

Final Report

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Development and Validation of Sterility Systems for Trees

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Steve Strauss, : 541-737-6578, Steve.Strauss@OregonState.edu
Olga Shevchenko, 541-737-8482, Olga.Shevchenko@OregonState.edu
Cathleen Ma, 541-737-8486, Caiping.Ma@OregonState.edu

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Oregon State University
Department of Forest Science
321 Richardson Hall
Corvallis, OR 97331-5752

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EXECUTIVE SUMMARY

The overall goal of this project was to develop and validate sterility systems in poplar with the ultimate goal of fulfilling the basic requirements for commercial use. For this, sterility must be complete and stable over multiple growing seasons, cause no detrimental effects on vegetative growth, and successful transformation events must be identifiable via molecular tests when trees are still juvenile. Because of the inherent difficulties in achieving and demonstrating complete sterility in trees, our approach was to study alternate sterility systems in *Arabidopsis* and/or early-flowering tree systems. The public benefit from this work is the capacity for containment of genes or exotic forms of trees so they can be of benefit for industry for production of wood, energy, and renewable products, while having minimal impact on wild populations of trees.

We tested three methods for engineering sterility: dominant negative mutant (DNM) proteins, floral tissue ablation, and RNA interference (RNAi) to suppress the expression of several floral regulatory genes. The ultimate goal of this work was to produce a number of transgenic poplars that could be outplanted to enable future assessments of the effectiveness of these transgenic sterility methods.

Our attempts to produce ablation constructs that did not interfere with tree health were partially successful. Using the poplar *LEAFY* gene promoter and the barnase/barstar system, we were able to regenerate plants that grew well in the greenhouse, but they showed poor health in the field. Four of seven DNM genes tested were considered promising enough, based on results in *Arabidopsis*, to produce transgenic poplars. Single, double, and triple RNAi genes were produced and transformed into poplar. Over all, we produced 1,964 PCR-confirmed transgenic events with 19 different kinds of sterility genes and several kinds of control genes. We propagated 5,640, 6,820, and 7,055 trees for each of three test poplar genotypes, and field plantings were begun in Spring of 2003 and will be finished in Spring 2007. Continued field studies and monitoring will be required to establish if any of the approaches we have taken will prove to be safe for tree health, stable, and provide reliable containment.

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Summary of Project Objectives

We have tested three methods for engineering sterility. For two methods, we also tested strategies to improve the genetic engineering efficiency for multiple transgenes by reducing the number of transcriptional units (i.e., a promoter::coding region::terminator unit) present in a construct.

- 1. Dominant Negative Mutant (DNM) Sterility Systems.** Complete analysis of *Arabidopsis* DNM transgenes in transgenic *Arabidopsis*.
- 2. Floral Ablation Sterility Systems.** This approach uses a floral promoter to direct the expression of a cytotoxin. We will evaluate the poplar *NZZ* promoter and the *PTAPI* promoter, as well as basic approaches to avoid tree toxicity.
- 3. RNAi Sterility Systems.** Double-stranded RNA (dsRNA) is a potent inducer of post-transcriptional gene silencing (PTGS), termed RNA interference (RNAi). Poplar RNAi constructs will be produced and tested in transgenic plants.
- 4. Redundant Sterility Systems.** Overlapping gene function, environmental influences on gene expression, and the potential instability of transgene expression or silencing make it difficult to be confident that sterility will be absolute throughout a trees's life span. Redundant sterility constructs may be able to overcome these difficulties. We will combine sterility genes and transform them together into poplar.
- 5. Establishment of Transgenic Poplar Field Plantings.** Field plantings will be initiated to enable additional studies after the grant period ends.

Overview of approach and modifications to work plan

We have isolated and characterized six poplar gene homologs (Table 1) of well-studied *Arabidopsis* genes that control the early stages of flower development (Ng and Yanofsky, 2000). All but *LFY/PTLF* belong to a family of transcription factors named after its highly conserved DNA binding and dimerization region, the MADS domain (Riechmann and Meyerowitz, 1997). We had planned to

Table 1. Names and functions of genes or their promoters that we studied.

| <i>Arabidopsis</i> Gene | Function in <i>Arabidopsis</i> | Poplar Homolog(s) |
|-------------------------|---|-------------------------|
| <i>AGAMOUS (AG)</i> | Stamen & carpel identity | <i>PTAG1, PTAG2</i> |
| <i>APETALA3 (AP3)</i> | Petal & stamen identity | <i>PTD</i> |
| <i>APETALA1 (API)</i> | Flower initiation; sepal & petal identity | <i>PTAPI-1, PTAPI-2</i> |
| <i>LEAFY (LFY)</i> | Flower initiation | <i>PTLF</i> |

isolate a poplar homolog of the newly discovered (i.e., at time of proposal submission) single-copy gene from *Arabidopsis NOZZLE (NZZ)*, which is necessary for both female and male fertility. It differs from the genes listed above in that it acts at very late stages of flower development (Schiefthaler et al., 1999; Yang et al., 1999). However, after intensive study we have concluded it is not a suitable candidate for near-term sterility systems due to its rapid evolution that makes isolation difficult, and its very weak expression that would make it a poor gene to drive an ablation construct. Thus, we stopped its development to ensure that the other goals of this proposal could be met.

Studies in *Arabidopsis* have identified moderately strong DNM transgenes, thus we are testing the same genes in poplar; the highly conserved amino acid sequence of these genes should enable DNMs to work across genera. Additional studies focus on the development of floral ablation and dsRNA-induced gene silencing (RNAi) sterility systems. Because overlapping gene function and the potential instability of transgene expression or silencing make it difficult to be confident that sterility will be absolute throughout a tree's life span, we also seek to produce multi-gene, "redundant" sterility constructs.

The long delay until the onset of flowering in most poplars (three to six years), and their recalcitrance to conventional flowering-induction treatments, has been the most important impediment to our research on engineered sterility. Transgenic trees must be planted in the field for several years before flowering starts, which is costly, time consuming, and restricted by GMO regulations.

The female *P. alba* clone (6K10) was obtained from Maurizio Sabatti (University of Tuscia, Viterbo, Italy) that had been shown to flower in less than one year in Italy under some conditions. We developed conditions for its propagation and maintenance *in vitro*, and produced 60 plants for initial experiments on floral induction. Treatments tested included elevated CO₂, fertilization, water stress, root chilling (4°C for six months), paclobutrazol (75 ppm, root drench), and controls. In a subsequent experiment, we applied various concentrations of paclobutrazol (0, 5, 10, 25, 50, and 75 ppm root drenches), and grew plants for two weeks in a greenhouse before transferring them to a chamber with photoperiodic control, to allow induction of dormancy. One month after transfer, three out of the five plants that had received either the water stress or the root chilling treatment produced reproductive buds (Figure 1). Six months of root chilling (Figure 2A) followed by short days and cool temperatures (to force buds to set) resulted in plants that flowered by the time they were nine months of age (Fig. 2B). Other studies were conducted to try and identify the optimal conditions for floral induction. Different durations in the water baths, short days (8-hr photoperiod) and cool temperatures (55 °C) were used to try and induce even earlier bud-set. Results from these experiments indicated that it is difficult to induce flowering in less than nine months; chilling roots for much less than six months was not sufficient to induce flowering. Unfortunately, subsequent trials with all of these methods for inducing flowering were found to give variable results. Nonetheless, use of this clone, which does begin to flower within three years in the field in Oregon, will be useful for broadening, and speeding,



Figure 1. Cross-section of a floral bud from a precocious clone of *Populus alba* approximately six months after floral induction treatment (root chilling) and one month of growth under short days.

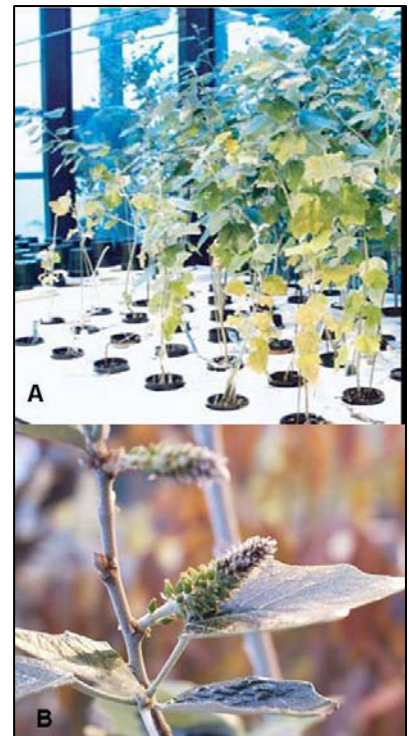
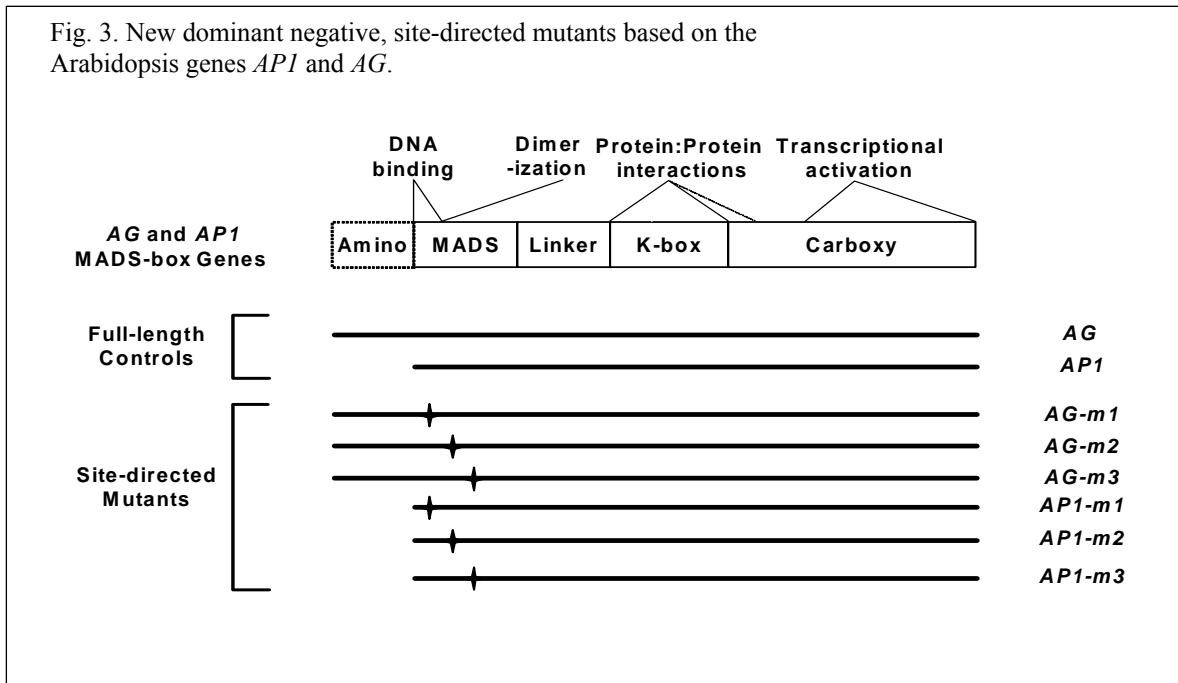


Fig. 2. Induction of flowering in *Populus alba* (clone 6K10) via root chilling. (A) Water bath used for inductive treatments. (B) Catkin on a nine-month-old plant in a glasshouse.

sterility assessments. A large number of transgenic events have been produced in this clone that will be outplanted in the field for long-term assessment.

