# Molecular Biology of Woody Plants
## Volume 1



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# OPTIONS FOR GENETIC ENGINEERING OF FLORAL STERILITY IN FOREST TREES


## 1. Abstract

Engineering of genetic sterility in transgenically modified trees destined for commercial uses will simplify compliance with regulatory guidelines and mitigate ecological concerns of transgene dispersal. It could also be a critical technology for reducing the rate of escape and invasive mobility of exotic plantation species. Added benefits may include increased biomass production by redirecting energy normally expended on reproduction, and elimination of nuisance pollen and fruits. We discuss the two basic strategies for genetically engineering reproductive sterility; 1) suppression of reproductive gene expression and 2) genetic cell ablation of floral structures through the use of cytotoxins or gene products whose overexpression is detrimental to cell function. We also discuss various cytotoxins and inhibitors thereof that could be used to reverse sterility and enable traditional breeding.


## 2. Introduction

Genetically modified trees containing foreign genes (transgenes) are subject to government regulatory guidelines in most countries. A major focus of regulation is the potential impact of transgene release into the environment. Because of the potential for wide dispersal of transgenes from trees, environmental impacts can be difficult to predict and control. By reducing dispersion of all genes, engineering reproductive sterility will help to simplify impact analysis and thus facilitate regulatory and public approval (reviewed in Strauss et al., 1995). In trees, significant amounts of energy and nutrients are allocated for reproductive development (Ledig and Linzer, 1978). Prevention of reproductive floral structure development may also have the added benefit of increasing growth rates if energy devoted to reproduction is redirected toward vegetative growth.

Reproductive sterility has other applications besides transgene containment. Many trees are utilized as crop species in areas of the world to which they are not indigenous. These untransformed trees sometimes behave as serious exotic weeds in these new environments (Hughes, 1994; Richardson, 1998). These crop trees can spread from plantations into the surrounding habitat, and sometimes dramatically alter local ecosystems. Engineered sterility would greatly reduce their rate of escape into


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surrounding ecosystems, and also prevent gene flow from the farmed exotic species to wild or feral relatives with which they can interbreed.

In this review, we focus on genetic mechanisms and options for engineering sterility. We focus on examples from research on poplar, however, also discuss comparable progress in other forest species. Poplars (genus *Populus* L.) are widely used as model systems for forest biotechnology due to their many favorable characteristics. These include fast growth, ease of clonal propagation, facile transformation, and small genome size. Commercial uses of poplar include pulp, energy, chip, and solid wood production, and more recently, bioremediation (Wright, 1994). A number of genes are available for introducing commercially desirable traits into the poplar genome. These include genes for herbicide and insect resistance, and modified wood chemistry (Tzfira et al., 1998).

## 3. Floral Sterility

Two basic strategies, gene suppression and genetic cell ablation, can be utilized for engineering floral sterility. Both methods require the isolation and characterization of floral genes. Gene suppression requires part or all of the gene coding region, while genetic cell ablation utilizes the floral gene's regulatory region. By cell ablation, we include disruption of floral development due to overexpression of deleterious genes, as well as cell death caused by tissue-specific expression of a cytotoxin gene. Gene directed mutagenesis would be an ideal means for engineering sterility; however, rates are too low for practical use in trees, and would require knockout of both alleles at a locus or rapid inbreeding to make mutagenized (recessive) alleles homozygous.

### 3.1. TARGET GENES FOR FLORAL CONTROL

Despite a general, conserved pattern of floral development, a great diversity exists in the final reproductive structures of higher plants. Floral development has been particularly well-studied in the model angiosperm *Arabidopsis*. This model plant provides a general framework for understanding floral gene interactions; however, a number of exceptions and elaborations have been observed in other systems (Ma, 1994). Three levels of control occur in floral development, which relate to the transitional fate of the apical meristems. During the transition to reproductive growth, vegetative meristems are initially converted into inflorescence meristems, and are subsequently converted into floral meristems. Finally, floral organs develop from the floral meristems (Weigel, 1995; Yanofsky, 1995).

The transition from vegetative to reproductive growth (flowering) is influenced by both environmental signals such as photoperiod and temperature, and by endogenous signals influenced by plant age. Redundant regulatory pathways exist that control the transition to flowering in plants; the interplay of developmental signals and environmental cues in these pathways lead to flowering (Weigel, 1995). More than twenty genes involved in these flowering time pathways have been identified in *Arabidopsis* through genetic

screens (reviewed in Haughn et al., 1995 and Amasino, 1996). These timing genes can be divided into two classes, those that promote (early-flowering) and those that repress (late-flowering) the phase transition from vegetative growth to inflorescence development. A number of these timing genes have recently been isolated and appear to encode regulatory proteins (Lee et al., 1994; Putterill et al., 1995; Bradley et al., 1996; Bradley et al., 1997; Cardon et al., 1997; Kania et al., 1997; MacKnight et al., 1997).

