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Title: Maintenance of Normal Vegetative Growth via Attenuation of Barnase as an Agent of Floral Tissue Ablation in *Populus*

Abstract approved:

Steven H. Strauss

To reduce the environmental impacts caused by gene flow from transgenic trees, reproductive sterility genes have been developed that use a floral regulatory element linked to a cell toxin. This results in dysfunction or ablation of floral tissues, causing sterility. However, floral promoters often permit low levels of expression in vegetative tissues, which can impair plant health. We therefore developed an attenuation system to avoid the deleterious effects from unintended cytotoxin gene expression in vegetative tissues, and tested it in transgenic poplars.

We used the promoter from the poplar ortholog of *LEAFY* (*PTLF*) to drive the barnase (ribonuclease) cytotoxin, and three heterologous promoters to drive the attenutation transgene, barstar. Barstar is a specific 1:1 inhibitor of barnase, and thus should protect vegetative tissues from low levels of barnase expression. The LEAFY gene was chosen is known to be an effective floral ablation agent, however, it also shows expression in vegetative tissues. The heterologous promoters included the cauliflower mosaic virus (CaMV) 35S basal promoter +5 to -72 fragment (35SBP), the CaMV 35S basal promoter fused to the 68 bp TMV omega element (35SBP omega), and the nopline synthase (*NOS*) promoter.

We first studied the expression properties of the promoters by evaluating promoter::GUS gene fusions in transgenic poplar (*Populus tremula* × *alba*) via fluorometric GUS assays. In leaves, the *NOS* promoter imparted the highest expression with a mean expression level five-fold higher than *PTLF*, and 9- and 14-fold higher than 35SBP omega and 35SBP, respectively. Only the *NOS* and *PTLF* promoter showed tissue-specific expression patterns with respect to shoots, leaves, stems, and roots. The *NOS* promoter exhibited the strongest expression in roots. The *PTLF* promoter specified highest expression in shoot tips.

Directed by the poplar floral promoter *PTLF*, the barnase gene was assembled into constructs harboring barstar driven by either 35SBP, 35SBP omega, or *NOS.* All constructs also contained flanking matrix-attachment regions (rb7) from tobacco to increase and stabilize transgene expression. An unattenuated *PTLF*::barnase construct (lacking barstar) failed to give rise to any transgenic plants, whereas the attenuated constructs had transformation efficiencies above four percent. However, their transformation rates were still significantly below that of constructs lacking barnase, which averaged 6.1%. Both absolute and relative growth rates of plants measured during a several month greenhouse trial were not statistically significant different between attenuated and transgenic or non-transgenic controls. However, four independent attenuation lines (7% of the attenuation lines), from two constructs, were identified that had very poor growth and significantly lower barstar:barnase RNA ratios than the other attenuated lines. Although the attenuation lines containing *NOS*::barstar had significantly higher barstar:barnase RNA ratios than the 35SBP::barstar or 35SBP Omega::barstar containing lines, no significant differences in relative growth rates were found.

A statistically significant positive linear association was found between relative growth rate and barstar:barnase ratio in the attenuated lines. Graphical analysis suggested a threshold for barstar to attenuate barnase, above which additional levels of barstar did not provide further attenuation nor impact plant growth. By enabling transformation and normal growth of transgenic plants, the attenuation system developed may be a valuable means to produce healthy, sterile trees.

Maintenance of Normal Vegetative Growth via Attenuation of Barnase as an Agent of Floral Tissue Ablation in *Populus*

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APPROVED:

Major professor, representing Genetics

Head of Genetics Program

Dean of Graduate School

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Hao Wei, Author

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CONTRIBUTION OF AUTHORS

Dr. Richard Meilan assisted with collecting growth data and plant tissue samples for confirmation study, designing the growth room and doing growth measurements for Chapter 3. Dr. Amy Brunner assisted with the comprehensive study design, floral promoter motif characterization, and writing of Chapter 1. Dr. Jeffrey Skinner was involved in the design of the study, and assisted with the fluorometric GUS assay for Chapter 2. Caiping Ma assisted with the plant transformation for both Chapter 2 and 3.

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CHAPTER 1

Introduction and literature review

Motivations to engineer tree sterility

As the main sources of wood and fiber production, forest trees face increasing demand for harvest. To minimize exploitation of wild stands, it is desirable to increase the rates of biological productivity of plantations (Sedjo and Botkin 1997). Genetic engineering can help improve plant productivity, and introduce commercially valuable traits into trees, such as herbicide and insect resistance (Riemenschneider et al. 1988; 1991; 1997; Meilan et al. 2000a; 2000b; 2002a; 2002b), and modification of wood chemistry (Tzfira et al. 1998). However, transgenes can disperse long distances via pollen and seed, which could lead to undesired ecological effects (DiFazio et al. In press). Environmental concerns and the attendant regulatory barriers are therefore substantial impediments to the deployment of genetically engineered plantations (Strauss et al. 1995; James et al. 1998).

Genetically engineered plant sterility has been proposed as an approach to greatly reduce ecological concerns over transgene dispersal from plantations (Strauss et al. 1995; Skinner et al. 1999; Meilan et al. 2001). In addition, abolishment of

reproductive development might save significant amounts of energy and nutrients for plant growth (Teich et al. 1975; Ledig and Linzer 1978), potentially promoting biomass production.

