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*Review article*



# Genetic engineering of reproductive sterility in forest trees

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Abstract

Containment of transgenes inserted into genetically engineered forest trees will probably be necessary before most commercial uses are possible. This is a consequence of (1) high rates of gene dispersal by pollen and seed, (2) proximity of engineered trees in plantations to natural or feral stands of interfertile species, and (3) potentially undesirable ecological effects if certain transgenes become widely dispersed. In addition to gene containment, engineering of complete or male sterility may stimulate faster wood production, reduce production of allergenic pollen, and facilitate hybrid breeding. We review the regulatory and ecological rationale for engineering sterility, potentially useful floral genes, strategies for creating sterility-causing transgenes, and problems peculiar to engineering sterility in forest trees. Each of the two primary options - ablating floral tissues via floral promoter-cytotoxin fusions, and disrupting expression of essential floral genes by various methods of gene suppression - has advantages and disadvantages. Because promoters from structural and enzymatic floral-specific genes often work well in heterologous species, ablation methods based on these genes probably will not require cloning of homologs from angiosperm trees. Methods that inhibit gene expression will require cloning of tree genes and may be more prone to epigenetic variability, but should allow assay of transgene efficacy in seedlings. Practical constraints include the requirement for vegetative propagation if complete sterility is engineered and the need for highly stable forms of sterility in long-lived trees. The latter may require suppression of more than one floral gene or employment of more than one genetic mechanism for sterility.

## Rationale for use of engineered, sterile trees

### *Incorporation of transgenic trees into operational forestry programs*

Genetic engineering, defined here as production of genetically altered crops through asexual transfer of genes, requires many steps before commercial use is possible (Fig. 1). Most research in genetic engineering of trees has focused on gene transfer and identification of genes governing commercially useful traits such as wood quality [41, 115 ]. Interest in sterility as a target trait arises mainly in considering one of the later steps in genetic engineering: ecological and biosafety analysis (Fig. 1). This probably is why it was not

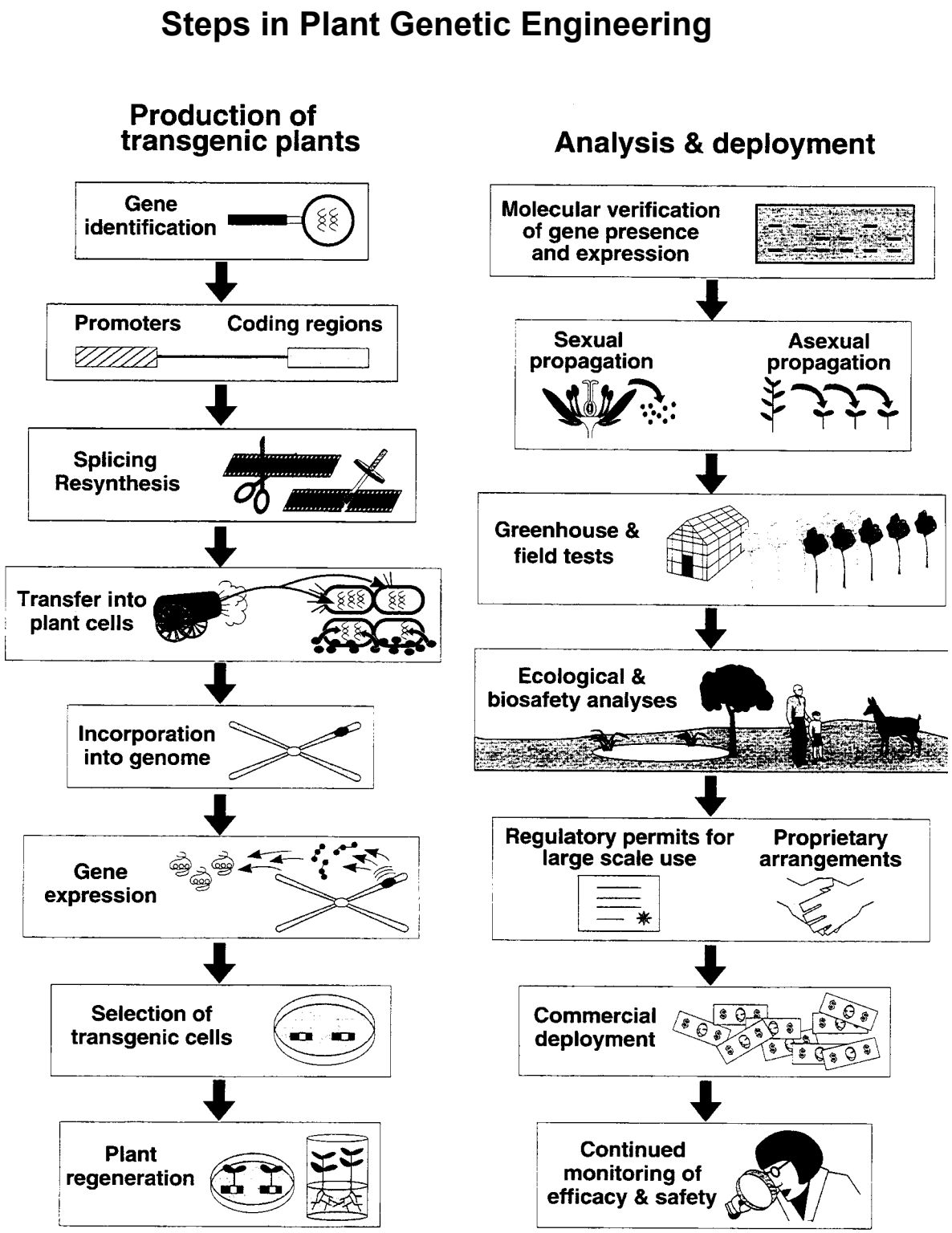


Fig. 1. A summary of the steps required to produce transgenic plants and bring them to the marketplace.

identified in an influential early analysis of goals for genetic engineering of forest trees [92].

Even in cases of genes known to be effective across many species, such as several genes for

insect and herbicide resistance, the path to commercial use of engineered trees is unclear [13]. The primary obstacles are (1) inefficient gene transfer and associated tissue culture systems for generation of transformed plants (nearly universal problems with woody plants); (2) inconsistent inheritance and expression of transgenes in sexually derived seed, resulting from segregation and changes in expression caused by methylation and other processes [e.g. 44] among progeny of heterozygous (hemizygous) primary transformants; and (3) economic and biological obstacles associated with incorporation and testing of transgenes in large numbers of genotypes. Many forestry programs, such as those for Douglas-fir in the Pacific Northwest of the USA, use large numbers of half- or full-sibling families in operational plantings.

For clonal programs such as are used with poplars, some eucalypts, and some pines, however, the issues are far less complex because one or a few primary transformants can be tested, multiplied, and deployed rapidly. Apart from the continuing need to develop efficient gene-transfer methods for commercially desirable genotypes, the major constraints to use of engineered trees are ecological safety and regulatory approval.

