

Transgenic sterility in *Populus*: expression properties of the poplar *PTLF*, *Agrobacterium NOS* and two minimal 35S promoters in vegetative tissues

HAO WEI,^{1,2} RICHARD MEILAN,³ AMY M. BRUNNER,^{1,4} JEFFREY S. SKINNER,⁵
CAIPING MA¹ and STEVEN H. STRAUSS^{1,6}

¹ Department of Forest Science, Oregon State University, 321 Richardson Hall, Corvallis, OR 97331-5752, USA

² Current address: Linus Pauling Institute, Oregon State University, 538 Weniger Hall, Corvallis, OR 97331-7304, USA

³ Department of Forestry and Natural Resources, Purdue University, G021E Pfendler Hall, 715 W. State Street, West Lafayette, IN 47907-2061, USA

⁴ Department of Forestry, Virginia Tech., Blacksburg, VA 24061, USA

⁵ Department of Horticulture, Oregon State University, 4017 Agriculture and Life Sciences Building, Corvallis, OR 97331-7304, USA

⁶ Corresponding author (steve.strauss@oregonstate.edu)

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Summary Transgenic sterility is a desirable trait for containment of many kinds of transgenes and exotic species. Genetically engineered floral sterility can be imparted by expression of a cytotoxin under the control of a predominantly floral-tissue-specific promoter. However, many otherwise desirable floral promoters impart substantial non-floral expression, which can impair plant health or make it impossible to regenerate transgenic plants. We are therefore developing a floral sterility system that is capable of attenuating undesired background vegetative expression. As a first step towards this goal, we compared the vegetative expression properties of the promoter of the poplar (*Populus trichocarpa* Torr. & Gray) homolog of the floral homeotic gene *LEAFY* (*PTLF*), which could be used to impart male and female flower sterility, to that of three candidate attenuator-gene promoters: the cauliflower mosaic virus (CaMV) 35S basal promoter, the CaMV 35S basal promoter fused to the TMV omega element and the nopaline synthase (*NOS*) promoter. The promoters were evaluated via promoter::*GUS* gene fusions in a transgenic poplar hybrid (*Populus tremula* L. × *P. alba* L.) by both histochemical and fluorometric *GUS* assays. In leaves, the *NOS* promoter conveyed the highest activity and had a mean expression level 5-fold higher than *PTLF*, whereas the CaMV 35S basal promoter fused to the omega element and the CaMV 35S basal promoter alone directed mean expression levels that were 0.5× and 0.35× that of *PTLF*, respectively. Differential expression in shoots, leaves, stems and roots was observed only for the *NOS* and *PTLF* promoters. Strongest expression was observed in roots for the *NOS* promoter, whereas the *PTLF* promoter directed highest expression in shoots. The *NOS* promoter appears best suited to counteract vegetative expression of a cytotoxin driven by the *PTLF* promoter where 1:1 toxin:attenuator expression is required.

Keywords: ablation, biosafety, biotechnology, containment, forestry, gene flow, genetic engineering, *GUS*, trees.

Introduction

Genetically engineered reproductive sterility is an important option for reducing the environmental impacts of transgene dispersal from trees (Strauss et al. 1995, Skinner et al. 2000, NRC 2004). One common method for engineering plant sterility is to employ a floral promoter to direct the expression of a cytotoxin, thus preventing the targeted floral tissues from completing their development (Mariani et al. 1990, Goldman et al. 1994, Block et al. 1997, Lemmetyinen et al. 2001, Skinner et al. 2003). However, in addition to the expected strong expression in reproductive tissues, many flowering-related genes display various degrees of “background” expression in vegetative tissues (Kyoizuka et al. 1997, Southerton et al. 1998a, 1998b, Brunner et al. 2000, Rottmann et al. 2000, Skinner et al. 2003). This implies that use of many floral promoters to direct cytotoxin expression may negatively impact vegetative growth or the ability to regenerate transgenic plants (Skinner et al. 2000, Lemmetyinen et al. 2004). We have observed that floral promoters from tobacco and *Brassica*, when directing two kinds of cytotoxins in transgenic poplars, resulted in significantly decreased tree growth in the field compared with transgenic trees lacking cytotoxins (Skinner et al. 2000). A system capable of attenuating such side effects of undesired vegetative expression would therefore be highly desirable.

We are developing an attenuation-based sterility system in which a floral promoter directing the cytotoxin barnase is cointroduced with the barnase inhibitor barstar (Hartley 1988) under the control of a weak to moderate, but widely expressed, “constitutive” promoter (Wei 2004). Undesired cytotoxin activity in vegetative tissues should be neutralized, enabling nor-

mal vegetative growth and yield, whereas the large spike of cytotoxin expression in floral tissues would overcome the low levels of cytotoxin inhibitor present, resulting in the desired ablation of floral structures. Mariani et al. (1992) used attenuation of barnase by barstar to restore fertility to male-sterile transgenic plants. Beals and Goldberg (1997) used barstar to attenuate barnase activity in a tissue-specific manner for control of anther dehiscence.

