
Poplar	*35S-ASCAD 35S-ASCOMT*	4	Field	V	4 yrs			Pilate et al. 2002
Potato	*Gus*, *nptII*	2	In vitro, greenhouse	V	2 yrs			Borkowska et al. 1995
Potato	*NptII*, *gus*, *ocs*, *rolA*, *and C*	4	Greenhouse	V	3 grs			Ottaviani et al. 1992
Sugarcane	*Ubi-bar*	1	Greenhouse	V	3 grs		Contained five copies	Gallo-Meagher and Irvine 1996
Sugarcane	*Pat*	1	Field	V	3 grs		Contained nine copies	Leibbrandt and Snyman 2003
Tall fescue	*Actin1-gus*	2	Growth room	V	5 grs			Bettany et al. 1998
Arabidopsis	*NOS-nptII*	7	In vitro	S	4 grs	Promoter methylation		Kilby et al. 1992
Arabidopsis	*35S-hpt*	28 (14)	In vitro	S	1 gr	Copy number		Scheid et al. 1991
Arabidopsis	*NOS-nptII*	111 (62)	In vitro, growth chamber	S	3 grs	Construct configuration, temperature	Copy number	Meza et al. 2001
Arabidopsis	*Fp1-dsFAD2*	1	Greenhouse	S	4 grs			Stoutjesdijk et al. 2002
Petunia	*35S-A1*	1	Field	S	1 yr	Promoter methylation, temperature, endogenous factors		Meyer et al. 1992
Rice	*35S-bar*, *35S-gusA*	12 (0–2)		S	3 grs	Presence of truncated transgene sequences	Copy number, position effect	Kohli et al. 1999
Rice	*Ltp2-gus*	3	Greenhouse	S	5 grs	Partial rearranged transgene		Morina et al. 1999
Tobacco	*NOS-nptII*	2	In vitro	S	3 grs			Müller et al. 1987
Tobacco	*NOS-nptII*	18 (5×10^{-5} ~5.9×10^{-4})[b]	In vitro	S	1 gr	Environmental stress	MAR	Conner et al. 1998
Tobacco	*35s-hpt*, *35s-cat*	4	In vitro	S	8 grs	T-DNA flanking sequences, position effect, extra vector sequence		Iglesias et al. 1997

V Vegetatively propagated, *S* sexually propagated, *gr(s)* generation(s), *yr(s)* year(s)
[a] Unstable events given in parentheses only where data on ten or more independent events reported
[b] Frequency of kanamycin-sensitive seedlings derived from each event

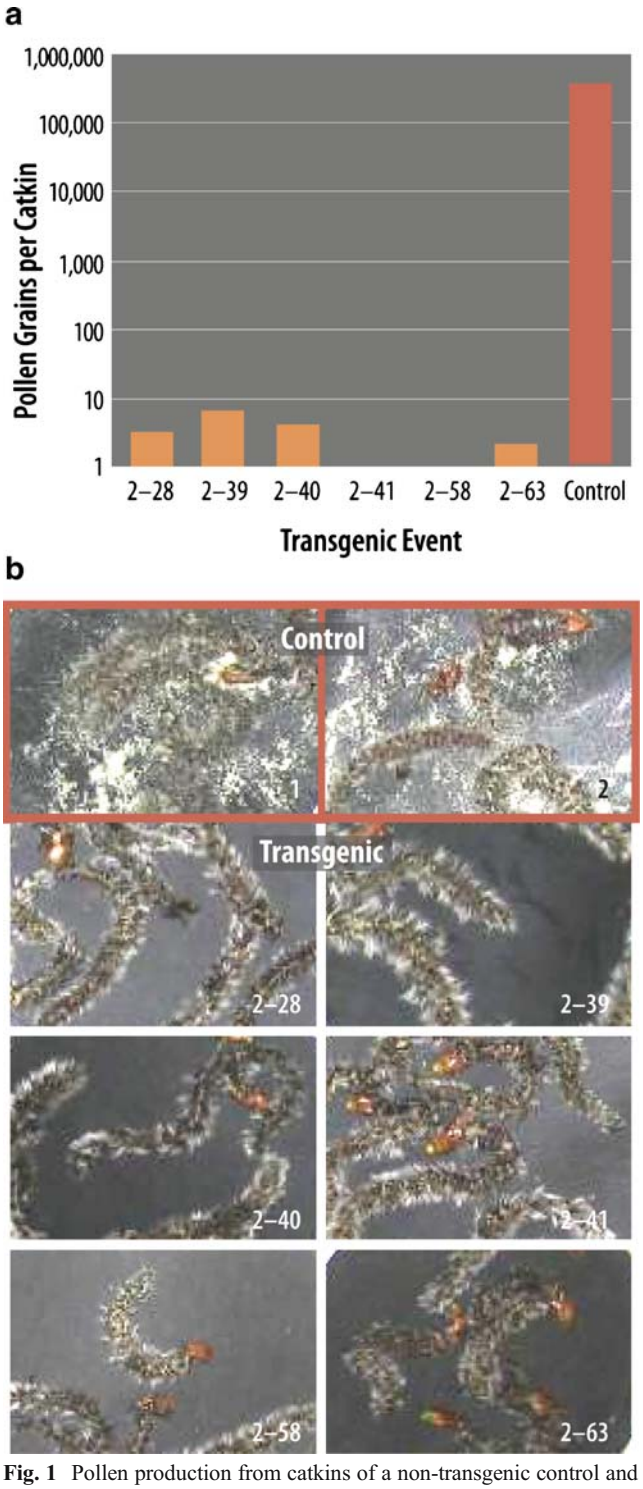


Fig. 1 Pollen production from catkins of a non-transgenic control and several transgenic trees that originated from different gene transfer events, after 10 years growth in the field in Oregon. **a** Pollen from mature catkins was allowed to dehisce and then forcibly discharged in Petri dishes in the laboratory. For each of the transgenic events, total pollen grains were counted under a dissecting microscope. Controls were diluted in water and counted using a hemacytometer. Between 3 and 22 catkins were analyzed from each tree, and the average number of pollen grains per catkin calculated. **b** Petri dishes after catkins were allowed to finish maturation and shedding of pollen. Note the apparent absence of pollen from the six different transgenic events sampled compared to the non-transgenic control samples

genes and approaches in various plant species that have been successfully used to impart sterility and/or restore fertility. This includes targeting of signal transduction proteins (Zhang et al. 2001, Poovaiah et al. 2002), amino acid metabolism (Dirks et al. 2001), choline biosynthesis (Mou et al. 2002), transcription factors (Preston et al. 2004; Smeekens et al. 2005), methylases or methyltransferases (Cigan and Albertsen 2002; Luo et al. 2005), and mitochondrial genes (Patell et al. 2003; Yui et al. 2003).

RNA interference and related methods

Double-stranded RNA (dsRNA) can induce a variety of sequence-specific gene suppression processes in plants, animals, and fungi (reviewed in Baulcombe 2004; Matzke and Birchler 2005). RNA-mediated gene suppression, also called RNA interference (RNAi), is now widely exploited to reduce the expression of specific genes (reviewed in Watson et al. 2005). Virus-induced gene silencing (VIGS) vectors are one option for inducing sequence-specific suppression and have great potential for functional genomics (Burch-Smith et al. 2004 and discussed below), but are not suited to stable introduction of a biosafety trait.

Stable transformation of transgenes containing an inverted repeat or hairpin sequence corresponding to a transcribed region of the target gene has been effective in a variety of plants, and posttranscriptional suppression has been shown to be stably inherited over several generations (Chuang and Meyerowitz 2000; Wesley et al. 2001). However, stability through rounds of vegetative propagation and across multiple years in field environments has not been extensively studied (discussed below). Inverted-repeat transgenes of promoter regions can induce methylation and transcriptional gene suppression of endogenous plant promoters, and this approach was used to engineer male sterility in maize (Cigan et al. 2005). Nonetheless, there have been relatively few studies, and thus, its utility as a gene suppression approach is uncertain. Moreover, it appears that promoters vary in their sensitivity to different types of cytosine methylation depending on their sequence composition (Matzke et al. 2004).

Multiple genes can be silenced by using a conserved region or by joining sequence segments of multiple genes together to create a compound RNAi transgene (reviewed in Watson et al. 2005). This capability is especially important for sterility systems where a redundant approach is desirable to produce a highly robust and reliable biosafety trait. Because of genetic redundancy in the regulation of flowering and many taxon-specific gene duplications and losses (Irish and Litt 2005), the extent and configuration of redundancy required for robust and effective RNAi suppression will vary between species.