*Ecological considerations and regulatory requirements in the USA*

The Coordinated Framework for the Regulation of Biotechnology [ 75 ] has established that introduction of transgenic plants into the environment would be regulated primarily by the Environmental Protection Agency (EPA) and the United States Department of Agriculture (USDA). The EPA regulates the use and testing of transgenic plants that produce a pesticidal substance. The Animal and Plant Health Inspection Service (APHIS) of the USDA regulates the release of transgenic plants that are potential plant pests. A transgenic plant will be regulated if the donor or recipient organism, the vector, or the vector agent is classified as or is suspected of being a plant pest. Because of the common use of *Agrobacte*

*rium tumefaciens* for plant transformation and of regulatory DNA sequences such as the cauliflower mosaic virus promoter, field testing of most genetically engineered plants falls under the jurisdiction of **APHIS.** Before approving a field test, APHIS not only assesses the potential of the transgenic plant to become a pest, but also evaluates the interactions of the trait, the plant, and the environment in a broader context. Issues determining whether a significant impact will occur include effects of the transgene, potential for transgene transfer, consequences if transfer occurs, probability of increased weediness, and the availability of agricultural practices to manage any consequences.

In contrast to small-scale field tests, commercial releases will cover much greater areas in more diverse environments and will lack strict confinement. Thus, unregulated cultivation of transgenic plants raises many new questions about the interactions between these plants and existing ecosystems [ 94]. What is acceptable in a field test may not be acceptable or practically achievable in widespread release. Risks will vary depending on the characteristics of the transgene, the recipient plant, and the environment of release. The tests and strategies employed to reduce the risks to acceptable levels before commercial release will therefore differ widely.

Concerns over the introduction of transgenic plants into the environment have centered on two problems: enhanced weediness and movement of transgenes by hybridization into gene pools of wild relatives. Both could produce offspring with increased invasiveness and such secondary impacts as loss of biological diversity through displacement of native species [23, 87, 104]. The EPA has indicated that it will issue a transgenic plant-pesticide rule that makes sexual isolation a primary criterion for exemption. This position was supported in a meeting of its Biotechnology Science Advisory Committee and the FIFRA Scientific Advisory Panel [70], which reasoned that genes encoding plant pesticides that are effectively contained within transgenic plants are unlikely to result in new environmental exposures.

Since most commercially grown forest trees are

in close proximity to interfertile populations of natural or feral origin, transgene containment is key to the use of genetically engineered trees. Gene flow within and among tree populations is usually extensive [ 1 ], which makes the probability of transgene escape from plantations high [84]. Though hybridization between cultivated and natural populations is not new, most engineered genes are novel; they may persist in natural populations by conferring a selective advantage. Genetic engineering of crops has to date emphasized three traits: herbicide resistance, insect resistance and viral resistance; all could confer a fitness advantage to a wild plant.

The possible biological effects of escaped transgenes for resistance traits have been widely discussed. The introduction of genes for insect resistance into wild populations could accelerate the evolution and spread of insects that are resistant to their effects [62, 83, 97], as well as affect endangered insect species and natural food chains. The introduction of herbicide-resistance genes could impair control of cultivated species or interfertile related populations [20, 31]. Viral resistance has been most commonly achieved in transgenic plants by expression of segments of viral genomes, such as capsid genes. Recombination between a transgene and an infecting virus, as well as heterologous encapsidation of other viral RNAs with an expressed coat protein, is known to occur, although the frequency and significance are unclear [25]. Transfer of native or recombined transgenes to wild relatives might increase the potential for a new viral strain or broadened host range, as well as alter the fitness of plant populations.

Predicting which transgenes are likely to persist is tenuous. Although characteristics associated with weediness have been identified, different weeds have different combinations of these traits, and weediness within a species varies widely among environments [43, 78]. Because intensive breeding usually severely impairs biological fitness, the introduction of transgenes into highly domesticated crops is unlikely to result in the evolution of new weeds. However, predicting the traits that may confer or enhance weediness in a less domesticated crop or in a wild relative is difficult. Thus, instances where probability of gene flow is high are likely to require strategies that minimize the chances of hybridization, even if the transgene does not provide an obvious selective advantage. Before a nonsterile transgenic crop can be deregulated, for example, detailed studies evaluating ecological performance, weediness, and invasiveness in a variety of natural habitats for the crop or for hybrids between the crop and wild relatives may be required [87].

Such comprehensive studies have been conducted for transgenic oilseed rape [ 16] and may be generally feasible for annual crops grown on large acreages and with large markets. They may be impractical for most forest trees, however, because of relatively limited economies of scale for individual transgenic genotypes; occurrence in a wide variety of ecosystems; slow growth, causing delayed expression of fitness differentials; and a long juvenile period before flowering and transgene dispersal. Consequently, significant ecological impacts are likely to be detectable only after very many years. In addition, the irreversibility of transgene escape dictates caution; once dispersed into natural or feral populations of trees, transgenes probably cannot, practically speaking, be retrieved. These factors suggest that methods for producing sterile trees will be necessary for most commercial uses of transgenic forest trees, at least until a record of safety is established. Although APHIS has issued two permits for field tests of transgenic poplars to date, in both cases the finding of no significant impact was based on the trees not being allowed to flower (USDA-APHIS Environmental Assessments for permits 89-109-03 and 93-039-02).

Genetic engineering of sterility may be critical to preventing escape of transgenes, but it too will have to undergo regulatory approval. This will be particularly relevant if sterility is achieved through a cytotoxic gene that disrupts cells involved in flowering, as discussed below. For example, APHIS would evaluate whether an expressed ribonuclease or ADP-ribosyl-transferase derived from the A chain of the diphtheria toxin gene (DTA) will harm beneficial organisms, espe

cially endangered species (S. van Wert, USDAAPHIS, personal communication). Because DTA is derived from a human pathogen, its use may be of particular concern to the public and thus used by special-interest groups to engender public opposition to genetic engineering. However, diphtheria toxin has been extensively studied; the A chain is considered safe because it lacks the B chain required for entry into eukaryotic cells [7, 76]. In this regard it differs little from the numerous other microbial protein toxins and toxin-encoding genes to which humans are exposed daily; they are harmless without a highly effective means for ingress into both organisms and cells. Recombinant fusion proteins that include DTA have been administered to cancer patients [50], and the targeted expression of the DTA gene is being actively pursued as a cancer therapeutic [60]. Thus, a strong case can be made for the safety of expressing the DTA gene and similarly disarmed cytotoxins in transgenic trees.

*Sterility and stimulation of wood production*

Diminished investment in reproductive tissues resulting from engineered sterility may increase wood production. An encouraging precedent is provided by agriculture, where the dramatic gains in yield from breeding of agronomic crops have come primarily from selection for increased allocation to more desirable plant organs, rather than from increased photosynthesis [24]. Little progress has been made on this front for forest trees, as most are propagated via seed. They may even have been inadvertently selected for precocity during selection of seed from flowering trees in the wild or during early phases of seed orchard production. If floral primordia can be ablated very early in development, which floral promoters from some homeotic genes should enable (discussed below), the reproductive drains of flower and fruit development may be largely avoided.

