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The *Populus PTD* promoter imparts floral-predominant expression and enables high levels of floral-organ ablation in *Populus*, *Nicotiana* and *Arabidopsis*

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Abstract

We evaluated the utility of the promoter from the *Populus PTD* gene—homologous to the MADS box genes *DEFICIENS* and *APETALA3*—to genetically engineer reproductive sterility. Floral-predominant expression was confirmed via GUS reporter assays in two heterologous species (*Arabidopsis* and tobacco) and in an early-flowering poplar genotype. Using the *PTD* promoter to direct expression of the disarmed cytotoxin DTA resulted in sterile plants with otherwise normal growth at high frequency in all three species. Biomass production in greenhouse-grown, morphologically normal tobacco cytotoxin lines was indistinguishable from lines lacking the cytotoxin gene, confirming strong floral specificity of the promoter. These results suggest that the poplar *PTD* promoter may prove useful for transgene confinement without detrimental effects on yield.

Introduction

Genetic engineering has been used to introduce novel, commercially valuable traits, such as pest resistance and herbicide tolerance, into a wide variety of agronomic crops (http://www.aphis.usda.gov/biotech/). The potential benefit of these introduced traits has also been demonstrated in transgenic trees (Meilan et al. 2002; Tzfira et al. 1998). A major concern over the use of transgenic trees, however, is the potential for extensive transgene dispersal through pollen and seeds (Ellstrand 2001; Kuvshinov et al. 2001; Mikkelsen et al. 1996; Nap et al. 1996). Most trees are cultivated in close proximity to wild or feral relatives, increasing the probability of transgene spread (Skinner et al. 2000; Strauss et al. 1995); engineered sterility could greatly mitigate gene flow.

A variety of approaches are being utilized to genetically engineer reproductive sterility in plants. Techniques currently under development include gene suppression, dominant negative mutants (DNMs), and cell ablation (Meilan et al. 2001; Skinner et al. 2000). Gene suppression strategies include antisense suppression, cosuppression, and RNA interference (RNAi). Suppression necessitates the use of gene sequences displaying high similarity to the target gene, frequently limiting the use of constructs to closely related species. DNM strategies work at the protein level and theoretically function across more divergent genera, but production of successful DNM transgenes is complex and currently relies on trial and error mutation of conserved amino acids or domains. Cell ablation strategies do not target specific genes and are therefore applicable across the broadest range of genera; however, they require the use of highly specific floral regulatory elements (i.e., promoters) to direct expression of deleterious genes.

Use of floral-specific promoters from tobacco (Koltunow et al. 1990; Wang et al. 1993) and *Brassica* (Hackett et al. 1992) to direct the expression of a ribosome inactivating protein (DTA) (Palmiter et al. 1987) or an extracellular ribonuclease (Beals and

Goldberg 1997; Hartley 1988) resulted in significantly decreased vegetative growth in poplar (Meilan et al. 2001; Skinner et al. 2000). This decrease was thought to be a result of 'leaky' cytotoxin expression in non-target, vegetative tissues due to the use of promoters from heterologous species. Such yield reductions would likely preclude commercial applications.

We hypothesized that the promoter from a native poplar floral gene might give lower levels of vegetative expression than heterologous promoters. RNA gel blots and *in situ* hybridization analysis indicated that the poplar *PTD* gene, a member of the *AP3/DEF* MADS box gene family, is expressed in a floral-specific pattern (Sheppard et al. 2000). Hill et al. (1998) demonstrated that *AP3* promoter sequence from −727 to +1 was sufficient to confer all aspects of normal *AP3* floral expression onto a gusA reporter gene in *Arabidopsis*; three CArG box motifs ($CC(A/T)_6A/GG$) were shown to be involved in controlling *AP3* expression patterns (Hill et al. 1998; Tilley et al. 1998). Nearly 2 Kb (1,915 bp) of sequence upstream of the *PTD* start codon has been determined (GenBank accession number AF057708 and US Patent No. 6,395,892 B1); it contains six CArG box-like motifs. To test whether this region of the *PTD* promoter would be useful for engineering sterility, the 1,915-bp fragment was subcloned in front of an intron-containing version of the reporter gene (gusA) (Vancanneyt et al. 1990). Preliminary studies with this construct in *Arabidopsis* showed floral-organ-specific expression (Sheppard et al. 2000). These results imply that the *PTD* promoter might be useful for engineering plant sterility. We report on the specificity of the *PTD* promoter when directing expression of GUS, and its effect on growth and floral morphology when driving a cytotoxin gene.

Materials and methods

Plasmid vectors

Assembly of the reporter-gene construct (pPTD::GUS) was previously described by Sheppard et al. (2000). The DTA::NOS_{term} fragment (cut with *Nco*I and blunt-ended with Klenow, then released with *Eco*RI) of pMKT17 (Thorsness et al. 1991) was subcloned into pBluescript-KS (Stratagene) digested with *Pst*I (T4 polymerase-blunted) and *Eco*RI, creating pDTA::NOS. The ablation-gene construct (pPTD::*DTA*) was produced by subcloning the 1.9-kb *Hin*dIII/*Avr*II fragment of the *PTD* regulatory region and the DTA::NOS_{term} fragment excised from pDTA::NOS (*Xba*I/*Eco*RI) into pBI101 (Clontech) digested with *Hind*III and *Eco*RI. Plasmid integrity was confirmed by sequencing across ligation junctions in both constructs. Basic recombinant manipulations followed Sambrook et al. (1989).

Plant materials and transgenic plant production

The pPTD::GUS and pPTD::*DTA* constructs were used to generate transgenic *Arabidopsis*, tobacco, and poplar lines. *Arabidopsis thaliana* (ecotype *Columbia*) was transformed via the *in planta* method of Bechtold et al. (1993); transgenic lines were selected as described by Rottmann et al. (2000). Transformation and regeneration of *Nicotiana tabacum* (var. Xanthi) was described previously by Han et al. (1997). Hybrid aspen (INRA 353-38; *Populus tremula* × *P. tremuloides*) was co-transformed with pDW151, a construct previously shown to cause early flowering (Weigel and Nilsson 1995), using the method of Han et al. (2000). Transgenic lines in each species were verified by PCR amplification of the appropriate diagnostic transgene. Lines transformed with pPTD::GUS were verified using a forward primer specific for the *PTD* promoter: V35-F5 (5′-AAGACTATTCTGGCTTCCTCTTAC-3′) and a GUS-specific reverse primer: GUS.003 (5′-CCAGACTGAATGCCCACAGGCC-3′). Lines transformed with pPTD::*DTA* were verified using V35-F5 and a *DTA*-specific reverse primer: DTA.001 (5′-GGTTTAGTCCCGTGG-3′). Co-transformed aspen lines were verified using one of the primer pairs just described along with a primer set to verify the 35S::*LEAFY* (*LFY*) operon of pDW151: 35S-F1 (5′-CACGTCTTCAAAGCAAGTGG-3′) and LFY.008 (5′-AAGAGCGTGATGAGTACC-3′). Only lines yielding appropriately sized amplification products for both the *PTD*-promoter construct and pDW151, after replicate tests that included negative control (non-transgenic) tissues, were considered co-transformed. Plants transformed with pPTD::GUS and pPTD::*DTA* will henceforth be referred to as PTD::GUS and PTD::*DTA* lines, respectively.