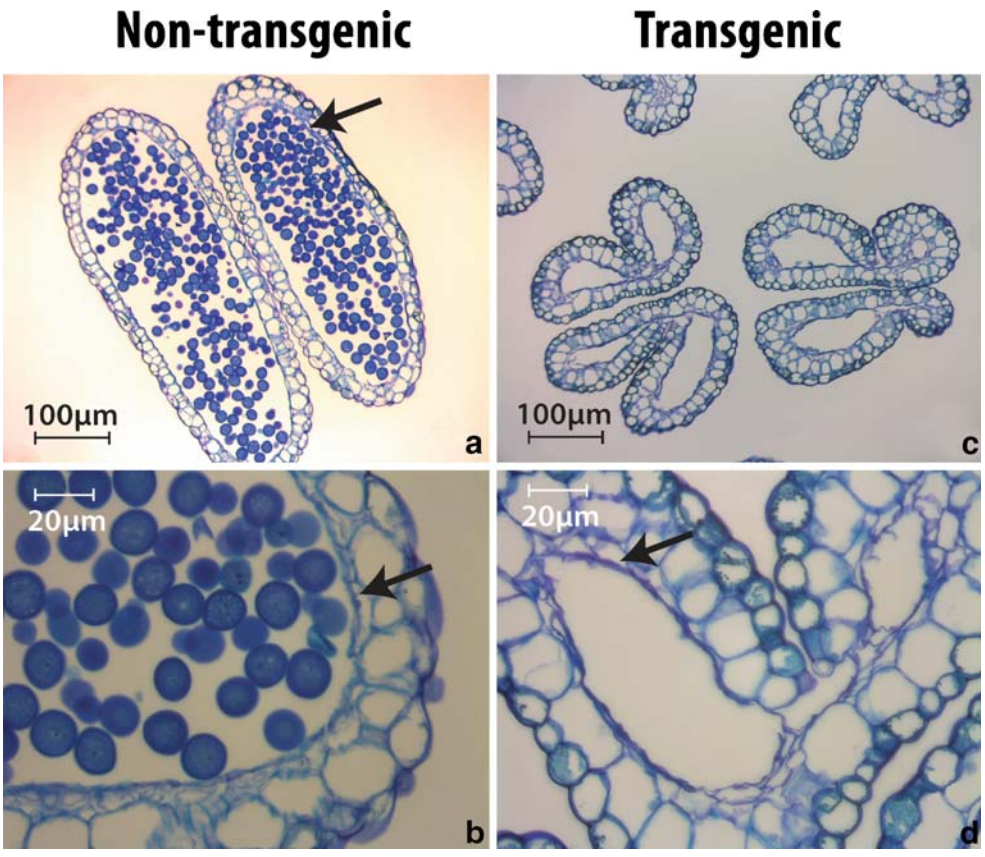


Fig. 2 Transverse sections of nearly mature anthers from a transgenic, putatively male sterile field-grown poplar and a non-transgenic control poplar of the same age. Slides in *top row* were taken at ×100 magnification, those below were taken at ×400 magnification. Samples were fixed, dehydrated, embedded in glycol GMA methacrylate plastic, sectioned, and mounted on slides. Sections were stained in 0.5% Toluidine Blue O in citrate buffer. Arrows point to tapetal layer (absent or disorganized in transgenics)

A population of transgenic events carrying the same RNAi transgene typically exhibit highly diverse levels of suppression. Although RNAi transgenics that phenocopy null mutations in floral regulatory and other genes have been obtained, strong suppression can be infrequent (Chuang and Meyerowitz 2000; Stoutjesdijk et al. 2002). In addition, the level of endogene suppression appears to be target-specific (Kerschen et al. 2004). The endogenous expression level of the target gene appears to influence the effectiveness of RNA-mediated silencing, but does not appear to be the only gene-specific determinant of RNAi effectiveness (Han et al. 2004, Kerschen et al. 2004, Wagner et al. 2005).

Possible additional determinants include spatiotemporal expression, RNA turnover, and sequence composition. Single-copy RNAi transgenics are preferable because multicopy events appear more variable with respect to level of suppression and stability, perhaps because multicopy transgenes are more susceptible to transcriptional gene suppression (Kerschen et al. 2004). For practical application, successful transformation events (i.e., those exhibiting strong suppression) must be identifiable via molecular tests when trees are still juvenile. This potentially limits the utility of this approach because many target genes are specifically or predominantly expressed in floral tissues. We have produced transgenic poplars carrying RNAi transgenes targeting various genes regulating floral onset and floral organ development. Using vegetative tissue from poplar transgenics still in tissue culture or the greenhouse, we have been able to identify events exhibiting strong target endogene suppression using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR; Fig. 4), suggesting that RNAi transgenic trees with greatly reduced fertility can be selected at an early, nonflowering stage.

Pleiotropic effects of RNAi methods can be significant. Nontarget effects of dsRNAs are well-known in animal systems (Jackson and Linsley 2004). However, this does not appear to be a common problem in plants for well-targeted dsRNAs, perhaps because both siRNAs and miRNAs require high levels of complementarity with their target (Watson et al. 2005; Schwab et al. 2005). Transitive suppression, whereby suppression spreads from the initiator sequence to an adjacent region, could potentially cause pleiotropic effects in plants. However, several plant studies have shown that transitive suppression occurred when the target was a transgene, but did not occur when an endogene was the target (Vaistij et al. 2002; Petersen and Albrechtsen 2005; Miki et al. 2005). Why transitive silencing appears to commonly occur with transgenes but not endogenes, is unknown. However, to date, a few studies have looked for transitive silencing with endogene targets.

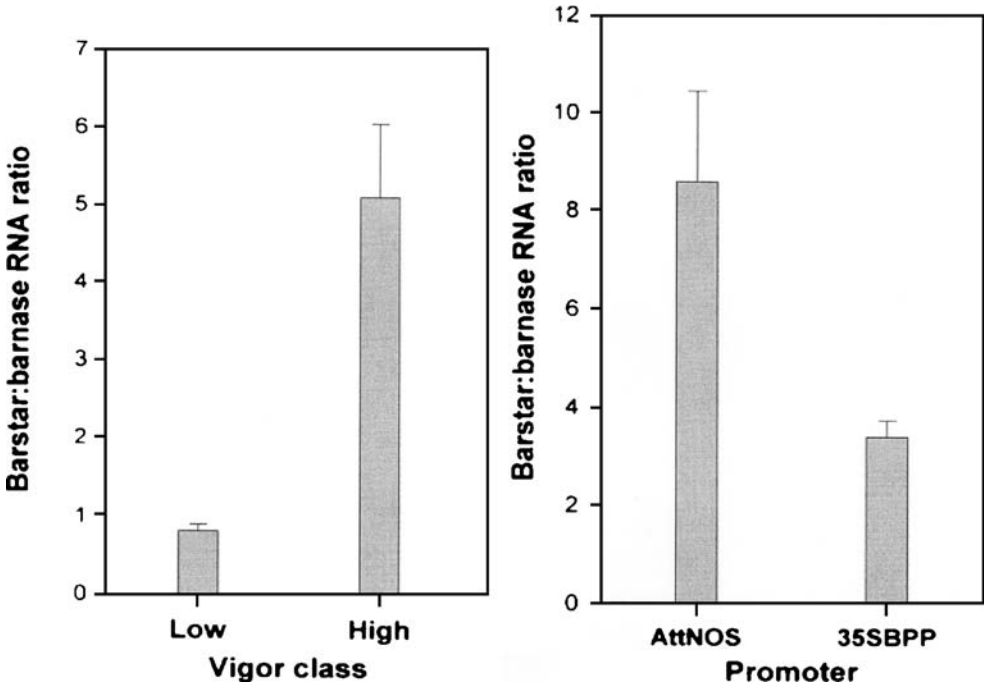


Fig. 3 Ratio of *barstar:barnase* RNA from shoot tips of greenhouse-grown trees with *barnase* driven by the poplar *LEAFY* (*PTLF*) gene promoter, and *barstar* driven by one of three promoters (Wei et al. 2006). **a** Transgenic events with the highest ratios had the greatest vegetative growth, and those with the lowest ratios tended to be stunted or have abnormal physiology. **b** The *NOS* promoter directed twice the level of *barstar* expression compared to the 35S basal promoter and the basal promoter with an omega enhancer element (mean shown). All data are expressed relative to *barnase* expression from a *pPTLF::barnase* gene

Dominant negative proteins

Alternative approaches to repressing floral genes include introduction of dominant negative mutant forms of the target endogene and artificial transcription factors. Several studies have identified dominant negative mutant forms of plant signal transduction proteins and transcription factors, including MADS box genes regulating floral development (e.g., Jeon et al. 2000; Dievart et al. 2003; Ferrario et al. 2004). Most dominant negative forms appear to exploit the modular nature of these proteins and that they often form multi-protein complexes. For example, a dominant negative protein might be able to interact with other proteins, but the protein complex cannot bind DNA. Based on studies of rice and mammalian MADS-box genes, we used site-specific mutagenesis to alter amino acids predicted to be necessary for dimerization and/or DNA binding in *AG* and *APETALA1(AP1)*. Constitutive expression induced strong loss-of-function phenotypes at a frequency of approximately 30% in primary *Arabidopsis* transformants, and these transgenes are now being evaluated in poplar and sweetgum (data not shown).