Poplars provide an ideal system for testing sterility systems imparted by genetic engineering. They are widely used as a model system for forest biotechnology due to their many favorable characteristics (Bradshaw et al. 2000; Brunner et al. 2004). These include fast growth, short time to flowering (about 4 to 5 years), ease of clonal propagation, facile transformation, small genome size, and extensive genome sequence resources (Brunner et al. 2004). Poplars also have the potential for direct commercial application of transgenic clones developed in research, which could lead to near-term economical benefits.

Cell ablation

Cell ablation is one of several approaches used to engineer plant sterility. It employs a specific promoter that is active only in certain cells or under certain conditions to direct expression of a lethal gene (Dotson et al. 1996; Baroux et al. 2001). This will cause death of the tissues within which the lethal gene is expressed and prevent the targeted organs from normal development. Floral ablation utilizes a floral promoter to direct cytotoxin gene expression (Mariani et al. 1990; Block et al. 1993; Goldman et al. 1994; Nilsson et al.1998). Promoters selected for this purpose show highly specific, though rarely exclusive, expression in reproductive tissues; low-level “leaky” expression in vegetative tissues is common. For example, Brunner et al. (2000) reported that *PTAG1* and *PTAG2*, the *Populus trichocarpa* homologous to the *Arabidopsis* floral homeotic gene *AGAMOUS* (*AG*), showed weak expression in vegetative buds, stems and leaves. Similar expression properties were found in *PTLF*,

the *Populus trichocarpa* ortholog of *LEAFY* (*LFY*) and *FLORICAULA* (Rottmann et al 2000), and promoters from the *Eucalyptus* floral genes *EAP1* and *EAP2* (Kyozuka et al. 1997), *ELF1* and *ELF2* (Southerton et al. 1998a), and *EGM1*, *EGM2*, and *EGM3* (Southerton et al. 1998b). Utilizing these promoters to direct cytotoxin expression may therefore result in abnormal vegetative growth, as was observed for transgenic poplars (Skinner et al. 1999; Meilan et al. 2001).

Barnase and barstar

Barnase, a well-studied extracellular ribonuclease of *Bacillus amyloliquefaciens*, is a cytotoxin that is commonly used for genetic cell ablation (Baroux et al. 2001; Bi et al. 2001; Leuchtenberger et al. 2001; Burgess et al 2002). It is a small protein consisting of 110 amino acids, without any non-peptide components or disulfide bonds (Hartley 1988; Mariani et al. 1990; Paddon and Hartley 1986). Paddon and Hartley (1987) demonstrated that barnase appeared to be an intracellular cytotoxin in *E.* coli, and can only be secreted if an *E. coli* signal peptidase site is fused to the N-terminus. Thus it is expected that native barnase expressed in plant cells will be cell-bound, and cause no harmful effects to tissues in which it is not expressed.

Barnase has been successfully used for floral tissue-specific ablation in transgenic plants. By fusion to a floral promoter, barnase has been used to successfully engineer male and female sterile plants (Mariani et al. 1990; 1992; Block et al. 1993; 1997; Goldman et al. 1994). Mariani et al. (1990) first demonstrated the commercial potential of this system by producing sterile tobacco transformed with a *TA29*::barnase gene fusion. Barstar is an intracellular inhibitor of barnase. It specifically inhibits barnase by combining with it in a 1:1 ratio to form an inactive complex (Hartley 1988). It therefore offers a means to attenuate barnase activity in

selected tissues (Mariani et al. 1992). Barstar has been reported to counteract barnase activity sufficiently to restore male-fertility during hybrid seed production (Mariani et al. 1992; 1997; Beals et al. 1997).

Attenuation system model

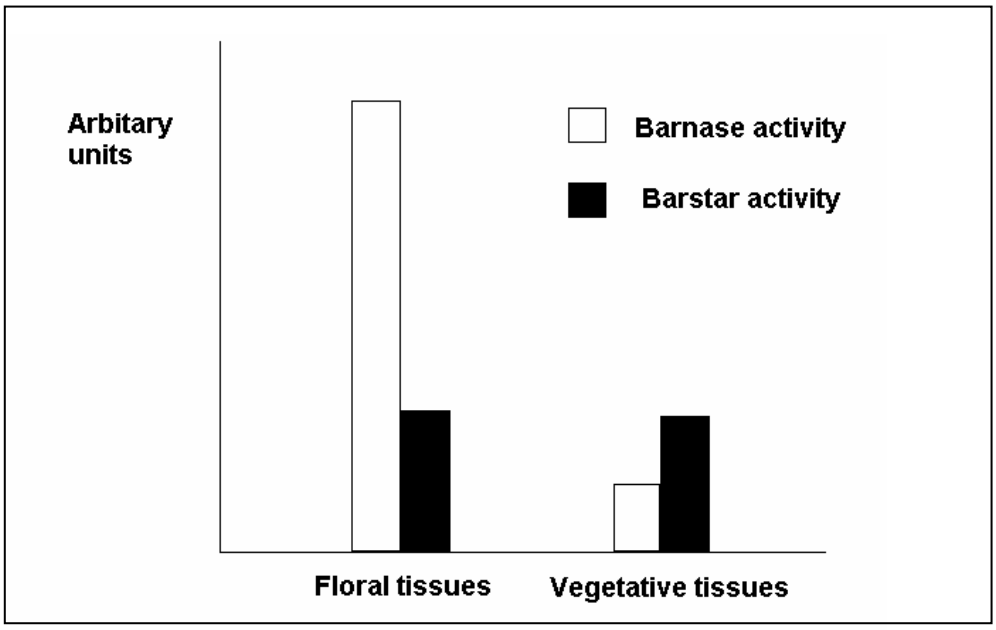

Figure 1. A model of activities of barnase (white bars) and its inhibitor barstar (dark bars) in plant floral and vegetative tissues. Under the control of a floral promoter, barnase shows high activity in floral tissues and low activity in vegetative tissues. Controlled by a constitutive weak or moderate promoter, barstar displays low activity in both floral tissues and vegetative tissues. In most transgenic regenerants, barstar activity is expected to be high enough to completely attenuate barnase in vegetative tissues, but not in floral tissues.