1. Dominant Negative Mutant (DNM) Constructs

The *Arabidopsis* genes *Agamous* (*AG*) and *Apetala1* (*AP1*) are flowering organ identity genes that belong to a family of transcription factors named after its highly conserved DNA binding and dimerization region, the MADS domain. Homologues of *Arabidopsis AG* and *AP1* have been isolated from the poplar genome. By manipulating expression levels of these genes it should be possible to produce sterile trees. DNMs usually disrupt the function of the wildtype (WT) endogene by producing altered peptides that inhibit the normal function of endogene.



We produced three site-directed mutants for each of *Arabidopsis AG* and *AP1* genes (Fig. 3), and 1 C-termini truncation construct for *AP1* gene, which has 3' transcriptional activation properties (Fig. 4). Two or three highly conserved amino acids have been replaced by ones with different physical /biochemical properties. Mutations were based on mammalian and rice MADS-box genes (Molkentin et al. 1996; Jeon et al. 2000), predicted to effect DNA binding (*AG-m2*, *AP-m2*), or both protein-protein interactions and DNA binding (*AG-m1*, *m3*; *AP-m1*, *m3*). The pAlter “Promega” system was used to create site-directed mutants. Full length sequence of *AG6* with N-termini and *AP1-6* were used as templates for the site-directed mutants (Fig. 6). The genes were expressed under an enhanced 35S promoter with an E9 terminator, and had flanking matrix attachment elements (Fig. 5).

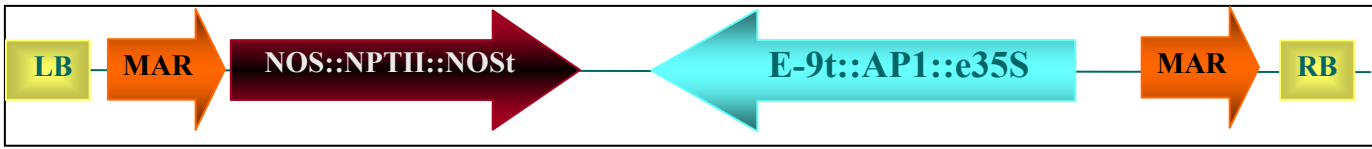
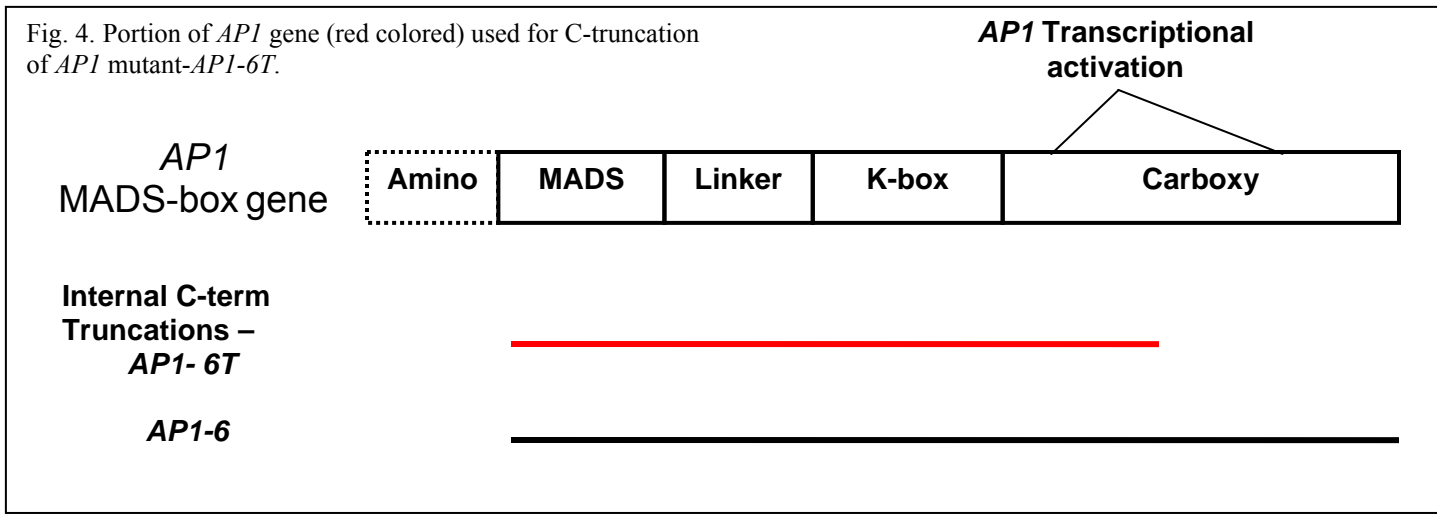


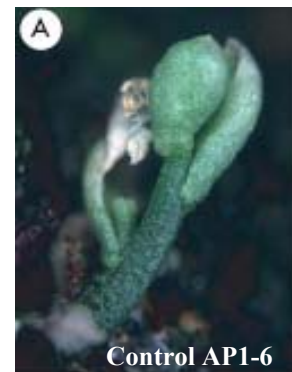
Fig. 5. Altered coding region of *AG* and *AP1* genes were driven by a 35S promoter with double enhancers, MARs have been fused on both side of DNM operon in tandem sequence; *NPTII*, gene conferring kanamycin resistance; LB/RB, left- and right-hand T-DNA borders.

All 7 constructs were first evaluated in *Arabidopsis*. T1 generation *Arabidopsis* plants were screened for abnormalities in flower phenotypes and vegetative morphology. *AG* and *AP1* are floral organ identity genes, ectopically expressed *AG* and *AP1* DNM often cause early flowering, reduction of vegetative phase, transition to inflorescence phase, and onset of a spectrum of morphological abnormalities, including defects in flower and leaf structures. Generally, the DNM mutant phenotypes resembled either loss-of-function or gain-of-function mutants (Table 2).

Table 2. *Arabidopsis* DNM T1 Transgenic Summary

| Transgene | No. T1 lines | No. mutants | No. sterile lines | General floral phenotype | Negative Pleiotropic Effects |
|---------------|--------------|-------------|-------------------|--------------------------|------------------------------|
| <i>API-6T</i> | 40 | 37 | 37 | Gain | Large |
| <i>API-m1</i> | 32 | 11 | 11 | Gain | Large |
| <i>API-m2</i> | 20 | 8 | 7 | Loss | Mild |
| <i>API-m3</i> | 18 | 9 | 6 | Loss | Mild |
| <i>AG-m1</i> | 25 | 21 | 9 | Gain | Large |
| <i>AG-m2</i> | 21 | 10 | 6 | Loss | Mild |
| <i>AG-m3</i> | 30 | 19 | 9 | Loss | Mild |

***API-6* control.** Most of the *API-6* control transgenic plants were extremely small, and many plants died after very little growth. Most of the surviving plants were mutant and flowered extremely early, producing only one or two leaves before flowering. Moreover, these plants remained very small, and inflorescence shoots were short and appeared to terminate prematurely in abnormal flowers. The abnormal floral phenotypes may be due in part to the extremely early-flowering phenotype, and may represent incomplete vegetative to floral transformations. This gene, coupled with the MAR elements and strong promoter, appear to be powerful elicitors of flowering in *Arabidopsis*.



Nearly all of the ***API-6T*** T1 DNM transgenics exhibited a diversity of mutant phenotypes. Most flowered early, though this was not as extreme as in the *API-6* transgenics. Many of the *API-6T* transgenics produced abnormal flowers that had characteristics similar to *API* gain-of-function mutants. Secondary flowers arose from primary flowers, and tertiary flowers from secondary flowers, indicating a partial conversion from flowers to indeterminate inflorescence shoots. Primary flowers were sometimes a complex mass of compound or tightly clustered flowers with abnormal floral organs and unusual numbers of floral organs. Stamens did not appear capable of producing pollen, and often exhibited transformation toward petals. Carpels were incompletely developed and not properly fused. None of the *API-6T* mutant plants have developed normal siliques or set seed.

***API-6T* (C termini truncation).** Caused dwarf phenotype, severe shortening of flowering time, only 2 rosette leaves (8-10 in WT), very short stem, compound flower on the main vegetative shoot, and solitary flowers with 5 petals at short lateral shoots (Fig.6).

API-m1. Most plants produced very strong *AP-1* gain-of-function phenotype similar to *AP1-6* (Fig. 6; tiny plants, 2 rosette leaves, early flowering, short primary inflorescence shoot converts into compound flower, lateral shoot convert into solitary flower).

API-m2 and **API-m3** showed similar *apl* loss-of-function phenotype. Mutants exhibit partial conversion of flowers into indeterminate inflorescence shoots; secondary flowers arise in the first-whorl (sepals) axils of primary flowers (Fig. 7; 4 small rosette leaves, long indeterminate inflorescence primary shoot, and a lot of indeterminate lateral inflorescence shoots).

Besides producing high frequency of DNM mutants (84%) we observed novel phenotypes for **AG-m1** strong *AG* gain-of-function mutants. Mutant T1 *Arabidopsis* plants exhibit a partial conversion of sepals to carpels (stigmatic papillae develop on the sepal), and in addition, the carpelloid sepals were fused, petals did not develop, and stamens were poorly developed too (Fig. 8; early flowering, lack of rosette leaves, small flowers, no petals, 4 sepals fused together, stigma structures on the top of sepals, lack of stamens, they are short, not quite developed and sterile, ectopic ovules)

AG-m2 and **AG-m3.** 30% of *Arabidopsis* transgenic lines produced a loss-of-function flower phenotype (Fig. 8; nested flower)

API-m2, *API-m3*, and *AG-m2* and *AG-m3* have been selected for poplar transformation because of minor pleiotropic effects and 30% of *Arabidopsis* plants appeared to be sterile.