β-glucuronidase assays

GusA reporter gene expression was localized by histochemical staining (Stomp 1992). GUS activity was quantified via the method of Jefferson et al. (1987).

Duplicate GUS assays were performed on each sample extract. The concentration of total protein in each extract was determined using a BioRad Protein Assay Kit. Final GUS activity was obtained by subtracting background GUS activity observed in corresponding untransformed samples and is expressed as pmoles 4-methylumbelliferone/minute/mg total protein. To quantify GUS activity in *Arabidopsis*, whole-organ tissue was sampled from leaf rosettes, inflorescence stem sections, stage 13 (bud open, petals visible, anthesis) to 14 (anthers extend above stigma) flowers, and from developing siliques (stage 17). *Arabidopsis* floral stages are described in Smyth et al. (1990). To quantify GUS activity in tobacco, whole leaves, stems, vegetative meristems, stage −3 (style elongating, stamen filament extension continues, anthers below stigma) to +1 (anthers and pistil fully differentiated and green) flowers (Type A), and stage 3 (corolla emerges from calyx) to 4 (sepals completely separated at top of calyx) flowers (Type B) were utilized. Tobacco floral-organ tissues were dissected from Type B and stage 10 (corolla limb beginning to open, petal tips becoming pink) to 11 (corolla limb halfway open, stigma and anthers visible) flowers (Type C). Numerical stages of tobacco flower development are described in Koltunow et al. (1990).

Biomass assays

All PTD::*DTA* tobacco lines failed to form seed (see Results); therefore, plants for the growth study were propagated using vegetative shoot cuttings (ramets) from primary transformants. Five Class III (defined below) PTD::*DTA* lines, three PTD::GUS lines, and two non-transgenic controls were propagated on rooting media. Eight rooted shoots of each line were transferred to potting soil (1:1 mixture of Sunshine Mix #2 [McConkey Company; Wilsonville, OR] and perlite) and grown using a randomized block design in a greenhouse supplemented with artificial light (16-hr photoperiod). Because rooted shoots initially varied in starting size and root mass, all plants were grown for 21 days to establish healthy root systems before being pruned to a 20 cm stem. One axillary bud was allowed to flush per plant, directing all growth to a single shoot (as other buds elongated, they were excised). Plants were allowed to grow until floral clusters were visible (35 days post-pruning) on a majority of the plants that flushed (flowers were visible on > 90% of plants; the remainder contained developing floral clusters that had not yet emerged from the apical leaf mass at the time of harvest). For all ramets that flushed, new shoot growth was excised; the vegetative tissues (floral clusters were removed and discarded) were dried at 70 °C for 48 hr in a forced-air oven before being weighed in a shielded balance. A Student's T Test was used to determine whether differences in growth were significant between control and transgenic lines, based on ramet means.

Quantitative real-time PCR

Real-time PCR was performed using SYBR® Green PCR Master mix (PE Biosystems; Foster City, CA) and primer pairs for *DTA* (transgene) and 18S rRNA (endogenous control for standardization) according to the manufacturer's guidelines. The forward and reverse primers for *DTA* were: DTA-RT.001 (5′-ACCCGCTCTCTGGAAAAGCT-3′) and DTA-RT.002 (5′-TTTAGTGCGAGAACCTTCGTCAG-3′), respectively. The 18S rRNA primers were: 18S-F (5′-AATTGTTGGTCTTCAACGAGGAA-3′) and 18S-R (5′-AAAGGGCAGGGACGTAGTCAA-3′). All primers were designed using Primer Express software (PE Biosystems; Foster City, CA). Total RNA was isolated from leaves and Type A flowers of the PTD::*DTA* tobacco lines using an RNeasy Plant Mini Kit (Qiagen; Valencia, CA), and used to prepare first-strand cDNA (Sambrook et al. 1989). Aliquots of test amplification mixtures were run on standard agarose gels to verify that they contained unique products of the expected size. Assays were performed in triplicate for each sample. Calculations for normalizing yield, standard deviation, and calibrator comparisons were done following the manufacturer's guidelines (User Bulletin #2; December 11, 1997; PE Biosystems; Foster City, CA). A Student's T Test was used to determine whether differences in expression were significant between control and transgenic lines, based on ramet means.

Results

Floral-predominant expression patterns are maintained in diverse species

Histochemical GUS staining revealed the gross expression pattern conferred by the 1.9-kb *PTD* regulatory fragment. Of 33 *Arabidopsis* PTD::GUS lines obtained during *in planta* transformation, 31 stained

positive for GUS activity; the two non-staining lines likely represent transformation events in which the PTD::GUS operon was silenced or otherwise inactivated. Staining whole plants and organs (leaf, inflorescence stem, and flowers) revealed that visible GUS activity was restricted to floral structures (Figure 1A). GUS activity was detected in stamens and petals, with expression typically stronger in stamens. Some activity was also visible at the base of flowers (receptacles), where the four organ whorls fuse. Three lines displayed weak staining in the lower portion of the sepals, although this may have represented diffusion from the floral base. Expression was observed in young flowers prior to emergence from buds, and before elongation of sepals, petals, and stamens was complete (Figure 1B). As fruit development progressed (through to stage 17), *PTD* promoter-directed GUS expression was maintained at the base of the developing silique (Figure 1C). GUS activity was not observed in the developing silique, including seeds.

Patterns of *PTD* promoter-directed GUS expression in tobacco were similar to those seen in *Arabidopsis*. A total of 15 PTD::GUS lines was initially analyzed for GUS activity in leaf, stem, root, and flower tissues. In 14 lines, GUS expression was limited to floral organs. In the remaining line, in addition to the strong activity in floral tissue, very weak GUS activity was observed in stem tissue. GUS activity was detected in young flowers (Figure 1D) and maintained throughout flower development.

The staining pattern in seven of the tobacco floral-specific PTD::GUS lines was analyzed in detail. Similar staining patterns were observed for each line, with GUS activity observed in the corolla, carpel and stamens, with the highest activity observed in stamens (Figure 1E). As with *Arabidopsis*, GUS activity was seen at the base of the flowers; however, this activity was more prominent in tobacco than in *Arabidopsis* and typically extended into the lower half of the carpel. As seeds developed, significant GUS activity was observed in the surrounding ovary tissue, but not the seeds themselves (Figure 1F).