Because poplars are commonly propagated by vegetative means and male and female sterility is desired for maximal gene containment, we sought the regulatory region of a gene that is critical for early bisexual stages of floral development. One such gene is *LEAFY* (*LFY*), which encodes a floral regulatory protein that is expressed at early stages of the differentiation of all floral organ primordia (Schultz and Haughn 1991, Blázquez and Weigel 2000). However, although *LFY* is predominantly expressed in floral tissues, it shows readily detectable background vegetative expression compared with many other flowering-related genes (Blázquez et al. 1997). This also appears to be true of *LFY* homologs in poplar (*PTLF*) and *Eucalyptus* (*ELF1*) trees (Southerton et al. 1998a, Rottmann et al. 2000). Nilsson et al. (1998) demonstrated that use of the regulatory region from the *Arabidopsis LFY* gene to drive the cytotoxin encoding gene *DTA* in transgenic *Arabidopsis* resulted in the complete ablation of floral organs; however, because of lack of an attenuation system, a large proportion of the transgenics were impaired in vegetative development, putatively because of cytotoxin expression in non-floral tissues. Such a condition is likely to be commercially unacceptable, especially in trees where impaired vegetative growth might not be observed for many years, as we have observed in poplars (Skinner et al. 2000).

We determined the vegetative background expression pattern conveyed by the *PTLF* promoter (5' flanking region) in detail by fusing it to the *GUS* reporter gene. In conjunction with the *PTLF* promoter analysis, we assayed the expression patterns and levels of three weak to moderately expressed constitutive promoters that were chosen as candidates for directing expression of cytotoxin attenuator genes. The attenuator regulatory candidates were the basal region of the cauliflower mosaic virus (CaMV) 35S promoter (-72 to +5) (Benfey et al. 1989), the CaMV 35S basal promoter fused to the tobacco mosaic virus (TMV) omega element (Holtorf et al. 1995) and the nopaline synthase (*NOS*) promoter (An et al. 1986). Matrix attachment region (MAR) elements were included in vectors because they are expected to increase, and possibly stabilize, transgene expression in poplar (Han et al. 1997). In associated studies, we found that the barstar gene driven by one of these promoters was required to enable recovery of transgenic poplars containing the barnase cytotoxin driven by the *PTLF* promoter and that it enabled normal growth rate in the greenhouse of a large majority of the regenerated plants (Wei 2004).

Material and methods

Promoter characteristics that guided construct assembly

We analyzed the location of putative functional elements in the

PTLF promoter by reference to published promoter studies and by motif searches with PLACE Web Signal Scan software (Prestridge 1991, Higo et al. 1999). There are two functional regulatory regions in the *Arabidopsis LFY* promoter (Blázquez and Weigel 2000). The first fragment is proximal to the translation start site (-373 to -246 bp) and is critical for *LFY* expression. The second is a distal fragment (-1782 to -1558 bp) which partially determines the expression level of *LFY*. Comparison of the *LFY* and *PTLF* promoter regions shows limited sequence similarity, but an 8-bp motif, CAACTGTC, is conserved and was identified as a GAMYB binding site by Gocal et al. (2001). This 8-bp motif is located at -249 to -242 bp in *Arabidopsis LFY* and -213 to -206 bp in *PTLF*.

The intact CaMV 35S promoter directs general, high-level expression in all plant tissues through the combinatorial properties specified by its multiple regulatory elements (Odell et al. 1985). The basal region of the 35S promoter (-72 to +5) is composed solely of a TATA box and a pair of putative CAAT boxes and is assumed to function as a weak "constitutive" promoter; in leaves, stems and roots of 15-day-old tobacco seedlings it directs less than 0.1% of the expression of a full-length version (Benfey et al. 1989). The TMV omega enhancer element acts post-transcriptionally in a quantitative non-tissue-specific manner by increasing the translational efficiency of plant mRNAs to which it is fused by up to 2- to 3-fold (Holtorf et al. 1995, Schmitz et al. 1996, Mannerlof and Tening 1997); fusion of the TMV omega element downstream of the 35S basal promoter should result in an approximate doubling of 35S basal promoter-directed product. Like the CaMV 35S promoter, the promoter of the *NOS* gene is expressed generally throughout plants (An et al. 1986), but at a considerably lower level (30× less) than the intact 35S promoter (Sanders et al. 1987, An et al. 1988). These characteristics suggested that these three regulatory regions would provide a diverse range of candidates for directing expression of a cytotoxin attenuator, depending on the level and detailed pattern of cytotoxin expression.

Plasmid assembly

Promoter::*GUS* fusions of four regulatory regions were generated in a plant binary transformation vector flanked by MAR elements (Figure 1). These were the *PTLF* floral promoter (*PTLF*::*GUS* construct, PGUS), a minimal CaMV 35S basal promoter (min35S::*GUS* construct, SGUS), the same minimal 35S basal promoter fragment fused to the TMV Omega translational enhancer (min35S::Omega::*GUS* construct, OGUS), and the *NOS* promoter (*NOS*::*GUS* construct, NGUS).

A fragment harboring an intron-containing *GUS* gene with an optimized translation initiation site that mimics the consensus plant translation initiation site was released from pPR97 (Szabados et al. 1995) by digestion with *Sac*I (T4 polymerase-blunted) and *Kpn*I, subcloned into the *Kpn*I and *Sma*I sites of an intermediate construct and used for the promoter::*GUS* assemblies. The intron-containing *GUS* version was used to eliminate confounding expression from any residual *Agrobacterium* presence (Shen et al. 1993). The promoter::*GUS* gene fusions were first assembled in pBluescript II SK(+) and

