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MODIFICATION OF FLOWERING IN TRANSGENIC TREES

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ABSTRACT

The manipulation of flowering can provide many benefits. First, it permits development of a strategy to genetically engineer reproductive sterility. This should help alleviate ecological concerns over the use of transgenic plantations. Additionally, sterility can reduce genetic pollution from plantations, promote vegetative growth, and eliminate nuisance tissues. Flowering control should also allow for shorter breeding cycles.

While each strategy for engineering sterility has advantages, it is unclear which method will work best with trees. Thus, we are testing a variety of techniques, such as tissue-specific ablation; dominant negative mutations; and post-transcriptional gene silencing, including RNA interference. Using the first approach, we have had success with the promoter from PTD, a poplar homolog of the *Arabidopsis* gene *APETALA3*. This promoter has directed expression of reporter and cytotoxin genes in floral tissues of *Arabidopsis*, tobacco, and poplar.

Despite indications that one or more of these strategies can be successfully applied to trees, we have not yet demonstrated that any single one fulfills the basic requirements for commercial use as a long-term containment measure. We are conducting research to determine whether sterility can be complete, stable over several rounds of propagation and growing seasons, successfully identified in juvenile trees, and cause no detrimental effects on growth.

Recently we have begun working with genes that affect the onset of flowering. Using real-time PCR to quantify expression of these genes in various tissues collected from female and male trees during different seasons and along an age gradient, we have detected expression changes that correlate with maturation. We are also beginning to experiment with various genomics approaches for identifying other genes that may be useful in engineering flowering control.

KEYWORDS

Ablation, dominant negative mutations, flowering control, gene silencing, *Populus*, RNA interference, sterility

INTRODUCTION

Being able to control flowering in trees is desirable for several reasons ". First, it will enable the development of trees that are incapable of producing sexual propagules. This would limit gene flow into the wild, helping to mitigate ecological concerns over establishment of transgenic plantations. Second, it is likely to prevent the growth reduction

control could lead to more rapid progress through conventional breeding as a result of shorter breeding cycles.

One of the more common ways to genetically engineer reproductive sterility is to ablate cells by expressing a deleterious gene in a tissue-specific fashion ². Floral tissue-specific promoters are fused to one of a variety of cytotoxin genes that lead to rapid and early death of the tissues within which the gene product is expressed ³. The most popular form of sterility employs an RNase gene that, although isolated from a bacterium, encodes an enzyme that is common in plants and animals ⁷.

A second way to genetically engineer flowering control is through the use of dominant negative mutations (DNMs). DNMs suppress the function of a gene at the protein level by overexpression of a mutant version of a protein ⁸. Inhibition is thought to occur by a variety of means, including formation of an inactive heterodimer, sequestration of protein cofactors, sequestration of metabolites, or stable binding to a DNA regulatory motif. The usefulness of this approach for floral control was demonstrated in *Arabidopsis* with DNM versions of the *AGAMOUS* (*AG*) gene ⁹. 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 third technique to control flowering involves post-transcriptional gene silencing (PTGS). Recent studies in a variety of eukaryotic organisms have shown that doublestranded RNA is a potent inducer of PTGS. This approach to induced silencing has been termed RNA interference (RNAi) ¹⁰⁻¹¹. Recent work in plants using inverted repeat transgenes showed that RNAi could provide a reliable means for engineering stable suppression of gene activity in plants ¹²⁻¹³.

All of these approaches rely on the use of genes that control floral development, either through the use of floral-specific promoters or coding sequences with high homology to native genes that are targeted for suppression/silencing. In addition, flowering-time genes provide a means of advancing or retarding the onset of reproductive growth. Thus, a major effort in our laboratory is directed at isolating and characterizing genes that regulate flowering time. This paper describes the recent progress we have made in various aspects of flowering control in trees.

