

## 3. Genetic containment of forest plantations

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“It is essential that new molecular gene-containment strategies... be developed and introduced.”

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### 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 trees (reviews in Strauss and Bradshaw, 2004). Genetically engineered, genetically modified or transgenic organisms, as used in this review paper, are defined as those that have been modified using recombinant DNA and asexual gene transfer methods – regardless of the source of the DNA employed.

Forest certification systems represent a growing mechanism for expression of social preferences in the marketplace (Cashore, Auld and Newsom, 2003). One major forestry certification system aimed at environmental and social compliance, that of the Forest Stewardship Council, bans all forms of genetically engineered 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 genetically engineered organisms (Snow *et al.*, 2005). It shows that social considerations can overwhelm technical innovations.

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Thus, containment systems may be required even for genes where no significant biological impact, or even a positive environmental effect, is 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 co-exist.

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 genetically engineered 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 with long-lived and open-grown trees. The powerful inverse association between forest stand density and degree of tree reproduction is widely known (Daniel, Helms and Baker, 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 genetically engineered 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 genetically engineered trees are great and occur for a number of reasons:

- laboratory cost of genetically engineered tree production, including production and study of many kinds of gene constructs and gene transfer events;
- ecological complexity in space and time and high stochastic variance in gene flow and related ecological processes, requiring many sites, environmental conditions, long time frames and large spatial scales;
- 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);
- cost of record keeping and compliance with regulations, which can be very demanding and legally risky for complex programmes that span many years and sites;
- 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;
- 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 non-commercial but sterile genotypes, or use of geographically distant planting environments);
- 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 genetically engineered 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 genetically engineered 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 Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA), which regulates all field research in the United States of America, 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 genetically engineering-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 genetically engineered 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 endeavour. 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, Ives and Andow, 2003). Obtaining licences to the set of patents that cover all of the elements of the best containment technology can also be very costly or impossible. At the same time, 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 vigour 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 AND 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 time of writing) scientific and patent (United States Patent and Trademark Office (US PTO)) literature and presented representative examples of developments. Tables 3-1 and 3-2 summarize the kinds of approaches being taken, nearly all of which are relevant to one kind of tree species or another.

TABLE 3-1

### Selected literature on genetic engineering of sterility published from 2000 onwards

Phenotype	Mechanism	Promoter	Active gene	Plant species	Reference
<b>Delayed flowering</b>					
Late flowering	Overexpression of <i>FLM</i>	35S CaMV	<i>Flowering Locus M</i>	<i>Arabidopsis</i>	Scortecci, Michaels and Amasino, 2001
	<i>AGL20</i> /shoot apical meristem	35S CaMV	<i>AGAMOUS LIKE 20</i>	<i>Arabidopsis</i>	Borner <i>et al.</i> , 2000
<b>Cell ablation</b>					
Male sterility	Altered pollen development	Endosperm specific promoter, <i>AGP2</i>	Fission yeast <i>cdc25</i>	Wheat	Chrimes <i>et al.</i> , 2005
	Pollen sterility	Rice tapetum promoter ( <i>TAP</i> )	Barnase/rice tapetum gene <i>rts</i>	Creeping bentgrass	Luo <i>et al.</i> , 2005
	Alteration in tapetal cells	<i>Tapetum A9</i> promoter	Chimeric gene in transgenic plant	<i>Arabidopsis</i>	Guerineau <i>et al.</i> , 2003
	Abnormal pollen	<i>BcA9</i>	<i>DTx-A</i>	<i>Brassica</i>	Lee <i>et al.</i> , 2003
	Tapetal dysfunction	<i>TA29</i> promoter	<i>RIP</i>	Tobacco	Cho <i>et al.</i> , 2001
	Reduced pollen viability	Pollen specific promoter <i>G9</i>	Chimeric genes <i>G9 uidA</i> and <i>G9-RNase</i>	Tobacco	Bernd-Souza <i>et al.</i> , 2000
Male and female sterility	Floral organ ablation with otherwise normal growth	<i>PopulusPTD</i>	<i>DTA</i>	Tobacco, poplar, <i>Arabidopsis</i>	Skinner <i>et al.</i> , 2003
Recoverable block of function (RBF)	Inducible fertility	Sulfhydryl endopeptidase, heat-shock promoter	<i>Barnase</i> (the blocking construct) and <i>barstar</i> (recovering construct)	Tobacco	Kuvshinov <i>et al.</i> , 2001
<b>Gene suppression</b>					
Male sterility	Distorted pollen morphology	Various	<i>AtMYB32 AtMYB4</i>	<i>Arabidopsis</i>	Preston <i>et al.</i> , 2004
	Temperature sensitive male sterility due to silencing choline biosynthesis	S-adenosyl-L-methionine	Phosphoethanolamine <i>N</i> -methyltransferase (PEAMT)	<i>Arabidopsis</i>	Mou <i>et al.</i> , 2002
	Mitochondrial dysfunction	Tapetum specific promoter	Antisense pyruvate dehydrogenase E1 $\alpha$ subunit	Sugar beet	Yui <i>et al.</i> , 2003
	Abnormal pollen	<i>Nin88</i> promoter	Antisense <i>Nin88</i>	Tobacco	Goetz <i>et al.</i> , 2001
	Abnormal pollen	Glutenin subunit gene promoter	Antisense sucrose non-fermenting-1-related (SnRK1) protein kinase	Barley	Zhang <i>et al.</i> , 2001
Restoration of fertility	Glucanase gene suppression	<i>pA9</i>	Sense and antisense PR glucanase	Tobacco	Hird <i>et al.</i> , 2000