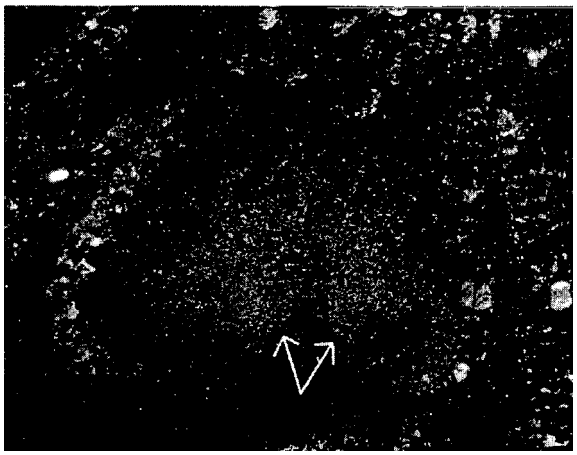
Once inflorescence growth has been initiated, a group of floral homeotic genes is activated that controls floral meristem fate through a regulatory hierarchy. These homeotic genes encode transcription factors and can be categorized into two basic groups: those that control the transition of an inflorescence meristem to a floral meristem, and those that control floral organ identity once a floral meristem has been established. Some of the genes, such as *APETALAI (API),* are involved in both functions (Weigel, 1995; Yanofsky, 1995). Examples of *Arabidopsis* meristem identity genes include *LEAFY (LM, API, APETALA2 (AP2),* and *CAULIFLOWER (CAL).* Genes involved in specifying floral organ identity are usually expressed in the floral meristem in a tissue-specific pattern. Members of this group include the *Arabidopsis* genes *API, AP2, APETALA3 (AP3), PISTILLATA (PI),* and *AGAMOUS (AG).* Homologs to most of these floral regulatory genes have been isolated from other species and have similar functions and/or expression patterns (reviewed in Weigel and Meyerowitz, 1994; below). Most of the floral homeotic genes belong to the evolutionarily conserved MADS box gene family. The MADS box is a highly conserved protein domain involved in DNA binding and protein dimerization, and is found in regulatory proteins of yeast, plants, and animals. A number of other MADS box genes, termed AGL for AGAMOUS-like, have been isolated, many of which show floralspecific expression patterns.

In a typical annual angiosperm such as *Arabidopsis,* all floral organs are derived from four concentric whorls of cells. The outermost whorl gives rise to the sepals, the second to petals, the third to stamens, and the fourth (innermost) to carpels. The whorled arrangement and specification of floral structures has been explained by a simple model termed the ABC model (Coen and Meyerowitz, 1991). The various organ identity genes can be classified into A-, B-, and C-function genes. Each class of organ-identity gene is expressed in two adjacent whorls and the location of its expression determines which floral organ type will be produced.

Isolation and analysis of genes involved in floral/reproductive development is currently being conducted in a few forest trees. These include poplars, eucalypts, pines, and spruces. Despite the differences in floral/reproductive development and form for these taxa, similarities in the regulatory genes involved, and their expression patterns, are observed (discussed below).

Floral development in Populus differs from that observed in model annual species (Boes and Strauss, 1994). Most poplars are dioecious, bearing only male or female floral structures on an individual tree, whereas species like the model plant *Arabidopsis* are

hermaphroditic, containing both male and female reproductive structures in the same flower. In both male and female poplar flowers, neither sepal or petal structures are present; instead a perianth cup subtends stamens or carpels. Despite the differences between poplar and model annual species, floral development patterns are generally conserved. Poplar cDNA and genomic-clone homologs to *LFY (PTLF), AP3 (PTD),* and *AG (PTAG1* and *PTAG2)* have been isolated in our laboratory (Sheppard et al., 1997; Brunner et al., 1998; Rottmann et al., 1998). In situ hybridization analysis of each of these genes has revealed floral meristem expression patterns similar to those seen with their gene homologs (Figure 1). Additionally, we have recently isolated the poplar homolog to *API (PTAPI-I* and *PTAPI-2),* and are beginning to characterize its expression (Brunner et al., unpublished).



*Figure].* Expression of PTAG1 in a cross-section of a developing female flower during carpel formation. PTAG1 expression (arrows) is visible in the carpels emerging from the central zone of the floral meristem. Photographed at 150X. Immature catkins were collected in late May, embedded, sectioned, and hybridized to in vitro-transcribed PTAG1 RNA.