Another option for dominant repression of transcription factor activity is the introduction of chimeric transgenes that are translational fusions of the selected transcription factor coding region and a repression domain such as the ERF amphiphilic repressor (EAR) motif (Hiratsu et al. 2003). Expression of EAR chimeras has proven to be useful for producing phenocopies of double knockouts in *Arabidopsis*, and thus, can overcome the problem of genetic redundancy among gene duplicates. Recently, Mitsuda et al. (2006) used this chimeric repressor approach with *AP3, AG*, *LEAFY*, and a floral expressed *MYB* gene, and reported very high levels of sterility in Arabidopsis and/or rice. Recent studies have also shown that synthetic zinc-finger domains fused to a transcriptional activation or repression domain are highly effective for manipulating the expression of specific genes (reviewed in Segal et al. 2003). By combining pre-defined zinc-finger modules appropriately, three-or six finger domains can be created that specifically bind to a selected 12 to 18 bp DNA sequences. For example, a transgene containing a human repression domain, fused to a zinc-finger module designed to bind to a site in the *AP3* promoter, was able to repress endogenous *AP3* expression and induce a loss-of-function phenotype (Guan et al. 2002).

It remains to be determined how these different methods of gene suppression compare with respect to frequency of transformants exhibiting strong repression/loss-of-function phenotypes, and stability over multiple years, in the field. It is also important to investigate whether pleiotropic effects are more common with certain methods. As discussed above, deleterious side-effects are not always evident under controlled conditions, but may appear as a cumulative effect of tree development, especially in the field. Although most studies have used strong constitutive promoters, tissue-specific promoters have been successfully used for RNAi and other repression methods. Promoters directing more restricted expression could reduce the occurrence of pleiotropic effects. However, they might be less effective at inducing strong, stable sterility.

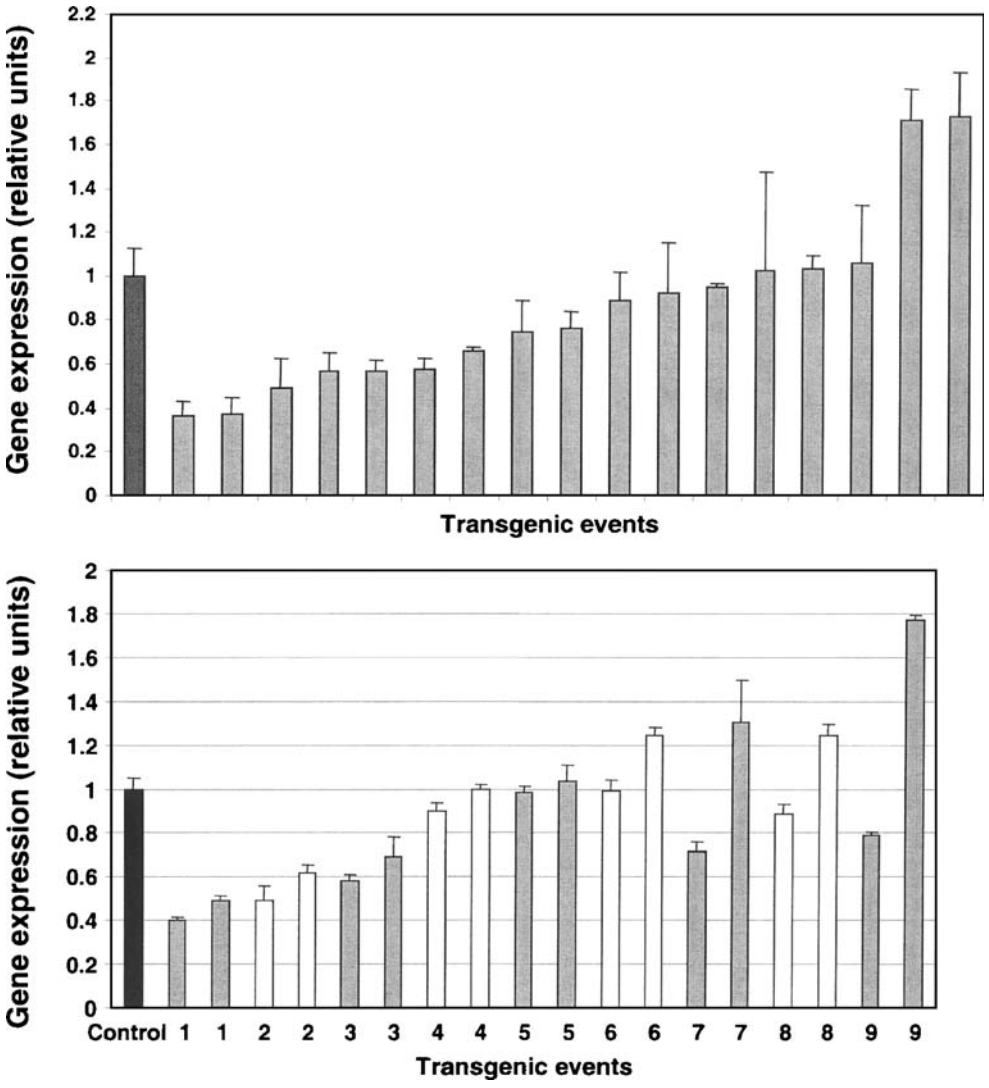


Fig. 4 Range of RNAi gene suppression (**a**, *top*) and repeatability among biological replicates (**b**, *bottom*) for floral genes expressed in vegetative tissues. **a** Relative expression level of native *PTLF* gene in selected poplar *PTLF*-RNAi transgenic trees and non-transgenic controls of poplar clone 353-53 (*Populus tremula x tremuloides*). Expression was determined by qRT-PCR analysis of native transcripts in vegetative shoots (a ubiquitin gene served as an internal control). Each datum represents a pool of total RNA from four to five ramets per transgenic event; *error bars* are standard deviations over three PCR technical replicates. **b** Relative expression level of native *Poplar SOC1(PSOC1)* gene in pairs of biological replicates (RNA extraction from different ramets) of selected *PSOC1*-RNAi transgenic trees and non-transgenic controls. qRT-PCR methods as in **a**. Data are means of independent qRT-PCR runs for two different ramets for single transgenic events; *error bars* are standard deviations over the average of two PCR technical replicates (r^2=0.41). Pairs in b (shading) show biological replicates per event

Targeted gene mutagenesis and replacement

The long sought-after goal of routinely creating precise deletions, insertions, or mutations with plant genes has been elusive, largely due to the propensity for random rather than homologous DNA recombination in plants. However, recent studies have demonstrated new strategies that achieve substantial improvements in the rate of targeted mutagenesis and gene replacement. By constitutively expressing the yeast *RAD54* gene, a member of the *SWI2/SNF2* chromatin remodeling gene family, Shaked et al. (2005) achieved gene targeting frequencies of 3 to 17% in *Arabidopsis*. Another approach employs the zinc-finger modules discussed above for targeted gene repression. In this case, the zinc-finger domain is fused to a nuclease to introduce double-strand breaks at specific genomic sites. In one study, zinc-finger nucleases (ZFN) were expressed in *Arabidopsis* to create breaks that were subsequently repaired by nonhomologous end joining, resulting in site-specific insertion/deletion mutations at frequencies of 2–20% (Lloyd et al. 2005). Using a ZFN to facilitate gene replacement via homologous recombination, Wright et al. (2005) achieved 10% gene targeting efficiency. Both *ZFN* and donor genes had been introduced into tobacco protoplasts via electroporation. In four of seven tobacco plants that were homozygous for the target reporter gene, the desired gene replacement occurred on both chromosomes; such a capability is critical for induction of sterility as loss of function effects are expected to be recessive, and breeding for homozygosity in trees is generally not feasible.