Poplars (aspen and cottonwood) have a juvenile period of five to seven years. A single male aspen clone (INRA 353-38) has been shown to flower precociously in as little as six months following transformation with a 35S::*LFY* operon (Rottmann et al. 2000; Weigel and Nilsson 1995). To speed analysis, this poplar genotype was co-transformed with a 35S::*LFY* construct, pDW151 (Weigel and Nilsson 1995), and pPTD::GUS. Poplar 353-38 transformed with pDW151 produces male flowers bearing multiple stamens (Figure 1G) (Rottmann et al. 2000); pre-

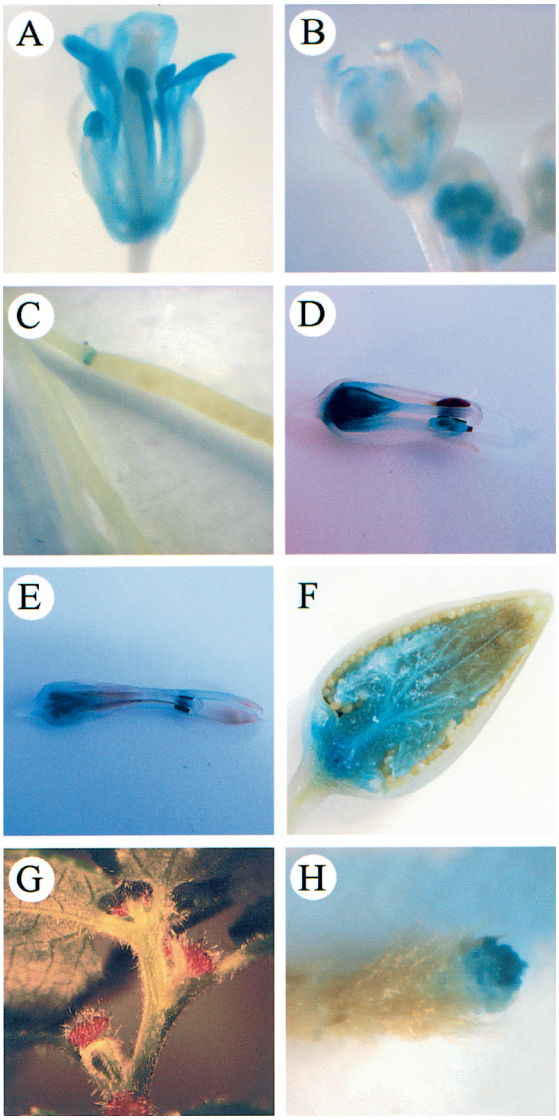

Figure 1. *PTD* promoter-directed GUS expression patterns in *Arabidopsis*, tobacco, and early-flowering poplar. PTD::GUS lines are shown in panels A–F and H. Panels A–C are *Arabidopsis*, D–F are tobacco, and G–H are aspen. A) Flower from line 26 (Stage 15) showing prominent staining in petals and stamens but not sepals or pistil. B) Floral cluster of line 46 showing staining of stamens and petals in very young flowers. C) Silique from line 29 (Stage 17) showing maintenance of staining at floral base. D) Type B flower of line 15 showing strong staining in anthers and carpels. E) Type C flower from line 15 showing similar staining to panel D. F) Seed pod with seeds of line 7 showing detail of carpel staining. G) Stamen clusters on flowers induced on hybrid aspen 353-38 transformed with 35S::*LFY*. H) Developing stamens in hybrid aspen line 255 co-transformed with 35S::*LFY*.

cocious flowers from poplar lines co-transformed with both pDW151 and pPTD::GUS (verified by PCR) were morphologically identical.

Leaf, stem and induced flower tissues from 15 co-transformed poplar lines that flowered precociously were stained for GUS expression. Activity was detected in floral tissue in 14 of the 15 lines, and was mainly confined to the stamens (Figure 1H). The remaining line did not display GUS activity in any of the assayed tissues and likely represents a silenced or otherwise inactive PTD::GUS operon. Expression was observed throughout stamen development and was maintained thereafter. The GUS activity occasionally extended into the short inflorescence stalk, although this was variable even among flowers from the same plant. In two of the lines, weak GUS activity was observed in leaves directly subtending the induced flowers, but not in leaves more distal to the flower (not shown).

Quantitative analysis of GUS expression

Although *PTD* promoter-directed GUS activity appeared to be floral-predominant in each species based on histochemical staining, expression may have been below the threshold of detectability for this assay, especially in vegetative tissues. Even low levels of cytotoxin expression in vegetative tissues could negatively impact growth. Thus, quantitative fluorometric GUS assays were performed on vegetative and floral tissues from a subset of lines transformed with PTD::GUS in an attempt to detect and quantify low-level expression.

GUS activity was measured in five randomly selected *Arabidopsis* PTD::GUS lines that displayed typical histochemical staining patterns. Rosette leaves, inflorescence stem sections, flowers (stages 13–14), and developing siliques (stage 17) were assayed. Total GUS activity varied among lines, but was maximal in flowers of all five lines, ranging from 22.6 to 70.3 (mean of 47.2) pmoles 4-MU/min/mg total protein. Expression levels for each tissue source were standardized to floral values (treated as 100%) (Figure 2A). Average percent normalized expression (%NE's) of 0.35 ± 0.12 and 0.87 ± 0.40 were observed in rosette leaves and in inflorescence stems, respectively (Figure 2A), showing that the PTD promoter does impart vegetative expression, but at a level approximately 100-fold below that in floral tissues. Consistent with the histochemical staining results, siliques contained much higher levels of GUS activity than vegetative organs, displaying an average %NE of 6.30 ± 2.53.

Seven tobacco PTD::GUS lines displaying representative histochemical staining patterns were examined in detail for GUS expression. Initially, leaves, stems, vegetative meristems (collected prior to floral transition), and whole Type A and B flowers (defined in Methods) were collected for quantitative analysis. For %NE standardizations of GUS activity, Type B flower values were set to 100%. GUS activity in Type B flowers ranged from 0.9 to 7.3 (mean of 2.5) pmoles 4-MU/min/mg total protein among the seven lines. In six of seven lines, GUS activity was maximal in Type B flowers; in the seventh line, activity was maximal in Type A flowers (1.3-fold that of Type B activity). Vegetative GUS expression was low relative to Type A and B flowers (Figure 2B). As with *Arabidopsis*, the *PTD* promoter directed levels of GUS expression in tobacco vegetative organs that were approximately 100-fold below that of floral tissues (Figure 2B). Leaves and stems had average %NE values of 1.33 ± 0.46 and 1.74 ± 0.67, respectively; vegetative meristems had a slightly higher %NE value of 3.06 ± 1.53.

To analyze individual organ expression, whorls from Type B and C flowers (defined in Methods) were removed and separated into five organ types: sepals, corolla, stamens, carpel, and the receptacle. In both Type B and C flowers, expression was maximal in stamens (Figure 2B), consistent with the histochemical staining. The activity estimates (pmoles 4-MU/min/mg total protein) in stamens were similar for both Type B and C flowers in a given line, so GUS activity was standardized by setting the stamen values to 100%. The average activity observed for each floral organ was similar for both Type B and C flowers (Figure 2B). Minimal GUS activity (< 2%, mean of 0.52%) was detected in sepals of Type B and C flowers (Figure 2B). Significantly higher levels of GUS activity were observed in carpels (< 26%, mean of 12.7%), petals (< 33%, mean of 18.4%), and receptacles (< 53%, mean of 20.3%), consistent with the histochemical staining patterns.