Substantial energy and nutrients are committed to reproductive development in trees [49]. Fielding [26] calculated that the energy invested in cones and pollen of radiata pine *(Pinus radiata)* is equivalent to a 16 % reduction of mean annual increment. Teich [ 101 ] observed that height growth in provenances of white spruce *(Picea glauca)* that produced cones early was 14% lower than in nonproducing provenances. Populations of knobcone pine *(Pinus attenuata),* a highly prolific and early-reproducing species, that initiated cone production a year earlier than average showed a 38 % reduction in stem volume at 10 years of age (S.H. Strauss, unpublished data). Morris [66] found that foliage production in cone-producing balsam firs *(Abies balsamea)* was one-quarter of that of nonproducing trees. The growth increment of Douglas-fir *(Pseudotsuga menziesii)* was an average of 16 % lower in conecrop years than in noncrop years [21]. ElKassaby and Barclay [22] found predominantly negative genetic and environmental correlations between increment growth and cone production in a Douglas-fir seedling seed orchard; the mean genetic correlation was -0.27 over 8 years of study, but -0.78 in the year with the heaviest cone crop. These diverse observations confirm the expectations that investment in reproduction diminishes vegetative growth of forest trees, and, therefore, that engineered sterility may increase wood production.

*Other uses for sterile trees*

As human populations continue to expand into forest and agricultural regions, the compatibility of trees with humans will grow in importance. Many people suffer from allergies caused by tree pollen. For example, in Japan many people suffer from allergies induced by the most commonly planted forest tree, the conifer sugi *(Cryptomeria japonica)* [38]. Sugi is clonally propagated, and efforts to select trees with much reduced pollen production are underway [98]. Introduction of genes that could entirely suppress pollen shed would be valued. Many genes that encode the production of pollen allergens from trees have been identified [e.g. 10], thus allowing for the

direct inhibition of allergenic proteins (discussed below), as well as reproductive sterility.

Concerns over genetic pollution of native populations by bred varieties of trees have been expressed many times [48]. However, this has not been a serious concern in most species as a result of limited domestication, substantial diversity in plantations, and the usually small differences in allele frequencies between production and native populations. More serious is the installation of large areas of markedly different genotypes, such as different provenances, exotic species, or novel hybrids, close to small native stands. Large influxes of foreign pollen or seeds might undermine the genetic integrity, diversity, or adaptedness of native populations. In such cases, engineered sterility would greatly reduce the impacts of intensively bred tree plantations on nearby stands.

## Genes useful for engineering sterility

There are several options available for generating sterile plants; chief among these are genetic ablation and inhibition of expression of genes essential for reproductive development. In genetic ablation, a cytotoxic gene is expressed under the control of a reproductive-specific promoter, killing all cells that follow that developmental pathway. Inhibition of gene expression can be accomplished by antisense RNA, sense suppression, or promoter-based suppression (reviewed below). In this paper, these three approaches (which may have multiple genetic mechanisms) are referred to collectively as `gene suppression' methods.

The transcriptional and structural information necessary for engineering sterility is contained in genes expressed in reproductive tissues. Key transcriptional features of useful genes are specificity of expression, which allows targeting of floral tissues without pleiotropic effects, and production of relatively high amounts of RNA, which allows a promoter to induce strong expression of an introduced gene. It is useful to differentiate between `floral-enhanced' genes, which are strongly expressed in floral tissues but are also expressed in vegetative tissues such as roots, stems and leaves, and `floral-specific' genes, which are not significantly expressed in nonreproductive tissues. One can further distinguish `gametogenic' genes - those specifically involved in formation of the stamens or pistils - from other floral genes, such as those expressed only in the perianth (sepals and petals), which are not therefore expected to be very useful for engineering sterility. Finally, despite high specificity, many genes may serve nonessential or redundant roles and are thus dispensable for flower development; their suppression would not lead to sterility [e.g. 106].

To lay the groundwork for a discussion of options for engineering sterility, we first briefly review the developmental genetics of flowering. We then describe a number of genes that could provide components for synthetic sterility constructs in both angiosperm and gymnosperm tree species. We emphasize the homeotic genes because they have received relatively little attention with respect to engineering of sterility (in contrast to structural and enzymatic floral genes [e.g. 30]), and because of their potential to act early in reproductive development.

### *Floral development and genetics*

As a plant matures, vegetative meristems become inflorescence meristems, which then become competent to produce floral primordia and floral organs. In a typical angiosperm flower, the meristem produces lateral organs in concentric whorls: the outermost whorl consists of sepals; the second, of petals; the third, of stamens; and the innermost, fourth whorl, of carpels. One important class of mutations that alter this normal developmental sequence involves the homeotic genes. The transcription factors encoded by these genes regulate sets of other genes that establish the transitions from vegetative to inflorescence to floral meristems and direct the formation of the floral whorls and organs.

Reproductive development in gymnosperms differs from that of angiosperms in some major

ways, and these are certain to be reflected in differing gene structure and expression. The gross differences in organization of seed cones versus angiosperm inflorescences, the lack of a carpet surrounding the ovules in gymnosperms, and the spiral, rather than whorled, arrangement of pollen-bearing organs in gymnosperms are some examples. However, as many features are also in common (e.g. the presence of a tapetum that nourishes pollen during development [3]), it is clear that many angiosperm floral genes, including transcription factors (see below), will have homologs that can be readily identified and used for engineering sterility once their modes of expression are known.

In addition to transcription factors that direct inflorescence and floral organ development, plants possess many genes that encode structural proteins and enzymes whose expression is restricted to reproductive tissues, usually during their later stages of differentiation. A number of the genes for structural proteins encode components of the specialized cell walls of floral organs [30]. Many of the enzymes characterized appear to be involved in defense against pathogens [28]. As a result of conservation of promoter or protein-coding regions, these and the regulatory genes provide several avenues for engineering tree sterility.

*Genes expressed during inflorescence development*

Genes that act very early in the development of floral structures, prior to gender differentiation, often affect formation of both male and female organs. Where complete sterility is the goal, such genes provide a major advantage over genderspecific genes, except in strictly dioecious species. Because of their early expression, engineering sterility with such genes might also be especially useful for enhancing wood production, as discussed above. We are aware of no reports of genes encoding structural proteins or enzymes that are specific to early stages of inflorescence or flower development; the many such genes characterized to date have been from highly differentiated tissues (reviewed below). However, homeotic genes that function early in floral development have been cloned and characterized.

The *Antirrhinum* gene SQUAMOSA (SQUA) and the *Arabidopsis* gene APETALA 1 (API) are homologues from the MAD S-box family of regulatory genes. The term `MADS box' describes a 57-amino-acid region conserved among plants, animals, and fungi that is thought to indicate a transcription factor. Multiple MADS genes have been found in all plant species studied, and several have been shown to be involved in flowering [e.g. 4]. *APl* and SQUA help to regulate the transition from an inflorescence meristem to a floral meristem [37, 56]. Mutants in SQUA develop additional inflorescence shoots instead of flowers, resulting in a mufti-stemmed structure. RNA from the gene is detected throughout the floral meristem at the early stages of flower development and continues to be expressed relatively strongly in the sepals and petals. The gene is weakly expressed in the basal region of the carpets, in the bracts subtending the flowers, and in the leaves immediately below the inflorescence [ 37]. *API* shows a similar, but more tightly regulated, expression pattern in *Arabidopsis* [56].