Like *Arabidopsis,* eucalypt flowers are hermaphroditic and are structurally similar to those of *Arabidopsis* early in development. However, a number of unique events ultimately lead to development of a distinctive flower. These include fusion of sepal and petal primordia to form a protective structure, termed the operculum, and initiation of several hundred primordia, which all give rise to stamens. Eucalypt homologs to *LFY (ELF]* and *ELF2)* and *API (F-4P]* and *EAP2)* have been isolated (Kyozuka et al., 1997; Southerton et al., 1998a). Additionally, three other florally-expressed MADS box genes have been isolated, two of which phylogenetically group with *AGL2 (EGMI* and *EGM3)* and one with PI *(EGM2)* (Southerton et al., 1998b). Ectopic expression of *ELF], -9P],* or *EAP2* in *Arabidopsis* yielded phenotypes similar to those seen for the corresponding

*Arabidopsis* homologs, suggesting at least partial functional conservation of activity (Kyozuka et al., 1997; Southerton et al., 1998x).

The reproductive development of the non-flowering gymnosperms differs from that of angiosperms in a number of ways. Examples include the spiraled, versus whorled, arrangement of pollen bearing cones, organization of the seed cone relative to an angiosperm inflorescence, and ovules that are not surrounded by a carpel. Despite these differences, a number of the genes involved in angiosperm reproductive development appear to have similar roles in gymnosperms. MADS box clones have been isolated from a variety of conifers, including pines, spruce, and fir (Nyers et al., 1993; Tandre et al., 1995; Liu and Podila, 1996; Wang et al., 1997; Mouradov et al., 1998x; Rutledge et al., 1998). Additionally, a homolog to the *Arabidopsis* gene *LFY,* named *NEEDLY (NLY)* has been isolated from P. *radiata* (Mouradov et al., 1998b), while a homeoboxcontaining gene has been isolated from Norway spruce (Sundas et al., 1993). A number of the products from these gene are expressed preferentially in the reproductive structures (Tandre et al., 1995; Mouradov et al., 1998x; Mouradov et al., 1998b; Rutledge et al., 1998; Tandre et al., 1998). Ectopic expression of *NLYl* or *AG* homologs from either Norway or black spruce in *Arabidopsis* yielded phenotypes similar to those seen for the corresponding *Arabidopsis* homologs (Mouradov et al., 1998b; Rutledge et al., 1998; Tandre et al., 1998). These results again suggest at least partial functional conservation of activity for these homologs from non-flowering plants.

## 3.2. MEANS OF ENGINEERING STERILITY

Two basic approaches are available to inhibit floral reproduction (reviewed in Strauss et al., 1995). The first technique suppresses the expression, accumulation, or function of a gene product required for reproductive development by utilizing part or all of the floralgene coding region. The other approach involves genetic ablation through the use of a floral-specific promoter that directs expression of a cytotoxin or other gene, disrupting targeted cell or organ development.

### *Gene Product Suppression*

Gene suppression strategies use versions of the targeted floral gene which prevent transcription, mRNA accumulation, or activity of the protein product. Both cosuppression and antisense suppression operate at the transcriptional or posttranscriptional level, while dominant negative mutants interfere with protein function.

*Cosuppression and Antisense Suppression.* Cosuppression, also termed sense suppression, occurs when introduced transgenes inhibit their own expression and/or that of the native gene (Flavell, 1994). Cosuppression can work at two levels, transcriptional and post-transcriptional (Mol et al., 1994; Gallie, 1998). Inhibition at the transcriptional level is usually associated with methylation. In transgenic plants, the introduced gene is usually the target of methylation. Suppression can occur in regulatory as well as coding regions; therefore use of an endogenous promoter fragment

alone could result in cosuppression. Cosuppression of a 35S promoter-driven transgene has been observed in transgenic lines that contain another copy of the 35S promoter as a result of promoter methylation (Park et al., 1996). Cosuppression at the posttranscriptional level is often associated with high rates of transcription and appears to act via degradation of mature mRNAs during or after export from the nucleus (Mol et al., 1994; Que et al., 1997). Both mechanisms of cosuppression require that the transgene be highly similar to the target gene at the nucleotide level, thus requiring introduction of a second copy of the native gene or a closely related gene. Untranslatable versions of transgenes appear to be more efficient in causing cosuppression (Smith et al., 1994).

Antisense suppression is induced via transcription of the noncoding strand, or complimentary strand, of a gene as RNA. Antisense RNA (AsRNA) appears to act posttranscriptionally, forming a hybrid molecule with the target gene's sense mRNA and typically results in decreased steady-state transcript levels of the native gene (Mol et al., 1994). There are two major modes by which AsRNA is thought to cause gene suppression after forming an antisense:: sense hybrid (Mol et al., 1994). First, the hybrid double-stranded (ds) RNA may provide a target for dsRNA-specific ribonucleases. Alternatively, the dsRNA hybrid may interfere with the process of RNA processing, transport, or translation. Each mode makes mRNA of the targeted gene unavailable for translation and results in reduction of gene product levels. Like cosuppression, AsRNA suppression requires that the transgene and the target gene share significant sequence similarity. The similarity does not need to be over the entire gene, however, as small AsRNA fragments have been shown to be effective at suppression (Cannon et al., 1990). Thus, distantly related homologs (e.g., from different plant families) can be used if regions of sufficient size show strong similarity.