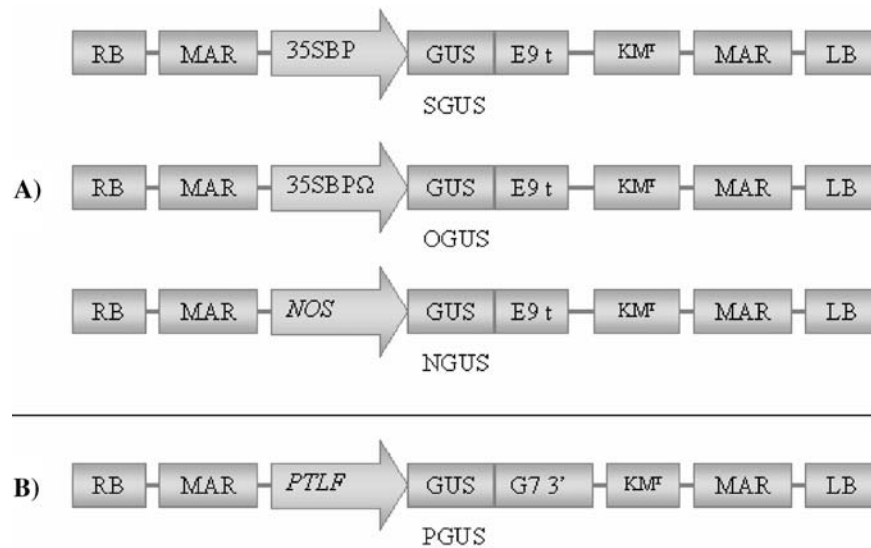


Figure 1. Key elements of DNA constructs. (A) Constructs for evaluating expression of three heterologous promoters. (B) Construct for studying expression imparted by the *PTLF* promoter. Arrows show direction of transcription. Abbreviations: 35SBP = cauliflower mosaic virus 35S basal promoter (+5 to -72 fragment); 35SBPΩ = 35SBP fused to tobacco mosaic virus omega element; E9 t = E9 terminator; G7 3' = gene 7 3' region (terminator); KM^f = kanamycin resistance operon; LB and RB = left- and right-hand borders of T-DNA in binary vector; MAR = matrix attachment region; NOS = NOS promoter; PTLF = promoter from the poplar floral homeotic gene *PTLF*; and GUS = reporter gene.

then subcloned into the binary vector pG3M. The pG3M binary vector was derived from pGreen II (Hellens et al. 2000) by first inserting *AscI* linkers at the *HpaI* and *StuI* sites and then cloning two copies of the 1.2-kb tobacco RB7 MAR (matrix attachment region) elements (Allen et al. 1996) as direct repeats at the *FspI* and *SapI* (blunted) sites that flank the poly-linker. The MAR elements (Spiker and Thompson 1996, Allen et al. 2000) have been shown to elevate transformation and transgene expression approximately 2-fold in regenerated transgenic poplar (J. Skinner and S. Strauss, unpublished data).

The 2.6-kb *PTLF* promoter fragment (-2630 to -20 relative to the translational start codon) used for *PTLF::GUS* construction was obtained by PCR amplification from a genomic clone (Rottmann et al. 2000) with the primers: 5'-AGCCGCGGTA-CTAAATAAATATATAAAC-3' and 5'-TGCGGCCGCGATC-TTTCACAGGTGCATGTC-3'; *SacII* and *NotI* sites (underlined) were incorporated at the 5' and 3' ends, respectively. For the min35S::*GUS* construction, the 35S basal promoter (-72 to +5 relative to the transcriptional start site) was PCR amplified from pEL301 (Mohamed et al. 2001) with the PCR primers: 5'-AGAATTCGGATGACGCACAATC-3' and 5'-AGGT-ACCCCGTGTCTCTCC-3' with *EcoRI* and *KpnI* sites (underlined) incorporated at the 5' and 3' ends, respectively. For the min35S::Omega::*GUS* construction, the same 35S basal promoter region fused to the TMV Omega translational enhancer was PCR amplified from pEL301 with the primers: 5'-AGAATTCGGATGACGCACAATC-3' and 5'-TGGTAC-CTGTAATTGTAAATA-3', which had *EcoRI* and *KpnI* sites (underlined) incorporated at their 5' and 3' ends, respectively. For the *NOS::GUS* construction, the *NOS* promoter region (-263 to +36 relative to the transcriptional start site) from a *NOS:nptII* selectable marker operon present in binary vector pBIGUS1 was PCR-amplified with the primers: 5'-AGAATTCGATCATGAGCGGAGA-3' and 5'-AGGTACCGGTGCA-GATTATT-3' with *EcoRI* and *KpnI* sites (underlined) incorporated at the 5' and 3' ends, respectively. All four promot-

er::*GUS* fusions were generated as transcriptional fusions within each promoter's 5' untranslated region (UTR) to the *GUS* gene. All cloned PCR-amplified fragments used in plasmid constructions were confirmed to be mutation free by sequencing.

Two transcriptional terminators were employed in assembly of these constructs. The 3' UTR of the pea ribulose biphosphate carboxylase (RuBP carboxylase) gene (E9 terminator) was fused downstream of the three weak promoter::*GUS* cassettes. The 3' UTR of gene 7 from *Agrobacterium tumefaciens* (G7 3') (Velten and Schell 1985) was fused downstream of the *PTLF::GUS* fusion. A *XhoI* fragment containing a full-length 35S promoter::*NPTII::NOS* terminator cassette was inserted downstream of each promoter::*GUS::terminator* operon. The assembled promoter::*GUS::terminator* fusions and the selectable marker cassette were excised from their respective intermediate constructs via *StuI* and *ClaI* digestion and subcloned into the *SmaI* and *ClaI* sites of pG3M between the flanking MAR elements (Figure 1). Further details on plasmid assembly are described by Wei (2004).