CELL ABLATION

In our early attempts at utilizing cell ablation with poplar, we relied on heterologous promoters, which had shown floral-specific expression in tobacco and *Arabidopsis4-16* to drive the expression of two cytotoxin genes, *DTA* ¹⁷ and *barnase* ¹⁸. When introduced into transgenic poplars, these fusions resulted in decreased vegetative growth (Fig. 1), suggesting leaky expression in non-target tissues.

Recently, we have begun experimenting with the promoter from a poplar gene, *PTD* (the *Populus trichocarpa* homolog of *DEFICIENS*), that appears to be floral-specific in its expression pattern ¹⁹. We have shown that *PTD* promoter directs expression of the *GUS* gene early in the development of floral organs in *Arabidopsis* and poplar (Fig. 2A & B). The latter was co-transformed with the *LEAFY* (*LFY*) gene from *Arabidopsis* under the control of the 35S promoter, which has been shown to induce early flowering in poplar ²⁰. A *PTD::DTA* fusion resulted in either ablated or perturbed development of petals and stamens in *Arabidopsis* (Fig. 2C), and the ablation of petals, stamens, and carpels in transgenic tobacco (Fig. 3A). *PTD::DTA* also appears to prevent flowers

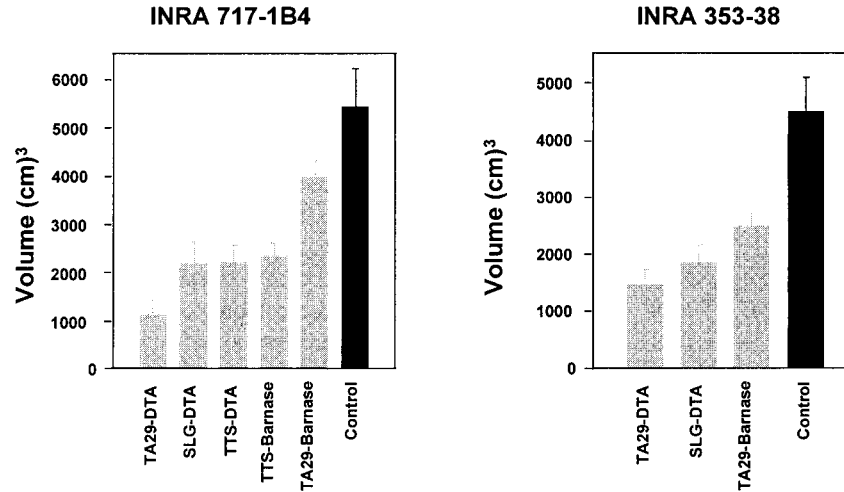


Figure 1. Effects of various sterility-inducing transgenes on growth. INRA 717-1B4 is a female *P. tremula* x *P. alba* clone; 353-38 is a male *P. tremula* x *P. tremuloides*.

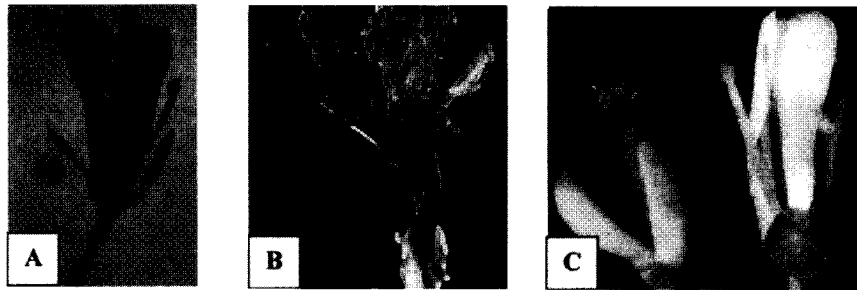


Figure 2. Specificity of PTD promoter. PTD::GUS expression in an *Arabidopsis* flower (A) and early-flowering (co-transformed with 35S::LFY) aspen (B). Dark zones indicate GUS expression. Effects of PTD::DTA on flower development in *Arabidopsis* (C); a transgenic flower is shown on the left, a non-transgenic control on the right.