GUS activity levels were also analyzed in leaves and flowers of seven poplar lines co-transformed with pDW151 and pPTD::GUS. A single line had very low absolute expression (below that of transgenic leaf tissue, suggestive of gene silencing or mislabeling), and was therefore excluded from normalization calculations (Figure 2C). The leaves of all six remaining lines displayed low levels of GUS activity relative to

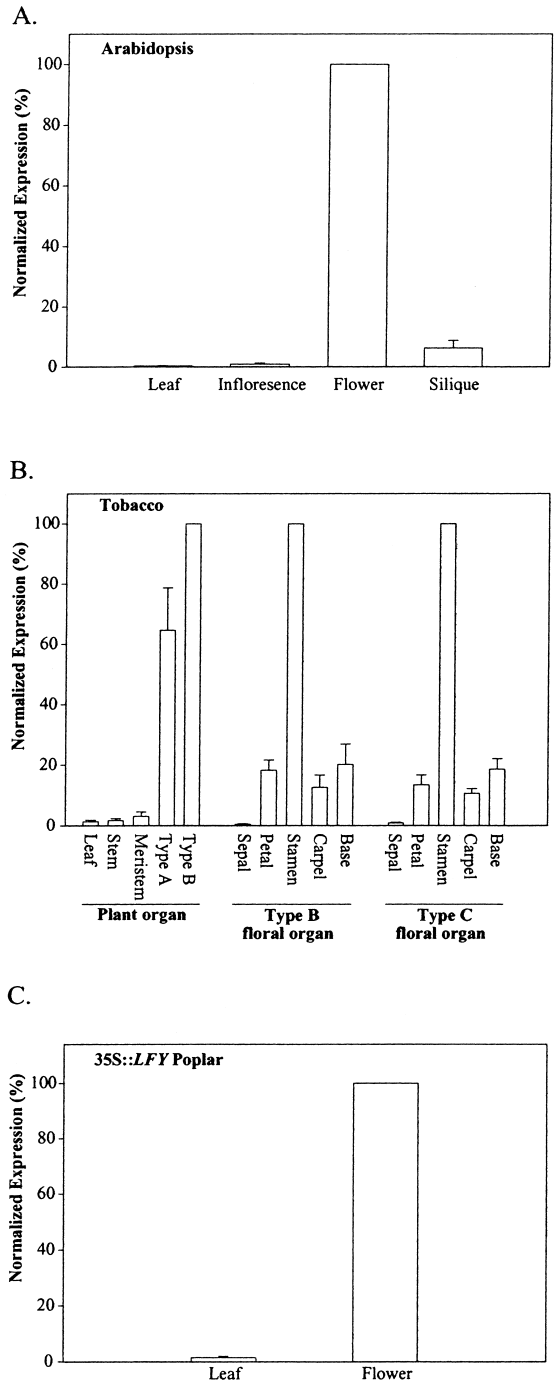

Figure 2. Normalized *PTD* promoter-directed GUS expression levels in transgenic plant organs. Percent normalized expression (%NE) was standardized to whole-flower values for plant parts and to stamen values for dissected flowers. Duplicate assays were performed on all tissues for each independent line. Average (across all lines) normalized *PTD* promoter-directed GUS activity in: A) leaves, inflorescence stems, flowers, and siliques for five independent transgenic *Arabidopsis* lines; B) plant parts (leaf, stem, meristem, and Type A and Type B flowers) and floral organs (sepal, petal, stamen, carpel, and receptacle) for six independent transgenic tobacco lines; and C) leaf and induced flowers of seven independent aspen lines co-transformed with 35S::*LFY*.

the levels observed for floral tissue; GUS activity in leaves averaged just over 1% of that in flowers (1.48 ± 0.51; Figure 2C), similar to results in *Arabidopsis* and tobacco.

Engineered sterility in diverse species

To determine if the *PTD* promoter would be useful for engineering sterility via cell ablation, a gene whose product disrupts protein synthesis (DTA) (Palmiter et al. 1987) was subcloned downstream of the *PTD* promoter fragment utilized in the PTD::GUS studies. This construct was tested in *Arabidopsis*, tobacco, and hybrid aspen (clone 353-38 co-transformed with pDW151).

A majority of *Arabidopsis* lines containing PTD::*DTA* displayed normal vegetative growth when compared to untransformed and PTD::GUS lines (Figure 3A). However, a wide range of petal and stamen aberrations was consistently observed. These varied from the complete absence of both organs to ablation and/or perturbation of one or the other organ (Figures 3B, C, D). Silique development was altered in all cases where abnormal floral development was observed (see below). Frequently, petals were ablated while stamen organogenesis proceeded normally (Figure 3C). Stamen elongation, however, was occasionally suppressed (Figure 3D). Where petal organogenesis occurred, development was usually abnormal and expansion was incomplete (Figure 3D). In no case were flowers observed that lacked stamens but contained petals. In other respects, inflorescence architecture was similar to that of wild-type flowers (Figures 3E, F). A variety of phenotypes were often observed among flowers on the same line, suggesting that precise timing of *DTA* expression, or environmentally-induced differences in toxicity thresholds among tissues, were important (see Discussion).

Siliques failed to elongate from virtually all abnormal flowers on PTD::*DTA* lines. On the left-hand side of Figure 3G is a flower from a transgenic plant at a position where petal expansion was occurring in non-transgenic plants of the same age (approximately 0.5 cm below inflorescence apex). The other three flowers/siliques were found approximately 17 cm from the inflorescence apex. The two middle (dwarf) 'siliques' were taken from PTD::*DTA* plants, whereas the normal-sized silique on the right came from a non-transgenic plant.

A total of 155 transgenic *Arabidopsis* PTD::*DTA* lines (based on resistance to kanamycin) were evalu-