TABLE 3-2  
Selected patents on genetic engineering of sterility published from 2000 onwards

Phenotype	Mechanism	Promoter	Active gene/Protein	Species	Reference
<b>Time of flowering</b>					
Altered floral development	Expression of floral meristem identity protein	Modified native promoter	<i>CAULIFLOWER (CAL)</i> , <i>APETELA 1 (API)</i> , <i>LEAFY (LFY)</i>	Angiosperm or gymnosperm	Yanofsky, 2000
<b>Cytotoxin ablation</b>					
Suicide gene to ablate gamete	Any of several cytotoxic genes expressed in gametes	Male- or female-specific promoter expressed in gamete	Various "suicide" genes ( <i>barnase</i> , <i>tasselseed2</i> , <i>diphtheria toxin A</i> )	Rice	Dellaporta and Moreno, 2004
Female sterility	Enhance fruit development or induce sterility	<i>DefH9</i> promoter	DNases, RNases, proteases, glucanases, lipases, toxins, etc.	Many	Spena <i>et al.</i> , 2002
<b>Gene suppression</b>					
Male sterility	Calcium/calmodulin-dependent protein kinase (CCaMK) expression	Developmental stage-specific anther promoter	Antisense RNA	Tobacco	Poovalah, Patil and Takezawa, 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	<i>Arabidopsis</i> , tobacco	Dirks <i>et al.</i> , 2001
Male sterility	Suppression of <i>ATH1</i> gene to control flowering time	35S CaMV	Antisense <i>ATH1</i>	<i>Arabidopsis</i>	Smeekeens, Weisbeek and Proveniers, 2005
Delayed flowering time	Loss of function of <i>SIN1</i> 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
<b>Floral promoters</b>					
Male sterility	Anther development-specific genes and promoters	Tapetum, pollen	Antisense RNA or any gene that compromises pollen viability	<i>Brassica</i> , <i>Arabidopsis</i> , tobacco	Knox, Singh and Xu, 2004
Female sterility	Regulatory region of corn silk/pistil genes	C3 promoter	Silk-specific gene, C3	Maize	Ouellet <i>et al.</i> , 2003
Restoration of fertility to cytoplasmic male sterile plants	Wild-type <i>atp6</i>	<i>AP3</i> promoter	Wild-type <i>atp6</i> gene fused to a mitochondrial transit peptide	<i>Brassica</i>	Brown, 2002
Conditional male sterility	Upon application of acetylated toxin	Stamen-selective promoters	Deacetylase	Wheat	Quandt, Bartsch and Knittel, 2002
Male and female sterility	Poplar floral homeotic genes and promoters	Native promoters	<i>PTLF</i> , <i>PTD</i> , <i>PTAG-1</i> , <i>PTAG-2</i>	Poplar	Strauss <i>et al.</i> , 2002
Male sterility	Recessive mutant causes sterility	<i>Ms41-A</i> promoter	<i>Ms41-A</i>	<i>Arabidopsis</i> , maize,	Baudot <i>et al.</i> , 2001
Male sterility	Absence of a functional callase enzyme	<i>MsMOS</i> promoter	<i>msMOS</i>	Soy	Davis, 2000

TABLE 3-2 (CONTINUED)

Phenotype	Mechanism	Promoter	Active gene/Protein	Species	Reference
<b>Protein interference</b>					
Reversible male sterility	Dominant negative genes under anther-promoter reversed by expression of a repressor	Anther-specific promoter and <i>lexA</i> operator	Any cytotoxic methylase or growth-inhibiting gene	Maize	Cigan and Albertsen, 2002
Cytoplasmic male sterility	ATP synthesis in mitochondria inhibited	Ubiquitin promoter	Unedited <i>Nad 9</i> gene	Rice, wheat, maize, soybean	Patell <i>et al.</i> , 2003
Male sterility	Biotin-binding polypeptide ablates male gamete tissue, fertility can be restored	Promoter regulated by the <i>LexA</i> operon expressed in anther	Botin-binding polypeptide and inhibitory proteins	<i>Arabidopsis</i> 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 <i>et al.</i> , 2001
Male sterility	Protein that disturbs metabolism, development and gene for reversibility	Stamen-specific promoter	A sterility RNA, protein or polypeptide	<i>Brassica</i> , maize, rice	Michiels, Botterman and Cornelissen, 2000
<b>Mitigation</b>					
Male sterile and dwarf	Unknown	Native promoter	<i>df11</i> gene	Safflower	Weisker, 1995
Dwarf plants	GA insensitive	Native promoter	Mutant of <i>GA1</i>	<i>Arabidopsis</i>	Harberd <i>et al.</i> , 2004
Dwarf plants	Rht mutant dominant allele causes GA-insensitivity	Native promoter	Mutant of <i>Rht</i> (D8)	Rice	Harberd, Richards and Peng, 2004