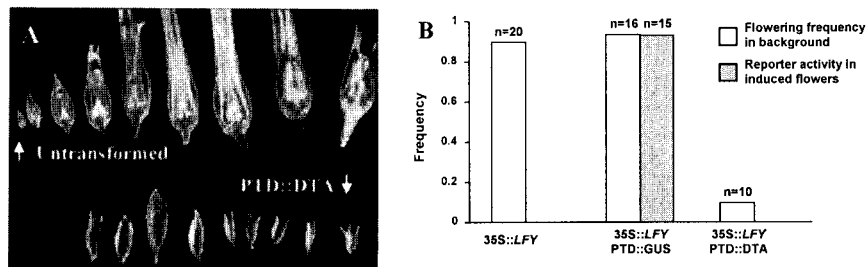


Figure 3. Effects of PTD::DTA on flower development in tobacco (A) and aspen (B). The upper panel in (A) shows normal flower development. Florets from transgenic tobacco plants consisted of only sepals [lower panel in (A)]. The 35S::LFY construct was used to induce flowering in aspen.

from forming on poplar co-transformed with 35S::LFY (Fig. 3B). Expression of PTD::DTA had no significant effects on growth in tobacco (data not shown).

DOMINANT NEGATIVE MUTATIONS

The approach taken for generating our DNM constructs is shown in Fig. 4. A key feature of these constructs is the promoter used to drive expression of the DNM transgene. For strong inhibition, the DNM protein should be present at a much higher level than that of the native protein. We are using hybrid promoters composed of two copies of the enhancer element from the 35S promoter (e35S) coupled to either the *ACTIN2* (*ACT2*) or *ACTIN11* (*ACTH*) promoters from *Arabidopsis*. The *ACT* promoters show strong expression in meristems, young growing tissues, and floral tissues. We have verified that the *2e35S:ACT2::GUS* and *2e35S:ACT11::GUS* constructs function in transgenic poplar, tobacco and *Arabidopsis*.

Guided by a study of an *AG* DNM η , we altered poplar and *Arabidopsis* *MADS-box* (a motif common to many floral homeotic genes) cDNAs via polymerase chain reaction (PCR) to encode proteins that lack the C-terminal domains (Fig. 4). Constructs containing full-length coding regions were also produced to provide positive controls for analysis of transgenic phenotypes. All constructs have been introduced into *Arabidopsis* via *in planta* transformation and co-transformed with 35S::LFY into poplar. Fifteen constructs containing *Arabidopsis* transgenes are currently under evaluation in transgenic *Arabidopsis*; five each for the *Arabidopsis* genes *API*, *AP3*, and *A G*. Though not a *MADS-box* gene, *PTLF* (the *P. trichocarpa* homolog of the *Arabidopsis* *LFY* gene) also has a modular structure α . Alignment of all known *LFY* homologs revealed a total of six putative protein domains, based on amino acid conservation (Fig. 4). However, the actual functions of these domains are unknown, so analysis of *LFY*