Plant transformation

All plasmids produced were transformed into *A. tumefaciens* strain C58 harboring the plasmid pSoup by the freeze-thaw method of Holsters et al. (1978). Hybrid poplar (*Populus tremula* L. × *P. alba* L.; INRA 717-1B4) was transformed by the method of Han et al. (2000). Transgenic events (i.e., independent gene insertions) harboring min35S::*GUS* and min35S::Omega::*GUS* were verified by PCR with a primer set specific for the *GUS* reporter gene (5'-TAAAAGGACAG-GGCCATC-3' and 5'-GTGATATCGTCCACCCA-3'). Transgenic *PTLF::GUS* and *NOS::GUS* events were verified via histochemical *GUS* staining of leaves during root induction in vitro. For each construct, four ramets of 10 independent transgenic events were used in experiments. Two-month-old plants were transferred to soil and maintained in a lighted growth room in Corvallis, Oregon, USA for one month before being

transferred to a greenhouse where the plants were maintained in a natural photoperiod. Tissue samples for reporter activity assays were collected during the spring and summer of 2003.

Histochemical and fluorometric GUS assays

Sixty days following transfer of plants to soil, shoot tips with two nodes and their expanding leaves were excised from greenhouse-grown transgenic plants and *GUS* reporter activity visualized by histochemical staining (Stomp et al. 1992). For quantitative fluorometric *GUS* assays (SPECTRAMax GEMINI XS, Molecular Devices Corporation), the following tissues were sampled: shoots (tips plus one node), leaves (fourth or fifth nodes below the apex), stems (between nodes three and four) and roots (including tips). Quantitative *GUS* assays were performed on two or four ramets per event per construct (see below) according to Jefferson et al. (1987). The concentration of total protein present in each extract was determined with a BioRad Protein Assay Kit (Hercules, CA). Activity of *GUS* is expressed as pmol 4-methylumbelliferone min⁻¹ mg⁻¹ total protein. Analogous samples were collected and assayed as above from identically treated untransformed (non-transgenic) control plants.

Statistical analysis and sampling of tissues

Student's *t* tests and one-way analysis of variance (ANOVA) of *GUS* activity means for each event were employed for sta-

tistical analysis of between- and within-construct comparisons. The *GUS* activity present in leaf tissue was used for between-construct comparisons, whereas *GUS* activities from shoots, leaves, stems and roots were used for within-construct comparisons. Tissue samples from four ramets for each of 10 independent events per construct were evaluated for the between-construct comparisons. Tissue samples from two ramets for each of 10 events per construct were evaluated for the within-construct comparisons.

Results

Sequence characteristics of PTLF promoter fragments

In addition to the 8-bp CAACTGTC motif previously described, analysis of the *PTLF* promoter region (-2630 to -20 bp) fused to the *GUS* reporter using the PLACE Web Signal Scan software package (Prestridge 1991, Higo et al. 1999) identified eight promoter segments that contained sequences matching known plant *cis*-acting regulatory element motifs. Two of the putative motifs corresponded to two different gibberellin-response elements, five corresponded to four different light regulatory element motifs and one to a sucrose-response element (Figure 2, Table 1).

Comparison of the promoter regions of *PTLF* and *PTAPI-1*, a *P. trichocarpa* Torr. & Gray homolog of the *Arabidopsis*



Figure 2. Putative regulatory DNA motifs in the poplar floral homeotic gene *PTLF* promoter region identified by PLACE Web Signal Scan software (Prestridge 1991, Higo et al. 1999; <http://www.dna.affrc.go.jp/htdocs/PLACE>). Identified motifs are in bold and underlined. The translation start codon ATG is in bold. Numbers designate the distances from the translation start site. The arrow above the T at position -41 indicates the transcriptional start site, based on 5' RACE experiments (A.M. Brunner and O. Shevchenko, Oregon State University, unpublished data).

Table 1. Putative transcription factor interaction motifs in the poplar floral homeotic gene *PTLF* promoter identified by PLACE Web Signal Scan software (Prestridge 1991, Higo et al. 1999). The positions of motifs are designated by their distance upstream of the translational start site. Abbreviation: *LFY* = *LEAFY* gene.

Motif name ¹	Position in <i>PTLF</i>	Position in <i>LFY</i>	Sequence	Related function	PubMed ref. no.
GARE2OSREP1	-2122	-	TAACGTA	Gibberellin-responsive	12787245
GT1CORE	-975	-2141, -606	GGTTAA	Light-response regulation	3243271
IBOXCORE	-1197	-2603, -1164, -989	GATAA	Light-response regulation	2902624
MYBGAHV	-2053	-663	TAACAAA	Gibberellin-responsive	8535141
REalphaLGLHCB21	-256, -1106	-585	AACCAA	Phytochrome regulation	8597658
REbetaLGLHCB21	-886	-	CGGATA	Phytochrome regulation	8597658
SURE2STPAT21	-1875	-	AATACTAAT	Sucrose-responsive element	8054988

¹ Motif designation as generated in the PLACE Signal Search output; see <http://www.dna.affrc.go.jp/htdocs/PLACE/> for original *cis*-element details.