We hypothesized that “leaky” expression of barnase in vegetative tissues could be attenuated by expression of barstar under the control of weak or moderately expressed “constitutive” promoters (Figure 1) (“constitutive” is defined as substantial expression in most major tissue-types, even if expression strength varies widely). Therefore, only in floral tissues, where barnase expression is sufficiently high, should its activity overcome barstar inhibition and lead to cell ablation. In vegetative tissues

barnase expression should be insufficient to exceed barstar inactivation. We therefore refer to promoters that impart these properties as “attenuation” promoters.

In previous research, Beals et al. (1997) demonstrated the feasibility of attenuation of barnase in designated tissues by specific expression of barstar. They used the tobacco *TA56* promoter which is active during dehiscence of anther tissues to drive barnase expression. Barstar was driven by three promoters, which showed activity in different anther tissues from *TA56*. Normal dehiscence was only observed when barstar expression overlapped with barnase expression imparted by the *TA56* promoter. Conversely, dehiscence was blocked when barstar expression was confined to the tissues in which the *TA56* promoter was not active. Therefore we expected that weak, non-specific expression of barstar could be employed to protect non-target tissues from undesired, low level expression of barnase. The major goal of this research is to test this concept with respect to use of the promoter of the poplar floral gene *PTLF* for floral ablation.

Promoters and other genetic elements used in research

PTLF promoter characteristics

PTLF is the *Populus trichocarpa* ortholog of *LEAFY* (*LFY*) and *FLORICAULA* (Schultz and Haughn 199; Coen et al. 1990). It shows strong expression in developing inflorescences, and low level expression in leaf primordia, very young leaves, and other vegetative tissues (Rottmann et al. 2000). *LFY* encodes a transcription factor that plays important roles in floral development (Chujo et al. 2003). The *LFY* promoter has been successfully employed to drive diphtheria toxin A chain (DTA) to engineer sterile *Arabidopsis* plants through floral ablation because of its floral predominant expression (Nilsson et al. 1998). *LFY* is regulated by the

gibberellin and light pathways, and also responds to sucrose (Blazquez et al. 1997; 1998).

Figure 2. Putative regulatory DNA motifs in the *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 bold and underlined. The translation start codon ATG is bold and boxed. Numbers designate the distances from the translation start site (+1). The arrow on T (-41) indicates the position of the transcription start site based on 5' RACE experiments (A. Brunner and O. Shevchenko, unpubl., data).

There are two conserved functional fragments in the *LFY* promoter region (Blazquez and Weigel 2000). The first is a proximal fragment from -373 to -246 bp upstream of the translation start site, which is an element critical for *LFY* expression.

The second is a distal fragment from -1782 to -1558 bp, which partially determines the quantitative expression level of *LFY*.

Table 1. Putative transcription factor interaction motifs in the *PTLF* promoter identified by PLACE Web Signal Scan software (Prestridge 1991; Higo et al. 1999). The position of motif is the distance upstream of the translation start site.

Motif name	Position in *PTLF*	Position in *LFY*	Sequence	Related function	PubMed Ref. #
GARE2OSREP1	-2122	-	TAACGTA	Gibberellin-responsive	12787245
GT1CORE	-975	-2141, -606	GGTTAA	Light-response regulation	3243271
IBOXCORE	-1197	-2603, -1164, -989	GATAA	Lght-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

Figure 3. Sequence alignment of a portion of the 5' flanking region of the *Arabidopsis LFY* gene (-309 ~ -153) and the poplar *PTLF* gene (-271 ~ -118) with several putatively conserved motifs in dark. A conserved 8 bp motif is outlined in black.

We analyzed the 5' flanking region of the *PTLF* gene using the software PLACE Web Signal Scan (Prestridge 1991; Higo et al. 1999), and identified several putative motifs in the *PTLF* promoter (Figure 2, Table 1). We found total 8 possible motifs; two of them were gibberellin response elements, five of them were possibly involved in the light-regulated pathway, and one was a sucrose response element.

Comparison of the *LFY* promoter region with *PTLF* promoter shows that there is only weak sequence similarity between the two promoters, but an 8 bp motif CAACTGTC is fully conserved (Blazquez and Weigel 2000), and has been identified as a GAMYB binding site by Gocal et al. (2001). This motif is from -249 to -242 bp in *LFY* and -213 to -206 bp in *PTLF* (Figure 3). In addition, using the PLACE Web Signal Scan software we identified in *PTLF* one MYBGAHV, three IBOXCORE, one REalphaLGLHCB21, and two GT1CORE motifs that were also found in the *LFY* promoter (Table 1). These possible motifs in the *LFY* and *PTLF* promoters suggest that the *PTLF* promoter, similar to the *LFY* promoter, integrates environmental and endogenous signals to control flowering.