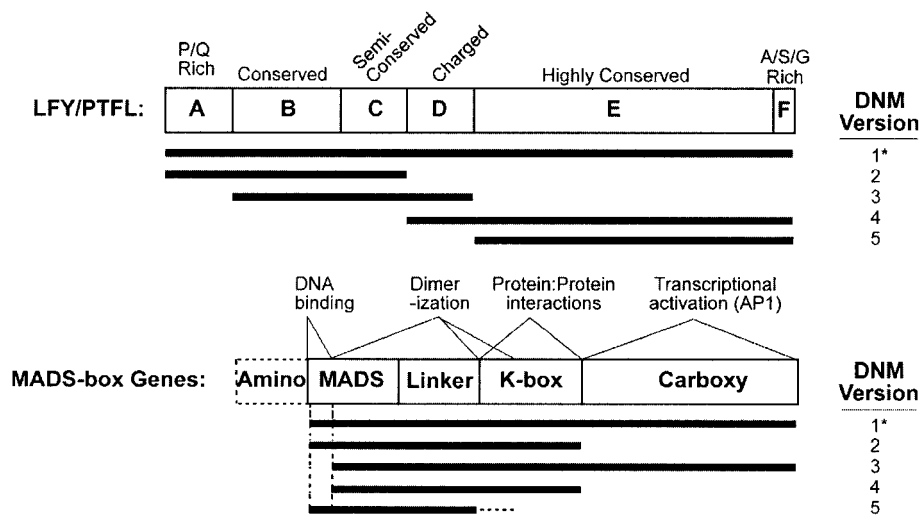


Figure 4. Domain structure and deletions used to generate dominant negative mutant versions of genes being tested in transgenic *Arabidopsis*, tobacco, or poplar. Constructs denoted with an asterisk are full-length versions.

genes lacking various domains was undertaken. Five *LFY* DNM transgenes have been introduced into *Arabidopsis* and tobacco; results from this will guide development of one or more *PTLF* DNMs for use in poplar.

In total, 27 different kinds of DNM and control constructs have been introduced into *Arabidopsis*. Preliminary results suggest that the hybrid promoter used in our first generation DNM constructs may not be exhibiting the desired expression pattern. Thus, we are now beginning to assemble another set of DNM constructs that will utilize a different promoter.

RNA INTERFERENCE

We have just begun to experiment with several options for suppressing flowering via RNAi of single and multiple floral homeotic genes. In these studies, we are using an early-flowering genotype of *P. alba* that allows us to test transgenes for their effects on flowering in a short time interval. We are focusing on several genes isolated in our laboratory, including poplar homologs to the *Arabidopsis* genes *LFY* (*PTLF*), *AG* (*PTAG*), *APETALA1* (*PTAPI*), and *APETALA3* (*PTD*).

Following studies by Chuang and Meyerowitz (2000) " with *AG*, the MADS-box region will be excluded from our constructs in order to avoid suppression of non-target MADS-box genes. Untranslated regions (UTRs) will also be excluded from the RNAi transgenes so that UTR-specific probes can be used to distinguish native gene and transgene expression while analyzing transgenic plants. An incomplete version of *PTLF* will be employed to ensure that it cannot encode a functional protein.

Constructs will be produced to suppress: 1) *PTLF*, 2) *PTAPI-1/PTAPI-2*, 3) *PTAG1/PTAG2*, and 4) *PTD*. While the first three are likely to give both male and female sterility, the phenotype of *PTD* is difficult to predict. *PTD* most resembles a B-function gene in sequence (required for stamen and petal development in most angiosperms); however, in poplar it is also expressed strongly in female flower primordia. We have characterized two *PTAPI*s and the two *PTAG*s²². Because each pair shares approximately 90% nucleotide identity in coding regions outside the MADS box, it is highly likely that an RNAi transgene-containing sequence from one of the pair (*e.g.*, *PTAPI-1*) will result in suppression of both (*e.g.*, *PTAPI-1* and *PTAPI-2*). RNAi and cosuppression studies both suggest that this level of nucleotide identity is sufficient for cross-suppression, which has been observed for sequences with as little as 84% identity¹¹. If this turns out to be untrue, we will make constructs containing sequences from both genes within each pair.

Because of functional redundancy, suppression of more than one floral regulatory gene is likely to be necessary to achieve complete sterility. Thus, we will also generate constructs that are designed to suppress the following pairs of genes: 1) *PTLF/PTAPI*, 2) *PTLF/PTAG*, 3) *PTAPI/PTAG*. One construct is designed to suppress three genes: *PTLF/PTAPI/PTAG*.

EXPRESSION STABILITY

Stable transgene expression over the lifetime of a tree, and in its vegetative propagules, is critical for all engineered sterility systems. We have therefore begun investigating the stability of transgene expression using reporter genes.