The homologs *LEAFY (LFY)* from *Arabidop*sis [113] and *FLORICAULA (FLO)* from *Antirrhinum [ 15* ] also regulate the transition from inflorescence to floral meristems. They too appear to be transcription factors, but do not contain MADS-boxes. Mutations cause multiply branched stems in place of flowers, similar to SQUA mutants [37]. These genes are expressed in the floral primordium at the earliest stage of development and as the floral organ primordia begin to form. Weak expression of *FLO* and *LFY* in nonfloral portions of the inflorescence has been noted [ 15, 113]. Significant vegetative expression is observed in *FLO/LFY* homologues from tobacco, eucalyptus, and poplar [ 15, 79, 88, 113, S. Southerton, personal communication].

*APETALA2* (AP2) represents a third type of homeotic gene, with no sequence similarity to either the MAD S or the *FLO/LFY* gene families. Its expression patterns have not yet been described in detail, but it is expressed in all organs

from the earliest stages of floral development and appears to regulate AGAMOUS (A G) (described below) [74]. It is also expressed at low levels in vegetative tissues.

*Genes expressed in both male and female floral organs*

The *Arabidopsis* gene *AG* has a major role in differentiation of both male and female floral tissues. Mutation of *AG* results in stamens being transformed to petals and carpets developing as new flowers in a repetitive fashion. The homologous *PLENA (PLE)* gene of *Antirrhinum* performs the same function, and close homologs have also been identified in tobacco, tomato, and maize [e.g. 81 ]. *AG* and *PLE* transcripts are first detectable as the sepal primordia emerge; they are present in all cell layers within the floral meristem, but not in sepal or petal primordia. When stamen and carpet primordia develop, however, they are expressed strongly and uniformly in these tissues (reviewed in [74]).

Self-incompatibility (SI), a mechanism by which the stigma recognizes pollen with a dissimilar genotype, is directed by genes whose promoters cause expression in both male and female organs (reviewed in [ 116]). Genes associated with gametophytic forms of SI become active in microspores only after meiosis [71]. Thus, a transgene containing an SI promoter would be active in only half of the pollen in hemizygous transformants, resulting in little reduction of fertility. Sporophytic SI in the Brassicaceae [68] is expected to be more useful than gametophytic SI because the genes are expressed in the tapetum and are thus able to affect all pollen. The *Brassica* S locus glycoprotein *(SLG)* promoter is expressed weakly in the tapetal wall of the anther and pollen 5 to 6 days before anthesis, and is expressed strongly in the stigma starting 3 days before anthesis. Related sequences at other loci have been found in the *Brassica* genes *SLRI* and *SLR2 (S* locus-related). Transcription of the *SLG* and *SLRI* promoters in a heterologous system, tobacco, shows that the site of male expression is limited to pollen [33, 102], following the pattern of gametophytic SI expression common to the Solanaceae.

A tobacco gene of unknown function, *TA20, is* expressed moderately strongly in the epidermis, wall, and connective tissue of the anther, and also in certain tissues of the pistil, such as the ovary wall, the tissue connecting ovules to the placenta, and the parenchyma of the style [46].

*Gender-sped genes*

In dioecious tree species such as Populus, genderspecific genes could be of direct use for engineering complete sterility in clones of known gender. In monoecious or hermaphroditic species, complete sterility could be produced by combining maleand female-specific sterility constructs in a single plant. Manipulation of genes that are gametophytically expressed only after meiosis is not expected to be effective for eliminating all pollen or ova, as discussed above (e.g. [67]). Consequently, this section will describe only male- and female-specific genes for which sporophytic expression has been conclusively demonstrated.

*Male-sped genes*

In addition to providing complete sterility for male clones of dioecious species, male-specific genes may be useful in generating male-sterile trees to be used in hybrid or single-cross breeding systems for monoecious or hermaphroditic species. The relative ease with which anthers and pollen can be separated from other floral tissues has enabled identification of a large number of male-specific cDNAs from a variety of herbaceous species (reviewed in [30, 61]). Most of these genes were identified by differential screening and encode enzymes or structural proteins involved in pollen development. In addition, some male-specific regulatory genes have been identified through genetic analysis of mutants and eventual molecular cloning (reviewed in [ 14, 74]).

One of the most well-characterized malespecific promoters belongs to the tobacco gene *TA29.* This gene is strongly expressed in the

anther and encodes a glycine-rich polypeptide that is probably involved in synthesis of the pollen cell wall [46]. The specificity of transcription from the TA29 promoter in transgenic plants has been tested in widely divergent species, and it appears to be expressed only in the tapetum, the secretory tissue that nourishes the developing pollen [46, 57, 86]. Several other anther-specific cDNAs have been cloned from tobacco, including lipid transfer proteins and a thiol endopeptidase [30]. Not all are specific for the tapetum. For example, the endopeptidase *TA56* is expressed in the connective tissue and stomium before their degeneration [46]. Several cDNAs encoding pollen proteins, including known allergens, have been cloned from angiosperm trees; however, the expression patterns of their genes are not yet known (e.g. [ 10]).

Mitochondria) genes have been implicated in male sterility in a variety of species; maize and petunia have been studied most intensively (reviewed in [34, 51]). The genes responsible for cytoplasmic male sterility (CMS) are abnormal versions of mitochondria) genes created by recombination. It is believed that they prevent the tapetum from achieving the high metabolic rate needed for complete development of the pollen [ 51 ]. Given the present difficulty of organelle transformation, mitochondria) genes used in transgenic plants need to function as nuclear genes; gene products therefore must be targeted to the mitochondria. This requires addition of a mitochondria) signal sequence to the coding region [8]. A greater problem is that the mechanism of action may be deleterious to plant health, as in the case of the maize CMS gene T-urfl3, expression of which can be lethal or cause susceptibility to a fungal toxin [ 51, 111 ].

Several regulatory genes are known to be involved in development of the male organs. The *Antirrhinum* genes *DEFICIENS (DEF* A) and *GLOBOSA (GLO)* and their respective counterparts in Arabidopsis, APETALA3 (AP3) and PIS*TILLATA (PI),* are MADS-box genes required for differentiation of petals and stamens (reviewed in [74]). Surprisingly, however, *green petal,* the putative homologue of *DEFA* from petunia, is not essential for stamen formation [ 108]. These genes have a similar expression pattern: earliest expression is in the region of the primordia for the petals, later spreading to the stamen primordia. Strong expression continues in these organs as they mature, but there is little or no expression in the sporogenous tissue. However, despite weak expression in carpets, when the *AP3* promoter is used to express a cytotoxic gene in Arabidopsis only the petals and stamens are affected [ 17]. This shows that weak expression in non-target organs does not preclude use of a gene for engineering ablation of specific organs; thus, *LFY* and other genes that show low levels of vegetative expression (discussed above) may be useful for engineering sterility.

*Female-sped genes*

A number of ovary- and pistil-specific genes have been characterized (reviewed in [28]). These include structural genes for cell wall components of the transmitting tissue, such as proline-rich proteins and extensin-like proteins, and enzymes that may help to defend the reproductive tissue from infection, such as P-glucanases, pectate lysases, chitinases, and proteinase inhibitors [6]. As a general rule, these proteins are strongly expressed and secreted. The gametophytic self-incompatibility locus found in the Solanaceae (reviewed in [71]) encodes glycoproteins with RNase activity that are expressed strongly in the transmitting tissue of the pistil.