Genetic redundancy further complicates introducing sterility via gene targeting (e.g., both alleles of two or more genes might need to be replaced/mutated). However, replacement of only one allele of one gene with a dominant suppression transgene might be more effective in achieving reliable sterility than random integration of the sterility transgene because it would reduce wild-type gene dosage

and may avoid position effects that can occur with random transgene integration. A key factor limiting the use of gene targeting is ease and efficiency of transformation in the species or genotype of interest. The feasibility of gene targeting is dependent of the combined frequencies of transformation and gene targeting and ease of transformation, regeneration, and selection. *In planta* transformation is routine for *Arabidopsis* and that allows production and screening of a large number of transgenics with little effort; no similar system exists for trees.

One caveat to gene mutation/deletion is that recent studies suggest the possibility that there might be cases where it is not permanent. *Arabidopsis* hothead (*hth*) mutants can inherit allele-specific DNA sequences at multiple loci that were not present in the genomes of their parents, but were present in an earlier ancestor (Lolle et al. 2005). Under certain environmental conditions, varieties of flax exhibit highly specific DNA changes at multiple loci from parents to progeny, including a large insertion that is found in natural populations, but is not present in the genome of the progenitor (Chen et al. 2005). To explain the non-Mendelian inheritance of *hth* mutants, Lolle et al. (2005) proposed that a cache of stable RNA serves as the template for extra-genomic DNA sequence reversion; however, others have posited alternative explanations (e.g., Comai and Cartwright 2005). It is unclear whether this type of reversion could occur somatically in trees (e.g., during vegetative propagation or under certain stressful conditions). Rates of transgene instability under vegetative growth appear to be considerably lower than under sexual reproduction (discussed below).

Repressors of flowering

The activities of some strong repressors of the transition to flowering are directly correlated with their expression level (reviewed in Boss et al. 2004). Thus, constitutive expression or overexpression of a floral repressor in appropriate tissues may be effective at long-term postponement of flowering. Because of the multiple pathways promoting flowering, this approach might delay, rather than prevent, the transition to flowering, but if flowering was delayed until long after harvest age, it still could be an effective biosafety approach. In addition, a floral repressor transgene could be combined with a different sterility transgene, such as one suppressing genes necessary for reproductive organ development, to provide redundancy. Overexpression of a floral repressor might be more likely to induce pleiotropic effects that, as discussed above, might not be apparent until trees are field-tested. Maintaining trees in a purely vegetative phase throughout their rotation cycle, whether by overexpression of a floral repressor, suppression of a floral promoter, or both, is highly desirable because this would completely prevent resource allocation to reproductive structures. However, depending on the tree taxon and environment, development of sterile reproductive structures might not be desirable, if for example, the plantation provides important habitat for birds or beneficial insects that feed on flower parts.

Reproductive gene molecular biology and genomics in trees

Analysis of floral gene homologs

Most published studies of genes controlling flowering in trees have described the isolation and gene expression patterns of homologs of genes known to control various stages of flowering in *Arabidopsis* (e.g., Southerton et al. 1998; Sheppard et al. 2000; Cseke et al. 2003). Results from heterologous overexpression in *Arabidopsis* and tobacco have also been reported, and these studies have usually shown a phenotype similar to that induced by overexpression of the *Arabidopsis* homolog (e.g., Kyozuka et al. 1997; Rutledge et al. 1998; Elo et al. 2001). Functional gene studies of flowering in trees are rare because of the lack of sufficiently efficient transformation systems to produce multiple-event transgenic populations for large numbers of target genes. In addition, the multiple-year nonflowering phase of trees requires long and costly time spans and large areas for field research. *LFY* and *AP1* and tree orthologs of *FT*, which accelerate flowering when overexpressed in *Arabidopsis*, have been shown to induce early flowering in poplar and/or citrus, potentially bypassing the long time delays to flowering (Weigel and Nilsson 1995; Rottmann et al. 2000; Pena et al. 2001; Endo et al. 2005; Böhlenius et al. 2006; Hsu et al. 2006). In some cases, however, the inflorescences have been abnormal or gametes inviable (Rottmann et al. 2000; Hsu et al. 2006); induction of at least some *FT* homologs may bypass this problem (Böhlenius et al. 2006).

Both overexpression and antisense constructs of the silver birch genes, *BpMADS1* and *BpMADS6*, homologs of *SEPALLATA3* and *AG*, were transformed into an early flowering birch genotype (Lemmetyinen et al. 2004b). Although mutant phenotypes were somewhat inconsistent or rare, suppression of *BpMADS1* appeared to cause some inflorescences to partially revert to vegetative shoots, and in two *BpMADS6* transgenics, some male inflorescences lacked stamens, suggesting functions similar to their *Arabidopsis* counterparts. In *PTLF* antisense poplar transgenics that flowered after several years in the field, some male transgenic events produced mutant flowers with homeotic conversion similar to *lfy* mutants (data not shown). Phenotypes were consistent between catkins from

a single transgenic event, but catkins typically displayed a basal to tip gradient with flowers at the tip having a more severe mutant phenotype; thus, basal flowers often produced stamens that were wild-type in appearance. However, in the transgenic event with the most severe mutant phenotype, few flowers with stamens were observed. RNAi transgenes have been reported to be more efficient at inducing suppression than antisense constructs (Wesley et al. 2001), suggesting that RNAi versions of *PTLF* now entering field trials (data not shown) might give a higher rate of sterility both within and between events.

Encouraging results were found with RNAi studies of *PCENL1*, a poplar homolog of the *Arabidopsis* floral repressor, *TERMINAL FLOWER 1*. Transgenic events that showed strong reduction in target endogene expression as determined by qRT-PCR initiated flowering earlier than wild-type in the field (Mohamed 2006); the extent of precocious flowering was significantly correlated with the level of endogene suppression (Fig. 5). These studies suggest that RNAi suppression of orthologs of *Arabidopsis* genes that promote flowering, and do not appear to have any role in vegetative development, can be an effective method for introducing biosafety traits. They also suggest that transgenic events will need to be carefully screened to select lines exhibiting strong suppression. Where vegetative tissue expression is detectable, it should be possible to screen for desirable events during seedling growth, saving years of study and reducing the costs and issues of screening large numbers of field-grown trees.

The extent of overlap in genes and pathways regulating reproductive development in angiosperms and gymnosperms is poorly known. Most studies have focused on MADS-box genes. For example, studies have identified *Picea*, *Ginkgo*, *Gnetum*, and *Cycas* genes belonging to the *AG* subfamily (Rutledge et al. 1998, Shindo et al. 1999, Jager et al. 2003, Zhang et al. 2004). The expression patterns of the gymnosperm *AG* homologs and phenotypes induced by heterologous ectopic expression or complementation of an *Arabidopsis ag* mutant support a conserved function in controlling reproductive organ development. Conifer homologs of the MADS-box B-class floral organ identity genes, the flowering time gene, *SOC1*, and *LEAFY* have also been identified (Tandre et al. 1995, Sundstrom et al. 1999, Mellerowicz et al. 1998, Mouradov et al. 1998). The Norway spruce gene *DAL10* belongs to a MADS-box subgroup that is possibly gymnosperm-specific and is specifically expressed in pollen and seed cones (Carlsbecker et al. 2003). Another spruce MADS-box gene, *DAL1*, belongs to the *AGL6* subfamily and its expression correlates with maturation to the adult or flowering phase (Carlsbecker et al. 2004).