There are five major approaches to containment. One approach, mitigation (e.g. Al-Ahmad, Galili and Gressel, 2004), is a directed form of plant domestication such that the fitness benefits of transgenes are effectively cancelled 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, Helms and Baker, 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 3-3 (Harberd, Richards and Peng, 2004; Harberd *et al.*, 2004), 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 genetic engineering approaches: ablation, where floral tissues are effectively destroyed or made non-functional



TABLE 3-3  
Summary of studies on stability of transgene expression in plants

Taxa	Gene	Number of events (unstable) <sup>1</sup>	Environment	Propagation	Generations or years	Associated factors	Non-associated factors	Reference
Chrysanthemum	<i>35S-gus</i>	17(0)	Greenhouse	Vegetative	1 generation			Pavingerová <i>et al.</i> , 1994
Citrus	<i>35S-uidA</i> , <i>NOS-nptII</i>	70 (0)	Screenhouse	Vegetative	4–5 years	Copy number	T-DNA rearrangements	Cervera <i>et al.</i> , 2000
Poplar	<i>FMV-cp4</i> , <i>FMV-gox</i>	40 (1)	Field	Vegetative	4 years			Meilan <i>et al.</i> , 2002
Poplar	<i>35S-rolC</i>	6–22 (2–6)	<i>In vitro</i> , greenhouse, field	Vegetative	5–6 years	T-DNA repeat formation, flanking AT-rich sequence		Kumar and Fladung, 2001
Poplar	<i>35S-uidA</i> , <i>EuCAD-uidA</i>	44 (0)	<i>In vitro</i> , greenhouse, field	Vegetative	6 years		Copy number, extra vector sequence	Hawkins <i>et al.</i> , 2003
Poplar	<i>35S-ASCAD</i> <i>35S-ASCOMT</i>	4	Field	Vegetative	4 years			Pilate <i>et al.</i> , 2002
Potato	<i>Gus</i> , <i>nptII</i>	2	<i>In vitro</i> , greenhouse	Vegetative	2 years			Borkowska <i>et al.</i> , 1995
Potato	<i>NptII</i> , <i>gus</i> , <i>ocs</i> , <i>rolA</i> , and <i>C</i>	4	Greenhouse	Vegetative	3 generations			Ottaviani, Hanisch ten Cate and Doting, 1992
Sugar cane	<i>Ubi-bar</i>	1	Greenhouse	Vegetative	3 generations		Contained five copies	Gallo-Meagher and Irvine, 1996
Sugar cane	<i>Pat</i>	1	Field	Vegetative	3 generations		Contained nine copies	Leibbrandt and Snyman, 2003
Tall fescue	<i>Actin1-gus</i>	2	Growth room	Vegetative	5 generations			Bettany <i>et al.</i> , 1998
<i>Arabidopsis</i>	<i>NOS-nptII</i>	7	<i>In vitro</i>	Sexual	4 generations	Promoter methylation		Kilby, Leyser and Furner, 1992
<i>Arabidopsis</i>	<i>35S-hpt</i>	28 (14)	<i>In vitro</i>	Sexual	1 generations	Copy number		Scheid, Paszkowski and Potrykus, 1991
<i>Arabidopsis</i>	<i>NOS-nptII</i>	111 (62)	<i>In vitro</i> , growth chamber	Sexual	3 generations	Construct configuration, temperature	Copy number	Meza <i>et al.</i> , 2001
<i>Arabidopsis</i>	<i>Fpl-dsFAD2</i>	1	Greenhouse	Sexual	4 generations			Stoutjesdijk <i>et al.</i> , 2002
Petunia	<i>35S-A1</i>	1	Field	Sexual	1 year	Promoter methylation, temperature, endogenous factors		Meyer <i>et al.</i> , 1992
Rice	<i>35S-bar</i> , <i>35S-gusA</i>	12 (0–2)		Sexual	3 generations	Presence of truncated transgene sequences	Copy number, position effect	Kohli <i>et al.</i> , 1999
Rice	<i>Ltp2-gus</i>	3	Greenhouse	Sexual	5 generations	Partial rearranged transgene		Morina, Olsen and Shimamoto, 1999
Tobacco	<i>NOS-nptII</i>	2	<i>In vitro</i>	Sexual	3 generations			Müller <i>et al.</i> , 1987
Tobacco	<i>NOS-nptII</i>	18 ( $5 \times 10^{-6}$ $\sim 5.9 \times 10^{-4}$ ) <sup>2</sup>	<i>In vitro</i>	Sexual	1 generation	Environmental stress	MAR	Conner <i>et al.</i> , 1998
Tobacco	<i>35S-hpt</i> , <i>35S-cat</i>	4	<i>In vitro</i>	Sexual	8 generations	T-DNA flanking sequences, position effect, extra vector sequence		Iglesias <i>et al.</i> , 1997

<sup>1</sup> Unstable events given in parentheses only where data on ten or more independent events reported.