Figure 4. Alignment of SINE in portion of *PTLF* and *PTAP1-1* promoters. PTLF sequence is from -625 to -380, and PTAP1-1 sequence is from -2597 to -2356. Target site duplication sequences that delineate the ends of the SINE are bold and boxed. Target site duplications have apparently degenerated in the *PTLF* SINE.

Recent studies in our laboratory based on 5' RACE experiments showed that the transcription start site of *PTLF* gene is at -41 bp upstream of translation start codon (Figure 2). The *PTLF* promoter region that we utilized is from -2630 to -20 bp upstream of the translation start codon, including the conserved 8 bp fragment, and thus should include most qualitative and quantitative expression elements present in *LFY*. Surprisingly, when *PTLF* was compared with *PTAP1-1*, the *Populus*

trichocarpa homolog of the *Arabidopsis AP1* gene, an approximately 200 bp conserved sequence was observed, which corresponds to a SINE (short interspersed element; Figure 4). The SINEs located upstream in *PTLF* and *PTAP1-1* promoter region might be functionally significant and thus partly contribute to the variation in expression pattern between *PTLF* and *LFY*, and *PTAP1-1* and *AP*1.

Attenuation promoters

An optimal attenuation promoter used to control barstar expression would be active in all major plant tissues at a low level sufficient to inactivate unintended barnase cytotoxity, but will not inhibit barnase-caused ablation of targeted floral tissues (Figure 1). The "sufficient but low" level required for complete attenuation of unintended barnase activity is not fixed, but depends on the floral promoter directing barnase expression. The CaMV 35S promoter is a well-studied plant virus promoter active in many plant tissues. However, the expression level of the CaMV 35S promoter is likely to be too high in target floral tissues, precluding its direct use to control barstar expression. However, promoters are composed of several regulatory elements, including a basal promoter region that is required for initiation of transcription, and several regulatory regions that specify quantitative, temporal- and/or cell-specific expression. Deletion of the regulatory elements from a full promoter therefore may generate a weak but effectively constitutive promoter suitable for directing barstar expression. Deletion of the CaMV 35S promoter to +5 to –72 fragment removes all putative tissue-specific and quantitative regulatory elements, converting it into a "basal promoter" that contains a TATA box and a pair of putative CAAT box elements. Similar fragments have been shown to direct low levels of

expression, retaining about 5% of full promoter activity (Odell et al. 1985; Fang et al. 1989).

The tobacco mosaic virus (TMV) enhancer element "omega element" has been identified as a post-transcriptional enhancer which can increase the translational efficiency of plant mRNAs approximately two- to 10-fold in various plants, including tobacco, potato, and *Arabidopsis* (Gallie et al. 1987; Gallie and Walbot 1992; Schmitz et al. 1996; Mannerlof and Tening 1997). In *Arabidopsis* Holtorf et al. (1995) showed that when fused with the TMV omega element, the CaMV 35S full promoter was enhanced by two- to three-fold without altering organ specificity. This suggests that fusion of the TMV omega element downstream of the CaMV 35S basal promoter might increase the steady-state level of protein products. Therefore inclusion of the TMV omega element might elevate the quantity of active barstar to a sufficient level to fully attenuate barnase activity should the expression of barstar from CaMV 35S basal promoter prove insufficient.

The nopaline synthase gene (*NOS*) promoter has been shown to be active in all tissues examined in tobacco (DeBlock et al. 1984; Horsch et al. 1984). It is also commonly used to drive the *kanamycin* resistance gene *NPTII* during regeneration of transformed plants. The *NOS* promoter directs substantial expression in a wide variety of tissues (An et al. 1986), however, it is weaker than the CaMV 35S full promoter. Sanders et al. (1987) compared transcriptional levels in transgenic petunia plants with the CaMV 35S promoter *vs.* the *NOS* promoter. The 35S full promoter was at least 30 times stronger than the *NOS* promoter.

MAR element

Gene silencing and other "position effects" (effects on transgene expression caused by different chromosomal positions of T-DNA integration) may inactivate transgenes after incorporation into the plant genome (Finnegan and McElroy 1994; Flavell 1994). Matrix attachment regions (MARs) are DNA sequences that bind to cells' proteinaceous nuclear matrix and form DNA loops (Spiker and Thompson 1996). A transgene flanked with MARs is expected to reduce the influences of the genetic elements located in adjacent chromatin, elevating a transgene's stability and expression. MARs have been shown to increase and stabilize transgene expression in transformed tobacco (Mlynarova et al. 1994; Mlynarova et al. 1995; Allen et al. 2000) and appeared to increase transformation frequency and transgene expression level by 10-fold in poplar (Han et al. 1997). We therefore modified the backbone binary vector pGreenII (Hellens et al. 2000) by the addition of directly repeated tobacco MAR elements (Allen et al. 1996) that flank the T-DNA region.

GUS reporter gene

To characterize the expression properties of the floral promoter and weak to moderate "constitutive" promoters, we employed the GUS reporter gene (Jefferson et al. 1987). The GUS gene has been widely used as a reporter to facilitate both qualitative and quantitative evaluation of transgene expression. The X-gluc histochemical GUS stain offers a means to visibly inspect gene expression patterns, while the MUG fluorometric enzymatic assay provides a method to sensitively quantify transgene expression (Jefferson et al. 1987; Gallagher 1992). The GUS reporter gene we used contains an intron to eliminate transient *Agrobacterium*-caused

expression, increasing the precision of promoter evaluation (Shen et al. 1993). In addition, an optimized translational initiation site (ACCAUGG) was incorporated into the GUS gene, which mimics the consensus plant translation initiation site (ACAAUGG: Szabados et al. 1995).