Two potential female-specific regulatory genes have been identified; however, detailed descriptions of the genes are not yet available. *AGLI* (AG-like 1) is a cloned Arabidopsis MADS-box gene closely related to A G. Its function is not yet known, but it is expressed only in the developing carpet, especially in the ovules [54]. *BELL (BELI ) is* a non-MADS-box regulatory gene that is required for normal ovule formation. Its expression pattern is somewhat similar to that of AGLI [85]. *FLO10* is known only by its mutant phenotype, in which the carpet is converted into additional stamens [91].

**Approaches to engineering plant sterility**

We will discuss the two major strategies, already mentioned above, that have been used for most cases of engineered sterility: inhibition of the expression of genes essential for reproductive development via antisense, sense, or promoter suppression; and ablation of floral tissues by expression of a cytotoxin-encoding gene under the control of a floral-specific promoter. Although a very wide variety of lytic genes could potentially function as cytotoxins if expressed highly and without subcellular localization, we focus only on those demonstrated to be of use for ablating floral cells.

There are several other strategies for engineering sterility that we have elected to ignore. Sterile plants have been produced *via* ectopic expression of endogenous floral genes using reproductive or constitutive promoters [55, 63, 81, 105]. Despite these successes, we do not regard this as a practical means for engineering tree sterility because of the propensity for pleiotropic effects and unpredictability when a gene is expressed in a novel manner. For example, when the tomato homolog of A G, TAG1, was ectopically expressed under the CaMV 35S promoter, abnormalities of vegetative, as well as reproductive, development were observed [ 81 ]. Methods based on mitochondria) genes [35] are excluded for reasons discussed above. The possibility of floral gene deletion or mutagenesis through homologous recombination (e.g. [73]) is ignored because its present low efficiency precludes practical application to trees and this method is likely to require disruption of both alleles at a locus.

*Disruption of genes essential for fertility*

Antisense and sense suppression are proven methods for impairing the expression of genes required for fertility. They depend on identifying expressed genes needed for development of reproductive organs, but do not depend on use of promoters that function exclusively in floral tis

sues. Transforming sequences need match only a portion of the target gene.

Antisense RNA acts either by reducing mRNA translation or by increasing mRNA degradation. It has provided strong inhibition of gene expression in diverse genes and species, although some fraction of the normal expression usually remains (reviewed in [47, 65]). Sense suppression, which is associated with the introduction of duplicate copies of either a native gene or transgene, reduces expression of the original gene, the newly introduced gene, or both (reviewed in [27, 39]). The mechanisms behind sense and antisense suppression are not yet defined. In some instances, sense suppression appears to be due to posttranscriptional degradation of the message. It is most strongly induced by a large number of transgene copies and overexpression of transgene RNA, and the effects appear to be heightened by modification of the transgene to render it untranslatable [52, 53].

Suppression of transcription can be a consequence solely of homology in the 5'-flanking region [ 12, 59]. In some instances of either sense or promoter suppression, the methylation state of the DNA is correlated with the level of gene expression; however, this is by no means a universal phenomenon [27]. A concern with respect to all forms of gene suppression is stability of the phenotype. Spontaneous reversion to a nonsuppressed state has been observed in many cases ([27], W. G. Dougherty, personal communication; R. Jorgensen, personal communication). The causes of reversion are not known, nor is there any information on its frequency during the vegetative life cycle of trees.

Antisense RNAs targeted against several floral genes have been used to generate sterile plants. Van der Meer *et al.* [ 109] used the cauliflower mosaic virus (CaMV) 35S promoter fused with an anther enhancer [ 110] to drive expression of an antisense chalcone synthase gene in petunia. Biosynthesis of flavonoids essential for pollen pigmentation and development was disrupted, resulting in male sterility. When an antisense version of TAG] (the tomato homologue of AG) driven by the CaMV 35S promoter was intro

duced into tomato, plants with aberrant maleand female-sterile flowers were obtained [ 81 ]. As expected, there were no detectable effects on vegetative organs (TAG] is expressed only in stamens and carpels). A similar strategy was employed in tomato to disrupt expression of TMS, another MADS-box gene believed to be involved in directing floral meristem and floral organ identity [80]. Slightly affected plants demonstrated partial fertility, while moderately and severely affected plants were completely sterile. After four cycles of vegetative propagation of one completely sterile line, no partial or complete reversions to fertility were observed. The MADS-box genes constitute a large gene family in tomato, and there was concern that antisense RNA targeted at one MADS-box gene might interfere with other MADS-box genes essential for plant development. However, antisense TM5 RNA did not appear to interfere with expression of three other MADS-box genes studied, and no phenotypic effects were observed in vegetative organs.

Sense suppression has also been used to induce sterility and interfere with floral development. Taylor and Jorgensen [ 100] generated petunia plants that were self-sterile, but partly cross-fertile, via sense suppression of chalcone synthase. Expression of a petunia floral homeotic gene that contains a MADS-box, *fbpl,* was inhibited when expression of sense transcripts was driven by the CaMV 35S promoter [5]. No *fbpl* mRNA was detected in developing flowers, an indication that suppression was complete; flowers were male- and female-sterile and no pleiotropic effects were noted in vegetative organs.

*Floral promoter-cytotoxin fusions*

Sterility may also be engineered by inserting genes controlled by floral-specific promoters that either ablate cells or disturb development of floral tissues. In order to avoid pleiotropic effects on vegetative organs, it is necessary to use promoters that are highly specific in their expression and to select transformants with transgenes in positions that allow this specificity to be retained. This technique is likely to provide stable sterility, especially if the promoter used is not endogenous to the transformed species (avoiding promoter suppression based on sequence identity) and RNA levels are not extremely high (avoiding posttranscriptional suppression). Four kinds of cytotoxic genes have been used in engineering sterility. RNase and ADP-ribosyl-transferase show strong effects and are likely to be useful in any tissue, while the others, rolC from *Agrobacterium* and glucanase, have thus far demonstrated usefulness only in male sterility.

*RNase*

Mariani *et al.* [57] fused the promoter of the tobacco tapetum-specific TA29 (Goldberg 1988) [29] with the coding regions from two genes encoding RNases: barnase, from *Bacillus amyloliquefaciens,* and the RNase-T1 gene from A*spergillus oryaae.* During anther development, the tapetum in two species of transgenic plants, tobacco and oil seed rape *(Brassica napus),* was selectively ablated, preventing pollen development. The plants were otherwise normal. Only one copy of the barnase construct was required to produce male sterility, whereas at least four copies of the RNase-T 1 gene were required. Denis *et al. [ 19]* observed that some male-sterile rapeseed plants reverted to fertility. This reversion occurred with both RNase genes. Instability of the RNase-T1 engineered sterility was correlated with temperatures higher than 25 °C. By screening a population of transgenic plants, however, they were able to select stable male-sterile plants.

The TA29-barnase construct has been used to engineer male sterility in a variety of agronomic species [58]. Reynaerts *et al.* [86] introduced TA29-RNase constructs into three agronomic species. In both cauliflower *(Brassica oleracea)* and witloof chicory *(Cichorium intybus),* barnase transformants were male-sterile, with only floral morphology and time of flowering altered. However, vigor was reduced in lettuce *(Lactuca sativa)* transformed with barnase, and plants transformed with the RNase-T1 construct were not completely male-sterile. These results show that a sterility constraint can have variable results

among species of a single plant family (Asteraceae).