Both cosuppression and antisense suppression have been demonstrated as effective means of inhibiting gene expression in plants. Cosuppression of chalcone synthase affects flower pigmentation, giving a broad range of phenotypes with variable levels of genetic and developmental stability (Jorgensen et al., 1996). Cosuppression of two petunia ovule-specific MADS box genes resulted in aberrant seed coat development (Columbo et al., 1997). Antisense suppression has been used to study gene function and to engineer sterility using numerous floral genes (van der Meer et al., 1992; Punch et al., 1994a; Pnueli et al., 1994b; Lee et al., 1996; Harrison et al., 1998). A drawback to these approaches is that spontaneous loss of the suppressed state, or changes in the degree of the suppression, can sometimes occur (Flavell, 1994; Strauss et al., 1995). In tobacco, cosuppression of a 8-1,3-glucanase transgene was observed to be maintained during vegetative growth and floral development, but expression was restored in developing seeds, specifically in tissues derived from meiotically dividing cells. Cosuppression was also maintained in plantlets taken through in vitro regeneration (Balandin and Castresana, 1997). This suggests that meiotic, rather than mitotic, cell division is more problematic with respect to reversal of gene silencing. Thus, gene

suppression may be a reliable means for engineering traits, including sterility, in vegetatively propagated species such as poplars.

Another shortcoming of gene suppression, however, is that the parameters for design of efficient sense or antisense strategies based on gene sequence are not known; many constructs and transformants must often be tested to identify a few lines displaying the desired phenotype. Palauqui et al. (1996) found that 19-25% of tobacco transformants displayed observable nitrate and nitrite reductase cosuppression phenotypes. This frequency would be much lower when isolation of very strong suppression phenotypes is desired, as would be needed for engineered sterility. Therefore, a large number of transgenics would need to be produced to isolate the few displaying the desired phenotypes. Additionally, maintaining the number of transgenic lines that would be needed to evaluate the stability of the sterility phenotype through to flowering would be costly. This is further complicated by the fact that transformation efficiency of trees, in general, is much lower than that of traditional model systems like *Arabidopsis* and tobacco. For instance, transformation of poplar varies by genotype. For aspen, transformation efficiencies can be as high as 30% (transgenic plants per explant cocultivated). However, for *Populus trichocarpa x P. deltoides* hybrid cottonwoods useful for forestry practices, efficiency ranges from zero to about 4% (Han et al., 1996; Strauss et al., 1997).

*Dominant Negative Mutants.* Dominant negative mutants (DNMs) suppress the function of a gene at the protein level by overexpression of a mutant version of the protein (Espeseth et al., 1993). Inhibition can occur by a variety of means, including formation of an inactive heterodimer, sequestration of protein cofactors, sequestration of metabolites; and stable binding to a DNA regulatory motif (Espeseth et al., 1993). The usefulness of this approach for floral control was demonstrated in *Arabidopsis* with DNM versions of the *AG* gene (Mizukami et al., 1996). Expression of a truncated AG protein in which the C-terminal region was deleted resulted in flowers phenotypically similar to those observed in *ag* mutants, suggesting that the truncated version of AG was inhibiting endogenous AG function. A DNM version in which both the K and C domains of AG were deleted resulted in flowers with increased numbers of stamens and carpels, suggesting that the meristem determinacy function of AG was partially inhibited, whereas the AG organ identity function was not.

Due to codon degeneracy and conservation of three dimensional structure, members of a protein family from widely separated taxonomic groups can have similar amino acid sequences and/or tertiary structure even when their respective nucleic acid sequences have diverged significantly. Because the DNM approach functions at the protein level, DNM constructs can be expected to function in a wider taxonomic context than gene suppression constructs. DNM phenotypes should also be more stable than gene suppression phenotypes because the unintended cosuppression of a DNM transgene and its native gene should still yield floral sterility. In contrast, phenotypic instability due to loss or reduction in gene suppression would give rise to fertile gametes. In cases where two or more genes with distinct DNA sequences have redundant functions, however,

cosuppression would not allow the retention of floral sterility. Thus, when feasible, DNMs should be targeted to genes with non-redundant functions.

*Genetic Cell Ablation*

Genetic cell ablation occurs by the directed expression of a cytotoxic transgene in a specific cell type, resulting in the death or disturbed development of those cells and any organs derived from them. It disrupts both the cell type(s) in which the promoter specifies expression, and any descendant cell types that arise either directly from, or rely on signals generated by, the ablated progenitor cells. In floral cells, this usually results in ablation of entire organs or ancillary structures. The effects of cytotoxic gene products are usually confined to the cells in which they are expressed. Plant cells are connected by plasmodesmata and the size of the pores are large enough to allow most of the gene products discussed to pass through. However, transport through plasmodesmata appears to be highly regulated. For example, although DTA is a relatively small molecule, it does not appear to pass through plasmodesmata to adjacent cells (Day et al., 1995). The cytotoxicity of the gene product must be considered in relation to the degree of promoter specificity. If leaky expression in nontarget tissues occurs for a potent cytotoxin, decreased vegetative growth and yield are likely (see below).