Forward-looking genomics approaches

Although comparative studies indicate that similar genes and pathways control reproductive development in angiosperms and to an extent in gymnosperms, taxon-specific gene duplications and losses, and subsequent subfunction-

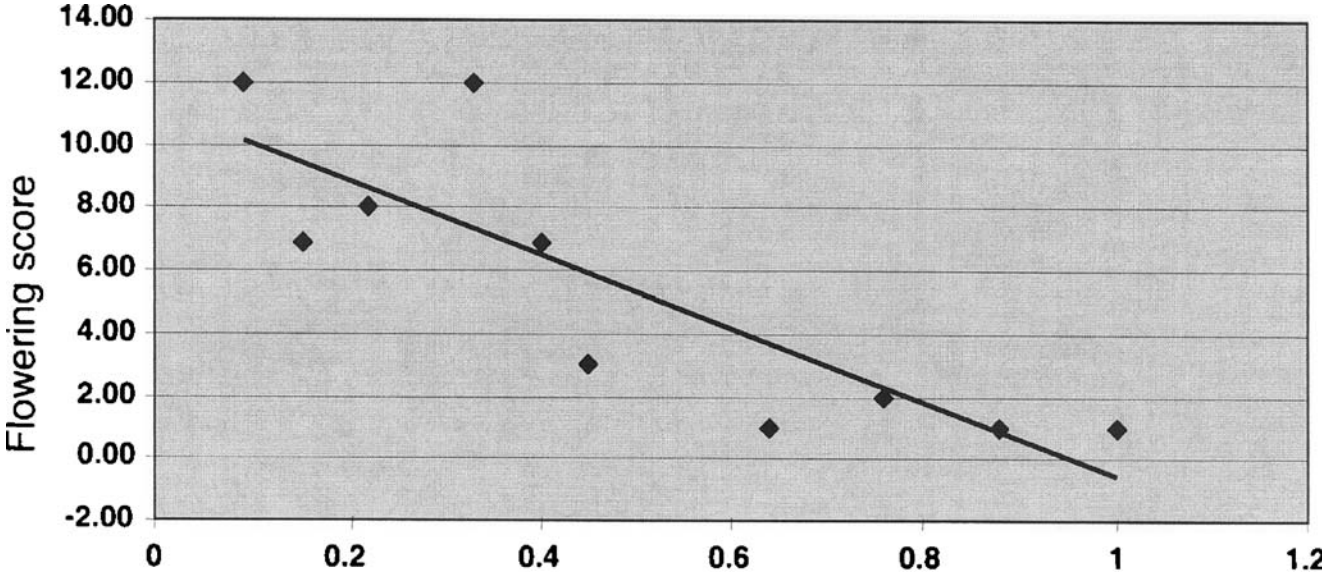


Fig. 5 Association of expression level of native *PCENL1* transcripts and flowering of field-grown *PCENL1* RNAi transgenic trees of poplar clone 717-1B4 (*P. tremula x alba*). Expression was measured by qRT-PCR as described in Fig. 4. Pools of RNA from two ramets per event were used for each assay. Final flower score was estimated as the number of flowering ramets per event × mean number of flowers for each event, rated using a scoring system for each tree (mean for an event) of 0: no flowers, 1:1 to 11 flowers, 2:11 to 30 flowers, and 3: >30 flowers. Only those transgenic events that showed evidence of gene suppression (estimated expression below that of non-transgenic control) were included (r^2=0.71, P<0.01)

alization and neofunctionalization, make predictions of gene function based solely on orthology or expression patterns problematic (Irish and Litt 2005). The poplar genome sequence and an increasing number of large expressed sequence tags (EST) datasets for various tree taxa greatly facilitates identification of tree homologs to various *Arabidopsis* genes regulating flowering and their lineage-specific gene duplications and losses (Brunner and Nilsson 2004). Moreover, the Floral Genome Project (http://www.floralgenome.org/; Albert et al. 2005) and other projects (e.g., Brenner et al. 2005) have developed extensive floral EST datasets from diverse plants including phylogenetically important eudicots, non-grass monocots, basal angiosperms, and gymnosperms. Although many of the floral EST sets are not from trees, comparative floral genomics studies are still informative because tree taxa occur in almost all eudicot orders (Groover 2005). These extensive sequence resources are beginning to reveal patterns of conservation and divergence of families of floral regulatory genes (e.g., Zahn et al. 2006).

Genomic platforms for analyzing gene networks controlling flowering in trees will enable selection of genes and design of sterility strategies with greater precision and effectiveness. Global expression analyses of *Arabidopsis* development, responses to floral induction stimuli, and spatial patterns in flowers of *Arabidopsis* mutants, have revealed tissue-predominant expression patterns and components of gene networks controlling floral initiation and floral organ development (Schmid et al. 2003, 2005; Wellmer et al. 2004). Bioinformatic analyses of co-expressed genes, chromatin immunoprecipitation studies, and comparison of regulatory regions of orthologous genes can identify cis-regulatory elements associated with a particular response or process (e.g., Li et al. 2005, Kreiman 2004, Rombauts et al. 2003). Yeast two-hybrid screens were used to develop a comprehensive interaction map of all *Arabidopsis* MADS domain proteins (de Folter et al. 2005). Combined with global expression analysis, protein interaction studies would be especially useful for selecting genes and sterility methods unlikely to have pleiotropic effects. Similar strategies are beginning to be applied to poplar, and a new USA National Science Foundation Plant Genome Project is studying the transition to flowering in poplar (http://www.poplargenomics.org/). This includes use of overexpression and RNAi poplar transgenics for transcriptome analyses.

Comprehensive study of gene expression is more difficult in trees than annuals due to complex developmental phase changes and increasing size and tissue complexity across years. We have observed that some genes showing floral-predominant expression in poplar show levels of vegetative expression that vary in intensity across an annual cycle of growth and dormancy (data not shown). Furthermore, trees are exposed to very variable abiotic and biotic conditions over many years that can markedly affect gene expression. For example, galling insects appear to induce ectopic organ developmental programs that are similar to reproductive development; *LEAFY*, *AP1*, and C-class MADS-box genes directing carpel development, but not B-class genes, are expressed during development of galls on grape vine leaves (J.C. Shultz, personal communication). This is especially problematic for ablation sterility systems where selection criteria for appropriate promoters are most stringent.

In addition to not having complete genome sequences, studies in most tree taxa are generally limited by lack of efficient transformation systems. Development of VIGS vectors for trees could be particularly valuable for studying genes controlling flowering. A VIGS vector has recently been developed for poplar (Naylor et al. 2005), but unfortunately, a poplar genotype that reliably flowers in the greenhouse in the absence of *FT* overexpression is not currently available. Some other tree species, such as eucalypts and apple, can be reliably induced to flower via use of plant hormones and cultural treatments.

As tree genomics tools and knowledge of candidate genes for flowering advance, it should be possible to clone genes that control onset of flowering using high-resolution quantitative trait locus (QTL) or association genetics approaches. This approach potentially allows discovery of mechanisms of reproductive development that are unique to trees, rather than relying on studies of herbaceous annual model plants for target gene identification. Liebhard et al. (2003) reported QTLs for juvenile phase in apple. Missiaggia et al. (2005) identified a QTL for very early flowering in eucalypts. For these studies, it will be essential to have large populations ready that include segregants with rare precious flowering. To prevent flowering, these genes could then be suppressed or mutated, as discussed above.

Stability of transgene expression

It is well-known that newly produced transgenic plants often exhibit instability in expression of transgenes, related endogenes, and their encoded traits. It is also widely known that the level of instability varies widely among constructs, species, and gene transfer methods. However, after field screening, gene insertion events with strong and stable expression are generally identified, and these are the ones that are focused on during research and commercial development. The ability to identify highly stable transgenic events has been firmly established by the hundreds of millions of hectares of GE crops that have been grown by farmers, which contain a variety of genetic constructs in a variety of genotypes and species. These include commer-

cialized trees (papaya, poplar), with traits induced via RNAi (papaya, tomato, squash) and with conventional transgene expression.

Questions remain, however, about the long-term stability of specific traits in vegetatively propagated crops, including containment traits and to what extent stable expression can be identified and delivered in an efficient manner in breeding programs with transgenics. It is also unclear how strong and stable a sterility phenotype must be to confer an adequate level of containment. A high level of stability of a leaf-expressed gene for herbicide resistance, imparted by genes derived from other species, does not guarantee that a native gene designed to suppress a floral meristem identity gene via RNAi will be sufficiently reliable for stringent, long-term containment goals. Because of the importance of stability of gene expression for genetic containment in trees, we review both what has been learned from studies in other vegetatively propagated crops, and then in the following section, consider how a modeling approach can help to identify how much trait instability (i.e., reversion to fertility) might be biologically acceptable.

Due to the long life cycles of forest trees and the complex environments they experience, stability of expression of GE-introduced traits in trees have received considerable debate (Fladung 1999; Hoenicka and Fladung 2006a). In addition, possible genome instability due to effects of the gene transfer process and interaction with plant genome sequences, adds to scientific uncertainties about long-term performance of primary transformants in the field. In an AFLP study with four *Agrobacterium*-transformed aspen transgenic lines carrying a *rolC* gene, 886 out of 889 (99.9%) of the amplified bands were common between the control and transgenics—suggesting very limited GE-associated genomic change compared to extensive wild AFLP polymorphism in poplar and most other tree species (reviewed in Hoenicka and Fladung 2006b). In agronomic crops, it also appears that genomic variation imparted by transformation is modest compared to the extensive genomic variation present in traditionally bred and wild plants (Bradford et al. 2005).