The same approach was used to engineer female sterility in tobacco. Goldman *et al.* [32] used differential screening to identify a stigma-specific gene, *STIGl,* expressed only in pistils and not in vegetative tissues. They fused its 5'-flanking region to *GUS* and introduced it into tobacco and *Arabidopsis.* In both species they recovered stigma-specific expression, suggesting that the promoter may have wide usefulness, at least among dicots. When the promoter was fused to barnase and introduced into tobacco, transgenic plants were female-sterile and had ablated stigmatic surfaces, but they had normal vegetative morphology and complete male fertility.

An attractive feature of employing barnase for engineering sterility is the existence of barstar, a protein from *B. amyloliquefaciens* that inhibits expression of barnase through formation of a stable complex. Crossing barnase male-sterile lines with transgenic *barstar* male-fertile lines restores fertility [ 18, 58], a necessity in agronomic crops where the products are seeds or fruit.

*ADP-ribosyl-transferase*

The DTA gene encodes a protein that inhibits translation through ADP-ribosylation of elongation factor 2 [76]. It is considered highly safe, as discussed above; the toxin is active only in the cell in which it is expressed, is not secreted, and is not internalized by eukaryotic cells in the absence of the diphtheria toxin B chain. When linked to a cell-specific promoter, the DTA gene is therefore an excellent candidate for targeted cell destruction and has been used to ablate cells in a wide variety of organisms, as well as to produce sterile plants.

Thorsness *et al. [ 102*] linked the DTA coding sequence with the promoter from the S-locus glycoprotein *(SLG)* of *Brassica oleracea* (reviewed in [69]). When this construct was introduced into tobacco plants, DTA was expressed in both pistils and pollen. The transmitting tissues of the stigmas in transformants were ablated and would not support pollination, rendering the plants female-sterile. The construct was expressed ga

metophytically in 50-75 % of pollen grains, consistent with integration of the toxin gene at one or two loci. Transgenic oilseed rape had a similar phenotype, though pollen sterility was incomplete [42]. *Arabidopsis,* however, a different genus of the Brassicaceae, showed altered floral morphology and self-sterility, but cross-fertility with wildtype pollen, when transformed with this construct [103]. SI promoters can therefore impart different phenotypes within the same plant family (as well as between families; reviewed above); the authors [ 103] caution against use of these genes for imparting sterility in heterologous systems.

*RoIC*

*Ro1C* is one of the T-DNA genes from *Agrobacterium rhizogenes.* When *ro1C* is introduced into tobacco plants controlled by the CaMV 35S promoter, expression causes male sterility and several other changes in plant development, including reduced plant height, apical dominance, and leaf pigmentation [90]. *RoIC* inhibits microspore development, leading to the production of nonfunctional pollen. Fertility can be restored to male-sterile tobacco plants through crosses with transgenic plants containing the rolC gene in an antisense orientation under control of the CaMV 35S promoter. In order to avoid pleiotropic effects, *rolC* would need to be expressed under the control of a floral-specific promoter, although it can impart male sterility even when driven by promoters with poor expression in anthers, such as the CaMV 35S and *rbcS* gene promoters [90].

*Glucanase*

During normal floral development in angiosperms a callose wall is secreted by the microsporocytes before and during meiosis. After meiosis, this wall is broken down by callase, a P-1,3-glucanase secreted by the tapetum, and the microspores are then released into the locule of the anther. Worrall *et al. [ 117* ] created transgenic tobacco plants that secreted a modified form of a pathogenesis-related (PR) fl-1,3-glucanase driven either by a double CaMV 35S promoter or by the tapetum-specific promoters from the *Arabidopsis A3* and *A9* genes [77]. The transgenic plants ex

pressing the glucanase under direction of the CaMV 35S promoter were phenotypically normal and male-fertile. Transformants containing the glucanase gene controlled by the tapetum-specific promoters exhibited premature dissolution of the callose wall and varying degrees of male fertility.

## Testing sterility genes in trees

### *Expression of reproductive-sped genes in trees*

Promoters for structural and enzymatic flowering genes, such as the tapetum- and style-specific genes described above, often have adequately conserved expression patterns when tested in other species. Thus, testing of promoter/cytotoxin constructs developed in herbaceous species seems logical either before or simultaneously with efforts to clone new genes from transformable angiosperm trees. Conifers, however, with their markedly different reproductive development, may require isolation of homologous genes or testing of many heterologous constructs before an effective one is identified.

Regulatory genes appear to show considerably greater differences among species than do the structural and enzymatic genes, both in expression and in their roles in guiding floral development. Mutants for homeotic genes that are clear homologs display substantially different phenotypes in different species and vary with respect to the timing and cellular localization of expression. *FLO,* for example, appears to be an excellent gene for engineering sterility on the basis of its mutant phenotype and specificity of expression in *Antirrhinum.* In *Arabidopsis,* however, even strong mutants of its homologue *LFY* do not show complete sterility [ 113 ], and in some species vegetative expression of *LFY/FLO* homologues is significant (reviewed above). This is unfortunate, as the early and bisexual expression of many of these regulatory genes provides a great deal of promise for use in trees. Use of these genes for engineering tree sterility will therefore probably have to rely on isolation and characterization of genes from species very closely related to the commercial targets, followed by careful study of its expression. We have isolated cDNA homologs of *LFY [88]* and *DEF A [ 95* ] from poplar; homologs to the homeotic genes can also be isolated readily from conifers, including spruce [ 89, 99], pine, and Douglas-fir [72] (Fig. 2).

Engineering sterility by interfering with expression of key floral genes through gene suppression relies on sequence homology, rather than on func-

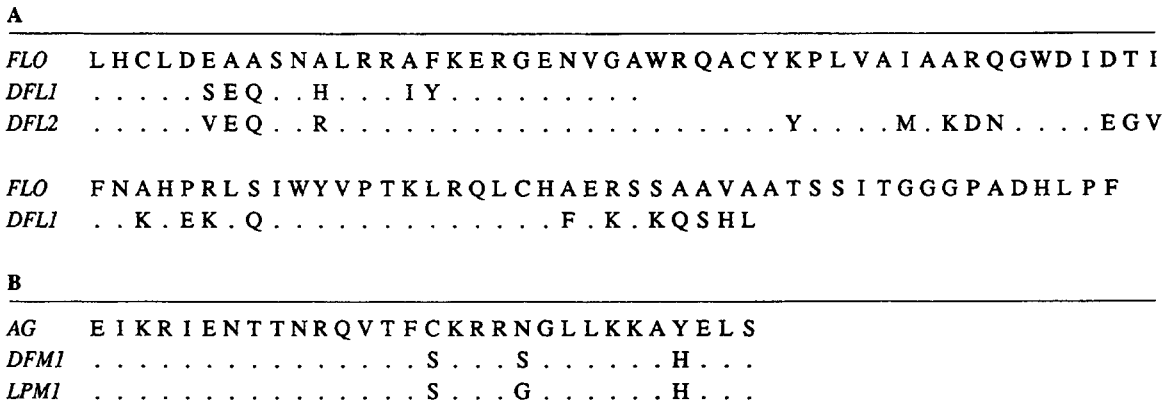


Fig. 2. PCR amplification and sequencing of conifer homeotic gene fragments. Primers to conserved regions were synthesized and used to amplify genomic or cDNA fragments from Douglas-fir and loblolly pine, which were then cloned and sequenced. Dots indicate amino acids conserved relative to the sequence of *FLO* or *A G.* A. *FLORICAULA/LEAFY (FLO-LFY)* fragments from Douglas-fir. DFL 1, Douglas-fir *FLO/LFY* homologue fragment cloned from PCR of genomic DNA. DFL2, Douglas-fir *FLO/LFY* homologue fragment from male bud cDNA. The full sequence of DFL2 included a poly(A) region, demonstrating that the gene was transcribed. B. MADS-box gene fragments from genomic DNA of Douglas-fir and loblolly pine. *AG, AGAMOUS;* DFM, Douglas-fir MADS-box clone; LPM, loblolly pine MADS-box clone.