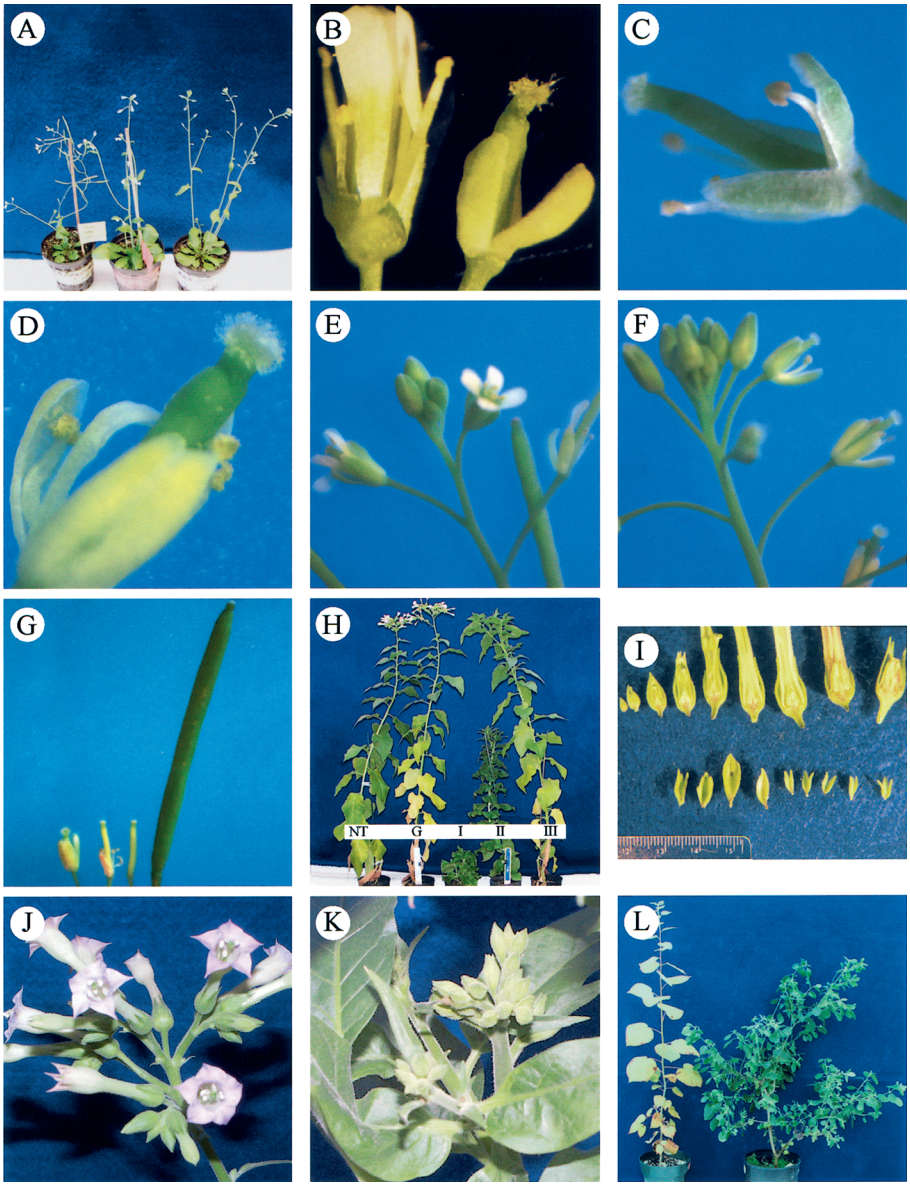

Figure 3. *PTD* promoter-directed ablation phenotypes in *Arabidopsis*, tobacco, and aspen. Plants or tissues are shown for *Arabidopsis* (panels A–G), tobacco (panels H–K), and poplar (panel L). A) Top view showing unaltered growth and rosette leaf development of representative PTD::*DTA* (line 13, right) relative to an untransformed (left) and PTD::GUS (line 4, middle) plants. B) PTD::*DTA* flower (line 1, right) showing complete ablation of petals and stamens vs. an untransformed flower (left, front sepal removed). C) PTD::*DTA* flower (line 28) showing petal ablation and partially elongated stamens. D) PTD::*DTA* flower (line 15) showing aberrant petal and stamen development. E) Floral cluster of untransformed plant showing the multiple stages of floral development visible within the first few cm of the floral apex. F) Floral cluster of a PTD::*DTA* (line 4) plant showing typical inflorescence architecture with variable PTD::*DTA* floral phenotypes. The first open flower on the right has ablated petals, the next flower down on the right has perturbed (unelongating) petals. G) Comparison of silique development in PTD::*DTA* (left three) and untransformed (right) plants. The PTD::*DTA* flower on the left was taken from a position where petal expansion was occurring in non-transgenic plants (Stage 15). The three siliques on the right were all taken from the same position on inflorescences of equivalent age. Petal and stamen remnants are intact on the flower at the middle left, and were removed from the flower at the middle right. H) Tobacco phenotypes. From left to right: typical non-transgenic (NT), PTD::GUS (G), and Class I (severe), Class II (moderate), and Class III (normal) PTD::*DTA* plants. I) Longitudinal cross-sections showing flowers from non-transgenic (top; developmental sequence shown) and from sepal-only PTD::*DTA* plants (bottom). J) Wild-type tobacco inflorescence phenotype. K) Close-up of line 22 inflorescence showing PTD::*DTA* flowers with the sepal-only phenotype; note that normal inflorescence architecture was maintained. L) Comparison of non-transgenic aspen (left) and aspen co-transformed with pDW151 (35S::*LFY*) and pPTD::*DTA* showing the typical branchy phenotype characteristic of *LFY* over-expression (Rottmann et al. 2000).

ated from four independent *in planta* transformation experiments, and phenotypes similar to those described above were observed in each case. Lines from the largest experiment (69 total) were analyzed in detail over 60 days of growth and grouped into three broad categories, based on floral phenotype. A majority of the lines (81%) produced aberrant flowers (Figure 4A); only six of the lines had a normal phenotype (flowers indistinguishable from those on control plants). Seven lines did not flower at all during the 60-day period of the trial and exhibited less vigorous vegetative growth (not shown), suggesting toxin expression in vegetative tissue. Lines with an abnormal floral phenotype displayed the range of ablated and/or abnormal floral morphologies described above; however, they generally showed normal vegetative growth (Figure 3A). The floral phenotypes were consistent within plants; individual plants could be decapitated and would reflower with the same range of phenotypes.

Results with PTD::*DTA* tobacco lines were similar to those seen with *Arabidopsis*. Two to four ramets were regenerated for each of 21 tobacco lines via vegetative cuttings and compared to four ramets from each of six PTD::GUS lines and a single untransformed control line. All ramets of any given line gave consistent vegetative and floral phenotypes, indicating that transgene expression was stable following vegetative propagation. Four phenotypic classes were identified based on vegetative and floral morphologies (Figure 3H). All of the PTD::GUS and non-transformed control lines exhibited normal floral development and vegetative growth. All PTD::*DTA* lines bore flowers with missing floral organs, and could be subdivided into three classes based on vegetative morphology: severely affected (I), moderately affected (II), and normal (III) (Figure 3H). Two PTD::*DTA* lines fell into Class I, three into Class II, and the remaining sixteen (76%) into Class III (Figure 4B). Apart from the ablated floral structures, Class III lines appeared normal, although leaves on some of the transgenic plants appeared to be slightly more lanceolate. In contrast to the range of floral phenotypes in *Arabidopsis*, flowers of PTD::*DTA* tobacco lines were uniform, consisting of sepals only; all internal floral organs (petals, stamens, carpel) were completely absent (Figure 3I). The sepal-only flowers occurred in normal-looking clusters (compare Figures 3J and 3K), indicating that the cytotoxin affected floral but not inflorescence meristems, like that seen in *Arabidopsis*. Florets were easily dislodged from the cluster, suggesting *DTA* expression was occurring near the base of the flower, consistent with the PTD::GUS expression patterns. The ablation phenotype was again stable; plants that were pruned displayed the same phenotype when the plant flowered a second time.

The effect of PTD::*DTA* on flowering frequency was also evaluated in poplar co-transformed with pDW151 (Figure 4C). Eighteen of 20 (90%) lines that were shown via PCR to be transformed with only 35S::*LFY* flowered. As noted above, 15 of 16 co-transformed (35S::*LFY* and PTD::GUS) lines (94%) displayed precocious flowering; 14 of these 15 flowering lines showed expression in the induced flowers. Thus, neither the co-transformation process nor the presence of PTD::GUS affected flowering frequency, and constitutive *LFY* expression did not prevent the *PTD* promoter from directing strongly floral-predominant expression (Figure 4C).