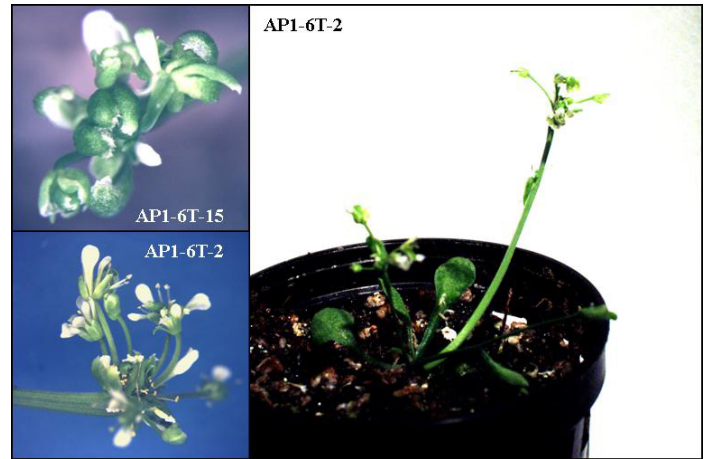


Fig 6. Dwarf phenotype of the *API-6T* transgenics.

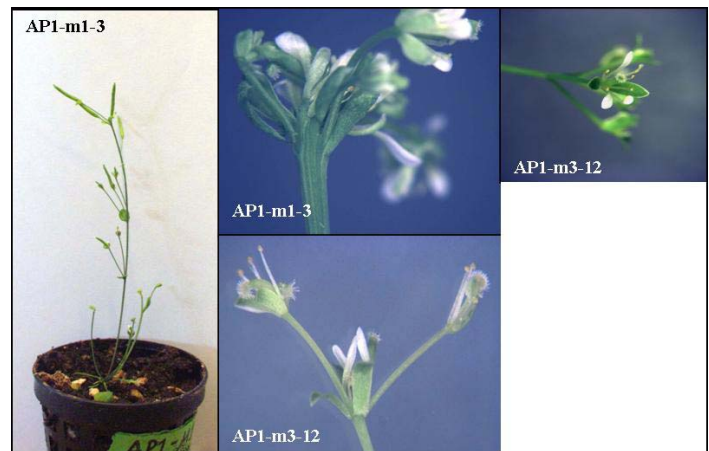


Fig. 7 *API-m1* and *API-m3* loss-of-function phenotype.

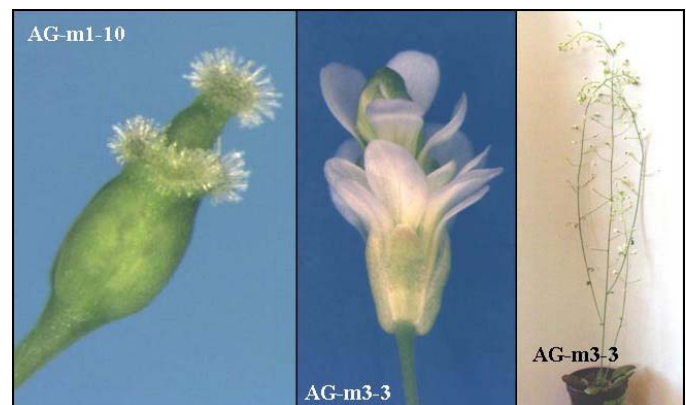


Fig. 8 Gain-of-function vs. loss-of-function in the *AG* transgenics.

| Construct | Clone 717♀ | Clone 353♂ | Clone 6K10♀ |
|------------------|-------------------|-------------------|--------------------|
| <i>API-M2</i> | 21 | 13 | 3 |
| <i>API-M3</i> | 27 | 31 | 25 |
| <i>AG-M2</i> | 17 | 7 | 1 |
| <i>AG-M3</i> | 22 | 28 | 17 |
| Total | 87 | 79 | 46 |

2. Floral Ablation Sterility System

Ablation refers to the use of a floral predominant promoter to drive expression of a cell toxin of some kind (e.g., the bacterial gene Barnase, an RNase), causing floral tissues to develop abnormally or to cease developing entirely. Because most promoters appear to be somewhat “leaky,” at least when inserted transgenically, low-level (vegetative) expression from the promoter occurs and can cause autotoxicity with highly toxic ablation proteins. Thus a focus of our work has been on characterizing promoter specificity, and developing means to “buffer” against promoter leakiness.

Attenuation systems. Barstar, a specific inhibitor of Barnase, has long been known from bacteria as an “insurance gene.” It provides protection of Barnase-containing bacteria against leaky expression. We therefore chose to test its value for this same purpose in plant cells using a variety of plant-expressed promoters. We produced several constructs (Fig. 9) that involved the poplar *LEAFY* (*PTLF*) gene. The bisexual function of *PTLF* makes it ideal for engineering complete sterility; however, it also shows significant vegetative expression. In all his constructs, a MAR element was included to improve and stabilize gene expression.



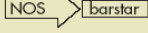
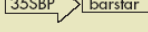
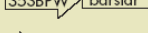
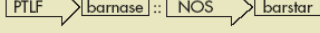
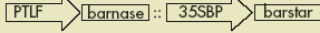
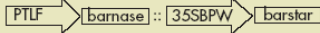
| Construct name | Explants Co-cultivated | Transformation efficiency (%) | Main elements of construct |
|----------------|------------------------|-------------------------------|--|
| pGPG | 388 | 5.2 |  |
| pGPB | 535 | 0.2 |  |
| pGNB | 355 | 3.4 |  |
| pGSB | 341 | 7.9 |  |
| pGOB | 339 | 5.9 |  |
| pGBNB | 391 | 3.1 |  |
| pGBSB | 420 | 3.3 |  |
| pGBOB | 353 | 2.5 |  |

Fig. 9. Overview of constructs that have been transformed into hybrid aspen clone 717-1B4 to test whether deleterious effects from vegetative expression of the cytotoxin barnase driven by the *PTLF* promoter can be attenuated by low-level expression of its specific inhibitor, barstar. Promoters are indicated by arrows, and the coding sequence by rectangles. All genes are flanked by MAR elements (not shown).

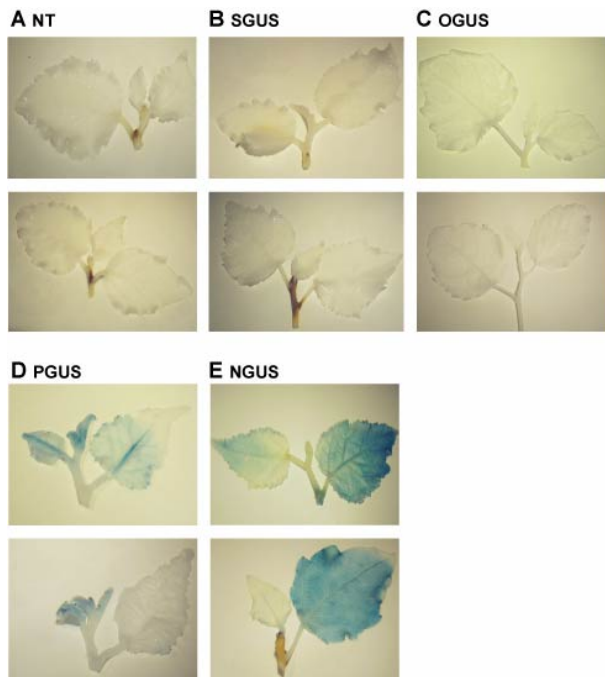


Fig. 10. Histochemical GUS staining of transgenic plants harboring promoter::GUS constructs. Two shoots are presented for each construct. The plants selected for this figure had GUS expression levels close to the mean level for each construct based on fluorometric GUS assay. A) non-transgenic, B) basal 35S, C) 35S-Omega, D) *PTLF*-GUS, E) *NOS* (described in text).

To understand the expression properties of both the *PTLF* promoter and “attenuation” promoters used to drive expression of Barstar, we fused them to the GUS reporter gene and generated transgenic poplars. Two of the four promoters caused visibly detectable GUS expression (Fig. 10: *NOS* = NGUS; *PTLF* = PGUS), whereas the basal promoter constructs did not (35S = SGUS; 35S-omega = OGUS). These results were verified in quantitative GUS analyses, where *PTLF* and *NOS* promoters showed the strongest expression, with the 35S basal promoters having only slightly higher expression than the non-transgenic control plants (Fig. 11A). When tissues were subsampled, we found that the majority of *PTLF* expression occurred in shoots, whereas the majority of *NOS* expression occurred in roots (Fig. 11B).

We then studied constructs where the three attenuation promoters were driving Barstar either alone (C35S, Comega, CNOS), or in tandem with a *PTLF*::Barnase gene (Att35S, AttOmega, AtNOS). We also produced an unattenuated construct where *PTLF* was driving Barnase and no Barstar gene was present; however, we were unable to recover any transgenic plants with this construct. The rate of transformation (transgenic shoots per explant cocultivated) was significantly (Chi-square test, $P < 0.05$) higher for the constructs that contained a Barstar gene without *PTLF*::Barnase (mean of 6.1%) than for those with both genes (mean of 4.2%), a reduction of nearly one-third. Thus, attenuation by Barstar provides insurance not just against poor growth, but for recovery of transgenic plants themselves.

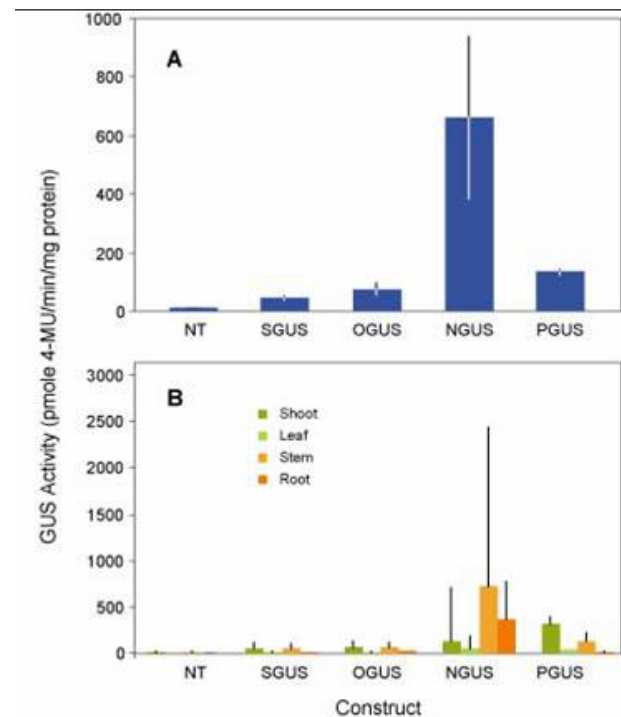


Fig. 11. GUS expression in leaf tissues of non-transgenic controls and transgenic plants harboring different GUS constructs. Bars show one standard error over line means. NT = Non-transgenic; other abbreviations are given in text. A) Construct means over tissues, B) Tissue means by construct.