tional conservation of promoter fragments. Although exact requirements for sequence homology can only be determined empirically, a minimum of 80-90 % is likely to be required for strong suppression (R. Jorgensen and W. Dougherty, personal communication). Thus, genes from at least the families, if not the genera, of commercial interest will probably be necessary. For example, an antisense version of TM5 from tomato had no effect in tobacco, a closely related member of the Solanaceae, despite having strong effects in tomato and 95 % amino acid similarity to a tobacco homolog of the gene [80].

*Establishing a commercially viable system*

The goal of engineered sterility is to greatly reduce the risk of transgene escape on a scale where it is likely to have undesirable ecological impacts. Initial field tests to study effectiveness should establish that transgenic trees remain sterile over multiple years, dormancy cycles, and environments. Sterility genes will also have to function stably in several tree genotypes if they are to be employed without close scrutiny for each new transgenic clone produced.

However, given the probability of mutation and epigenetic effects when transgenic materials are commercially deployed over large acreages and many years, both cytotoxin and gene suppression approaches are expected to lead to occasional reversions to fertility. Only with homologous recombination, which will allow sections of essential floral genes to be permanently deleted, will the probability of reversion become negligible. However, provided that reversions are rare and can be identified and eliminated, and that the genes released are not expected to have serious effects in extremely small numbers, occasional reversion should not pose an important problem. Study of large commercial plantings are likely to be needed to estimate reversion frequency. Monitoring for sterility as well as other traits would be desirable for the first large commercial plantings of transgenic trees.

*Use of model genotypes*

In tree taxa that have not been previously studied, sterility constructs should first be tested in species or clones that are readily transformed and can be induced to flower early. This may save a great deal of time and effort compared with attempts to directly use clones of commercial importance. Work on engineered sterility in crop plants has indicated that promoter-cytotoxin constructs based on structural or enzymatic floral genes can work across reasonably broad taxonomic categories (reviewed above). For gene suppression methods, genes cloned from genera of interest are likely to be sufficiently similar in sequence to be effective in a number of congeneric species. Thus, once effective sterility is demonstrated in one genotype, a smaller number of transgenics of the commercial genotypes can probably be produced and monitored without intensive study.

To establish the reliability of engineered sterility over a number of years, it would be desirable to allow trees to flower in field tests. This may present regulatory problems, however, because flowering will potentiate escape of transgenes should sterility fail. For small-scale field tests, physical isolation from native stands may be possible. However, complete isolation requires impractically large distances for wind-pollinated trees. Alternatively, genotypes could be used that are sexually incompatible with native species in the vicinity of field tests, yet sufficiently closely related to have similar reproductive development. For example, in the Willamette Valley of Oregon we intend to use aspen hybrids (section Leuce) as models for commercially important cottonwood hybrids (sections Aeigeros and Tacamahaca). Aspens are effectively incompatible with the native cottonwood species and hybrids that will grow in proximity to our low-elevation field tests [82, 96]. They will also be sexually isolated by phenology and distance from native aspens, which occur at relatively high elevations. The only compatible trees are a small number of planted shade trees. However, aspens are so poorly adapted to the area that invasiveness of transgenic propagules is extremely unlikely. Poor

adaptedness is, though, the major drawback to this approach, as exotic species may be unable to survive and grow normally in the test environment.

Another option for precluding gene escape is the use of naturally sterile trees as hosts for transgenes. Triploids are common among both aspen and cottonwood hybrids, are often of commercial value, and are considered effectively sterile [e.g. 9]. Because sterility is a consequence of chromosomal imbalance expressed during growth and development of gametes, it should be possible to observe the morphological effects of other introduced sterility genes, as most affect earlier stages of reproductive development. Should sterility genes fail, the formation of viable progeny will still be prevented.

Finally, if fertile genotypes must be used that are sexually compatible with native populations, it may be necessary to use isolation bags on flowers, or to harvest flower buds before expansion for study in contained environments. For example, poplar flower buds can be harvested before expansion and induced to shed pollen and develop seeds in the greenhouse. For extremely rapidly growing trees, however, such as hybrid cottonwoods in the northwestern USA (heights while flowering may exceed 25 m), harvest or bagging of all flower buds on trees in multi-acre field tests would require an enormous effort. It should therefore be pursued only if other options do not exist and the transgenes pose a clear ecological risk.

*Early flower induction*

A key experimental technology for studying sterility genes in trees is induction of early flowering. Although techniques are well developed in many conifer species because of their long history of commercial breeding, they are poorly developed in many angiosperm forest tree species, such as poplars. Nonetheless, even with effective treatments, flowering will often take two years or more, slowing assessment of sterility phenotypes. Manipulation of environmental and chemical factors has been used successfully to hasten maturation and intensify flowering in a variety of woody dicots, particularly fruit trees. Successful treatments include root restriction, girdling, drought stress, fertilization, extension of photoperiod, control of temperature, and application of growth retardants. Selection of precocious genotypes can also be highly effective (reviewed in [93]). With limited research, tree juvenility probably can be overcome as a serious obstacle to studying sterility in angiosperm forest species.

*Predicting gene effectiveness*

Developing a system with a high probability for inducing sterility in as many transformants as possible is important. This is necessary because (1) producing many transformants probably always will be difficult in many commercially important tree clones; (2) sterility cannot be assessed for many months to years, significantly delaying transgene evaluation; and (3) this delay causes considerable expense because of the substantial size of even young trees if many of them must be brought to sexual maturity.

Present methods for genetic engineering of most crop species rely on producing and screening a large number of transgenics, only a fraction of which express and inherit the transgene satisfactorily and are free of somaclonal variation. However, in juvenile trees it may be difficult to ensure transgene expression adequate to impart sterility when sexual maturity occurs months to years later.

Predicting transgene efficacy of floral promotercytotoxin methods is especially problematic, as the promoter is not expected to confer expression until flowering occurs. Here there are at least four options, none very satisfactory: (1) assessment of methylation in the promoter region (which, if present, would indicate the gene is likely to be silenced); (2) assessment of gene copy number, preferring a single or low number of copies to reduce chances of sense suppression [39]; (3) assessment of transgene structure, avoiding inserts with multiple inverted copies [36]; and (4) measurement of expression of closely linked transgenes derived from the same plasmid, assuming correlation of position effects (though this is not always the case [ 114]).