To test the value of the *PTD* promoter for inducing sterility in poplar, 10 co-transformed lines were generated. Based on the above frequencies, early flowering would be expected in at least nine of the 10 lines, and PTD::*DTA* expression in at least eight of those lines. However, flowering was only observed in one of the 10 lines (Figure 4C), implying that cytotoxin expression caused floral-organ ablation prior to observable development (explained in Discussion). Poplar lines co-transformed with pDW151 and pPTD::*DTA* displayed a branchy phenotype similar to that reported by Rottmann et al. (2000) for aspen 353-38 containing 35S::*LFY* alone (Figure 3L), suggesting that it was not cytotoxin expression that disturbed vegetative growth. The single line that flowered was phenotypically identical to lines transformed with pDW151 alone, indicating the 35S::*LFY* operon was active; this line was shown, via PCR, to contain the PTD::*DTA* operon.

Biomass potential maintained

To determine if there were significant differences in biomass for normal-appearing transgenic plants, as might be selected for commercial use, we compared growth of several Class III PTD::*DTA* tobacco lines with non-transgenic and PTD::GUS lines. Eight ramets each of two untransformed, three PTD::GUS, and six Class III PTD::*DTA* lines were propagated *in vitro* and transferred to soil. Following pruning (see Methods), between five and eight ramets per line regrew. Thirty-five days after decapitation, flower ini-

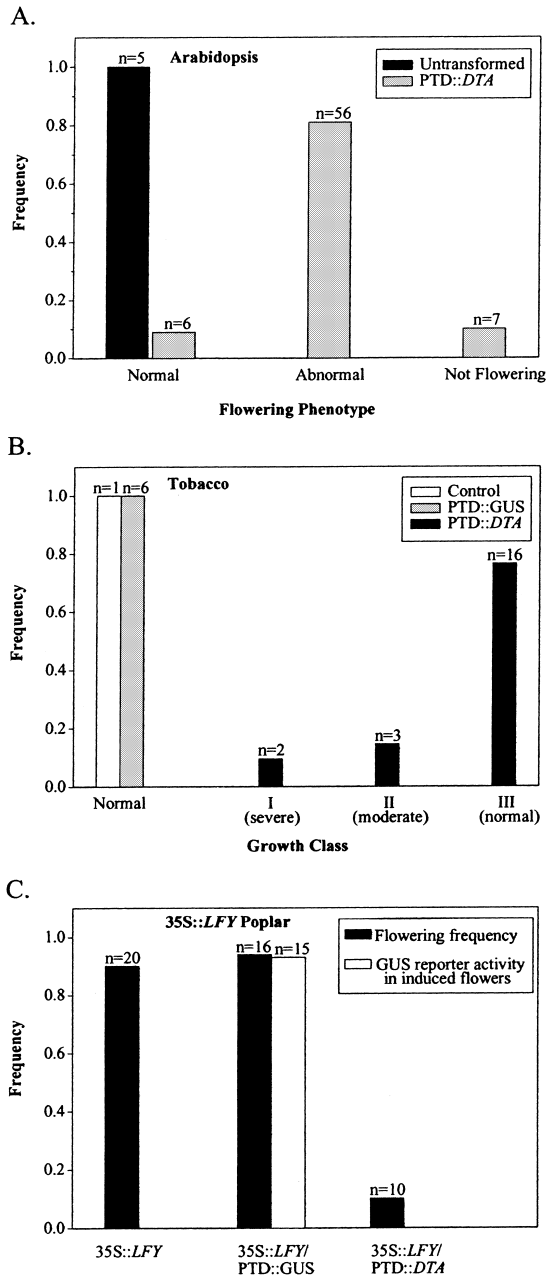

Figure 4. Phenotype frequencies of plants transformed with PTD::*DTA*. A) Flowering class distribution of PTD::*DTA Arabidopsis* lines. 'Normal' plants were indistinguishable from non-transgenic plants. 'Abnormal' denotes plants with ablated and/or aberrant floral organs. B) Frequency of normal flowering plants among morphological classes of tobacco transgenics. C) Frequency of normally flowering plants among aspen co-transformed with 35S::*LFY*. The 35S::*LFY* construct (pDW151) induced flowering in 353-38 at high frequency both on its own and in the presence of a second transgene (PTD::GUS), and PTD::GUS targeted expression to 35S::*LFY*-induced 353-38 flowers at high frequency. Co-transformation of 35S::*LFY* and PTD::*DTA* reduced the frequency of flowering plants from 90% to 10%. For each panel the number of lines (n) contributing to each mean is indicated above bars.

tiation was visible on > 90% of the plants; the remaining plants were found to have initiated unemerged floral clusters following harvest of vegetative tissues. No obvious differences in flowering time were observed among genotypes.

All ramets of the six PTD::*DTA* lines displayed normal vegetative growth. Dissection of flowers from each ramet of the PTD::*DTA* lines revealed that they consisted of only sepals, verifying that the PTD::*DTA* operon had been reactivated and the Class III phenotype was maintained. Dry weight measurements of the regrowth failed to show a statistically significant difference (Student's T Test, P = 0.18) between non-transgenics and plants transformed with PTD::GUS, compared with those harboring the cytotoxin construct (Figure 5). Therefore, the low levels of *PTD*-specified vegetative expression determined via quantitative GUS assays were not sufficient to negatively impact growth in this environment.

Cytotoxin expression in Class I and III tobacco lines

Real-time PCR was performed on a subset of Class I and III PTD::*DTA* tobacco lines to determine if phenotypic differences were due to cytotoxin expression in the non-target tissues of Class I lines. Leaf tissue from two Class I and three Class III lines were assayed (Figure 6). Relative levels of cytotoxin-gene mRNA in newly developing flowers (1–4 mm in size) were also determined for the three Class III lines. PTD::*DTA* line 5 (Class III) had the lowest leaf *DTA* value and was arbitrarily set to 1.0 for standardization. The levels of PTD promoter-directed expression in leaf tissue of the Class I lines were not significantly different from those observed in the Class III lines (Student's-t test, P = 0.29; Figure 6). Although the highest leaf *DTA* mRNA level was observed for a Class I line, it was only two-fold greater than the highest Class III leaf level; this Class III line had a *DTA* mRNA level that was 1.9-fold greater than the other Class I line. The same three Class III lines were also used for biomass determinations, which failed to detect any detrimental effects on growth (Figure 5). The three Class III lines showed increased *DTA* message levels in floral tissue relative to leaf tissue, but the increase was not as marked as that seen in the GUS assays (~ 10- to 40-fold vs. ~ 100-fold).