In contrast to the transformation results, the rate of growth of regenerated plants after several months in the greenhouse was very similar among the gene constructs and did not differ from that of the non-transgenic controls (Fig 12). At least 17 independent transformation events (lines) and 5 randomized ramets were included in the greenhouse trials. The large majority of the plants had normal morphology for all constructs (Fig. 13A); however, two lines in both the Att35S and AttNOS constructs were observed that were clearly weak and poor growing (Fig. 13B). When these four lines were pooled into an “abnormal” class and compared with the other lines, their mean growth rate was 2-fold less and their mean *Barnase:Barstar* expression ratio was 6-fold greater. Thus, despite highly effective attenuation for the majority of lines, it is important to screen and remove weak lines that arise from aberrant position effects.

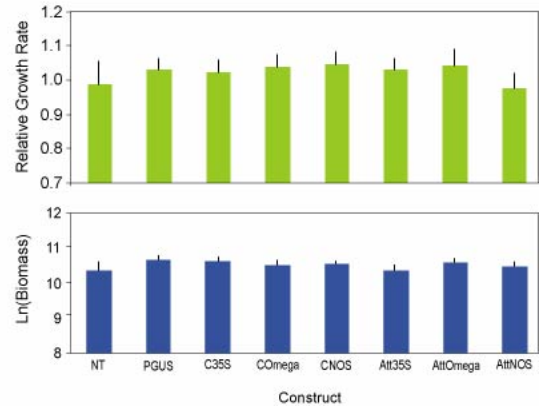


Fig 12. Mean growth rates of transformants harboring attenuated and control constructs. Bars show one standard error over line means.

We also examined the relationship between *Barstar:Barstar* ratio and growth rate in a larger sample of lines using relative growth rate, rather than absolute size, as an indicator of vigor. Relative growth was based on the ratio of final and beginning size during the greenhouse trial. A statistically significant correlation between relative growth rate and *Barstar:Barstar* ratio was observed (not shown). However, the population appeared to be composed of two groups, within which no correlation was obtained. The slow growing group consisted of 8 lines, and the fast growing group consisted of 23 lines. Semi-logarithmic ($r^2 = 0.42$) and quadratic ($r^2 = 0.33$) regressions explained the associations better than did simple linear-linear regression ($r^2 = 0.28$), though all were statistically significant. The groups appeared to separate at a ratio of about 1.2, above which no association was apparent. This suggests that increased levels of *Barstar*



Fig. 13. Morphology of abnormal and normal attenuated and transgenic plants at the time of final growth measurements. Healthy plants are approximately 1 m in height. Normal transgenic (A) and non-transgenic control plants (B) Representative abnormal attenuated transgenic plants compared with a normal transgenic plant.

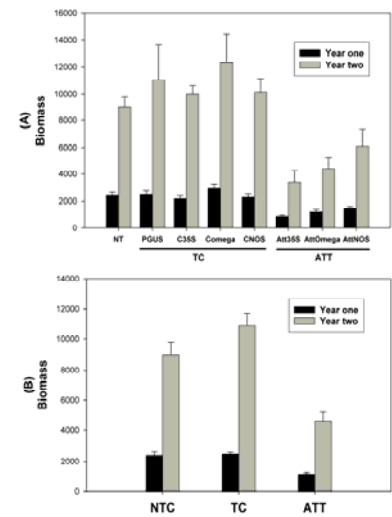


Fig. 14. Biomass index (height x diameter²) of transgenic plants in field trial. A) Biomass of plants harboring attenuated, *barstar* only, and control constructs in field trial. B) Mean biomass of pooled non-transgenic control (NT-control), transgenic control (TC, n=24 events), and attenuated plants (ATT, n=59 events) in field trial. Bars show one standard error over event means. Black and gray color indicate the biomass data collected in years one and two, respectively.

provide no benefit or detriment once Barnase expression is fully attenuated at the expected ratio near to unity.

Subsets of trees from the greenhouse study, totaling 390 trees, were randomly planted in two two-tree plots in September of 2003. The entire planting was surrounded by one or two rows of unmeasured border trees. For the *pPTLF::GUS* and *pPTLF::barstar*- constructs (*PGUS*, *C35S*, *COmega*, and *CNOS*) there were six events, and for the three attenuation constructs (*Att35S*, *AttOmega*, and *AttNOS*) there were by 21, 17, and 19 events, respectively. The non-transgenic controls were planted in 9 two-tree plots.

After only a single year in the field, a pattern emerged that was markedly different than seen in the greenhouse, and which remained consistent after a second year of growth. Although the non-transgenic and transgenic control trees continued to perform similarly within and between groups, the attenuated trees had markedly reduced performance. Plants containing the attenuation constructs had significantly ($P < 5\%$) lower mean Biomass Index (BI) than did the transgenic and non-transgenic control plants in both years (Fig. 14). When the transgenic and non-transgenic controls were pooled into a single group and compared to a group composed of plants containing attenuation constructs, the growth of the attenuated trees was approximately 50% that of the control means. When comparing the three attenuation constructs, only the difference between *AttNOS* vs. *Att35S* was statistically significant at the 5% level, but only in year one ($P = 0.02$). However, the ranking of growth for plants containing the three attenuation constructs was consistent between both years, and correlated with the strength of the promoter used to drive *barstar* expression (Wei et al. 2006). Plants containing *pNOS*, by far the strongest promoter, accumulated the most biomass, and the promoter with intermediate levels of expression, omega-enhanced 35S, resulted in intermediate growth.

The lack of full attenuation was also expressed in leaf coloration. At the end of the growing season but prior to leaf senescence, a number of plants showed signs of chlorosis (Fig. 15), and the attenuated plants had obviously lighter foliage than did the pooled transgenic and non-transgenic controls ($P < 0.001$ via T-test and Fisher's Exact Test; Fig. 16). There was no difference between the transgenic and non-transgenic controls. The *Att35S* and the *AttOmega* plants had the most chlorosis,

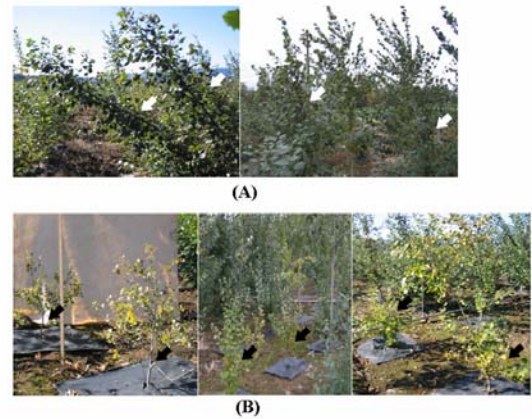


Fig. 15. Examples of poorly growing, chlorotic transgenic trees (B) compared to normal trees (A) in field trial.

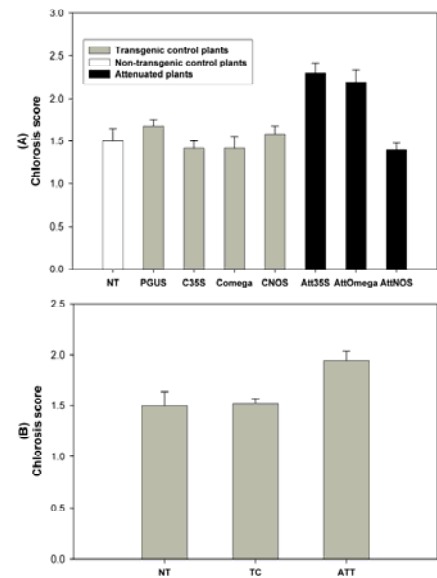


Fig. 16. Chlorosis score of transgenic plants in field trial, where higher scores indicate greater chlorosis. (A) Mean chlorosis score of plants harboring attenuated, *barstar* only, and control constructs in field trial. (B) Mean chlorosis score of pooled non-transgenic control (NT-control), transgenic control (TC, n=24 events), and attenuated plants (ATT, n=59 events) in the field trial. Chlorosis scores were collected only in year two. Bars show one standard error over event means.

whereas the *AttNOS* plants had little, and were significantly less chlorotic than plants containing the other two attenuation constructs ($P < 1 \times 10^{-5}$ via T-test and $P < 0.001$ via Fisher's Exact Test). Again, *barstar* promoter strength was correlated with extent of chlorosis, and the *pNOS* plants showed the lowest degree of chlorosis.

Within constructs, some events accumulated biomass to the same extent as control plants (Fig. 17), suggesting that some transgenic events may have sufficient *barstar* expression for full attenuation. As in the greenhouse study, there was a very strong association between growth and transgene expression ($P \sim 0.001$): the best growing events tended to have the highest *barstar:barnase* ratio (Fig. 18). However, a threshold, where full attenuation might be occurring, was more difficult to identify, and may require a *barstar:barnase* ratio of 8.0 or more. These results demonstrate the importance of field testing during early phases of research to identify pleiotropic effects of transgenic sterility genes in trees.

Although we observed early flowering in nearly one-quarter of the trees in the test (77 of 372 trees), and in at least one tree in nearly half of the events (43 of 105 events), we were unable to allow them to mature to see if they were sterile because of USDA APHIS restrictions. In a small sample analyzed, however, the catkins on trees containing *PTLF:Barnase* transgenes did not appear substantially different in length and form from wild-type trees. They attenuated trees, however, appeared to have stigmas that were abnormally small (Table 4). We are applying to USDA APHIS for an enlarged permit that we hope will lead to permission for these trees to flower, and to set seed, in 2007 so that we can see if this change in morphology translates into reduced fertility. We will also more carefully study their internal morphology if they flower next year. Included in the trial were also a number *PTLF:GUS* transgenic trees. We observed strong and highly floral organ specific patterns of expression in a small sample that we analyzed via histochemical GUS staining. Expression tended to be strongest in the stigma and ovary, and nearly absent in the cup and flower stalk (Figure 19).