Antisense and sense suppression methods are notoriously unpredictable; many transformants usually must be screened in order to find a few with strongly and stably reduced gene expression, and inhibition can be spontaneously released (reviewed in [40]). Although recent work with matrix- (or scaffold-) attachment sequences (DNAs that bind to chromatin proteins) has shown great promise in reducing the importance of position effects [2, 11, 64], they do not appear to impart particularly high levels of expression when *Agrobacterium* is used as the .vector [ 11 ]; high transcription rates may be critical for sense suppression [53].

Antisense and sense suppression methods offer an important advantage, however; because genes specific to flowering are targeted, strong constitutive promoters can be used to drive transgenes, the expression of which can be monitored in vegetative tissue of young transformants. For sense suppression, transformants likely to show strong gene suppression will be those with high levels of transcription, as determined by nuclear run-on experiments, but low steady-state RNA levels, as determined by northern blots [ 53 ], indicating activation of whatever cellular mechanism causes suppression. However, high levels of transcription are not necessary for suppression [65, 107], and in practice very low steady-state RNA levels are themselves good predictors of suppression (W. Dougherty, personal communication). Thus, in contrast to methods based on floral promoters, gene-suppression methods might allow early, direct predictions of transgene efficacy at imparting sterility by study of transgene suppression in vegetative tissues. Otherwise, sterility might have to be assessed by flowering tests for each new transformant, possibly delaying commercial uses for several months to years.

This method for predicting inhibition of floral genes assumes that impairment of transgene expression in vegetative tissues will predict later impairment of endogenous floral-specific genes. This has not been widely tested but is supported by at least one study of floral gene suppression [ 5 ]. The petunia homeotic gene *fbpl,* which is normally expressed in petals and stamens, can be cosuppressed by insertion of multiple sense copies under control of the CaMV 35S promoter. Impairment of expression in the transgene was observed in tissues such as leaves and early inflorescences, which include cells in which 35 S should be expressed well but in which *fbpl* is not expressed. Nonetheless, complete suppression was observed there as well as in petals and stamens, indicating that expression of the endogenous gene is not required for stimulation of sense suppression.

For both the cytotoxin and gene-suppression strategies, it would be advisable to build in some redundancy to increase confidence that sterility will be stable over the lifetime of vegetative clones. For cytotoxins, the main concern is likely to be gene shutdown or down-regulation caused by methylation or position effects, respectively. Insertion of more than one construct, preferably using different promoters and coding regions to avoid cosuppression, should enhance stability. For gene suppression, impairing expression of more than one floral gene would be advisable. For example, targeting both an early and a late homeotic gene, such as *LFY* and *A* G, is expected to give a strong sterility phenotype. Targeting more than one gene can be accomplished either by constructing single fusion genes or by including genes driven by separate, unrelated promoters. It may also be useful to insert both gene-suppression- and cytotoxinbased constructs into single plants.

*Engineered male sterility and hybrid breeding*

Hybrids are highly valuable in a number of genera of monoecious or hermaphroditic forest trees, including *Eucalyptus, Pinus,* and *Larix [ 119]*. Because the cost of seed production is a major constraint to use of hybrids in species that are difficult to propagate vegetatively, engineered male sterility could allow many new uses of hybrids in forestry. The progeny of a genetically engineered male sterile tree would contain transgenes, however, so such a system may require additional steps to prevent transgene escape through seeds. This might include use of a female sterility gene in the pollen parent or a repressible female sterility gene in the female parent. In the former case,

the female sterility gene would need to be linked with a different selectable marker from that delivered on the male-sterility construct to allow completely sterile progeny to be selected in juveniles. For repressible female sterility, the gene would be present but not expressed during breeding. Expression could, for example, be controlled by application of an inductive chemical (see below). However, we anticipate that engineered trees whose only transgenes are selectable marker, reporter, and sterility genes should eventually be relieved of regulatory burdens (discussed below), paving the way for routine use of engineered male sterility in breeding.

*Restoration of fertility*

In some species it will be desirable both to transform valuable clones with sterility genes for commercial use and to use them in crosses and further breeding. This could be accomplished by using versions of the clone that lack sterility genes and then retransforming select progeny. However, as transformation of trees and selection of transformants with normal phenotypes and desired transgene expression may often be the primary bottleneck in genetic engineering, avoiding retransformation may be preferable when possible.

It should be feasible to devise sterility constructs that allow restoration of fertility under specific conditions. In contrast to male sterility systems in hybrid breeding of agronomic crops, restorer genes cannot simply be delivered into cells of completely sterile trees by sexual crossing (cf. [ 58 ] ). Thus, some kind of environmentally induced gene that restores fertility, but does not interfere with normal plant development, is needed. It is beyond the scope of this paper to describe in detail how such a system might work; however, several advances have been made on chemically induced gene expression in plants in recent years (reviewed in [ 112]). These studies have included exogenous compounds, such as steroids, tetracycline, and copper, and endogenous compounds, such as salicylic acid. Inductive treatments could be applied specifically to allow crosses to be made. Fertility could be reversibly restored by inducing restorer genes like barstar [58] or antisense RNA to cytotoxic genes. Pollen made sterile by inhibiting chalcone synthase expression has been restored to fertility by supplementing with flavonols [ 118]. Restoration of fertility can be achieved by activating recombinases that target inverted sequences flanking coding regions of any sterility gene (reviewed in [45]).

*Long-term prospects of the need for sterility*

We believe that the need for sterility in transgenic trees may be temporary, at least when considered on the scale of years to decades, and at least for some combinations of genes, species, and environments. With increasing familiarity and field test experience, many transgenes that are now strictly contained in the USA should be authorized for unrestricted release in plants (e.g. reporter and selectable marker genes with pathogen derived promoters and terminators, and genes that are likely only to impair biological fitness, such as those for sterility). In addition, we expect that many genes, including some for insect resistance and herbicide resistance, will be permitted to be released once some ecological and risk studies are completed, if done in association with a monitoring program that will allow problems to be detected. However, given the negative publicity that some special-interest groups are working to attach to plant genetic engineering, it may be very many years before regulatory authorities relax requirements, scientific and economic arguments notwithstanding.

## Conclusions

Over the past several years, dramatic progress in the molecular biology of flowering has provided many options for genetic engineering of sterility in plants. The two main options are floral promotercytoxin gene fusions and any of several methods of floral gene suppression. In tree species that can be clonally propagated, the main obstacles to engineering of sterility are a lack of information

about floral gene sequences and expression, and inefficient gene transfer systems. To provide the high levels of stability that may be needed for forest trees, redundant constructs would be desirable.

In addition to the practical goals of genetic engineering, isolation and study of the expression of floral gene homologs in forest trees will give new insights into flower development and evolution. This is most clear for study of the floral regulatory genes in gymnosperms. As a consequence of a phylogenetically primitive mode of development, they should reveal the basic regulatory circuitry from which the angiosperm flower evolved. However, new insights will also be gained from study of woody angiosperms, the reproductive morphology of which often is distinct from that of Arabidopsis and other commonly studied species as a result of wind pollination, dioecy, and phylogenetic distance. Thus, imparting sterility by genetic engineering not only will be a large step toward the safe use of transgenic forest trees; it also will generate mutant phenotypes likely to yield fresh insights into the molecular diversity of floral development.

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