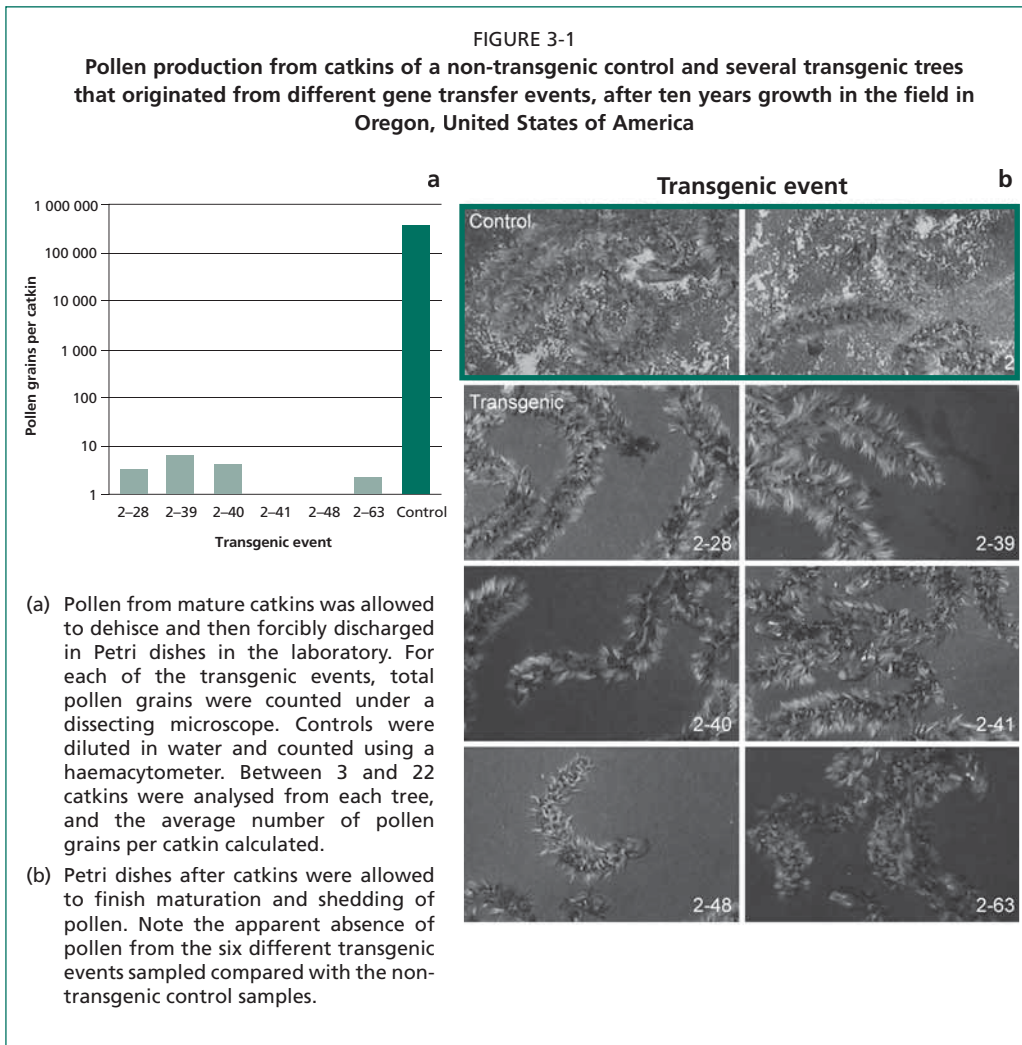
<sup>2</sup> Frequency of kanamycin-sensitive seedlings derived from each event.



by a cytotoxin; excision, where some or all functional transgenes are removed from gametes before their release; gene suppression, where the activity of one or more genes essential for reproduction are impaired at the DNA, RNA or protein levels; and 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.