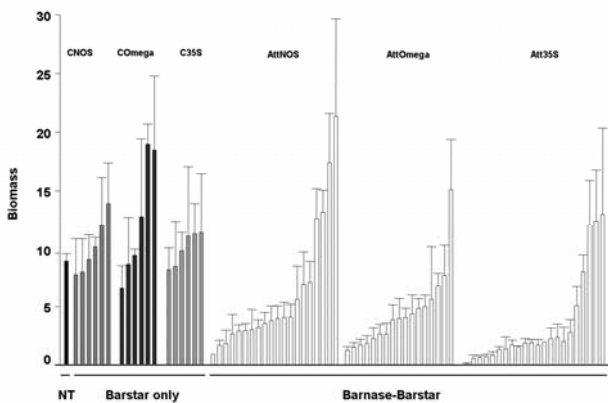
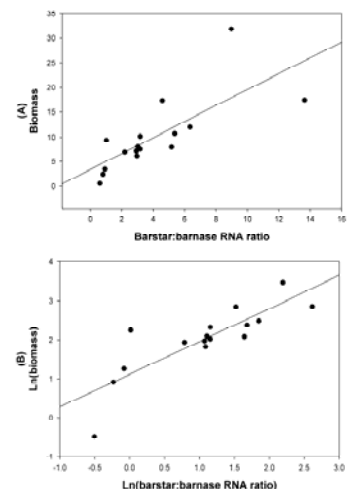


Fig. 17: Mean first-year growth (biomass index, height x diameter²) by event in field trial. The units on the y axis are biomass indices in cm³ multiplied by 10³.

Fig. 18. Linear regressions of biomass index for plants in the field trial after the first year of growth on *barstar:barnase* RNA ratio. A) $y = 1.61x + 3.43$, $R^2 = 0.56$, $P = 0.001$. B) $\ln(\text{biomass})$ on $\ln(\text{barstar:barnase RNA ratio})$: $y = 0.84x + 1.11$, $R^2 = 0.69$, $P < 0.0001$.



PTAPI promoter. The *PTAPI* promoter was evaluated for using for ablation construct. Homologs of the *APETALA1 (API)* gene exist in the poplar genome as a recently duplicated pair, referred to as *PTAPI-1* and *PTAPI-2*. The genes are of interest because their overexpression causes early flowering in *Arabidopsis* and citrus trees, and their suppression reduces fertility. The expression of these genes in poplar also appears to be more highly floral-specific than other genes we have studied, such as poplar *AGAMOUS (PTAG)* and *LEAFY (PTLF)*. *PTAPI* is expressed throughout female and male floral meristems at their earliest stages of development, as well as being strongly expressed in the developing vasculature of the inflorescence stem. This suggests that *PTAPI*-directed ablation may severely disrupt the development of the entire catkin, possibly providing a useful tool for floral ablation. To determine whether the *PTAPI* promoter might be effective in driving cytotoxic genes, we have begun studying several aspects of its behavior. We sequenced approximately 2 kb upstream from the translation start site of *PTAPI-1*. Comparison of this poplar sequence with the same region of *Arabidopsis API* revealed many areas of short, modest conservation, but no large regions of striking similarity. A variety of putative *cis* elements were identified, including CAAT, GATA, and AGAA (pollen-specific) boxes, along with many others. The 2-kb region upstream of the *PTAPI-1* gene was fused separately to the *GUS* reporter gene and the cytotoxic barnase gene to study promoter expression and cytotoxin effects on plant growth and flowering. The two constructs were transformed into *Arabidopsis* and two poplar genotypes (female hybrid aspen clone 717-1B4 (*Populus tremula* x *P. alba*), and early flowering *Populus alba* clone 6K10).

| Line | Area of stigma (cm ²) |
|--------------------|-----------------------------------|
| Control | 3.9 ± 1.2 |
| Transgenics | |
| Bob 15-2 | 1.5 ± 0.5 |
| Bob 39-3 | 2.3 ± 0.7 |
| Bob 49-1 | 0.5 ± 0.2 |
| Bob 91-1 | 0.7 ± 0.0 |

For *PTAPI-1::GUS Arabidopsis* T1 (first transgenic generation) progeny a strong inflorescence-specific staining pattern was observed (Fig. 20). We did not observe any GUS activity in the rosette leaves and only a hint of activity in the vasculature of the inflorescence stems and its cauline (flower-subtending) leaves, but strong expression was seen in the developing flower. We generated five *PTAPI-1* constructs; three reporter gene constructs, one for *PTAPI-1* driving barnase (*PTAPI-*

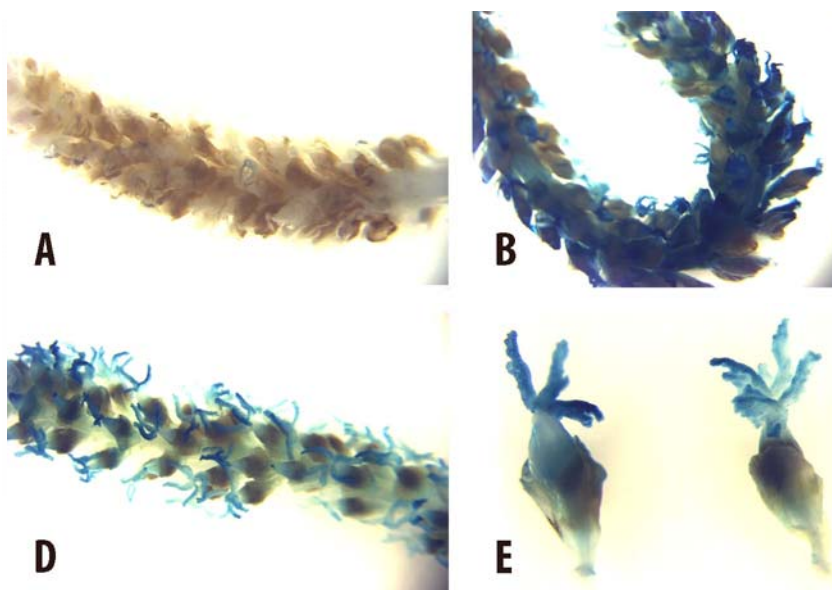


Fig. 19. Histochemical GUS-stained catkins and isolated flowers taken from the field in spring 2006 containing the *PTLF::GUS* reporter gene (lines 18-4, 21-1). (A) Stained control catkins; (B) catkins of 18-4; (C) flower of 18-4; (D) catkin of 21-1; (E) flower of 21-1.

I::barnase), and one attenuation construct (35S basal promoter::*barstar*) with MARs flanking both sides of the gene. Unfortunately, we also found strong and consistent expression from all three types of *PTAPI* promoters in vegetative tissues, a likely reason that no transgenic trees could be produced for *PTAPI-1::barnase* or *PTAPI-1* attenuation constructs (Table 5).

NOZZLE studies. We attempted to isolate poplar homologs to a gene called *NOZZLE* (*NZZ*) from *Arabidopsis*. *NZZ* is a single-copy gene in *Arabidopsis* that is necessary for both female and male fertility, and, in contrast to the poplar floral genes we have been studying, it acts at a very late stage in flower development. Loss-of-function mutation in this gene in *Arabidopsis* leads to male and female sterility in the later stages of flower development (e.g., flowers appear fully developed but they do not produce gametes). Maintenance of floral structures may be most important for insect/animal pollinated species whose flowers serve a nutritional function. Thus, this gene and its regulatory sequences could present a valuable tool for engineering flower sterility in species where floral development is important for ecological reasons, or to provide redundancy with other sterility mechanisms. Unfortunately, because of the low level of expression and rapid rate of evolution of this gene, it is rarely identified in DNA databases and thus difficult to clone.

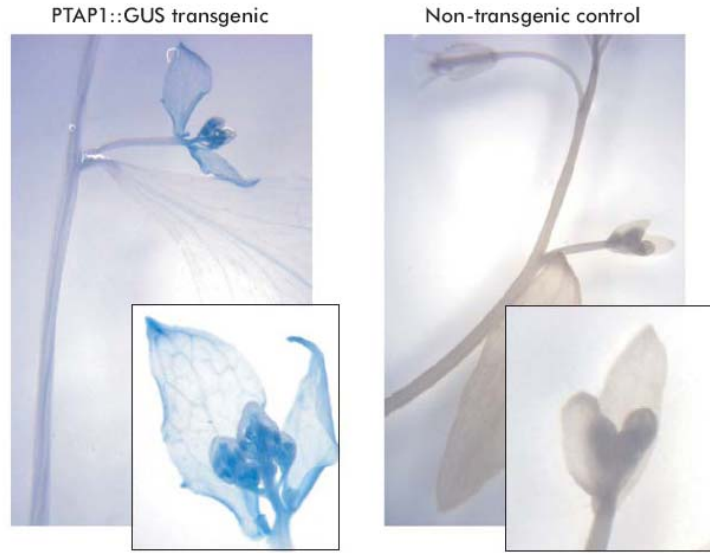


Fig. 20. GUS expression pattern directed by the *PTAPI-1* promoter intragenic *Arabidopsis*. Close-ups (insets) are of the floral bud clusters shown in the accompanying lower-magnification photos.

| Table 5: Summary of Transgenic Events | | | | |
|---------------------------------------|-----------------------------|--------------------------|------|-------|
| Construct name | <i>Arabidopsis</i> T1 lines | Poplar transgenic events | | |
| | | 717♀ | 353♂ | 6K10♀ |
| <i>PTAPI-1::GUS, MARs</i> | | 9 | NA | NA |
| <i>PTAPI-1L::GUS</i> | | 20 | 20 | 16 |
| <i>PTAPI-1L::GUS, MARs</i> | | 20 | 20 | NA |
| <i>PTAPI-1::Barnase</i> | 0 | 0 | NA | NA |
| <i>PTAPI-1::Bar, 35SBP::barstar</i> | 0 | 0 | 0 | 0 |

We identified two putative poplar homologs to *NOZZLE* which we called *PNZZL1*, and *PNZZL2*. The genes show a number of very short, though regularly spaced, stretches that are similar. There are also many large insertions and deletions between the conserved regions. The strongest similarities occur in a region near the N-terminus that seems to encode a basic region followed by an α protein helix (Fig. 21). After intensive study we have concluded it is not a suitable candidate for near-term sterility systems due to its rapid evolution that makes isolation difficult, and due to its very weak expression making it poor for driving ablation genes.