Two classes of cytotoxic genes can be used for cell ablation. One class is composed of genes whose normal function results in cell death. The most commonly utilized members of this class are the A subunit of diphtheria toxin (DTA) and the ribonuclease barnase. The other class is composed of genes whose normal role does not necessarily cause cytotoxic effects, but whose inappropriate expression can be manipulated to result in cell death or cell dysfunction.

*DTA and* TOxfl. The DTA gene product of *Corynebacterium diphtheria* causes cell death by catalyzing the NAD+-dependent ADP-ribosylation of elongation factor-2 (EF2). In this form, the EF-2 subunit is unable to perform protein translation. The native diphtheria toxin is composed of three regions; an export signal peptide, the A chain, and the B chain. Only the A chain is needed to catalyze the ribosylation event (Greenfield et al., 1983). The B chain is needed for cellular receptor recognition and import of the A chain into foreign cells. By utilizing a genetically engineered version of the diphtheria toxin gene that only encodes the A chain (DTA), the gene product's activity is restricted to the cell in which it is expressed (Palmiter et al., 1987). Intracellularly expressed DTA cannot be taken up by adjacent plant cells or by organisms feeding on the plant tissue. It has therefore been used widely in research without the requirement for stringent containment measures.

A cytotoxin that behaves in an identical manner to DTA is the *Pseudomonas* Exotoxin A (toxA) gene (Day and Irish, 1997). The ToxA gene product of *Pseudomonas* is also an ADP-ribosyl-trasferase, catalyzing the same reaction as DTA. Use of ToxA might avoid political concerns over commercial use of a molecule originating from the causative agent of diphtheria. However, both DTA and ToxA act enzymatically, where

one molecule is capable of inactivating a large number of EF-2 subunits. In mammalian cells, one molecule of DTA is sufficient to cause cell death (Yamaizumi et al., 1978), but its toxicity to plant cells appears to be lower (Nilsson et al., 1998). A number of other ADP-ribosyltransferase toxins that occur naturally might also be useful for engineering cytotoxicity in plant cells, though their ribosylation target and corresponding mode of action differ from that of DTA and ToxA (Table 1).

TABLE 1. Bacteria Producing ADP-Ribosyltransferase Toxins

| Bacteria[1] | Toxin | Substrate |
|---|---|---|
| *Bordetella pertussis* | PT toxin | G protein |
| *Clostridium botulinum* | C2 toxin | actin |
| *Clostridium difficile* | *C. difficile* transferase | actin |
| *Clostridium perfringens* | iota toxin | actin |
| *Clostridium spiroforme* | *C. spiroforme* toxin | actin |
| *Corynebacterium diptheria* | DT toxin | EF-2 subunit |
| *Escherichia coli* | LT1 and LT2 toxins | G protein |
| *Pseudomonas aeruginosa* | ToxA | EF-2 subunit |
| *Vibrio cholerae* | CT toxin | G protein |

[1]Adapted from Domenighini et al. (1995)

The usefulness of DTA and ToxA for ablating several kinds of plant organs has been demonstrated (Day and Irish, 1997). Czako and An (1991) showed that expression of DTA was toxic to tobacco cells. Cell lineages of seed, pollen, and floral structures have been ablated using DTA in a variety of plant species. (Thorsness et al., 1991; Czako et al., 1992; Kandasamy et al., 1993; Thorsness et al., 1993; Day et al., 1995; Roberts et al., 1995; Twell, 1995; van der Geest et al., 1995). ToxA was shown to arrest embryo development in *Brassica napus* and tobacco when expressed under control of napin regulatory sequences (Koning et al., 1992). These previous studies were successful in ablating a portion of the floral structures, some derivatives of which resulted in sterility. Recently, fusion of the *LFY* regulatory sequences to DTA was demonstrated to cause complete ablation of all floral structures (Nilsson et al., 1998).

*Barnase, RNase TI, and Sarnase.* Another commonly utilized cytotoxin is the *Bacillus* amylolquefaciens-derived enzyme barnase, a ribonuclease (Hartley, 1988). Expression of barnase within a cell causes degradation of that cell's RNA, resulting in cell death. Unlike DTA and ToxA, barnase is active in prokaryotic cells; therefore, *B. amylolquefaciens also* encodes an inhibitor of barnase, the gene product barstar, which forms a specific complex with barnase, avoiding autotoxicity. Barstar is also used to protect transformation vectors (e.g., *Agrobacterium tumefaciens)* from the toxic effects of barnase. RNase T1 of *Aspergillus oryzae* has been used in a similar fashion to barnase (Quaas et al., 1988), but it does not have a known specific inhibitor. However, an RNase and its corresponding inhibitor have also been isolated from *Streptomyces*

*aureofaciens* strain 808/26, and designated sarnase and sarstar, respectively, which display similar activities to barnase and barstar (Nazarov et al., 1993).