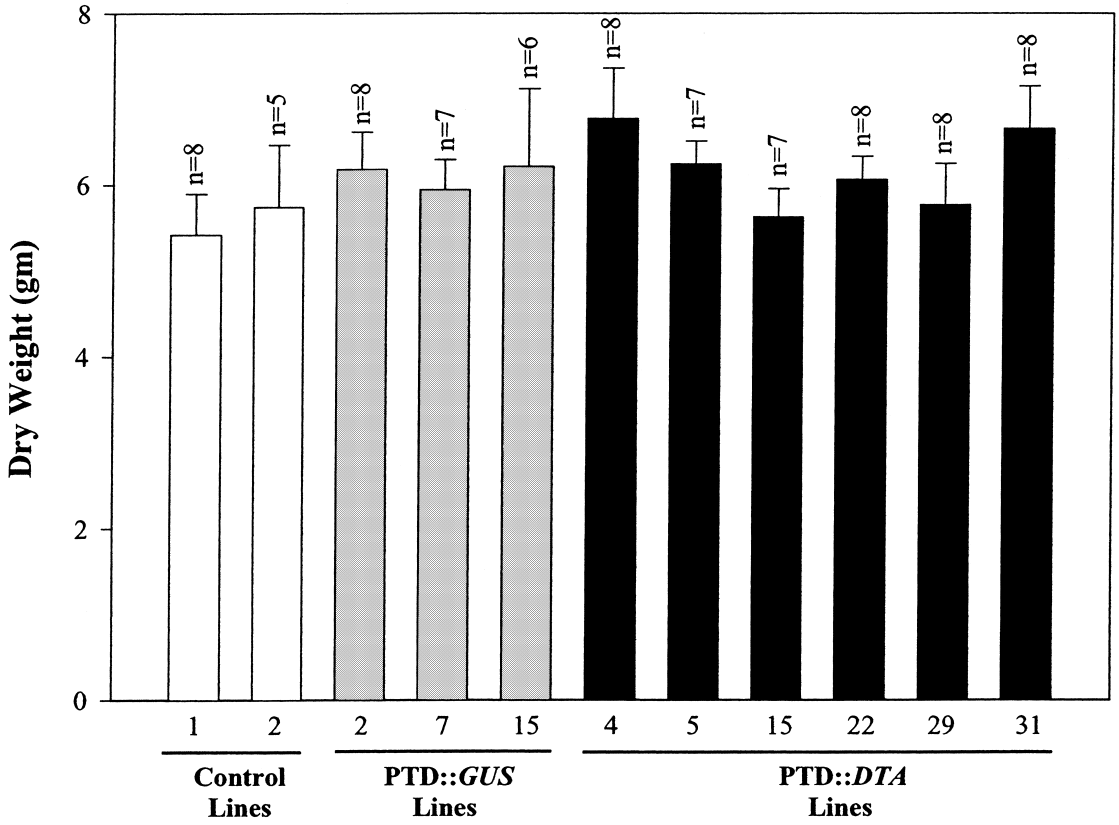

Figure 5. Effect of PTD::*DTA* on growth in morphologically normal tobacco plants (Class III). Accumulated biomass for two untransformed (Control), three PTD::GUS, and six Class III PTD::*DTA* lines was determined as described in Methods. The number of surviving ramets (vegetative propagules) used to determine the mean for each line is indicated above the bars (n). Line numbers are indicated below each bar. Error bars represent one standard error of the mean.

Discussion

Transgene confinement is a major concern for the public and government agencies that regulate genetically modified trees. In the Pacific Northwestern USA, transgene dispersal from fertile genetically modified poplars is highly probable because poplar plantations are frequently grown in close proximity to wild or feral relatives, and the trees are only modestly domesticated. Including a sterility-inducing transgene in genetically modified poplars would provide a means to dramatically reduce the potential for gene flow. Our results suggest that the *PTD* promoter may be useful for meeting this goal without detrimental effects on biomass production.

Several sequence features may contribute to the regulatory properties of the *PTD* promoter. First, it contains a homeobox motif (Sheppard et al. 2000). Second, it contains three large A/T-rich clusters (each > 150 bp and > 87% A/T) and four large repeats (approximately 100 bp each). The A/T-rich regions likely contribute to the strength of the *PTD* promoter, but not its tissue specificity. A/T-rich motifs are commonly associated with positive regulatory functions, affecting expression in a quantitative, not a tissue-specific, manner (Sandhu et al. 1998). Finally, six sequence motifs matching at least eight to nine of the 10-base consensus CArG box occur within the 1,900-bp fragment that has been sequenced; two of the six are a perfect match. Based on studies with the *Arabidopsis AP3* regulatory region (Hill et al. 1998; Tilley et al. 1998), one or more of these CArG-box motifs are thought to play a key role in the floral-specific, B function-like expression pattern of *PTD*.

In addition to poplar, the *PTD* promoter directed floral expression in species from two other plant families. In both cases, very low levels of GUS expression were detected in vegetative tissues compared to flowers. Within whole flowers, a B-function-gene expression pattern was observed, consistent with the na-