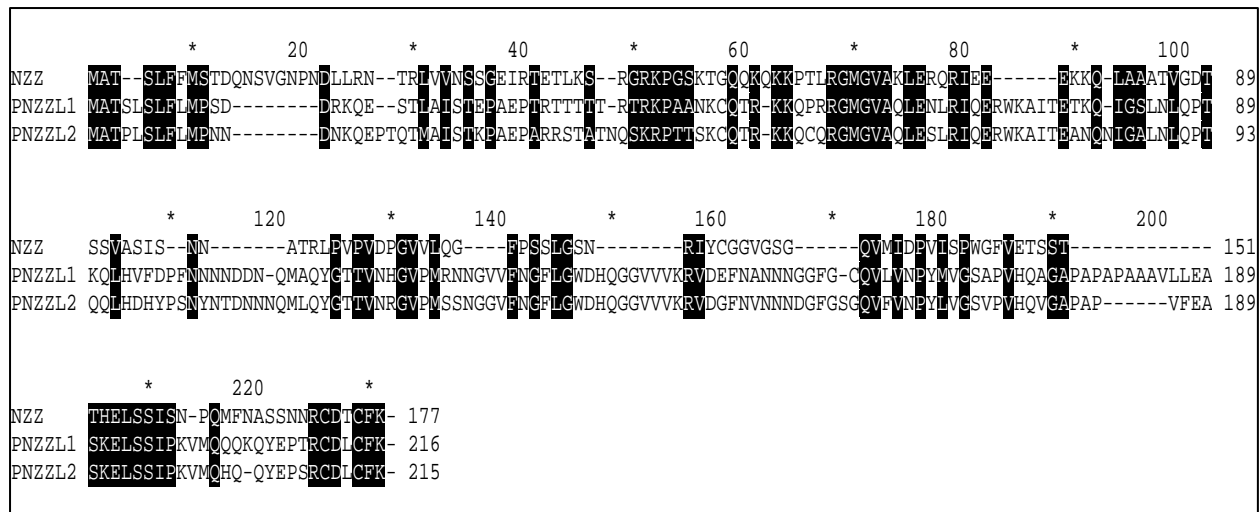


Fig. 21. Alignments of Arabidopsis *NOZZLE* with two putative *NOZZLE* homologs identified in the poplar genome sequence. Insertions/deletions are shown with dashes. Regions of identical (black) amino acids are shown.

3. RNAi Sterility Systems & 4. Redundant Sterility Systems

The major focus of the studying is the effectiveness of RNAi (RNA interference), employing double-stranded DNA versions of selected floral genes from poplar causing sterility. We tried to identify those transgenic events with the greatest extent of RNAi gene suppression via molecular tests when trees are still juvenile. The major limit of this approach is that targeted floral genes must show low levels of natural vegetative expression.

Despite this, we have been able to identify events exhibiting strong target endogene suppression via quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), using vegetative tissue from poplar transgenics still in tissue culture or the greenhouse. We have shown that the target floral genes can be consistently amplified in vegetative tissues and that there appears to be a wide range of variation in the level of suppression compared to control samples (Fig. 22). The results suggest that RNAi transgenic trees with greatly reduced fertility can be selected at an early, non-flowering stage.

Overlapping gene function, environmental influences on gene expression, and the potential instability of transgene

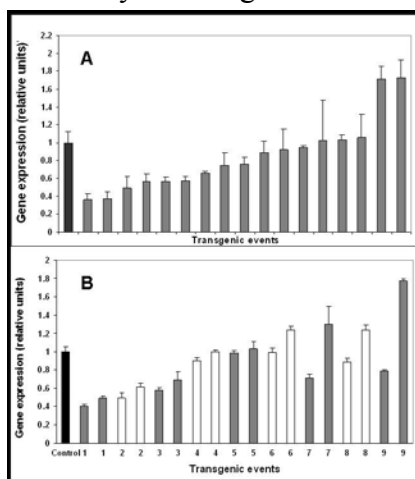


Fig. 22. Range of RNAi gene suppression (a, top) and repeatability among biological replicates (b, bottom) for floral genes expressed in vegetative tissues. (A) Relative expression level of native *PTLF* gene in selected poplar *PTLF*-RNAi transgenic trees and non-transgenic controls of poplar clone 353-53 (*Populus tremula x tremuloides*). Expression was determined by qRT-PCR analysis of native transcripts in vegetative shoots (an ubiquitin gene served as an internal control). Each datum represents a pool of total RNA from four to five ramets per transgenic event; error bars are standard deviations over three PCR technical replicates. (B) Relative expression level of native Poplar *AGL20* (*PAGL20*) gene in pairs of biological replicates (RNA extraction from different ramets) of selected *PAGL20*-RNAi transgenic trees and nontransgenic controls. qRT-PCR methods as in a. Data are means of independent qRT-PCR runs for two different ramets for single transgenic events; error bars are standard deviations over the average of two PCR technical replicates ($r^2=0.41$)

expression or silencing make it difficult to be confident that sterility will be absolute throughout a tree's life span. Redundant sterility constructs may be able to overcome these difficulties. A total of 18 different RNAi constructs have been designed to engineer poplar flower sterility. To impart strong sterility, a strong constitutive 35S promoter was used to drive all RNAi constructs (Fig. 23). Among these, 10 RNAi constructs were designed to suppress poplar floral homeotic genes (*PTAP*, *PTLF*, *PTAG* and *PTD*). Three RNAi constructs were designed to silence two separate floral genes with pairs of inverted repeats (*PTLF/PTAG*, *PTLF/PTAG*, and *PTAP1/PTAG*); both genes are separated by an intron and driven by a single promoter. For the *PTLF/PTAG* genes, an additional RNAi construct was assembled using independent inverted repeats driven by their own 35S promoters. The final sterility RNAi construct was made to simultaneously silence all three floral homeotic genes (*PTAP*, *PTAG*, *PTLF*) using a single operon. The construct design for the *PTAG IR* used MARS fused in tandem on both sides of the *PTAG IR* operon.

Postponing flowering until harvest may be an effective gene confinement strategy; therefore, constructs which would delay flowering of trees have been created for several genes that help to control the onset of flowering in *Arabidopsis*. These include RNAi versions of poplar homologs of the *Arabidopsis* flowering genes *Flowering Locus T (FT)*, *FPP1*, (*PPFL1* and *PPFL2*), *SOCI/AGL20 (PAGL20)*, *ALG24(PAGL24)*, *MFT (PMFT)*. Floral repressor members of the *TFL1* gene family, *PCEN-L*, and additional overexpression and knock-out versions of constructs were created for *PCEN-L*, *PMFT* and *PAGL24*.

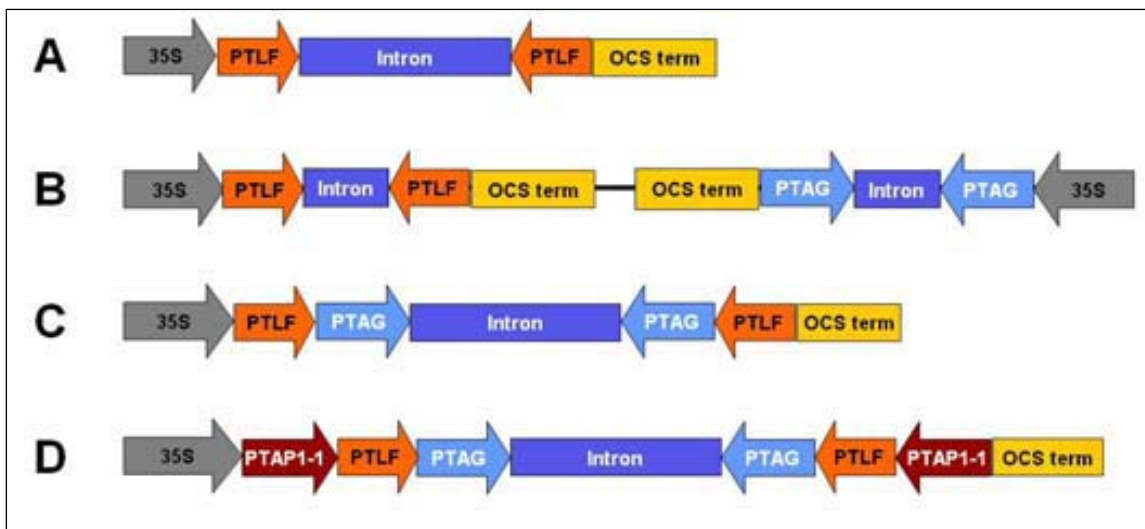


Fig. 23. Structure of RNAi constructs designed to silence poplar homologs of floral homeotic genes *PTLF* (*LEAFY*), *PTAG* (*AGAMOUS*), and *PTAP1* or *PTAP3* (*APETALA1,3*). Shown are examples of constructs designed (A) to target a single gene; to target two genes simultaneously using (B) tandemly arranged single-gene units or (C) a complex unit containing sequences for two genes separated by an intron; and (D) a triplex unit targeting three genes.

Detailed Studies of *CEN/TFL* Homologs. The *CEN/TFL1* family is a small gene family whose members have diverse influences on the onset of flowering. The *Arabidopsis* genome contains six genes belonging to this family, and members of different sub-groups have been shown to have opposite effects on the floral transition. While *TFL1* acts as a repressor of flowering, *FT* is a key promoter of flowering. Thus, altering the expression of genes in this family in trees may provide a means both to delay or prevent the onset of flowering to induce effective sterility, and to accelerate flowering to speed breeding and research. *CEN* is a snapdragon gene closely related to *TFL1* and was the first member of this family that was cloned. We isolated a close poplar homolog of *TFL1* and *CEN* named Poplar *CEN-Like1* (*PCENL1*), and a homolog of the functionally uncharacterized *Arabidopsis* gene *MOTHER OF FT* (*MFT*), named *PMFT*. Based on RT-PCR studies it was shown that both of these genes were expressed in floral tissues, but *PMFT* was expressed most strongly in expanded inflorescences (Fig. 24). *PCEN-L* was most strongly expressed in vegetative tissues and had a very strong seasonal pattern of expression, peaking in buds prior to their flushing. In contrast, *PMFT* appeared to increase most as buds formed and became dormant (Fig. 25). Based on these expression patterns and a phylogenetic analysis of the gene sequences, we hypothesized that *PCEN-L* might inhibit flowering and *PMFT* might promote flowering. The suppression of these genes should do the converse.