Both RNaseTl and barnase have been used in a genetic cell ablation system with tobacco and oilseed rape to engineer male sterility. A tapetal-specific promoter caused ablation of the tapetal cell layer, thus preventing pollen formation (Mariam et al., 1990; Denis et al., 1993). The work was performed in tandem with RNase T1 and barnase, and similar results were obtained, including occasional fertility reversion. RNase T1mediated sterility became increasingly unstable at temperatures over 25 C (Denis et al., 1993). Male or female sterile lines of plants have been produced by targeting barnase expression at various floral cell types, including those within the anther, stigma, and seed coat (Mariam et al., 1990; Mariam et al., 1992; Denis et al., 1993; Block and Debrouwer, 1993; Schmulling et al., 1993; Goldman et al., 1994; Block et al., 1997; Colombo et al., 1997; Beals and Goldberg, 1997).

Beals and Goldberg (1997) demonstrated the versatility of barnase inactivation using barstar. Barnase was placed under control of the tobacco TA56 promoter, which is active in tissues of the anther involved in dehiscence. Barstar was placed under control of three separate promoters that are expressed in various anther cell types. Normal dehiscence occurred when barstar expression overlapped with the cell types in which the TA56 promoter drove barnase expression. Conversely, dehiscence was prevented when barstar expression did not overlap with that of the TA56 promoter. This suggests a possible strategy for avoiding barnase-induced toxicity from leaky promoters. Weak, non-specific expression of barstar could be utilized to protect against inadvertent barnase expression in nontarget tissues.

*Other Cytotoxins. A* number of other genes are available that can be manipulated to generate cell death. These gene products can result in toxicity in a variety of ways, including cell lysis or reduced viability. The ricin toxin A chain inhibits translation by depurinating 28S ribosomal RNA. Ricin has been shown to be an effective cytotoxin in animals, but may not be toxic to plant ribosomes, possibly making it unusable for plant studies (Sehnke, 1994; Thorsness and Nasrallah, 1995; Day and Irish, 1997). Overexpression of a general DNase activity should result in degradation of cellular DNA, ultimately causing cell death. Likewise, expression of a bacterial restriction enzyme should lead to cellular DNA fragmentation, reducing cell viability. Pectate lyase genes degrade the pectin components of plant cell walls, resulting in cell lysis. The cloned *pelf* gene of the plant pathogen *Erwinia chrysanthemi,* when expressed in *E. coli* allows the latter to behave as a plant pathogen and cause plant cell lysis (Keen and Tamaki, 1986). Expression of the *pelf* gene directly in plant cells would be expected to lead to cell lysis as well. The CytA toxin of B. *thuringiensis* subsp. *israelensis* causes insect cell membrane disruption and might cause disruption of plant cell membranes if expressed in plants (McLean and Whitely, 1987). Callase, a p-1,3glucanase, is secreted by the tapetum to breakdown a tapetal callose cell wall, releasing the microspores into the anther locules. Transgenic tobacco plants expressing a

modified form of p-1,3-glucanase under control of tapetum-specific promoters displayed premature.callose wall breakdown and varying degrees of male fertility (Worrall, 1992).

Expression of a recombinase in a plant cell, such as the Gin recombinase of phage Mu, would result in extensive rearrangement of the plant genome and cause decreased cell viability, ultimately leading to cell death (Maeser and Kahmann, 1991). In a similar fashion, overexpression of a methylase would be expected to result in a hypermethylated state of the cell's DNA and cause a general decrease in gene expression, thus impairing cell viability (Cigan and Albertsen, 1997). The URF13 mitochondrial protein of maize interacts with a family of fungal toxins, causing permeability and disruption of the inner mitochondrial membrane (Braun et al., 1990). Expression of this gene in desired cell types would lead to cell death through mitochondrial disruption when the plant was exposed to the fungal toxin, or infected with a pathogenic fungal strain that produced this toxin. Cell ablation would be selectively induced by exposure of the transgenic plants to fungal strains producing the toxin or exogenous application of isolated toxin. Manipulating the availability of plant metabolites or phytohormones are another means of affecting cell viability. The P. *syringae* subsp. *savastanoi iaaL* gene conjugates lysine to indolacetic acid (IAA). Expression of this gene in plants resulted in abnormal development by depletion of IAA (Spena et al., 1991). Strong overexpression of the *iaaL* gene product in a targeted cell type could also be expected to lead to reduced viability of that cell.