A number of factors have been implicated in transgene silencing, including insert number, chromosomal environment (position effect), T-DNA structure, environmental stress, and endogenous factors (Table 3). Unfortunately, most of these factors do not seem to be consistent predictors of long-term stability. For example, there appears to be little association between insert number and instability, even though single-copy transgenes are widely assumed to be important for obtaining stable gene expression. Where transgene structure was studied, however, instability was often associated with transgene repeat structure, truncation, or other rearrangements at or near transgene insertion sites (Table 3).

Transgene stability under vegetative propagation has been studied in poplar, citrus, tall fescue, sugarcane, chrysanthemum, and potato. Transgene expression appears far less stable over sexually propagated generations than over vegetatively propagated generations (Table 3). Unfortunately, most studies have used a small number of transgenic events (<20), and thus, are of limited relevance to commercial transformation and breeding programs—which often screen many dozens or hundreds of events. Moreover, many of the published studies on stability of transgene expression have focused on unstable events observed in preliminary screens, and are thus biased with respect to the levels of instability expected in commercial programs.

In a study similar to what a tree breeding program might address, Meilan et al. (2002) reported high stability of herbicide resistance genes in 40 independent poplar transgenic events over 4 years in the field. Hawkins et al. (2003) reported stable expression of a *GUS* reporter gene in 44 independent poplar transgenic events over a period of 6 years under in vitro, greenhouse, and field conditions. Histological *GUS* analysis in 70 transgenic events showed similar patterns of *GUS* expression over a period of 4–5 years in citrus (Cervera et al. 2000). In contrast, in a study of 22 transgenic events carrying the morphological marker gene, *rolC*, phenotypic alteration or reversion was observed for up to one-third of the events during vegetative growth in either in vitro, greenhouse, or field conditions (Kumar and Fladung 2001). In biolistically transformed pine, Wagner et al. (2005) reported that the level of silencing of a *CAD* gene during embryogenic propagation was associated with expression level.

Variation in stability of transgene expression among studies can result from uncontrolled differences in experimental protocols, as demonstrated by James et al. (2004). Because native and introduced genes show stochastic (Raser and O'Shea 2004) and developmental variation in expression, it is important to pick a suitable control gene. For example, the strong and deleterious effects of variable expression of the *rol*C gene discussed above might be similar to the normal variation that occurs with many endogenes and transgenes, but its gene product is so powerful and toxic that its effect on development is amplified. In contrast, no such consequence, nor possibly any phenotypic effect at all, would be expected for similar levels of variation in a transgene encoding insect or herbicide resistance.

We have performed three stability studies using different transgene constructs (unpublished data). In one study, the *BAR* herbicide resistance gene was transferred into two poplar clones, and 32 transgenic events produced. The expression of the *BAR* gene was monitored on 384 plants over a period of 8 years of repeated coppicing in the field.

No instability or loss of the initial resistance phenotype was observed based on visualized herbicide damage and protein ELISA assays. In another study, the reporter genes *GFP* and *BAR* were assembled in the same binary vector, and transferred into two poplar clones. The expression levels were measured on 2,256 transgenic poplar trees generated from 404 independent transgenic events over 3 years in the greenhouse and the field. The expression of both genes was highly stable over 3 years, with no cases of gene silencing observed. However, the physical loss of transgene sequences was observed in three of the 80 transgenic events after they were regenerated via a second round of organogenesis in tissue culture.

In a third study, we examined the stability of RNAi silencing of a resident *BAR* gene in transgenic poplars that had been retransformed with inverted repeats (IR) of either a section of the coding sequence or the promoter sequence of the *BAR* gene. RNAi silencing efficiency and stability were studied in 56 RNAi transgenic events over 2 years in the field. The results suggested that dsRNA of the *BAR* coding sequence was highly efficient in suppressing *BAR* expression; 80% of the events showed more than 90% gene suppression. However, dsRNA of the *BAR* promoter sequence was much less efficient; only 6% of the events showed more than 90% suppression. Most importantly for gene containment, the degree of RNAi suppression appeared to be stable for both constructs over 2 years (Fig. 6). These studies, plus the reporter gene studies described above, suggest that instability of gene expression may only rarely be a problem in vegetatively propagated trees, though longer-term studies are desirable.

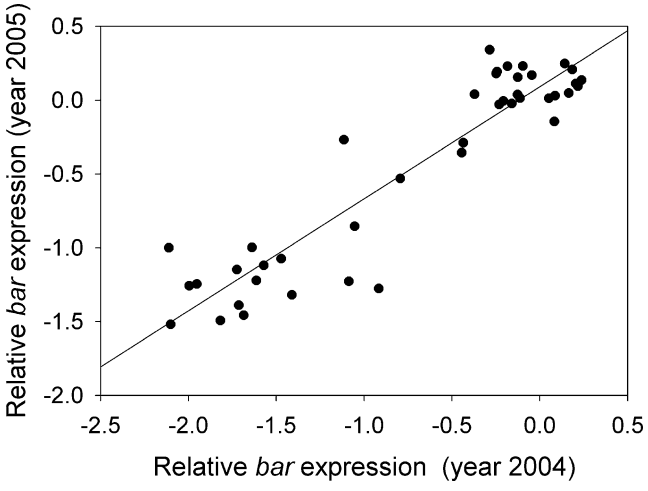


Fig. 6 Correlation of RNAi suppression in shoots of field grown trees between year 2004 and year 2005. Expression of the targeted *bar* transgene for 42 gene insertion events were quantified with real-time RT-PCR (ubiquitin gene used as internal control) and then expressed relative to that of the parent transgenic genotypes and log transformed (r^2=0.47, p<0.001)

Sterility as a quantitative trait: how much do we need?

Complete prevention of sexual reproduction with 100% certainty is a daunting technical and social challenge. The long time frames and large numbers of potential reproductive meristems in transgenic tree plantations provide many opportunities for reversion to fertility, such that rare events become probable. Furthermore, transgenic approaches to sterility will incur added economic and regulatory costs and social resistance (discussed above). It is therefore critical to define if sterility is needed at all for biological or social reasons, and if so, what level and form is required. However, there does not seem to have been any serious field studies, *in any crop*, sufficient to estimate the operational effectiveness of containment genes (Ellstrand 2003). Until many such studies are published, it would be unwise to assume that genes can be fully and safely contained in the near future. Conventional approaches to fertility reduction, including the use of hybrids or aneuploid germplasm (Bradshaw and Stettler 1993), also generally do not provide complete containment. However, they could provide an option for deployment of some transgenes in breeding programs that use ploidy-modified trees. However, such genotypes are rare in most forest tree breeding programs.

Poplar and some other tree species are capable of dispersal and establishment of vegetative propagules, thereby potentially bypassing most containment measures based on sexual sterility. Though local spread from plantings can usually be managed, some degree of long distance vegetative spread can occur through adventitious rooting from broken or abscised branches (Rood et al. 2003). If transgene containment is an important goal, it is important to explore the consequences of all of the different modes and levels of reproduction under realistic ecological scenarios. This is best addressed in the context of a risk assessment and is facilitated by the use of ecological modeling.

Risk assessment includes hazard identification, exposure assessment, consequence assessment, risk characterization, and delineation of mitigation options (Hill 2005). Risk from transgene dispersal is sometimes treated as synonymous with the exposure portion of the process, and demonstrations of potential distributions of transgenic propagules are treated as examples of the inherent risks of forest biotechnology (e.g., Williams 2005). However, the mere presence of transgenic propagules does not automatically constitute a negative endpoint (Stewart et al. 2003). Production and dispersal of transgenic seed and pollen constitute the first steps in a network of processes contributing to introgression of transgenes to wild populations. Even with the extensive dispersal distances expected for forest trees (Nathan et al. 2002), realized transgene introgression could still be extremely low due to sexual

incompatibility with wild trees, lack of availability of safe sites for establishment, negative fitness effects of transgenes or domestication genes in a wild setting, and extensive dilution from non-transgenic planted and wild stands (Pilson and Prendeville 2004; Hails and Morley 2005). As discussed above, transgene dispersal could also have large net ecological benefits.