For both *PCENL1* and *PMFT*, we produced overexpression transgenes and RNAi (gene silencing) transgenes. Overexpression transgenes were also introduced into *Arabidopsis*, and the two poplar genes induced opposite phenotypes in *Arabidopsis* T1 transgenics. Five out of twenty-two 35S::*PCENL1* lines never flowered. These lines continued to produce only rosette leaves long after wild-type plants had bolted and produced flowers. At 44 days, the wild-type plant shown in figure 26 had flowered, set seed, and would soon undergo senescence, while the 35S::*PCENL1* line continued to produce only rosette leaves. The 35S::*PCENL1* line never formed flowers, but did produce short inflorescence shoots with “leafy flowers” after 60 days in soil. A few of the lines that never produced flowers did form secondary rosettes in the axils of leaves in the primary rosette and short inflorescence-like shoots. However, these shoots bore only whorls of leaves (“leafy flowers”).

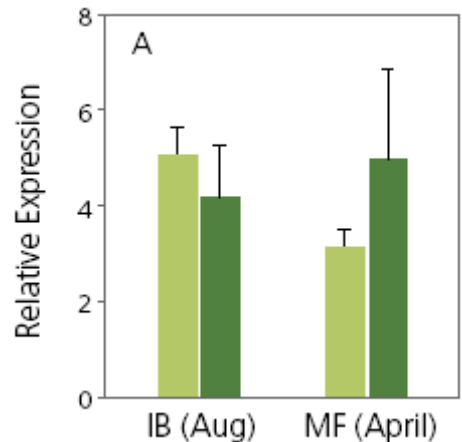


Fig. 24. Native gene expression in floral buds and expanded inflorescences of *P.trichocarpa x deltoids* hybrid trees for *PCEN-L* (bars to left) and *PMFT* (bars to right). Data is based on RT-PCR; bars are standard deviations over four replicates. IB = inflorescence bud, MF = mature inflorescence floral tissues.

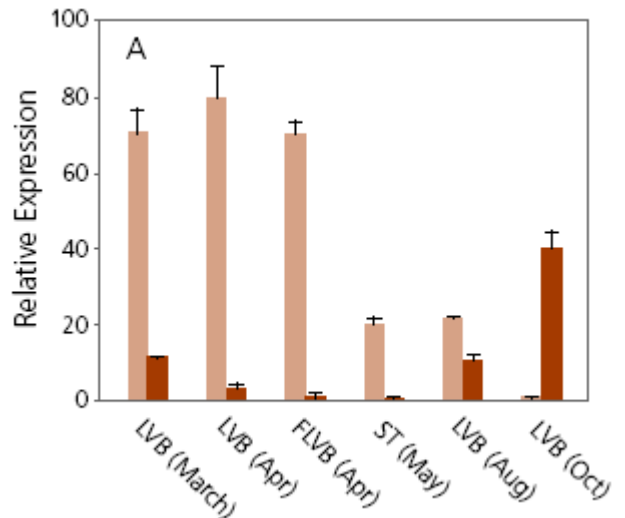


Fig. 25. Native gene expression over a seasonal cycle in lateral vegetative buds and associated tissues of *P. trichocarpa x deltoids* hybrid trees for *PCEN-L* (bars to left) and *PMFT* (bars to right). Data is based on RT-PCR; bars are standard deviations over four replicates. LVB = lateral vegetative bud, FLVB = flushed lateral vegetative bud, ST = shoot tip.

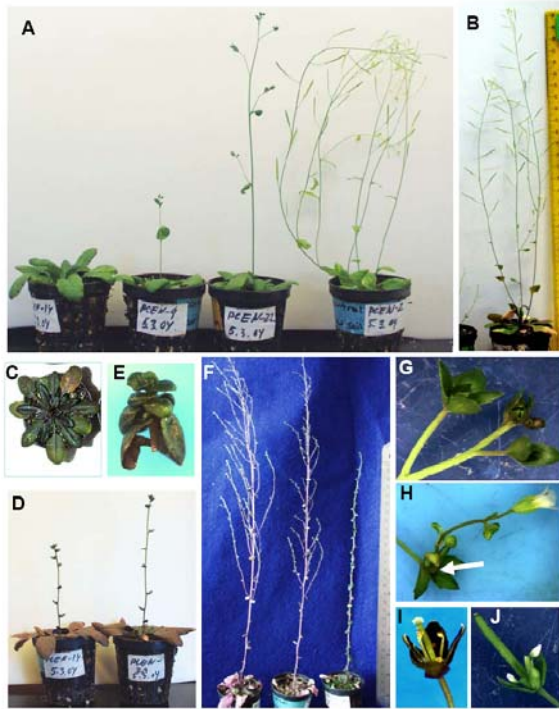


Fig. 26. Ectopic expression of *PtCENL-1* in wild-type *Arabidopsis*. (A) The flowering and late/non-flowering groups (first plant on the left) of *35S::PtCENL-1* plants at 30 d after planting. Note the absence of subtending cauline leaves on the upper parts of the third plant from the left. (B) Wild-type Columbia already setting seed pods at 40 d, for comparison. (C) Top view of a representative late flowering *35S::PtCENL-1* event that has an increased number in rosette leaf, and has not yet bolted at 40 d. (D) Two *35S::PtCENL-1* transformants with extreme phenotypes at 60 d; already bolted and producing leaf-like structures on the top of the shoots. (E) The cabbage-like shoot top from one extreme *35S::PtCENL-1* plant, showing compacted leaves that are thick, shiny, and succulent. Trichomes are absent from the leaves. (F) Morphology of three extreme events after 120 d; the main stem elongated and axillary shoots were produced later in development. (G) Leaf-like structures at floral positions on a representative event from the flowering group of *35S::PtCENL-1* plants. (H) A new shoot emerging from the leaf-like structure (arrow) bearing clusters of floral buds or flowers. (I) and (J) Abnormalities in floral organs observed in some of the *35S::PtCENL-1* flowering events. Plants were grown in plastic containers with a rim diameter of 5 cm.

In contrast, *35S::PMFT* plants flowered earlier than wild-type plants (Fig.27). To see whether similar effects might occur in poplar, these constructs have been introduced into poplar clone 717, and the transgenic plants were put in field trials during spring 2003.

Overexpression of *PCEN-L*, but neither *PMFT* nor RNAi for either gene, had effects on vegetative growth. The trees with the strongest expression tended to flush their buds later in spring (Fig. 28), showing the *PCEN-L* may have suppressive effects on vegetative as well as floral bud development. The association of gene expression with flushing was very strong and essentially linear on logarithmic scales (Fig. 29; $r = 0.87$, $P < 0.001$). The association with growth was weaker but in the expected negative direction for trees with a shorter growing season as a result of their late budburst ($r = -0.40$, $P < 0.10$).

We also observed early flowering after 2 years in the field in two of the *PCEN-L* RNAi transgenic events with most suppressed *PCEN-L* endogene based on qRT-PCR data. The structure of the flowers appears abnormal, vegetative shoots were only partly converted to inflorescence shoots, the shoots retained their woody nature, flowers were widely spaced, and vegetative shoots were also present on them. The catkins also dehisced prematurely despite a lack of pollination; they were sterile, releasing “cotton” but not seeds (Fig. 30).

On the following spring 2006, we found that these two events, and others with strong *PCENL* suppression, also flowered early and strongly, but during the time of normal flowering for this clone. They also had normal-appearing flowers (Fig. 31). The events with the strongest suppression, based on RT-PCR, had the largest number of flowers, and the relationship between intensity of flowering and endogene suppression appeared to be strong and highly linear (Fig. 32; $r^2 = 0.71$). For these studies expression was measured by quantitative RT-PCR in vegetative shoots collected in summer 2005; pools of RNA from 2 ramets per event were used for each assay, and the poplar ubiquitin gene was used an internal control for normalization. These results suggest that *PCENL* is a normal regulator of

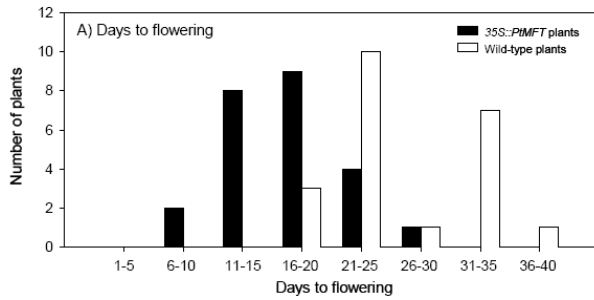


Fig. 27. Timing of flowering and transgene levels in *Arabidopsis* transformants. 35S::*PtMFT* and wild-type plants after transplanting into soil.

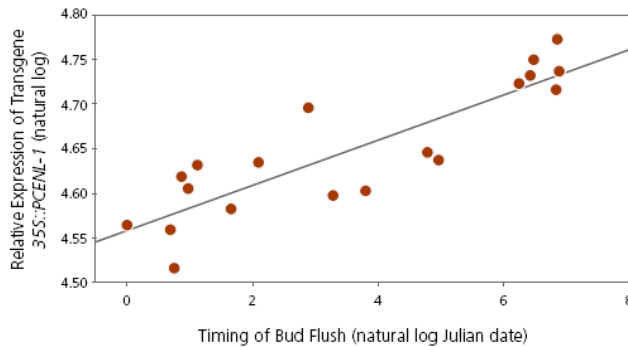


Fig. 29: Scattergram showing log-log linear relationship of *PCEN-L* transgene expression to bud flush in two-year-old, field grown poplars ($r = 0.87$, $p < 0.001$).

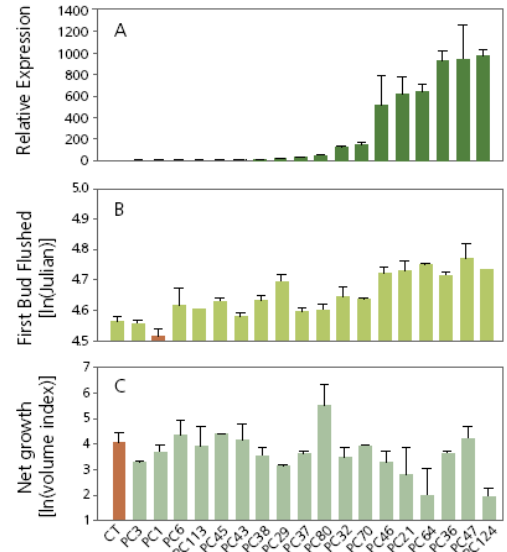


Fig. 28. Relationship of transgene expression for 35S:*PCEN-L* and tree growth in the field after two years. Data is based on RT-PCR studies of RNA from newly flushed leaves and flushing data in 2005. Bars are one standard deviation from at least four replicates (RT-PCR reactions or trees recorded in the field). CT = control, non-transgenic trees. (A) Relative gene expression for transgenic events. (B) Days to bud flush. (C) Tree growth in (diameter squared times height).

flowering onset in poplars, a major new discovery and the first time this has been reported for any tree species. This confirms our observation that the extent of gene suppression in floral meristems can be usefully predicted from RT-PCR studies of vegetative tissues, and it should be possible to screen desirable events during seedling growth, saving years of study and reducing the costs and issues of screening large numbers of field-grown trees.