The vector to be used for studying stability will consist of two reporter genes, green fluorescent protein (GFP) and a herbicide resistance gene, which were selected for

economy and speed of assay. Glufosinate is a contact herbicide that inhibits glutamine synthetase. Glufosinate resistance is conferred by the *bar* gene from *Streptomyces hygroscopicus*, which encodes phosphinothricin acetyltransferase ²³. Both genes can be used for scanning entire plants for sectors that have undergone gene silencing--one using a UV light source (GFP) and the other using low concentrations of glufosinate containing herbicide, which acts in a non-systemic manner.

Because gene silencing may be very different with a native versus a foreign promoter, we are using a different promoter with each reporter gene. We have chosen the *rbcS* promoter to drive expression of the *bar* gene because it exhibits strong leaf expression. An *Arabidopsis rbcS* promoter fused to *bar* has been shown to confer high levels of glufosinate resistance in poplar ²⁴, a result that we have repeated with transgenic poplars generated in our laboratory and grown in local field trials (unpubl. data). The GFP reporter gene will be driven by the 35S promoter, which has been used widely in transgenic poplars and is known to yield high levels of expression in leaves.

Poplar lines containing these reporter-gene constructs will be subjected to various stresses and grown in the laboratory and the field for several years to observe expression.

FLOWERING-TIME GENES

We are also identifying genes that regulate the transition from the vegetative to the reproductive phases in trees. We are trying to determine the degree of correspondence between the genetic control of phase transition in poplar, which has a juvenile period of four to six years, and that of the herbaceous annual plant *Arabidopsis*, which initiates flowering after a juvenile period that is only weeks in length. Both genera belong to the same clade of the eudicots ²⁵, and the *Populus* genome is small ²⁶, facilitating gene-to-gene comparisons. In addition, we are trying to relate vernalization, the induction of flowering by cold treatment, to the chilling temperatures needed to end dormancy in temperate zone trees, and to determine whether changes in DNA methylation are involved in post-dormancy changes in gene expression.

We are identifying candidate genes for study by isolating genes from poplar based on sequence homology with known flowering-time genes in *Arabidopsis* and other annual plants. To evaluate whether these candidate genes have important regulatory roles, we are taking advantage of a few characteristics that have established *Populus* as a model system for genetic and molecular analyses of woody plants. First, the ease of vegetative propagation, and the fidelity of juvenile characteristics in vegetative propagules, provides a continuous age gradient of a single genotype through the first year of flowering. This facilitates intensive study of quantitative and cell-specific changes in gene expression in relation to phase transition. Moreover, the ease with which poplars can be transformed has made them the tree taxa of choice for transgenic studies worldwide ^{27-3*}. This allows direct functional tests of the roles of specific genes via RNAi and overexpression.

We have already conducted expression analysis on several candidate genes in relation to maturation and seasonal changes in expression. We collected various tissues at different seasonal times (Fig. 5) from one female and one male *P. trichocarpa* x *P. deltoides* genotype. Ramets of each clone were represented in a continuous age gradient of one to six years (*i.e.*, they had been through one to six growing seasons when we began our collections). For both genotypes, inflorescences were first initiated at age four (*i.e.*, during their fifth growing season). In total, more than 60 RNA samples

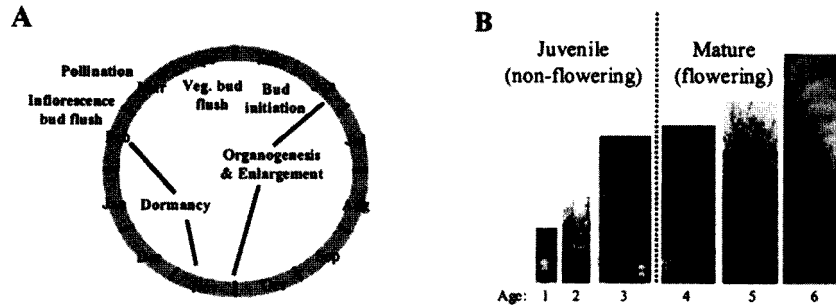


Figure 5. Seasonal cycle for *P. trichocarpa* x *P. deltoides* floral development in western Oregon (A). An age gradient of the female poplar clone from which tissues were collected (B).

were isolated. For accurate quantification of the modest expression levels shown by most of these genes, real-time quantitative RT-PCR was employed.