APETALA1 gene (A.M. Brunner and V.B. Busov, Michigan Tech. University, unpublished data), revealed an approximately 200-bp conserved region (Figure 3). A search of the *P. trichocarpa* genome sequence (<http://genome.jgi-psf.org/poplar/>) indicated that this is a dispersed repetitive element; homologous sequences were also found in a 95-kb region of the *P. deltoides* genome (Accession no. AJ416708). Further inspection of this repetitive sequence identified features characteristic of a short interspersed nuclear element (SINE), including conserved promoter motifs (A and B boxes) recognized by RNA polymerase III and a 3' poly A region (reviewed by Feschotte et al. 2002); we term this SINE family *PSI* (*Populus* SINE1). For *PTLF*, the SINE starts at -619 and ends at -400 (from translation start site) and for *PTAPI-1* the SINE starts at -2592 ends at -2378.

Histochemical evaluation of transgene expression

Among the four constructs tested, only transformants containing NGUS and PGUS showed visible blue staining (Figure 4). In all 10 lines transformed with PGUS, faint blue staining was found along leaf veins and blue spots were observed along the margins. Stems stained less intensely than leaves, and the strongest staining was found in the shoot tips, including newly expanding leaves and leaf primordia. In NGUS transformants, leaves displayed stronger staining than did shoot tips. Leaf expression from lines containing NGUS was stronger than from PGUS. No visible differences could be observed among

SGUS, OGUS and the non-transgenic control plants; all lacked any histochemically detectable expression.

Quantitative evaluation of transgene expression

Fluorometric assays were employed to quantify *GUS* expression levels of the three attenuator promoters and *PTLF*. The results were in strong agreement with those from histochemical staining (cf. Figure 4 and Table 2). In leaves, the *NOS* promoter conferred the strongest expression, the *PTLF* promoter the second strongest, followed by the min35S::Omega and min35S promoters (Figure 5). Based on ANOVA and Student's *t* tests, there were statistically significant differences in leaf expression between NGUS and the other constructs ($P < 0.01$) (Figure 5). The mean leaf expression level was about 5-fold greater for NGUS than for PGUS and 9- and 14-fold stronger than for OGUS and SGUS, respectively. Based on Student's *t* tests, there were also significant differences between PGUS and the other two weak promoter constructs. The mean expression level of PGUS was 1.8-fold higher than that of OGUS and 3-fold higher than that of SGUS (two-tailed *t* tests; $P = 0.01$ and 0.00, respectively). No significant differences were detected between OGUS and SGUS (two-tailed *t* test, $P = 0.17$), although the TMV omega enhancer-supplemented version tended to be expressed at a higher level, as expected.

The constructs varied widely in their patterns of expression (Figure 6, Table 2). The *PTLF* promoter showed statistically



Figure 3. Alignment of the short interspersed nuclear element in poplar floral homeotic gene *PTLF* (*PTLF*) and in the *P. trichocarpa* homolog of the *Arabidopsis APETALA1* gene (*PTAPI-1*) promoters. *PTLF* sequence is from -625 to -380 and *PTAPI-1* sequence (GenBank Accession no. AY616-

522) is from -2597 to -2356. Target site duplication sequences (13 bp direct repeats) that appear to delineate the ends of the *PSI* element in the 5' flanking region of *PTAPI-1* are in bold; the target site duplications for the *PSI* element present in the *PTLF* promoter have apparently degenerated.