Surprisingly, overexpression and RNAi forms of *PMFT* transgenic poplars did not produce any distinct phenotypes and were not significantly different from control plants in growth or timing of budflush (Fig. 33). Earlier, we had shown that *PMFT* is a functional gene; *Arabidopsis* plants ectopically expressing *PMFT* flowered early. The overexpressed transgenic events did exhibit high levels of transgene *PMFT* transcripts, and many of the RNAi transgenic events showed low levels of native *PMFT* transcripts, indicating that changes from transgene expression, at least at the RNA level, were substantial.

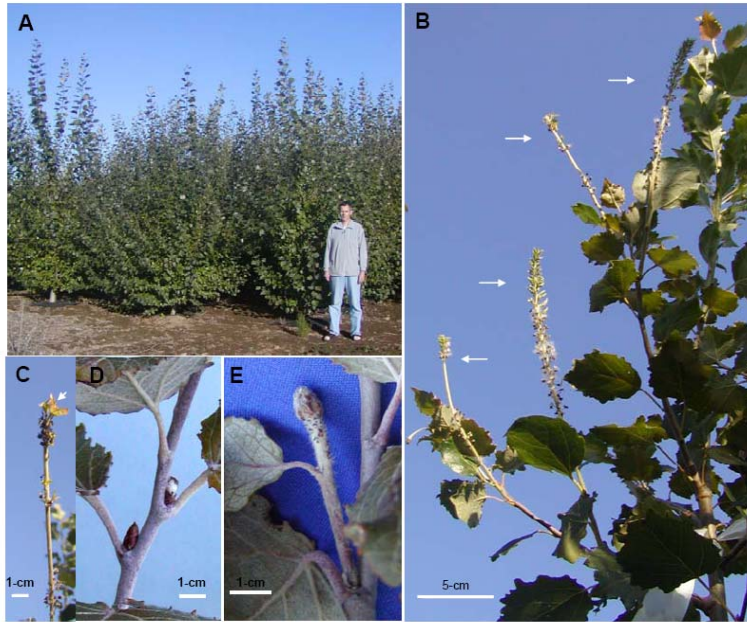


Fig. 30. Early-flowering phenotypes of transgenic *Populus tremula* x *P. alba* carrying an RNAi construct for suppressing endogenous *PtCENL-1* expression. (A) Two-year-old transgenic poplars growing in the field. (B) Female flowers borne on upward-pointing shoots on long straight catkins (arrows). (C) Close-up view of a dehiscid catkin with a new vegetative shoot growing on its tip. (D) Floral buds resting in the axils; the upper bud is beginning to expand. (E) A young inflorescence that had flushed a week earlier but failed to develop further. Pictures B to D were taken from transgenic *PtCENL-1* RNAi event 191 on September 5, 2005. Picture E was taken from the same tree two weeks later.



Fig. 31. Early flowering in the field of trees that showed strong RNAi-based *PCENL* suppression based on RT-PCR studies of vegetative tissues. (A) View of treetop (line 183-3); and (B) close-up of catkins (line 183-3).

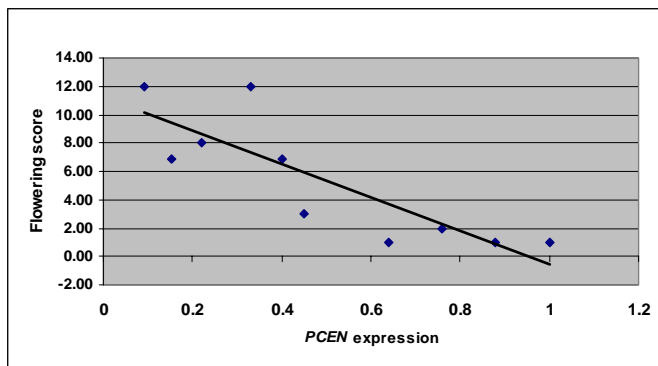


Fig. 32. Association of expression level of native *PCENL* transcripts with flowering in field-grown *PCENL*-RNAi transgenic trees that showed gene suppression. Flowering score was estimated from number of flowering ramets per event mean number of flowers for each event using a scoring system for each tree: 0 = no flowers, 1 = 1 to 11 flowers, 2 = 11 to 30 flowers, and 3 = >30 flowers. $r^2 = 0.71$.

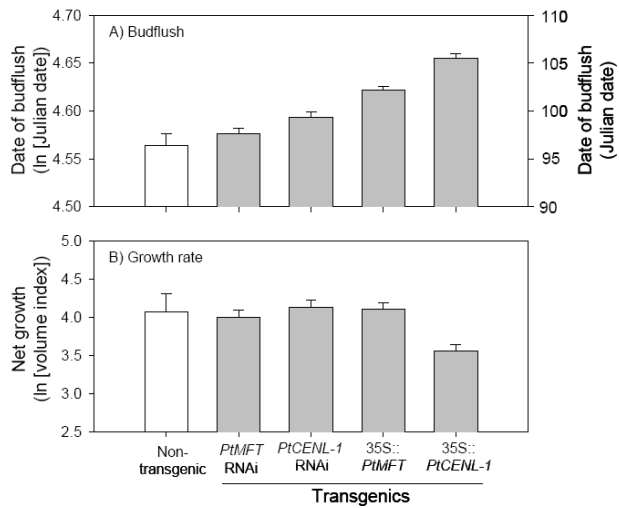


Fig. 33. Mean timing of budflush and mean net growth of field-grown transgenic *Populus tremula* x *P. alba* carrying four different constructs compared to nontransgenic controls. Data are means (in natural logarithm) from all events in each construct type. Standard errors for construct means are shown above bars.

5. Establishment of Transgenic Poplar Field Plantings

All constructs have been inserted into the female INRA 717-IB4 and male INRA 353-38 hybrid aspen clones to evaluate sterility effectiveness of constructs on both genders. These clones were chosen as they are easily transformed with *Agrobacterium*. We also used an early flowering female *Poplar alba* clone 6K10 to test some of the sterility and flowering time constructs.

We have produced nearly 1,964 PCR confirmed transgenic events (Table 6). We have propagated 5,640, 6,820, and 7,055 trees for the 6K10, 717 and 353 clones respectively. Field trials have been already established in the Spring of 2003 for some of these constructs (Table 1, data in parentheses). Some trees began to flower in the Spring of 2006. Flowering scores were estimated for early flowering *PCEN-L1* RNAi transgenic trees, bud flush was also scored for *PCEN-L*, *PMFT* RNAi and overexpression construct trees. Flower phenotypes were checked by light microscope, and flowers from reporter gene constructs were additionally GUS stained. Because land for these plantings is limited, we identified those transgenic events with the greatest extent of RNAi gene suppression from RT-PR studies of vegetative tissues, where the target genes show low levels of natural vegetative expression. An additional field planting containing the remaining sterility transgenics is scheduled to go into the field June 2007.

Table 6: Summary of Sterility Transgenics in or entering field plantings (2003 – 2007)

Summary of confirmed independent transformation events with sterility of floral reporter control genes for field testing. Number of transgenic events planted in 2003 are shown in parenthesis, NP – Not planned

| Sterility mechanism | Construct type/No. of constructs | Gene targeted | Clone 717♀ | Clone 353♂ | Clone 6K10♀ |
|----------------------------|---|-------------------------------------|-------------------|-------------------|--------------------|
| Floral Sterility | RNAi (9 constructs) | | | | |
| | 4 single genes | <i>PTD</i> | 23 | 22 | 28 |
| | | <i>PTAG</i> | 24 | 24 | 25 |
| | | <i>PTAPI</i> | 20 | 20 | 23 |
| | | <i>PTLF</i> | 25 | 21 | 22 |
| | 4 double genes | <i>PTLF, PTAG(2 designs)</i> | 47 | 45 | 56 |
| | | <i>PTLF, PTAP</i> | 22 | 22 | 41 |
| | | <i>PTAP, PTAG</i> | 24 | 28 | 25 |
| | 1 triple | <i>PTAG, PTLF, PTAP</i> | 20 | 20 | 29 |
| | Ablation (5 constructs) | | | | |
| | | <i>PTLF:barnase, 35SBP:barstar</i> | 21(21) | 2 | 6 |
| | | <i>PTLF:barnase, 35SBPΩ:barstar</i> | 17(17) | 2 | NP |
| | | <i>PTLF:barnase, NOS: barstar</i> | 19(19) | 0 | NP |
| | | <i>PTAPI:barnase, 35SBP:barstar</i> | 0 | 0 | NP |
| | | <i>PTD:barnase, 35SBP:barstar</i> | 8 | 8 | 7 |
| Flowering time | RNAi (8 constructs) | | | | |
| | 7 single gene | <i>PFT</i> | 20 | 27 | 28 |
| | | <i>PAGL20</i> | 24 | 17 | 26 |
| | | <i>PAGL24</i> | 22 | 28 | 28 |
| | | <i>PFPFL1</i> | 21 | 29 | NP |
| | | <i>PFPFL2</i> | 21 | 29 | 24 |
| | | <i>PMFT</i> | 15(15) | NP | 28 |
| | | <i>PCEN</i> | 15(15) | 27 | 26 |
| | 1 double genes | <i>PFT, PAGL20</i> | 13 | 17 | NP |
| | Overexpression (4 constructs) | | | | |
| | | <i>PMFT</i> | 19 (19) | NP | 26 |
| | | <i>PCEN</i> | 19 (19) | 27 | 23 |
| | | <i>PAGL24</i> | 32 | 29 | 39 |
| | <i>PSVP</i> | 30 | 26 | 41 | |
| Reporter GUS gene | 3 constructs | | | | |
| | | <i>PTAPI:GUS</i> | 56 | 45 | 26 |
| | | <i>PTLF:GUS</i> | 6 (6) | 29 | 13 |
| Total | 29 constructs | | 681 | 635 | 648 |

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