CHAPTER 2

Expression properties of the poplar *PTLF*, *Agrobacterium NOS*, and two minimal 35S promoters in transgenic poplar

Abstract

To help establish an effective floral sterility system for poplars, we studied the expression properties of the poplar *LEAFY* (*PTLF*) promoter and three heterologous promoters. The heterologous promoters included the cauliflower mosaic virus (CaMV) 35S basal promoter +5 to -72 fragment (35SBP), the CaMV 35S basal promoter fused to the 68 bp TMV omega element (35SBP omega), and the 300 bp nopline synthase (*NOS*) promoter. All the promoters were studied by evaluating expression of promoter::GUS gene fusions in transgenic poplar (*Populus tremula* × *alba*) via histochemical and fluorometric assay. In leaves, the *NOS* promoter conveyed the highest expression with a mean expression level five-fold higher than *PTLF*, and 9- and 14-fold higher than 35SBP omega and 35SBP, respectively. Only the *NOS* and *PTLF* promoter directed tissue-specific expression when shoot, leaf, stem and root, were sampled for fluorometric GUS assay. The *NOS* promoter exhibited the strongest root expression. The *PTLF* promoter specified highest expression in shoot tips.

Introduction

Genetically engineered sterility is an important option for reducing the environmental impacts of transgene dispersal from transgenic trees (Strauss et al. 1995). A common means for engineering plant sterility is to employ a floral promoter to direct expression of the cytotoxin, and hence prevent the targeted floral tissues

from formation (Mariani et al. 1990; Goldman et al. 1994; Block et al. 1997). In addition to the expected expression in reproductive tissues, however, many flowering-related genes display detectable expression in vegetative tissues (Kyozuka et al. 1997; Southerton et al. 1998a; Southerton et al. 1998b; Brunner et al. 2000; Rottmann et al. 2000), suggesting that direct use of floral promoters to control cytotoxin expression may result in a failure to regenerate plants, or decreased vegetative growth (Skinner et al. 2000). A system for attenuating undesired effects of cytotoxins on vegetative tissues is therefore highly desirable.

We are developing an attenuation system whereby a floral promoter directing a cytotoxin is coupled with a specific inhibitor of the cytotoxin under the control of weak to moderate, but widely expressed "constitutive" promoter. Unintended expression of the cytotoxin in vegetative tissues should be attenuated, enabling normal vegetative growth. The goal of this chapter is to describe the expression properties of the promoters used in this attenuation system. The effectiveness of the system for enabling transformation and normal vegetative growth when employing barnase and barstar (Mariani et al. 1990; 1992) are described in Chapter 3.

The floral promoter tested for direction of cytotoxin expression was the 5' flanking region of the *Populus trichocarpa* ortholog of *LEAFY* (*LFY*), called *PTLF*. The *LFY* gene is required for normal floral organ differentiation (Schultz and Haughn 1991). The *PTLF* gene shows strong expression in developing inflorescences and weak expression in leaves and other vegetative tissues (Rottmann et al. 2000). Three heterologous constitutive promoters were selected as candidates to direct cytotoxin attenuation. They were the cauliflower mosaic virus (CaMV) 35S basal promoter +5

to -72 fragment, the CaMV 35S basal promoter fused to the tobacco mosaic virus (TMV) omega element, and the nopaline synthase promoter.

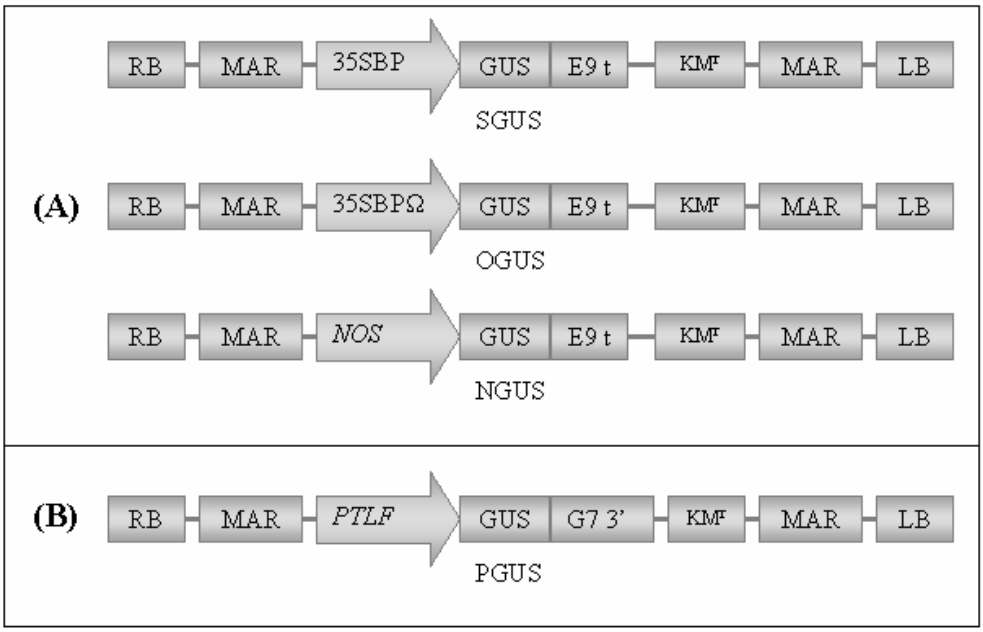

Figure 1. Key elements of DNA constructs. A) Constructs for evaluating expression of three heterologous promoters. B) Construct for studying expression imparted by the *PTLF* promoter. Arrows show direction of transcription. Abbreviations: 35SBP: CaMV 35S basal promoter (+5 to -72 fragment), 35SBPΩ: 35S BP fused to TMV omega element, E9 t: E9 terminator, G7 3': Gene 7 3' region (terminator), KM^r: *Kanamycin* resistance operon, LB or RB: Left or right border of T-DNA in binary vector, MAR: Matrix attachment region, NOS: *NOS* promoter, *PTLF*: Promoter of floral gene *PTLF*.