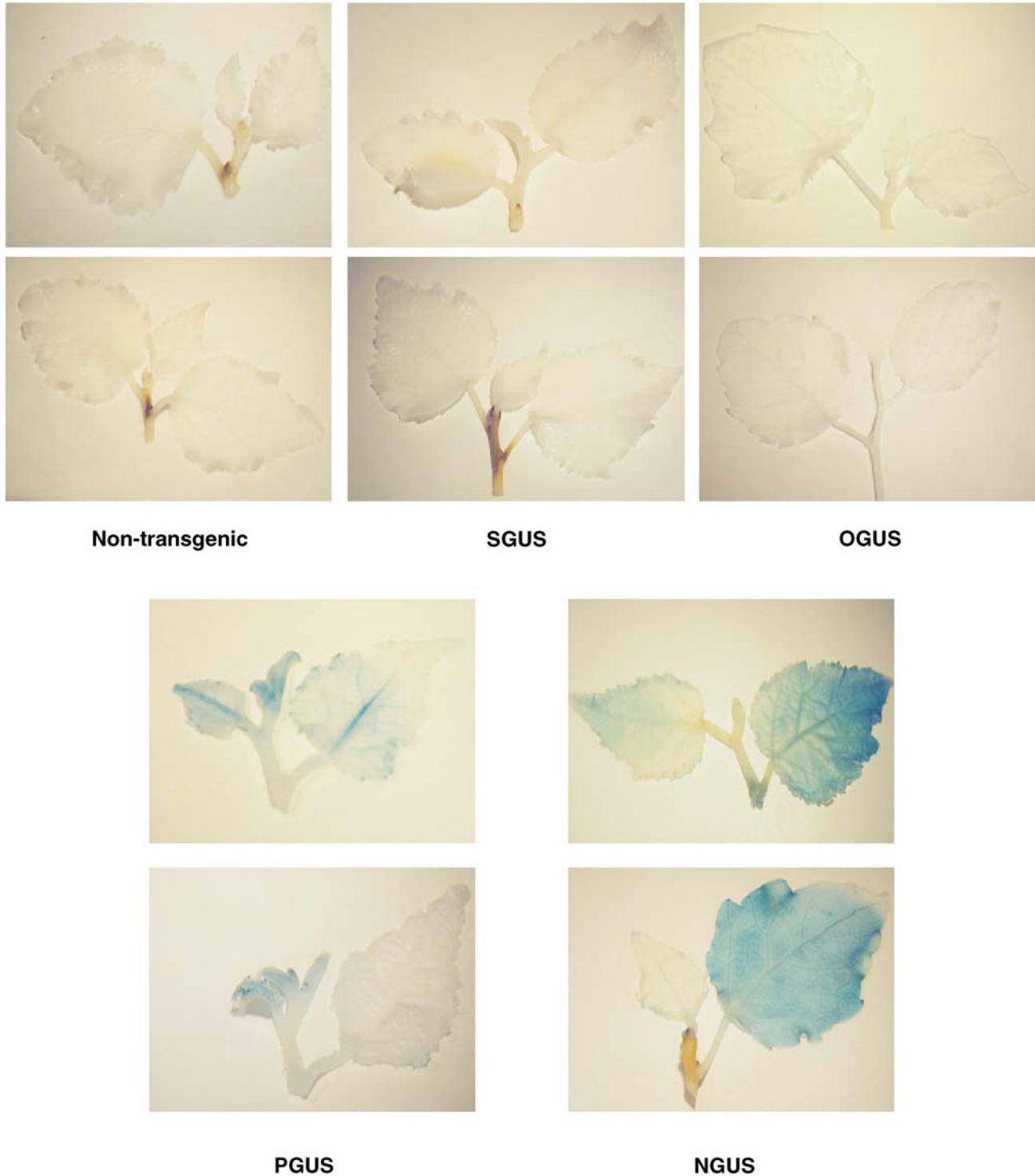


Figure 4. Histochemical GUS staining of transgenic plants. Each photo is of a different, representative (i.e., approximating the mean) transgenic event. Abbreviations: SGUS = min35S::*GUS* construct; OGUS = min35S::Omega::*GUS* construct; PGUS = *PTLF*::*GUS* construct; and NGUS = *NOS*::*GUS* construct.

significant ($P < 0.01$) variation in expression among tissue types. The difference between the strongest (shoot) and weakest (stem) expressing tissue was about 4.0-fold, whereas GUS activity in shoots was 2.3-fold higher than in leaves. There

were no statistically significant differences between roots and stems (two-tailed t test, $P = 0.63$); their measured GUS expression, however, was 1.7-fold lower than that of leaves.

There were no significant differences in expression levels

Table 2. Tissue-specific fluorometric GUS activities (pmol 4-MU min⁻¹ mg⁻¹ protein) among constructs and tissues (see also Figure 6). Data shown are means and standard errors over event means based on 10 independent events and two ramets per event. Abbreviations: SGUS = min35S::*GUS* construct; OGUS = min35S::Omega::*GUS* construct; NGUS = *NOS*::*GUS* construct; and PGUS = *PTLF*::*GUS* construct.

Construct	Shoot	Leaf	Stem	Root
Non-transgenic	10.85 (± 4.179)	10.36 (± 1.855)	12.75 (± 0.180)	12.88 (± 2.850)
SGUS	54.81 (± 12.20)	46.16 (± 6.656)	58.93 (± 10.09)	56.35 (± 9.089)
OGUS	63.69 (± 11.58)	66.68 (± 20.93)	72.81 (± 12.28)	57.74 (± 10.68)
NGUS	134.9 (± 49.23)	717.2 (± 361.6)	575.5 (± 128.1)	1719 (± 415.0)
PGUS	317.6 (± 33.90)	135.4 (± 9.685)	79.91 (± 9.460)	86.99 (± 10.85)

among SGUS and OGUS transformants (one-way ANOVA; $P = 0.90$ and 0.81 , respectively) among the tissue types tested. In SGUS transformants, the highest measured expression level was in stems, which was only 1.3-fold higher than in leaves, which had the lowest mean level. In OGUS transformants, the highest (stem) and lowest (root) expression levels only differed 1.3-fold. Although this difference was not significant, the mean expression of the OGUS construct tended to be consistently greater than that of the SGUS construct for each tissue type. Both 35S basal promoter variants were weakly expressed relative to the *NOS* promoter (Table 2). Stem and root expression for both 35S basal promoter constructs were similar to *PTLF*-directed expression, whereas leaf expression was approximately one-third of the stem and roots levels. Shoot expression of the two 35S basal promoter constructs was about 0.2-fold that of *PTLF*::*GUS*.

In contrast to the two 35S basal promoter-based transformants, the *NOS* promoter transformants exhibited preferential organ expression, in agreement with the histochemical staining. Strongest expression was found in roots, with a mean expression level 2.4-fold higher than in leaves. The weakest expression occurred in shoots, where mean expression was 5.0-fold lower than in leaves and slightly less than half of the *PTLF* promoter-based expression. The difference in mean ex-

pression between the highest (root) and the lowest tissues (shoot) was 12.7-fold. For the remaining three tissues (leaf, stem and root), *NOS*-directed expression exceeded *PTLF*-directed expression.

Discussion

Sequence analysis of the *PTLF* 5' regulatory fragment used in this study revealed the presence of multiple elements that match consensus plant *cis*-element binding sites (Table 1). One of these is an 8-bp CAACTGTC motif of the *LFY* promoter, which conforms to a consensus binding site for a MYB transcription factor. It interacts with an *Arabidopsis* MYB gene (*AtMYB33*) to putatively mediate a gibberellic acid-based signal to flower (Blázquez and Weigel 2000, Gocal et al. 2001).