Trees create special challenges for generating the data necessary for assessing potential introgression. Very large temporal and spatial scales must be considered for movement of tree pollen and seeds (Nathan et al. 2002; Smouse and Sork 2004). Furthermore, long-distance gene flow is a disproportionately important determinant of rates of spread of introduced organisms or genes (Higgins and Richardson 1999), and this process is subject to stochastic influences that make accurate measurement extremely challenging, if not impossible (Clark et al. 2003). This difficulty is magnified when one considers the network of interacting, highly variable factors that determine establishment and spread in wild systems (Parker and Kareiva 1996; Pilson and Prendeville 2004). Therefore, realistic, replicated experiments cannot be performed at appropriate scales and time frames for predicting introgression of transgenes (Parker and Kareiva 1996). However, data from non-transgenic populations can be used in simulations to provide useful estimates of what is likely to occur under various deployment situations and environments (Dunning et al. 1995; Pilson and Prendeville 2004).

Simulation approaches have been used successfully to investigate factors affecting the spread of transgenic insect-resistant oilseed rape varieties (Kelly et al. 2005) and to investigate factors affecting fitness of transgenic fish with enhanced growth (Howard et al. 2004). However, many of these kinds of studies have not taken into account realistic spatial distributions of transgenic organisms on the landscape relative to wild and managed habitats. The spatial dimensions of gene flow are an essential component of introgression because habitat availability and competition from wild relatives are likely to be two of the primary factors inhibiting spread of partially fertile transgenic trees, and these will be determined by management regimes and locations of wild populations on the landscape.

Many different types of models have been used for simulating dispersal and gene flow across a landscape (Nathan et al. 2003). One approach is to devise mechanistic models of pollen and seed dispersal based on the physical properties of the propagules and the environment (Katul et al. 2005; Nathan et al. 2002; Clark et al. 2003). Such models have a distinct advantage in that they are easily parameterized for a large number of species because flight characteristics of pollen and seeds are readily measured, detailed microclimatic data can be obtained for many sites, and the physics of dispersal by abiotic agents are fairly well characterized. Disadvantages include the large number of parameters that require estimation (particularly if realized gene flow is to be modeled) and the high computational requirements that limit the extent of the area and time frame that can be modeled (Nathan et al. 2002).

An alternative approach is to model gene flow phenomenologically based on field observations of dispersal and demographic processes. A common method is to use reaction-diffusion models to depict the movement of an 'invasion front' using a diffusion approximation and logistic growth models (Fisher 1937; Shigesada and Kawasaki 1997). Alternatively, probability density functions of propagule movement and/or reproductive success can be used to determine the probability of dispersal between points on a lattice of habitat cells (Higgins and Cain 2002; Lavorel et al. 1999). This approach has the advantage of being easily parameterized from historical data (e.g., a chronosequence of air photos or survey data) and readily integrated with geographic information systems (GIS). A major disadvantage is the difficulty of measuring contemporaneous realized gene flow on appropriate space and time scales to parameterize the models.

As an example of the latter approach, we developed a spatially explicit model of gene flow from hybrid poplar plantations based on observations of realized gene flow in wild populations (DiFazio 2002, Slavov et al. 2004). The model, called Simulation of Transgene Effects in a Variable Environment (STEVE), was applied to a landscape grid in northwest Oregon (23 km×37 km, 100 m^2 cells) containing information about elevation, habitat type, and poplar populations. The simulation has an annual time step, with modules to simulate creation and conversion of poplar patches, growth, reproduction, dispersal, and competition within poplar cohorts. The primary objective of this model was to produce a framework for virtual experiments that could accommodate the diverse silvicultural, agronomic, and ecological settings in which transgenic trees might be released, and to incorporate many different types of transgenic traits.

The findings of the STEVE model most germane to discussions of reproductive sterility come from simulations with different levels of innate fertility of transgenics and with various probabilities of reversion to fertility. Relative pollen production was calculated for each genotype within each sexually mature cohort of trees in each poplar cell. Representation of pollen and seed was entirely relative because the most important quantity is the ratio of transgenic to conventional genotypes in the propagule pools. Therefore, pollen production was directly proportional to the basal area of each genotype in a particular location on the landscape.

Relative fertility varied annually based on a user-defined standard deviation determined from annual field

observations of flowering in plantations. In addition, transgenics with reduced fertility could have their fertility partially restored according to a user-defined probability. Vegetative propagule production was also stochastic and proportional to basal area. Pollen was dispersed within the immediate vicinity of male trees and across the landscape according to empirically determined dispersal kernels (Slavov et al. 2004), and transgenic and conventional seed production was determined by the proportion of pollen of each genotype dispersed to female trees, modified by relative fertility factors.

As expected, fertility of transgenic trees had a strong effect on rate of gene flow from transgenic plantations. With highly reduced fertility, gene flow was at some of the lowest levels observed for all scenarios tested: between 0.1 and 0.2%, compared to approximately 5% for fully fertile transgenic plantations. In addition, transgene flow rates were not distinguishable within the range of 0 to 1% of wild fertility, indicating that complete sterility was not required to attain maximum gene containment (Fig. 7a). Thus, the reductions in fertility of approximately 10^5 that we have observed in the field (Fig. 1) would appear to be far in excess of the level needed for effective mitigation in this scenario. (In practice, only the pollenless events might be chosen for commercial purposes.) The low level of gene flow that we observed for fully sterile plantations was due to movement of vegetative propagules in the vicinity of plantations. However, transgenic gene flow remained very low under a wide range of rates of vegetative establishment (Fig. 7b), and gene flow rates were insensitive to changes in rates of vegetative establishment and shapes of vegetative dispersal curves (data not shown). Sexual fertility was therefore much more important than vegetative establishment in controlling gene flow in this system. Nearly 50% of the gene flow with low-fertility transgenics (fertility <0.1) was due to sexual reproduction, as demonstrated by simulations with vegetative establishment eliminated (Fig. 7b).

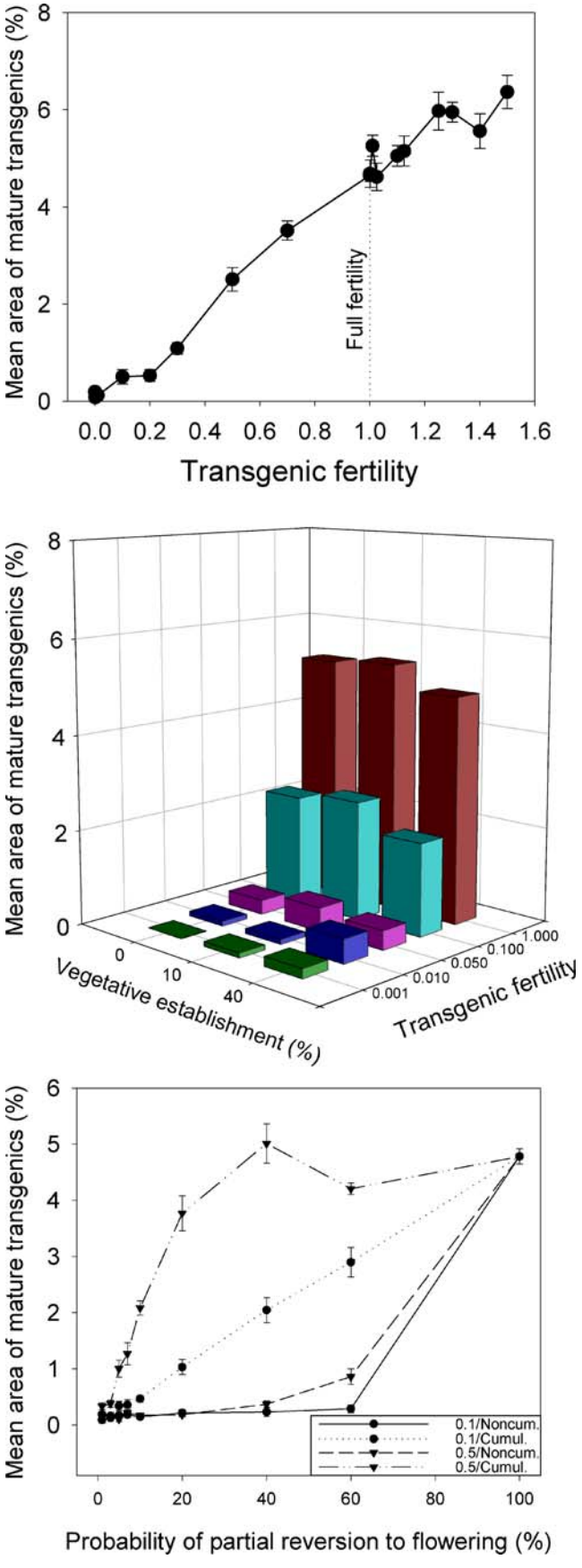
Other investigations have also identified fertility as a major factor limiting plant spread. For example, a reduction of fertility of as little as 75% was projected to limit the spread of scotch broom (*Cytisus scoparius* L), based on insect-protection assays and simulations (Rees and Paynter 1997). Density of pines spreading from plantations in South Africa was sensitive to fecundity and age of reproductive maturity in spatially explicit simulations (Higgins et al. 1996). Spread of feral oilseed rape was hypothesized to be limited by seed input based on patterns of establishment along shipping (i.e., dispersal) routes (Crawley and Brown 1995), and simulation modeling implicated seed viability as a major factor limiting spread of transgenic oilseed rape (Kelly et al. 2005). Therefore, the effectiveness of partial sterility in attenuating gene flow is not surprising, but the model is useful in demonstrating the importance of different modes of reproduction (vegetative vs various degrees of sexual reproduction).