The vegetative expression levels of *PTLF* differed significantly between juvenile and mature ramets in vegetative buds initiated during the current season (shoot apical meristem leaf primordia, and bud scales were removed) (Fig. 6). This difference persisted, although to a lesser degree, into autumn, but all ramets showed a uniformly low level of *PTLF* transcript in post-dormancy vegetative buds. The expression of *P. trichocarpa* *IDI-LIKES* (*PTIDILS*) was markedly upregulated in newly expanding shoots (shoot apical meristem, leaves, internode) from mature ramets that would soon initiate inflorescences (Fig. 6).

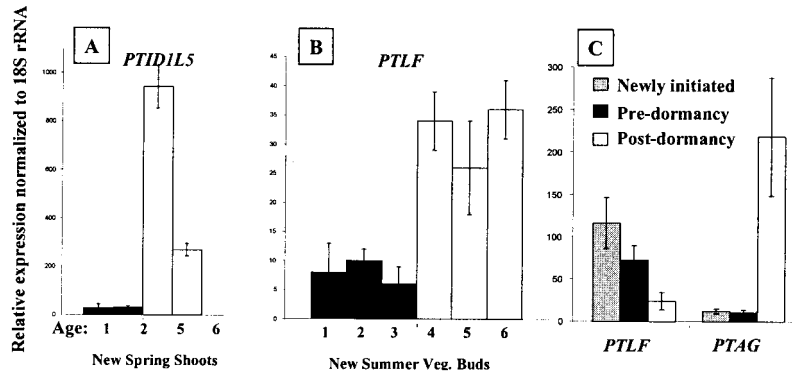


Figure 6. Variation in gene expression with age in a male poplar clone for poplar homologs of *INDETERMINATE*] (A) and *LEAFY* (B). Darkened bars indicate juvenile trees, open bars indicate sexually active trees. (C) Seasonal variation in vegetative bud gene expression in a mature female ramet for the poplar *LEAFY* gene (left) and *AGAMOUS* gene (right) at five years of age. Bars show one standard deviation based on three replicate measurements.

That *PTLF* vegetative bud expression was highest in the newly initiated vegetative buds of mature trees during long days is consistent with expectations based on the expression characteristics of *LFY*. Similarly, the expression pattern for *PTIDIL5* corresponds well with that of the maize gene *ID 1*³¹. *PTAG2* vegetative bud expression did not correlate with maturation, but was instead expressed at surprisingly high levels in ramets of all ages. It also exhibited a striking pattern of seasonal variation, in contrast to that seen with *PTLF* in mature ramets (Fig. 6). *PTAG2* was expressed at low levels in newly initiated vegetative buds and pre-dormancy, autumn vegetative buds. However, it was dramatically upregulated in post-dormancy vegetative buds. This result is particularly interesting given that the methylation and expression level of *AG is* altered in *Arabidopsis* lines with an overall decrease in methylation, and that the *CURLY-LEAF* gene acts to prevent *AG* vegetative expression³². The *PTA G2* pattern suggests the possibility that dormancy causes a transient change in its epigenetic regulation.

GENOMICS

Another way in which we will identify genes whose expression changes with maturation is via hybridization of poplar expressed sequence tag (EST) microarrays. With the aid of our collaborators in Sweden and France, we will use various RNA collections to screen microarrays for additional genes whose expression is correlated with maturation, and confirm expression patterns of selected genes using real-time PCR. Based on sequence homologies and expression patterns, we will begin to study the function of several genes via transformation using RNAi suppression and overexpression. All constructs will be tested in the early-flowering *P. alba* clone.