For some floral regulatory genes, the regions controlling expression are located both inter- and intragenically (Sieburth and Meyerowitz 1997). Based on the expression results we obtained, however, the *PTLF* promoter fragment we studied appears to contain all of the regulatory elements needed to mimic the vegetative expression profile of the corresponding endogene (Rottmann et al. 2000). This suggests that the major regu-

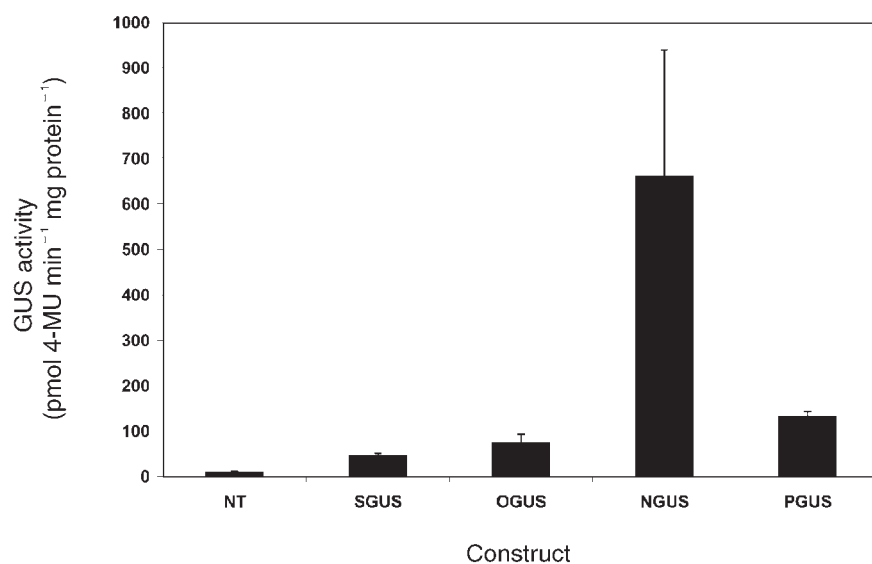


Figure 5. GUS expression in leaves of non-transgenic and transgenic plants. Bars show one standard error over event means based on 10 events and four ramets per event. Abbreviations: NT = non-transgenic; SGUS = min35S::*GUS* construct; OGUS = min35S::Omega::*GUS* construct; NGUS = *NOS*::*GUS* construct; PGUS = *PTLF*::*GUS* construct; *NOS* = *NOS* promoter; and *PTLF* = promoter from the poplar floral homeotic gene *PTLF* (see Figure 1).

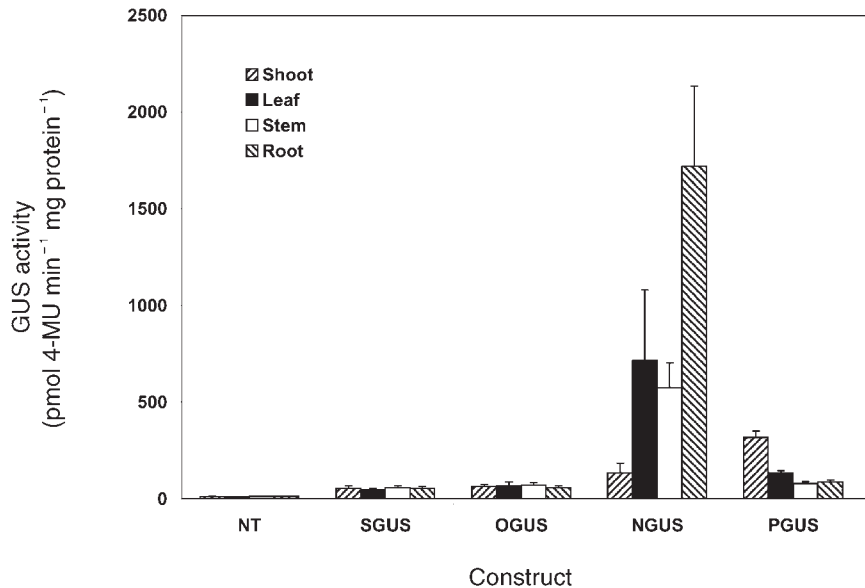


Figure 6. Variation in GUS expression among tissues and constructs. Bars show one standard error over event means. Abbreviations: NT = non-transgenic; SGUS = min35S::GUS construct; OGUS = min35S::Omega::GUS construct; NGUS = NOS::GUS construct; PGUS = *PTLF*::GUS construct; NOS = NOS promoter; and *PTLF* = promoter from the poplar floral homeotic gene *PTLF* (see Figure 1).

latory elements of the *PTLF* promoter are confined to the 5' flanking region.

The *PTLF* promoter conveyed strongest expression in leaf primordia and newly emerging leaves, with weak expression in veins, margins of older leaves and stems. Quantitative analysis of reporter gene activity confirmed the histochemical patterns observed (Table 2). Vegetative expression of *ELF1*, the eucalypt homolog to *PTLF* and *LFY*, is also strongest in young leaves and leaf primordia of shoot tips (Southerton et al. 1998a). These results suggest that the promoter activity of *LFY* homologs in vegetative organs is highest in young and newly differentiating tissues.