The model was also useful for exploring implications of unstable sterility. We simulated this by allowing some restoration of fertility for trees that began the simulations with highly reduced fertility (fertility level of 0.01 compared to wild-type trees) (Fig. 7c). These simulations had three important parameters: the probability of reversion to fertility (sampled from a normal distribution), the level of fertility restoration for each reversion event (10 or 50%, sampled from a normal distribution), and the duration of the restoration (cumulative or permanent restoration vs noncumulative or transient restoration, with reversion to the original fertility level each year). With a permanent restoration level of 50% per reversion event, a 20% probability of reversion was required for gene flow levels to approach those of fully fertile trees. With a permanent restoration level of 10%, gene flow was considerably less than full fertility, and this was true even with reversion rates as high as 60%. Gene flow with reversion rates up to 3% were nearly indistinguishable from that of trees with stable sterility. If reversion was not cumulative (i.e., fertility was reset to 0.01 each year for each tree), gene flow was still greatly reduced compared to wild trees and was marginally greater than for trees with stable sterility. These results were manifested across a broad range of probabilities of reversion. Reversion rates that we have observed under vegetative propagation for transgenic *Populus* (reported above) appear to be considerably below the rates required for significant effects on modeled transgene flow. In addition, such high rates of reversion would likely be detected with moderate pre-commercial screening and post-release monitoring efforts. The simulations discussed above dealt with sterility in relation to spread of neutral transgenes. Transgenes that enhanced the competitiveness of trees in wild settings caused greatly enhanced gene flow for fully fertile transgenic trees, but a tightly linked sterility gene was very effective at attenuating spread, even in the face of a strong selective advantage and incomplete sterility (DiFazio 2002).

Conclusions

> *"In theory, there is no difference between theory and practice. In practice, there is."* Andrew S. Tannenbaum, TIGR (The Institute for Genomic Research)

There are many genes of interest for commercial purposes that are likely to present very low risks, either because they are very similar to native genes, because they



◀**Fig. 7** Simulated effects of transgenic fertility on transgene flow based on the STEVE model (DiFazio 2002). Simulations were conducted over a 50-year time period, and gene flow was indexed by the proportion of 100 m^2 *Populus* cohorts greater than 10 years of age that contained at least one transgenic tree outside of plantations (Mean Area of Mature Transgenics). Responses were averaged over the final 25 years of the simulation to simplify presentation of results (responses stabilized by age 25 for the simulations shown). **a** Effects of fertility of transgenic trees relative to non-transgenics. **b** Interaction between vegetative establishment and fertility. Vegetative establishment is the proportion of established individuals in a new cohort that are derived from vegetative propagules. Variation in vegetative establishment had little overall effect on transgene flow, although a minor effect is apparent at low levels of fertility. **c** Effects of unstable sterility on transgene flow. Probability of sterility breakdown is the probability of a reversion to fertility (*x*-axis), which is then restored with a fertility level of 0.1 or 0.5, sampled from a normal distribution with a standard deviation of 0.05 or 0.25, respectively. Reversion was permanent and cumulative (Cumul.) for each tree through time, or fertility was transient and reset to the original value each succeeding year of the simulation (Noncum.). Low values of instability had little effect on gene flow; a cumulative reversion rate of about 20%, with 50% fertility restoration, would be required for gene flow levels to approach those of fully fertile transgenic trees

will reduce fitness or be neutral in the wild, or because their benefits outweigh their detriments. On the other hand, there may be crops, such as forms of bioindustrial crops that encode novel and potentially ecotoxic compounds, for which very strong biological containment would be clearly warranted. Nonetheless, the loudest social resistance seems to focus not on the products, traits, and their benefits vs risks, but on *perception* of "contamination" by GMOs generally. Indeed, because of the long-known propensity for long distance movement of pollen and/or seed from most tree species, if *complete* containment is the *social* goal, there is unlikely to be any place for GE trees in forestry plantation or horticulture—at least not for many decades. The technologies and simulations presented assume that some level of transgene dispersal could be socially and biologically acceptable—much like dispersal of new or modified genes and chromosomes introduced by breeding continues to have high social acceptance.

It has often been said that plant sterility should be an easy trait to engineer; after all, there are dozens of ways to damage a motor so it does not work. Unfortunately, motors do not have the redundancy and resilience of biological systems that have evolved to reproduce "at all costs," nor do vandalism-leaning auto mechanics face the large biological and social obstacles that researchers and companies do when trying to conduct field-relevant research with GE trees. To arrive at efficient, reliable, effective sterility systems, we make the following suggestions:

1. *Functional genomics in trees*. Much more basic functional genomics is required in model taxa that

represent the major forestry species. In this research, the main candidate genes based on studies in *Arabidopsis* and other model plant species, combined with newly discovered genes from trees identified in QTL, EST, or microarray studies of trees, would be repressed or overexpressed and their functions identified in the field or the greenhouse, hopefully under conditions of accelerated flowering. This should allow the most important genes and promoters to be identified, thus, informing efforts to combine genes in redundant, reliable systems. It is hoped that inducible systems that make use of the *FT* gene might provide the much needed acceleration in production of normal flowers (Böhlenius et al. 2006).

2. *Transformation technology improvements*. Gene transfer, gene targeting, and highly specific recombinase technology needs to be greatly improved if mutagenesis of floral genes, and efficient addition or removal of sterility genes in many genotypes, is to become feasible. This requires much basic research on innovative transformation, excision, and homologous recombination methods—first in model plant species; but then, considerable work will be required to transfer these systems to trees.
3. *Regulatory and intellectual property constraints*. Candidate sterility cassettes based on the results of suggestions 1 and 2 need to be designed to meet regulatory standards and have freedom to operate with respect to intellectual property. They must then be tested in a diversity of commercially relevant environments and genotypes for stability and pleiotropic effects. These should be combined with predictive assays where possible to enable their effectiveness and pleiotropy to be forecast from a young age. The current "anti-commons" (Boettiger and Bennett 2006), where the licenses for each genetic and construct element, and basic transformation technology, are owned by parties different from those bearing the costs and risks of this long-term research, appear to provide large disincentives to moving forward. High regulatory and licensing costs and market stigmas impede the "adaptive management" approaches so common in forestry (where research and commercial development go hand-in-hand, a result of the high costs and long time frames for forestry research).
4. *Transparency*. Containment research, due to its cost, long-time frame, and high level of scrutiny from society, should ideally be conducted by non-commercial third parties. A similar model is applied for all environmental research by Weyerhaeuser Company because of the need for independent validation of results for social acceptance (P. Farnum, personal communication). It is doubtful that company-based research, where only selected results are presented to the public, will be trusted, yet this model continues to be followed by some biotechnology companies. Ironically, the "eco"-vandalism that is still common in Europe, and continues to be a concern in the USA, limits the extent to which the details of field and laboratory research can be safely disclosed. It appears that both vandalism risks to companies and Forest Stewardship Council exclusion of GE trees from field trials—both motivated by ecological concerns over appropriate uses of forest biotechnology—are delaying, rather than promoting, the development of ecologically sound GE technologies.

Because of the rapid rate of growth of genetic information and technological innovations, we believe that highly efficient containment systems can be developed and their reliability established. Without such systems, which will require testing over many years, it appears that many kinds of transgenes may never obtain regulatory or social approval in many countries—greatly limiting the benefits that transgenic biotechnologies are likely to be capable of providing.

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