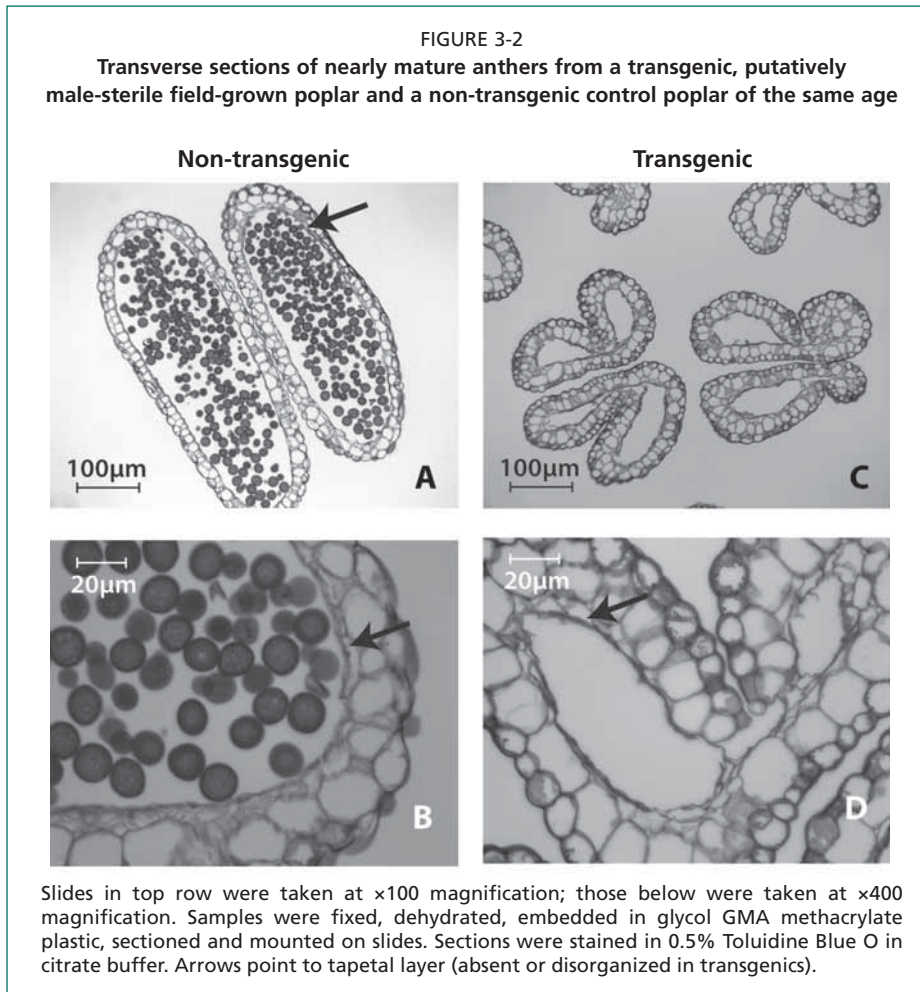
### Ablation approaches

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 3-1), 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 (Figures 3-1 and 3-2). However, Wei *et al.* (2007), studying poplar, and Lemmetyinen, Keinonen and Sopenan (2004) and Lännepää *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, barstar, a specific inhibitor of barnase, was co-expressed in transgenic poplars using various promoters, and it was found that gene insertion events with low ratios of barstar to barnase activity had abnormal growth and morphology (Figure 3-3), 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.*, 2007). 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) 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.

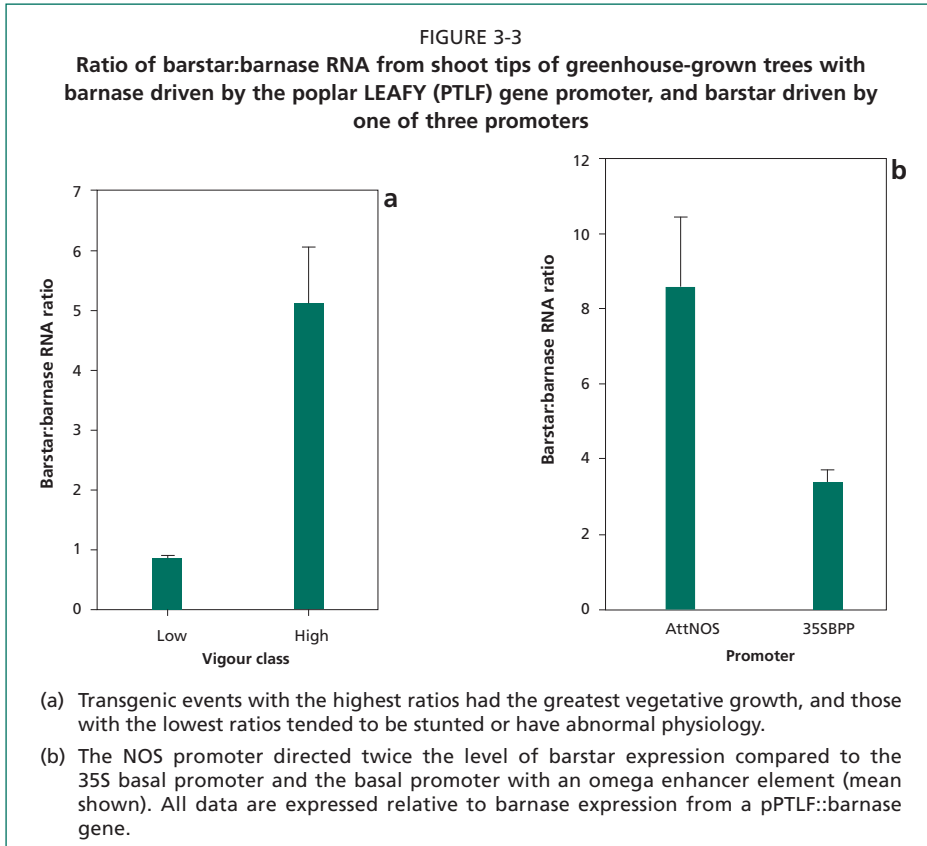


### Gene excision approaches

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, Conner and Nap (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 3.2 and 3.3, there have been a great variety of 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, Patil and Takezawa, 2002), amino acid metabolism (Dirks *et al.*, 2001), choline biosynthesis (Mou *et al.*, 2002), transcription factors (Preston *et al.*, 2004; Smeekens, Weisbeek and Proveniers, 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 post-transcriptional 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, Unger-Wallace and Haug-Collet, 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.