To study the expression properties of the selected promoters, we evaluated the expression of promoter::GUS gene fusions in transgenic poplars (Figure 1). We used an intron containing GUS reporter gene to eliminate transient expression of the genes in residual *Agrobacterium* (Shen et al. 1993). In addition, an optimized translation initiation site (ACCAUGG) was incorporated into the GUS gene, mimicing the consensus plant translation initiation site (ACAAUGG: Szabados et al. 1995). We employed tobacco matrix attachment regions (MARs) to enhance gene expression

level and stability (Allen et al. 2000). The MARs employed have been shown to elevate transformation and transgene expression approximately 10-fold in poplar (Han et al. 1997). We demonstrate that the *PTLF* promoter accompanied by MARs imparts substantial vegetative expression, and that the *PTLF* and *NOS* promoters impart different tissue-specific expression patterns.

There are two conserved motifs in the *Arabidopsis LEAFY* promoter (Blazquez and Weigel 2000). The first one is a proximal fragment from -373 to -246 bp upstream of the translation start site, which is a redundant element critical for *LEAFY* expression. The second one is a distal fragment (-1782 to -1558 bp), which partially determines the expression level of *LEAFY*. Comparison of the *LEAFY* promoter region with *PTLF* promoter (accession number U93196) shows that there is little sequence similarity between the two promoters, but an 8 bp motif CAACTGTC is conserved. This 8 bp motif is -249 to -242 bp in *Arabidopsis LEAFY* and -213 to -206 bp in *PTLF*.

The CaMV 35S promoter drives expression in many plant tissues. Deletion of the CaMV 35S promoter to the 35SBP removes all tissue-specific and quantitative regulatory elements, making it a “constitutive” basal promoter. It contains a TATA box and a pair of putative CAAT boxes. The similar promoter fragment has been reported to direct low levels of expression in *Nicotiana tabacum*, retaining 5% of the full CaMV 35S promoter activity (Odell et al. 1985; Fang et al. 1989). The tobacco mosaic virus enhancer element (the omega element) acts post-transcriptionally, increasing the translational efficiency of plants. It has been shown to elevate translation of mRNAs 2- to 3-fold in tobacco and *Arabidopsis* (Holtorf et al. 1995; Schmitz et al. 1996; Mannerlof and Tening 1997). Fusion of the TMV omega element

downstream to the 35SBP should therefore increase the amount of the expressed protein. The nopaline synthase (*NOS*) promoter is expressed in several plant tissues (An et al. 1986), but at a considerably lower level than the CaMV 35S full promoter (An et al. 1988). The 35S full promoter is at least 30 times stronger than the *NOS* promoter (Sanders et al. 1987).

Materials and methods

Plasmid assembly

Promoter::GUS gene fusions were first assembled in *pBluescript II SK (+)*, and then moved into the binary vector pG3M. The pG3M is a vector derived from pGreen II (Helens et al. 2000) by first inserting two *Asc*I linkers at *Hpa*I and *Stu*I sites, and then inserting two 1.2-kb tobacco RB7 MARs (Allen et al. 1996) as direct repeats at *Fsp*I and *Sap*I (blunted) sites that flank the polylinker. An intron-containing GUS gene with an optimized translation initiation site was originally constructed in plasmid pPR97 (Szabados et al. 1995). The 1.47-kb GUS fragment was released by digesting pPR97 with *Sac*I (T4 polymerase-blunted) and *Kpn*I, and subcloned into *Kpn*I / *Sma*I sites of an intermediate construct.

Promoters were obtained as follows: For PGUS, the 2.6-kp *PTLF* promoter (-2630 to -20 bp fragment, including the conserved 8 bp motif) was amplified from a genomic clone (Rottmann et al. 2000) by PCR using the primers: 5’-AGCCGCGGTACTAAATAAATATATAAAC-3’ and 5’-TGCGGCCGCGATCTTTCACAGGTGCATGTC-3’ with *Sac*II and *Not*I sites (underlined) incorporated at the 5’ and 3’ ends, respectively. For CGUS, the CaMV 35S basal promoter +5 to -72 fragment (35SBP) was PCR amplified from the EL301 plasmid (Mohamed et al. 2000) using primers: 5’-AGAATTCGGATGACGCACAATC-3’ and 5’-AGGTACC CCGTGTTCTCTCC-

3’ with *EcoR*I and *Kpn*I sites (underlined) incorporated at the -72 and +5 end, respectively. For OGUS, the 35SBP omega fusion was PCR amplified from EL301 using primers: 5’-AGAATTCGGATGACGCACAATC-3’ and 5’-TGGT ACCTGT AATTGTAAATA-3’ with *EcoR*I and *Kpn*I sites (underlined) incorporated at the 5’ and 3’ ends, respectively. For NGUS, the *NOS* promoter region was PCR amplified using primers: 5’-AGAATTCGATCATGAGCGGAGA-3’ and 5’-AGGTACCGG TGCAGATTATT-3’ with *EcoR*I and *Kpn*I sites (underlined) incorporated at 5’ and 3’ ends, respectively. All the amplified fragments were sequence confirmed.