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REFERENCES

1. Strauss, S.H., W.H. Rottmann, A.M. Brunner & L.A. Sheppard, Genetic engineering of reproductive sterility in forest trees, *Mol. Breed.*, 1995, 1, 5-26.
2. Brunner, A.M., R. Mohamed, R. Meilan, L.A. Sheppard, W.H. Rottmann & S.H. Strauss, Genetic engineering of sexual sterility in shade trees, *Journal of Arboriculture*, 1998, 24(5), 263-273.
3. Skinner, J.S., R. Meilan, A.M. Brunner & S.H. Strauss, Options for Genetic Engineering of Floral Sterility in Forest Trees, In: *Molecular Biology of Woody Plants*, S.M. Jain & S.C. Minocha (eds.), Kluwer Academic Publishers, Dordrecht,

4. Eis, S., E.H. Garman & L.F. Ebell, Relation between cone production and diameter increment of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], Grand fir [*Abies grandis* (Dougl.) Lindl.] and western white pine [*Pinus monticola* (Dougl.)]. *Can. J. Bot.*, 1965, 43, 1553-1559.
5. Tappeiner, J.C., Effect of cone production on branch, needle and xylem ring growth of Sierra Nevada Douglas-fir, *For. Sci.*, 1969, 15, 171-74.
6. Teich, A.H., Growth reduction due to cone crops on precocious white spruce provenances, *Environ. Canada Bi-monthly Res. Notes*, 1975, 31, 6.
7. Mariani, C., M. DeBeuckeleer, J. Truettner, J. Leemans & R.B. Goldberg, Induction of male sterility in plants by a chimaeric ribonuclease gene, *Nature*, 1990, 347, 737741.
8. Espeseth, A.S., A.L. Darrow & E. Linney, Signal transduction systems: Dominant negative strategies and mechanisms, *Mol. Cell. Diff.*, 1993, 1, 111-161.
9. Mizukami, Y., H. Huang, M. Tudor, Y. Hu & H. Ma, Functional domains of the floral regulator AGAMOUS: Characterization of the DNA binding domain and analysis of dominant negative mutations, *Plant Cell*, 1996, 8, 831-845.
10. Fire, A., RNA-triggered gene silencing, *Trends Genet.*, 1999, 15, 358-363.
11. Boshier, J.M. & M. Labouesse, RNA interference: Genetic wand and genetic watchdog, *Nature Cell Biol.*, 2000, 2, E31-E36.
12. Waterhouse, P.M., M.W. Graham & M.-B. Wang, Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA, *Proc. Natl. Acad. Sci. USA*, 1998, 95, 13959-13964.
13. Chuang, C.-F. & E.M. Meyerowitz, Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*, *Proc. Natl. Acad. Sci. USA*, 2000, 97, 4985-4990.
14. Koltunow, A.M., J. Truettner, K.H. Cox, M. Wallroth & R.B. Goldberg, Different temporal and spatial gene expression patterns occur during anther development, *Plant Cell*, 1990, 2, 1201-1224.
15. Hackett, R.M., M.J. Lawrence & C.H. Franklin, A *Brassica* S-locus related gene promoter directs expression in both pollen and pistil of tobacco, *Plant J*, 1992, 2, 613-617.
16. Wang, H., H.M. Wu & A.Y. Cheng, Development and pollination regulated accumulation and glycosylation of a stylar transmitting tissue-specific proline-rich protein, *Plant Cell*, 1993, 5, 1639-1650.
17. Greenfield, L., M.J. Bjorn, G. Horn, D. Fong, G.A. Buck, R.J. Collier & D.A. Kaplan, Nucleotide sequence of the structural gene for diphtheria toxin carried by corynebacteriophage ϕ 3, *Proc. Natl. Acad. Sci. USA*, 1983, 80, 6853-6857.
18. Hartley, R.W., Barnase and barstar: Expression of its cloned inhibitor permits expression of a cloned ribonuclease, *J Mol. Biol.*, 1988, 202, 913-915.
19. Sheppard, L.A., A.M. Brunner, K.V. Krutovskii, W.H. Rottmann, J.S. Skinner, S.S. Vollmer & S.H. Strauss, A *DEFICIENS* homolog from the dioecious tree *Populus trichocarpa* is expressed in both female and male floral meristems of its twowhorled, unisexual flowers, *Plant Physiol.*, 2000, 124, 627-639.
20. Weigel, D. & O. Nilsson, A developmental switch sufficient for flowering initiation in diverse plants, *Nature*, 1995, 378, 495-500.
21. Rottmann, W.H., R. Meilan, L.A. Sheppard, A.M. Brunner, J.S. Skinner, C. Ma, S. Cheng, L. Jouanin, G. Pillate & S.H. Strauss, Diverse effects of overexpression of *LEAFY* and *PTLF*, the poplar homolog of *LEAFY/FLORICAULA*, in transgenic poplar (*Populus trichocarpa*) and *Arabidopsis*, *Plant J*, 2000, 22, 235-245.