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, H. Griffiths and D. Grierson, 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; Figure 3-4), suggesting that RNAi transgenic trees with greatly reduced fertility can be selected at an early, non-flowering stage.

Pleiotropic effects of RNAi methods can be significant. Non-target 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, Jones and Baulcombe, 2002; Petersen and Albrechtsen, 2005; Miki, Itoh and Shimamoto, 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.

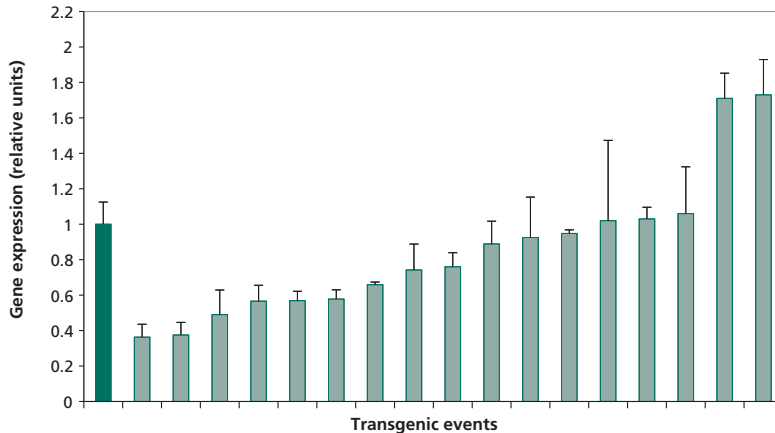
### **DOMINANT NEGATIVE PROTEINS**

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 multiprotein 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 *APE-TALAI(API)*. 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).

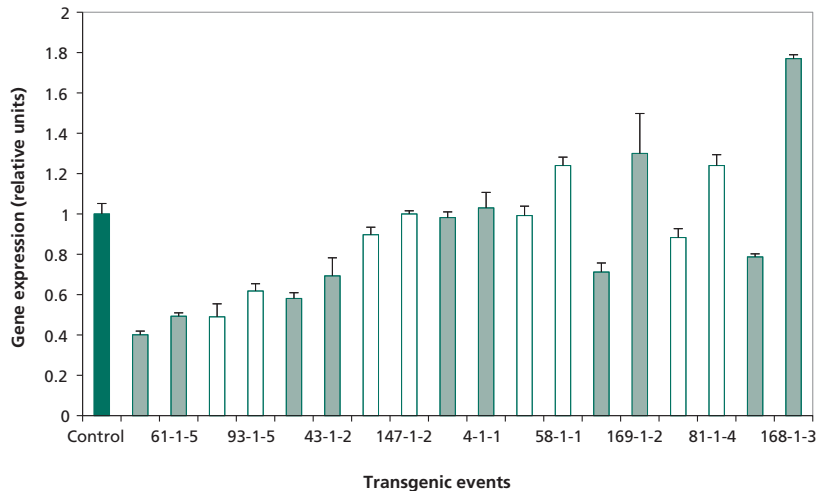
Another option for dominant repression of transcription factor activity is the introduction of chimeric transgenes that are translational fusions of the selected transcription factor coding region and a repression domain such as the ERF amphiphilic repressor (EAR) motif (Hiratsu *et al.*, 2003). Expression 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



FIGURE 3-4  
Range of RNAi gene suppression (top) and repeatability among biological replicates (bottom) for floral genes expressed in vegetative tissues



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* × *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.



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 top graph. 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 (shading) show biological replicates per event.



genes (reviewed in Segal, Stege and Barbas, 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 sequence. 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 or 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.

### 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 remodelling gene family, Shaked, Melamed-Bessudo and Levy (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 non-homologous 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 or 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 or 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, Schneeberger and Cullis, 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 were 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