Two different terminators were used. The 3’ untranslated region of ribulose bisphosphate carboxylase (RuBP Carboxylase) gene of pea (E9 terminator) was fused downstream of the weak promoter::GUS cassettes. The 3’ untranslated region of gene 7 from *Agrobacterium tumefaciens* (G7 3’) (Velten and Schell 1985) was fused downstream of the *PTLF*::GUS fusion. The *Xho*I fragment containing CaMV 35S promoter::*NPT II*::*NOS* terminator as selectable marker was inserted downstream of the promoter::GUS::terminator region. The assembled promoter::GUS::terminator gene fusions and the selectable marker were excised from intermediate constructs by *Stu*I and *Cla*I digestion and subcloned into the pG3M *Sma*I / *Cla*I sites. The T-DNA regions and construct name are given in Figure1.

Plant transformation

All plasmids produced were transformed into *Agrobacterium tumefaciens* strain C58 harboring the plasmid pSoup using the freeze-thaw method (Holsters et al. 1978). Hybrid poplar (*Populus tremula × P. alba: INRA-France 717-1B4)* was transformed with all DNA constructs using methods essentially as described by Han et al. (2000). Transgenic lines from SGUS and OGUS constructs were verified by

PCR using primers specific for the GUS reporter gene (5'-TAAAAGGACAGGG CCATC-3', and 5'-GTGATATCGTCCACCCA-3'). Transgenic lines from NGUS and PGUS constructs were verified by histochemical GUS assay of leaves during root induction *in vitro*. For each construct, four ramets of ten independent transgenic events (lines) were studied. Two month-old plants were transferred into soil and maintained in a lighted growth room in Corvallis, Oregon, USA for one month and then moved into the greenhouse, and maintained under a natural day-night cycle. Tissues were sampled for gene expression during spring-summer 2003.

Histochemical and fluorometric GUS assays

Sixty days after plants were transferred into soil, shoot tips with two nodes and their expanding leaves were excised from greenhouse grown transgenic plants and subjected to histochemical GUS assay according to Stomp et al. (1992). Tissue samples included shoots (shoot tip with one node), leaves (from nodes four or five below the apex), stems (between nodes three and four), and roots (including root tips). Fluorometric GUS assays were performed according to Jefferson et al. (1987). Protein was measured via the BioRad (Hercules, California) protein assay kit. GUS activities were determined by enzymic conversion of 4-methylumbelliferyl glucuronide to 4-methyllumbelliferone, expressed as pmoles 4-methylumbelliferone /minute/mg protein.

Statistical analysis and sampling of tissues

We compared fluorometric GUS expression levels between constructs based on leaf expression, and between tissues within constructs, using student's t-tests and one-way analysis of variance of line means. To compare expression between

constructs, leaf samples were collected from four ramets of one independent line and 10 lines for each construct. To compare tissue-specific expression within constructs, shoot, leaf, stem, and root samples were collected from two ramets for each of 10 lines for each construct.