*Promoter Types*

In tissue ablation studies, the promoter utilized to drive cytotoxin gene expression should be expressed specifically and strongly in the desired tissue type(s). Optimally, expression should not occur in non-target tissues, although low levels of background expression may be tolerated (see below). Most promoters used for engineering sterility to date have targeted expression in either male or female reproductive structures, resulting in male or female sterility, respectively (Day and Irish, 1997). The earlier in floral development a promoter directs expression, the more complete the ablation of all floral structures will be. In a dioecious species like poplar, utilizing a promoter for a gene that is active early in floral development, and thus functions in both sexes, would be advantageous.

Low levels of non-floral expression may be tolerable, depending on the cytotoxin utilized. For example, the toxicity of DTA appears to be lower in plant cells compared to animals. The LFY promoter of *Arabidopsis* gives high levels of expression in floral meristems, but also displays low level vegetative expression (Weigel et al., 1992; Blazquez et al., 1997). Nilsson et al. (1998) constructed transgenic *Arabidopsis* lines containing a LFYP,om::DTA construct. The majority of lines displayed complete floral tissue ablation, while vegetative growth appeared to be normal based on *visual* inspection. This suggests that low levels of background expression may be tolerable for DTA (but see below). For barnase, low-level background expression could be attenuated by expressing barstar under the control of a weak constitutive promoter. Thus, only in

tissues where barnase expression was strong, would barnase activity be able to overcome barstar inactivation and cause cellular toxicity. For other toxins, manipulated overexpression is often needed to convey cytotoxic properties, suggesting that leakiness will not have major phenotypic effects.

The origin of the promoter (endogenous vs. heterologous) can influence the extent to which expression is regulated. Heterologous promoters may not give identical expression patterns relative to that observed in their endogenous backgrounds. This may be especially true when utilizing characterized floral promoters from an annual (e.g., tobacco or Arabidopsis) that is phylogenetically distant to a perennial tree species. In experiments utilizing a series of heterologous floral-specific promoter:: cytotoxin fusions, a decrease in vegetative growth of transgenic poplar was observed (Figure 2; R. Meilan, unpublished results). The large majority of these slow growing trees appeared morphologically normal, in contrast to the endogenous LFYpRoM-DTA results described above by Nilsson et al. (1998). Because the trees are still juvenile (three years of age), the effects on flowering have not yet been observed.

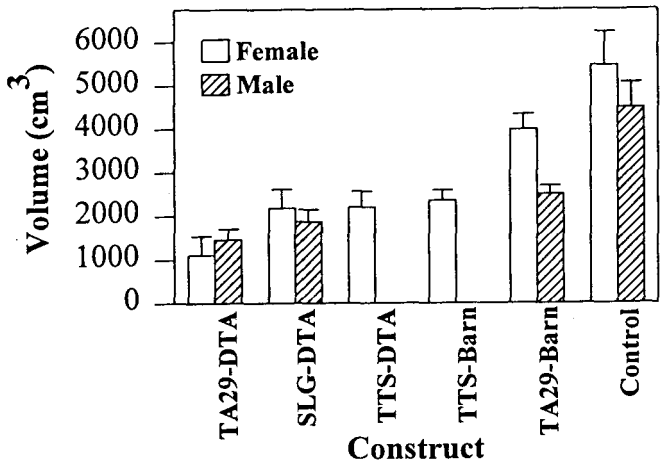



*Figure 2.* Vegetative growth of transgenic hybrid aspen trees engineered for reproductive sterility. The heterologous tobacco tapetum-specific TA29 promoter (Koltunow et al., 1990), a tobacco transmitting tissue specific (TTS) promoter (Wang et al., 1993), or a *Brassica S-locus* glycoprotein (SLG) promoter (Hacket et al., 1992) fused to Bamase (Barn) and/or DTA were introduced into female (INRA 717-1134, Populus *tremula x P. alba*) or male (INRA 353-38, P. *tremula x P. tremuloides*) clones of aspen. Vegetative growth (height x diameter squared) of transgenic and non-transgenic trees was measured after two growing seasons in the field near Corvallis, OR. Trees averaged 4.93 meters in height. Means are based on three or ten (control) ramets per transgenic line and between 5 to 22 lines per construct; standard errors are based on variation among line means.

In an effort to avoid the possible leakiness of heterologous promoters, we have isolated four different poplar floral homeotic gene homologs (above) and their corresponding

upstream regulatory regions. Both *PTLF* and PTAG1/PTAG2 display some vegetative expression in addition to strong floral expression. We are currently characterizing PTAPI-I and PTAPI-2 expression patterns. PTD expression, however, appears to be confined to floral tissues, and has thus been our focus for promoter studies. When tested in Arabidopsis, fusions of the PTD promoter to the GUS reporter gene have shown reporter activity in the petals and stamens, similar to what is observed with the AP3 promoter (Jack et al., 1992; Day et al., 1995; Hill et al., 1998); no vegetative expression was observed. Versions in which the PTD promoter is transcriptionally fused to DTA are being generated to analyze tissue ablation patterns (J. Skinner, unpublished data). When the AP3 promoter was fused to DTA, ablation of petals and stamens was observed in Arabidopsis (Day et al., 1995).