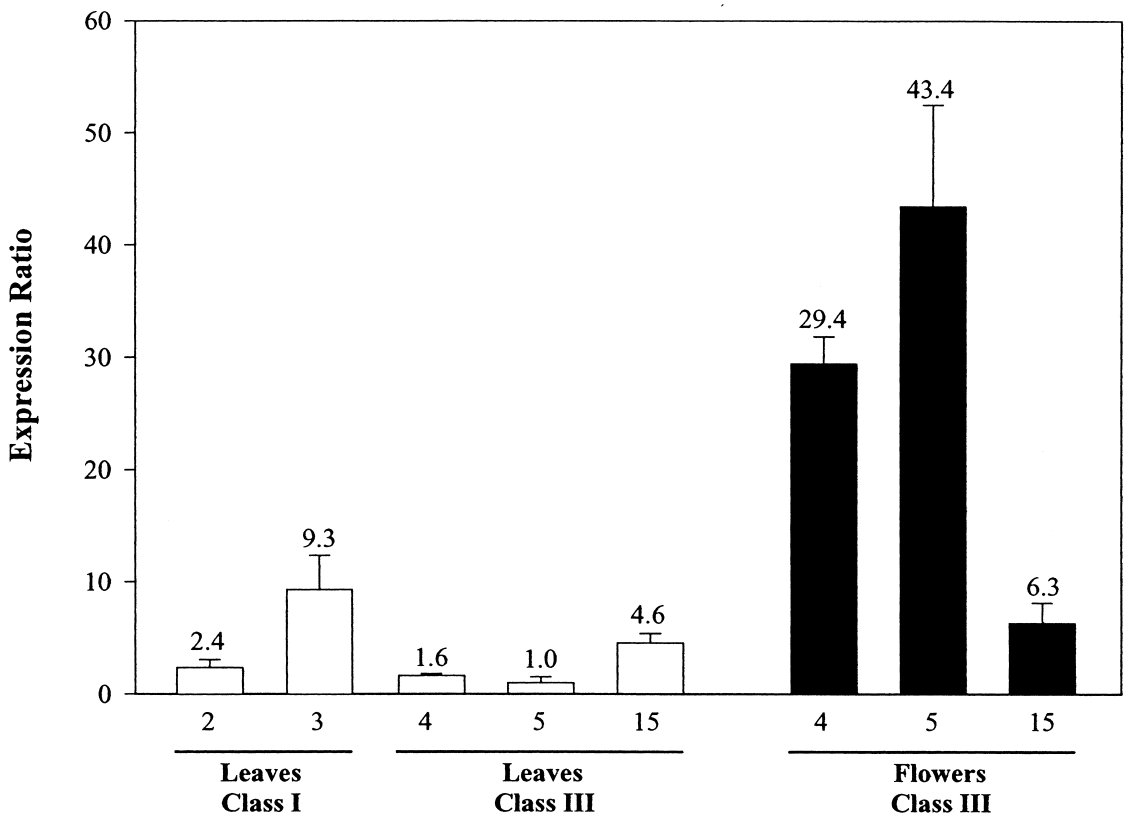

Figure 6. Real-time PCR analysis of *PTD* promoter-directed *DTA* expression in Class I and III tobacco lines. Expression levels were determined from triplicate subsamples. Error bars are one standard error of the mean. For leaves only, DTA levels are in relation to the lowest value (i.e., PTD::*DTA* line 5 value was set to 1.0) and are therefore presented as the fold increase over line 5 leaf expression (open bars; numerical average of fold-values shown above bars). Lines 2 and 3 (Class I) displayed highly disturbed vegetative phenotypes; lines 4, 5, and 15 (Class III) had normal vegetative growth (Figures 3H, 5). Floral *DTA* expression levels (black bars) of the Class III lines are expressed as the fold increase in *DTA* expression for floral tissues relative to leaf tissue of the same line (floral value/leaf value). Line numbers are indicated below each bar, ratios are shown above bars.

tive *PTD* gene's expression pattern in poplar, and its phylogenetic assignment to the *AP3* gene sub-family of MADS box genes (Sheppard et al. 2000). In *Arabidopsis*, expression was mostly confined to petals and stamens, with a band of activity at the base of the flower that was maintained during silique development. In tobacco, expression was also seen in the lower half of the carpel. In both herbaceous species, expression was strongest in stamens. As with *AP3* expression in *Arabidopsis* (Jack et al. 1992), *PTD* promoter-directed GUS activity was maintained throughout floral development in tobacco.

The GUS expression patterns were closely correlated with the developmental effects seen in PTD::*DTA* lines. In both tobacco and *Arabidopsis*, the majority of PTD::*DTA* lines exhibited floral abnormalities but had normal vegetative growth (Figures 3A, 3H). Defective flowers were arranged normally on their inflorescence in both tobacco and *Arabidopsis*, indicating that cytotoxic effects began during floral meristem, rather during inflorescence meristem, differentiation. In every PTD::*DTA* tobacco line, cytotoxin expression resulted in the complete ablation of all floral organs in which GUS expression had been observed, resulting in flowers comprised solely of sepals.

The cytotoxin effects in *Arabidopsis* were not nearly as severe as those seen in tobacco. Although complete organ ablation did occasionally occur, *DTA* expression mainly resulted in perturbed development of petals and/or stamens, and affected flowers were self-sterile. Surprisingly, petals appeared more susceptible than stamens to cytotoxin effects, despite the stronger GUS activity observed in stamens (Figure 1B). When using a translational fusion of its promoter and the first 54 amino acids of *AP3* to direct *DTA* ex-

pression in *Arabidopsis*, Day et al. (1995) observed complete ablation of petals and stamens among the six lines evaluated.

The wide range of floral ablation phenotypes observed in *Arabidopsis* may be a result of variation in toxicity thresholds and timing of expression among tissues. A critical level of *PTD* promoter-directed toxin expression may not have been reached prior to completion of petal/stamen differentiation. The frequency with which petals failed to elongate could be explained by this hypothesis if *PTD* promoter-directed *DTA* levels reached critical levels only after a majority of petal progenitor cells had already formed, but prior to elongation. Day et al. (1995) observed a varying degree of cytotoxin effects on integument development in *Arabidopsis*, even though all lines displayed identical patterns of stamen/petal ablation. The more severe effect of the AP3::*DTA* construct in stamen/petals vs. integuments may have resulted from regulatory elements in the *Arabidopsis* promoter enabling higher expression levels in the former tissues. Alternatively, the translational fusion employed may have been more efficient in stamen/petal progenitor cells. The greater phenotypic diversity seen in *Arabidopsis* compared to tobacco may also result from higher tolerance to *DTA* in *Arabidopsis*. Poplar might also be relatively insensitive to DTA, at least in vegetative tissues. Despite *LFY*'s significant level of vegetative expression, Nilsson et al. (1998) successfully used the *LFY* promoter controlling *DTA* expression to produce morphologically normal *Arabidopsis* plants with ablated flowers.

There were no obvious signs of cytotoxin expression in vegetative tissues in a majority of our *Arabidopsis* and tobacco PTD::*DTA* lines. While impractical for *Arabidopsis*, vegetative effects were evaluated quantitatively in the transgenic tobacco. The Class III PTD::*DTA* lines, which appeared vegetatively normal, differed only from the non-transformed plants in their disturbed floral development, and had similar levels of biomass accumulation (Figure 5). The severe vegetative phenotype of the two Class I lines did not appear to result from increased vegetative *DTA* expression because one Class III line had higher *DTA* mRNA levels than a corresponding Class I line. Instead, the perturbed vegetative growth of Class I (and II) lines may be due to position effects, where their insertion site gave rise to higher levels of expression in key vegetative cells or organs. It is also possible that the aberrant phenotype was caused by transgene insertion in a native gene. In addition to reduced size, these plants showed disturbed morphology from a very early age (during *in vitro* regeneration). There were elevated levels of *DTA* mRNA in flowers of Class III lines (Figure 6) compared to leaves; however, the difference was not as marked as seen with the GUS assays. Assuming equivalent levels of normalized *PTD*-directed expression of GUS and *DTA*, this suggests that at the time of sampling in the *DTA* lines the cells with the highest levels of *PTD* promoter-directed expression may have already been selectively eliminated.

Because poplars have a long juvenile period, we used a genotype that could be induced to flower early by *LFY* (Rottmann et al. 2000) in order to study *PTD* promoter-directed *DTA* effects. GUS activity in early-flowering PTD::GUS lines occurred at a high frequency (Figure 4C) and was confined to floral tissues, with the highest activity in stamens. Co-expression of the PTD::*DTA* and 35S::*LFY* operons severely reduced flowering frequency, implying that expression of *DTA* caused ablation of the floral tissues before flower development was observable. Because *LFY*-containing early-flowering lines are vegetatively abnormal (Rottmann et al. 2000), we have also generated poplar lines containing only PTD::*DTA* in both 353-38 and a female clone (INRA 717-1B4). These are being tested for biomass effects and sterility during the onset of normal flowering. To date, PTD::*DTA* poplars appear morphologically normal following a single year of growth (unpub. data).

Our results demonstrate that the *PTD* promoter may be useful for engineering reproductive sterility in several plant species. While the *DTA* gene is an excellent tool for testing candidate promoters during research and appears highly safe (Skinner et al. 2000; Strauss et al. 1995), commercial uses would likely employ other cell ablation genes such as ribonucleases (Hartley 1988), which provide opportunities to attenuate cytotoxin effects (Beals and Goldberg 1997; Skinner et al. 2000).

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