We examined the expression profiles of three candidate promoters for their ability to direct low to moderate levels of a cytotoxin attenuator throughout plants. Although the CaMV 35S basal promoter (-72 to +5) directed low levels of expression to all major vegetative organs (Table 2), we were unable to determine if expression was uniform throughout the various cell types within each organ because GUS levels were too low to detect by histochemical assays, even when using the TMV omega translational enhancer. The nearly uniform expression levels among the various vegetative organs by the 35S basal promoter fragments confirms that all of the major tissue-specific and quantitative regulatory regions of the wildtype (full-length) 35S promoter had been removed.

Inclusion of the TMV omega translational enhancer boosted expression (~1.5-fold), but not as strongly as the expected 2–3-fold amount based on previous reports (Holtorf et al. 1995, Mannerlof and Tening 1997). The inability of the TMV omega element to elevate translational efficiency substantially could be related to the low level of transcription caused by the basal 35S promoter, or to a lack of compatibility with the translational machinery of poplar. Nonetheless, even a 50% increase in expression could enable an effective attenuation threshold to be reached for floral promoters that give low lev-

els of background vegetative expression, such as that of the poplar *DEFICIENS* homolog *PTD* (Sheppard et al. 2000, Skinner et al. 2003). Alternatively, the 35S basal promoter expression could be boosted through creation of a hybrid promoter, such as by inclusion of non-tissue-specific quantitative enhancers like AT-rich elements (Sandhu et al. 1998).

In contrast to the two 35S basal promoter variants, the *NOS* promoter showed detectable levels of reporter activity by histochemical staining in all of the plant tissues examined. Its strong expression compared with the basal promoters was expected given its widespread use for directing expression of selectable markers during plant transformation. In mature leaves, stems and roots, *NOS*-directed expression exceeded that of the *PTLF* promoter at least 5-fold. However, *PTLF*-directed expression was slightly more than 2-fold that of the *NOS* promoter in shoots, implying that attenuation may not be as effective in this organ. Moreover, based on studies in tobacco, there are three additional concerns about use of the *NOS* promoter for attenuation: (1) it is wound and auxin inducible (An 1990); (2) expression in vegetative organs diminishes on flowering (An et al. 1988); and (3) it shows elevated expression in floral organs (An et al. 1988). Each of these might negatively impact a cytotoxin-based sterility system that employs a floral organ-predominant promoter, depending on their specific levels and patterns of expression. *PTLF*-driven cytotoxins, however, are likely to be affected by an increase in attenuator expression in floral organs; the peak of *PTLF* expression occurs in floral meristems before significant organ differentiation (Rottmann et al. 2000).

In contrast to *NOS*, the two 35S basal promoters appear to provide insufficient expression to attenuate vegetative expression from *PTLF*. Although stem and root expression levels were comparable between PGUS, SGUS and OGUS, *PTLF*-based expression in shoots and leaves was significantly greater than from either basal 35S form. Thus, the comparatively high

level of *NOS* promoter expression, coupled with an apparent absence of deleterious effects of barstar attenuator expression (Wei 2004), suggest that the *NOS* promoter may be the best candidate of those evaluated for attenuating unintended cytotoxin expression. This conclusion, however, assumes that a 1:1 ratio of cytotoxin to attenuator is required for full attenuation. In our study on the poplar *PTD* floral promoter directing an unattenuated cytotoxin, we found that despite background vegetative expression of ~1% relative to floral expression, we were able to obtain transgenic sterile tobacco without negatively impacting vegetative growth for the majority of transgenic events (Skinner et al. 2003). This implies that there is a basal level of cytotoxin activity that plants can tolerate, and that only activity above this threshold must be countered. In support of this view, we found that all three candidate attenuator-driving promoters, when driving barstar, adequately attenuated *PTLF*-directed barnase activity on plantlet regeneration; without one of these attenuation genes, however, we were unable to regenerate any *PTLF*::barnase transgenic plants (Wei 2004).

Although this report focused on the vegetative expression properties of the *PTLF* promoter, these levels are low overall compared with that in developing inflorescences where *PTLF* is maximally expressed. Rottmann et al. (2000), using competitive reverse transcription-PCR, determined that endogenous *PTLF* mRNA levels in shoots (which had the highest expression levels in this study) were less than 1% of the levels present in developing inflorescences. This implies that, upon transition to an inflorescence meristem, *PTLF*-directed cytotoxin should overwhelm any attenuator present. This is being tested by transformation of an early flowering poplar genotype (Meilan et al. 2004) and by greenhouse and field trials of normal transgenic poplars (Wei 2004).

Our inability to regenerate plants using an unattenuated *PTLF*::barnase transgene (Wei 2004) suggests that where complete floral tissue (vs. floral organ-specific) ablation is desired, an attenuation system may be essential. The failure to regenerate plants is likely a result of the less specific expression of inflorescence floral meristem identity genes compared with genes whose expression is restricted to highly differentiated tissues (e.g., anther tapetum-specific sterility genes: Mariani et al. 1990). Because they can ablate entire inflorescence cell lineages (Nilsson et al. 1998), meristem identity promoters are likely to be more desirable than reproductive organ-specific genes for inducing bisexual sterility and for bolstering vegetative growth (Strauss et al. 1995). An attenuation system would also allow a wider range of reproductive predominant promoters to be employed (i.e., chosen without regard for their background expression patterns). This should enable functionally redundant constructs to be more readily deployed where highly robust sterility systems are needed (NRC 2004), or where tissue disruption needs to be customized to the commercial and ecological needs of particular plant species and production systems.

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