### 3.3. REVERSIBILE STERILITY

In some cases, reversible engineered sterility would be desirable. For example, this would allow for crossing of transgenic lines to avoid the need for retransformation of newly bred genotypes. It might also allow for use of seed-based propagation systems if sterility was restored in progeny. In all cases, fertility/sterility reversibility will require an inducible promoter to activate genes during vegetative development.

Under an ablation system, fertility could be restored by induced expression of an antisense or DNM version of the cytotoxin. When using the barnase gene for sterility, barstar expression could be induced, as has been demonstrated for restoration of male sterility (Mariana et al, 1992). Alternatively, a site directed recombinase could be used to delete or invert specific parts of a sterility transgene (Kilby et al., 1993, Oliver et al., 1998). In this system, target sites for an inducible recombinase are placed in direct orientation where deletion is desired, or inverted orientation where inversion is desired. A promoter bordered by target sites could be removed or reoriented with respect to the coding region.

A series of inducible promoters are available for plant systems (Gatz and Lenk, 1998). Among these are tetracycline-, copper-, glucocorticoid-, and ethanol-regulated promoters (Gatz et al., 1992; Mett et al., 1993; Aoyama and Chua, 1997; Caddick et al., 1998). Some of these inducible promoters have limitations that would preclude their use with a crop tree. The tetracycline and glucocorticoid promoters would probably not be commercially feasible, mainly due to cost and environmental concerns associated with the inducing agent. For example, tetracycline is light sensitive, would require multiple applications, and its use might raise medical concerns. Additionally, it has not worked in all species tested (Gatz, 1996). The copper regulated promoter is induced by use of a high-copper nutrient solution either taken up by the roots or absorbed through foliar sprays (Mett et al, 1993). A limitation is that copper concentrations in the soil and plant could become high enough (due to fertilization or natural concentration) to compromise sterility unexpectedly, and even modest application levels may cause plant toxicity (R. Mohammed, unpublished observations). Our results indicate that the copper responsive promoter does not function in an inducible manner in poplar (R. Mohammed,

unpublished observations), and an improved version was needed for functional inducibility in other species (Mett et al., 1996).

An inducible system that may meet both biological requirements and commercial feasibility is the recently characterized ethanol-regulated promoter (Caddick et al., 1998). The inducing agent ethanol is affordable, displays low phytotoxicity, and does not persist in the environment. The promoter appears to have negligible levels of leaky expression in tobacco, and the system was not induced by endogenous alcohol production in response to waterlogging. However, the regulatory` properties )f this promoter still need to be verified in species other than tobacco.

A hybrid promoter based on prokaryotic regulatory elements has been tested and verified in tobacco, and may also prove useful in other plant species (Moore et al., 1998). To prevent background expression, the system relies on unique DNA regulatory motifs that are not activated by endogenous plant transcription factors. The hybrid promoter was constructed by cloning two prokaryotic lac operator copies upstream of a eukaryotic minimal promoter. To activate transcription from the hybrid promoter, a chimeric transcription factor consisting of the *S. cerevisiae Ga14* transcriptional activation domain fused to a mutant version of the lac-repressor with high binding affinity is needed. No detectable reporter gene expression was observed in the absence of the transcriptional activator. Inducible expression would be provided by placing the chimeric transcription factor under the control of an inducible promoter (e.g., ethanol system).

For the recombination approach, recombinase activity would need to be induced to allow reversible sterility. This approach has the advantage that it could be done at any point in the tree's life cycle. For example, it could be activated after a clone had been designated to have superior traits worth crossing into other lines. A disadvantage is that the recombinase would need to be re-activated in progeny to restore sterility. For the hybrid lac promoter, the hybrid transcription factor would need to be supplied by activating its expression. Likewise, use of a strategy to express a product (i.e., DNM, antisense, barstar, etc.) that would inhibit the sterility mechanism would need to be under control of an inducible promoter. For these various systems, repression of the sterility system would need to be initiated at an appropriate time in the tree's life cycle; this might extend for several months during the floral induction period. Identification of an efficient induction system needs to be a central part of any strategy for sterility reversal.

## 4. Summary

Investigations of flowering in model annual plant species have provided major insights into the genetic and molecular mechanisms governing flowering. Floral . genes characterized in these species have allowed isolation of their homologs from poplar and other tree species. Several strategies for engineering sterility are available and a

redundant approach that employs more than one mechanism might enhance phenotypic stability over the multiple growth and dormancy cycles through which a tree passes prior to harvest. Verification of these techniques in poplar will help establish methods suitable for generating sterility in other tree species, and facilitate widespread use of transgenic plantations.

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