22. Brunner, A.M., W.H. Rottmann, L.A. Sheppard, K. Krutovskii, S.P. DiFazio, S. Leonardi & S.H. Strauss, Structure and expression of duplicate AGAMOUS orthologs in poplar, *Plant Mol. Biol.*, **2000**, 44 (5), 619-634.
23. Riemenschneider, D.E., Genetic engineering of horticultural and forestry crops for herbicide tolerance, In: *Biotechnology of Ornamental Plants*, R.L. Geneve, J.E. Preece & S.A. Merkle (eds.), CAB International, 1997, pp. 367-384.
24. DeBlock, M.D., Factors influencing the tissue culture and the *Agrobacterium tumefaciens*-mediated transformation of hybrid aspen and poplar clones, *Plant Physiol.*, 1990, 93, 1110-1116.
25. Soltis, P.S., D.E. Soltis & M.W. Chase, Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology, *Nature*, 1999, 402, 402-403.
26. Bradshaw Jr., H.D., Case history in genetics of long-lived plants: Molecular approaches to domestication of a fast-growing forest tree: *Populus*, In: *Molecular Dissection of Complex Traits*, A.H. Paterson (ed.), CRC Press, NY, 1998, pp. 219-228.
27. Tsai C-J., G.K. Podila & V.L. Chiang, *Agrobacterium*-mediated transformation of quaking aspen (*Populus tremuloides*) and regeneration of transgenic plants, *Plant Cell Rep.*, 1994, 14, 94-97.
28. Tzfira, T., C.S. Jensen, W. Wang, A. Zuker, B. Vincour, A. Altman & A. Vainstein, Transgenic *Populus tremula*: A step-by-step protocol for its *Agrobacterium*-mediated transformation, *Plant Mol. Biol. Rep.*, 1997, 15, 219-235.
29. Kim, M.-S., N.B. Klopfenstein & Y.W. Chun, *Agrobacterium*-mediated transformation of *Populus* species. In: *Micropropagation, Genetic Engineering, and Molecular Biology of Populus*, N.B. Klopfenstein, Y.W. Chun, M.-S. Kim & M.R. Ahuja (eds.), Gen. Tech. Rep. RM-GTR-297. U.S. Dept. of Agric. Forest Service, Fort Collins, CO, 1997, pp. 51-59.
30. Han, K.-H., R. Meilan, C. Ma & S.H. Strauss, An *Agrobacterium* transformation protocol effective in a variety of cottonwood hybrids (genus *Populus*), *Plant Cell Rep.*, **2000**, 19, 315-320.
31. Colasanti, J., Z. Yuan & V. Sundaresan, The indeterminate gene encodes a zinc finger protein and regulates a leaf-generated signal required for the transition to flowering in maize, *Cell*, 1998, 93, 593-603.
32. Goodrich, J., P. Puangsomlee, M. Martin, D. Long, E.M. Meyerowitz & G. Couland, A polycomb-group gene regulates homeotic gene expression in *Arabidopsis*, *Nature*, 1997, 386, 44-50.