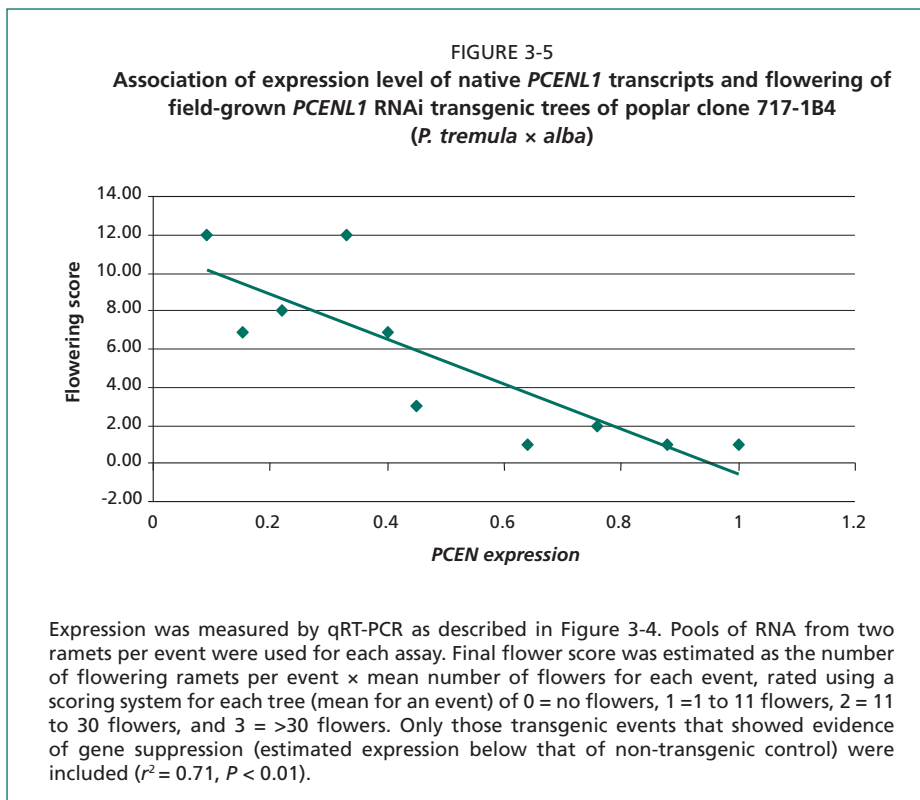
Most published studies of genes controlling flowering in trees have described the isolation and gene expression patterns of homologs of genes known to control various stages of flowering in *Arabidopsis* (e.g. Southerton *et al.*, 1998; Sheppard *et al.*, 2000; Cseke, Zheng and Podila, 2003). Results 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. Kyojuka *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 non-flowering 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 (Lemmetäinen *et al.*, 2004). 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, *TERMINALFLOWER 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 (Figure 3-5). 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 subfunctionalization 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 ([www.floralgenome.org](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 analysing 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). Bio-informatic 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, Zhong and Wong, 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 United States of America National Science Foundation Plant Genome Project is studying the transition to flowering in poplar. 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 programmes that are similar to reproductive development; *LEAFY*, *API* 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, Piacuzzi and Grattapaglia (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 precocious 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 focused upon 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 genetically engineered crops that have been grown by farmers, which contain a variety of genetic constructs in a variety of genotypes and species. These include commercialized 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 programmes 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 modelling 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 genetically engineered-introduced traits in trees has 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 genetic engineering-associated genomic change compared with 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 3-3). 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 re-arrangements at or near transgene insertion sites (Table 3-3).

Transgene stability under vegetative propagation has been studied in poplar, citrus, tall fescue, sugar cane, chrysanthemum and potato. Transgene expression appears far less stable over sexually propagated generations than over vegetatively propagated generations (Table 3-3). Unfortunately, most studies have used a small number of transgenic events (<20), and are thus of limited relevance to commercial transformation and breeding programmes, 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 programmes.

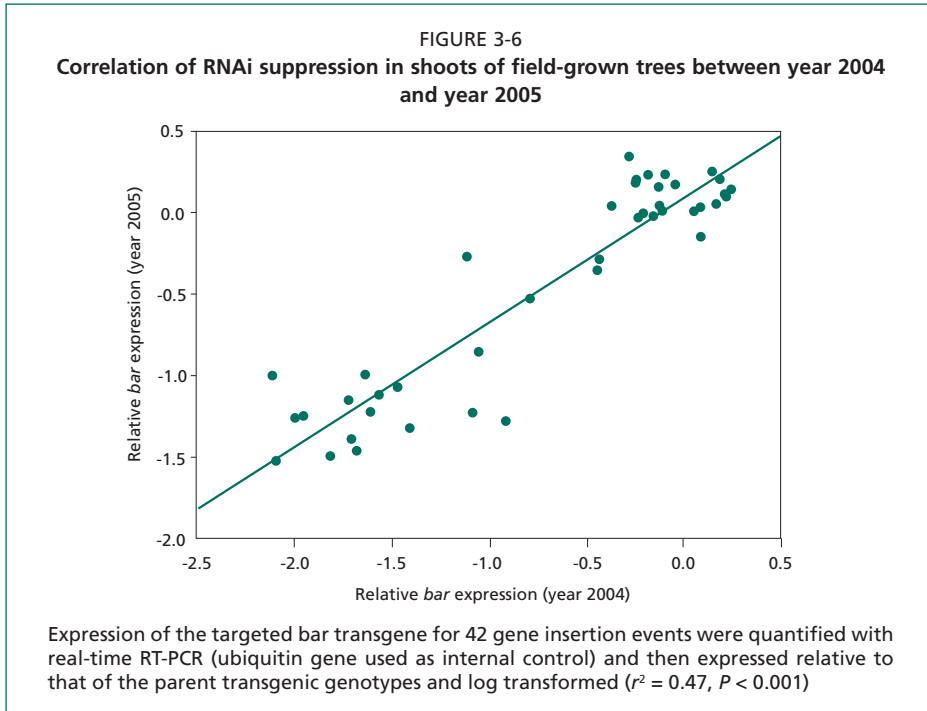


In a study similar to what a tree breeding programme might address, Meilan *et al.* (2002) reported high stability of herbicide resistance genes in 40 independent poplar transgenic events over four years in the field. Hawkins *et al.* (2003) reported stable expression of a GUS reporter gene in 44 independent poplar transgenic events over a period of six 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 four to five 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 cinnamyl alcohol dehydrogenase (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 *rolC* 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 eight 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 enzyme-linked immunosorbent assays (ELISA). 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 three years in the greenhouse and the field. The expression of both genes was highly stable over three 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 re-transformed 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 two 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 two years (Figure 3-6). 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.

### **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 programmes that use ploidy-modified trees. However, such genotypes are rare in most forest tree breeding programmes. 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 modelling.

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, Halfhill and Warwick, 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, DeWoody and Muir, 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 modelled) and the high computational requirements that limit the extent of the area and time frame that can be modelled (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, Smith and Reid, 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 geographical 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, DiFazio and Strauss, 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<sup>2</sup> 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.

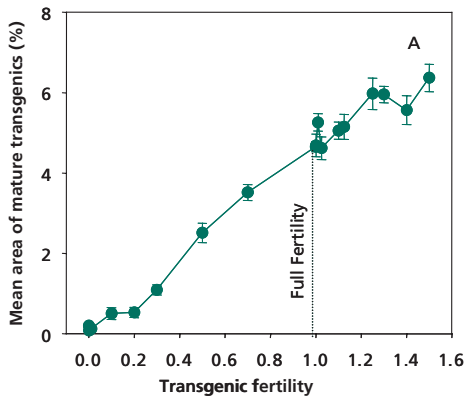
Relative fertility varied annually based on a user-defined standard deviation determined from annual field observations of flowering in plantations. In addition, transgenics with reduced fertility could have their fertility partially restored according to a user-defined probability. Vegetative propagule 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, DiFazio and Strauss, 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 with 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 (Figure 3-7a). Thus, the reductions in fertility of approximately  $10^5$  that we have observed in the field (Figure 3-1) 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 (Figure 3-7b), 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 (Figure 3-7b).

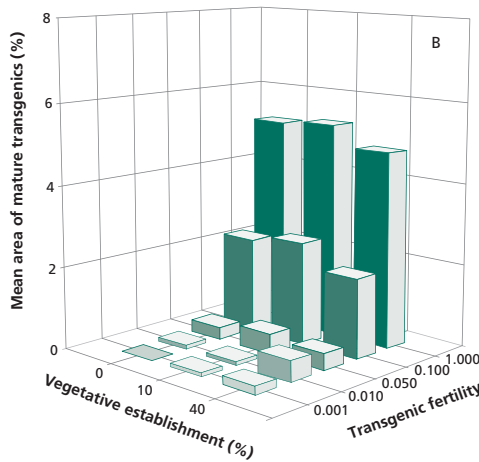
Other investigations have also identified fertility as a major factor limiting plant spread. For example, a reduction of fertility of as little as 75% was projected to limit the spread of scotch broom (*Cytisus scoparius* L.), based on insect-protection assays and simulations (Rees and Paynter, 1997). Density of pines spreading from plantations in South Africa was sensitive to fecundity and age of reproductive maturity in spatially explicit simulations (Higgins, Richardson and Cowling, 1996). Spread of feral oilseed rape was hypothesized to be limited by seed input

FIGURE 3-7  
**Simulated effects of transgenic fertility on transgene flow based on the STEVE model**

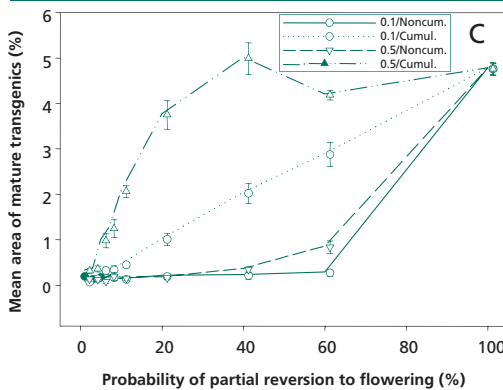
Simulations were conducted over a 50-year time period, and gene flow was indexed by the proportion of 100 m<sup>2</sup> *Populus* cohorts greater than ten years of age that contained at least one transgenic tree outside of plantations (Mean Area of Mature Transgenics). Responses were averaged over the 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.

Source: DiFazio, 2002.

based on patterns of establishment along shipping (i.e. dispersal) routes (Crawley and Brown, 1995), and simulation modelling 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) (Figure 3-7c). 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 non-cumulative 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 modelled 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 will reduce fitness or be neutral in the wild, or because their benefits outweigh their detriments. At the same time, there may be crops, such as forms of bio-industrial 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 genetically engineered 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 genetically engineered 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 over-expressed 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 licences 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 North America, 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 genetically engineered 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 genetic engineering 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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