Results

Histochemical GUS staining

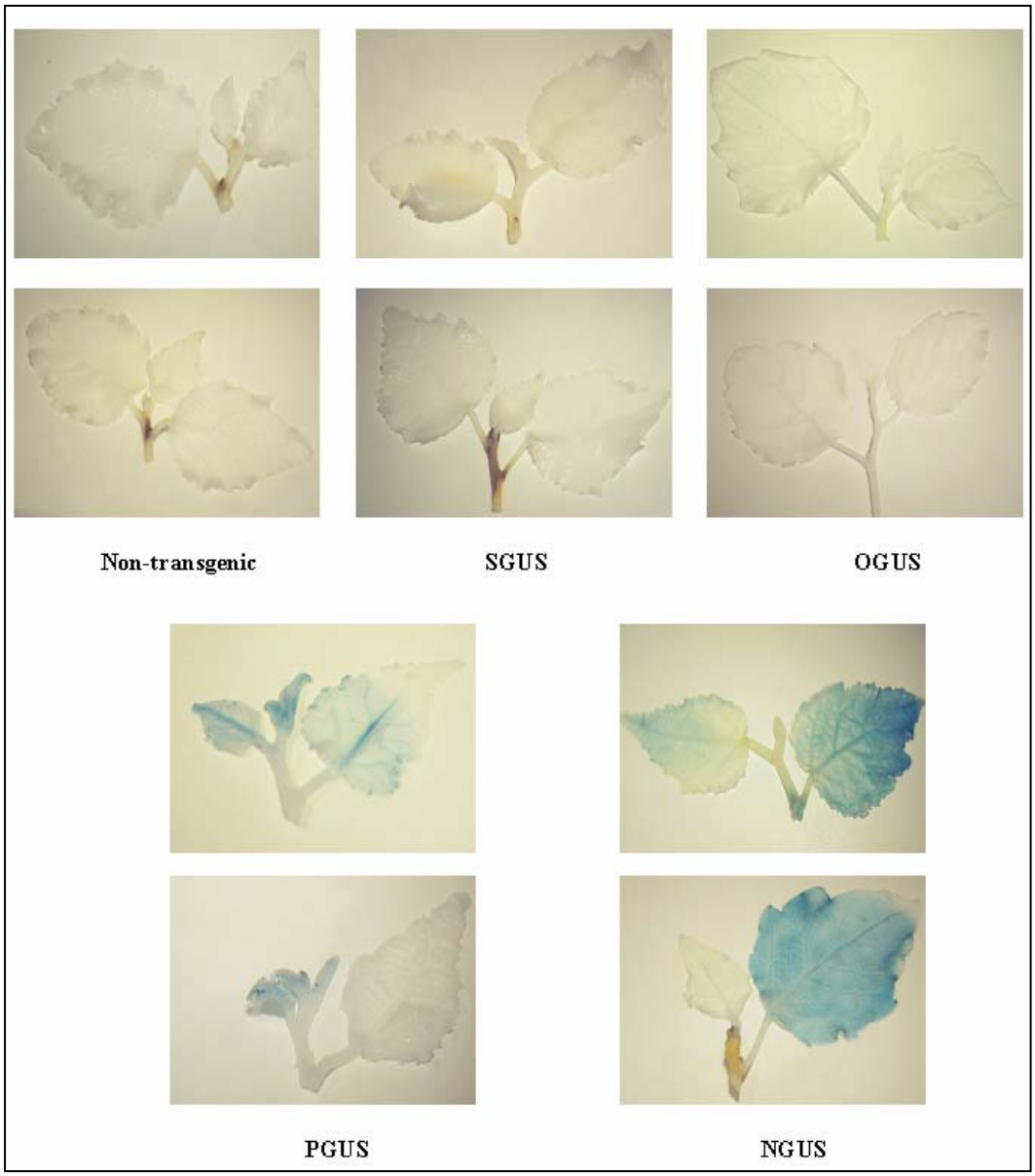

Figure 2. Histochemical GUS staining of transgenic plants. Each photo is of a different, representative (approximating the average) transgenic line.

Among four constructs tested, only NGUS and PGUS transformants showed visible blue staining; no trace of blue staining were found in any of the SGUS and OGUS transformants (Figure 2). In 10 lines of PGUS transformants, faint blue staining was found along leaf veins, and blue spots were observed along leaf edges. The stems stained less intensely than leaves, and the strongest staining was found in the shoot tips, including in the newly expanding leaves and leaf primordia. In NGUS transformants, leaves displayed stronger staining than did shoot tips. Leaf expression in NGUS was stronger than in PGUS. No visible differences could be observed among SGUS, OGUS, and the non-transgenic control plants; all failed to show detectable histochemical expression.

Quantitative GUS assay

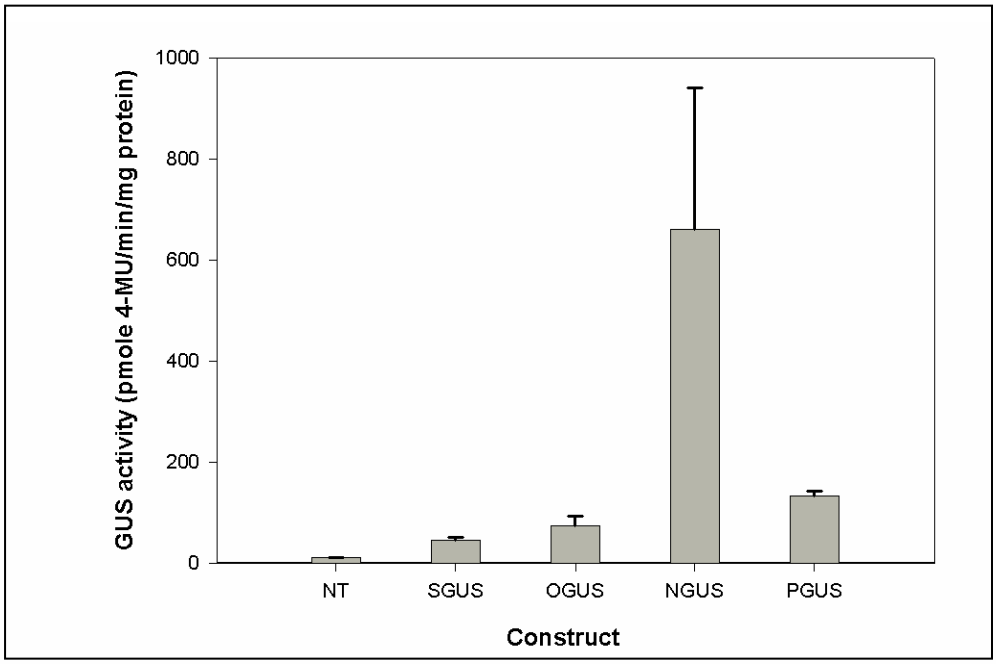

Figure 3. GUS expression in leaves of non-transgenic and transgenic plants. Bars show one standard error over line means based on 10 lines and four ramets per line. NT = Non-transgenic; other construct abbreviations are given in Figure 1.

Table 1. Tissue-specific fluorometric GUS activities (pmol 4-MU/min/mg protein) among constructs and tissues (see also Figure 4). Data shown are means and standard errors over line means based on 10 lines and 2 ramets per line.

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)

Fluorometric GUS assays were performed to quantitatively evaluate GUS expression levels (Table 1). In leaves, the *NOS* promoter conferred the strongest expression, the *PTLF* promoter was the second strongest, followed by the 35SBP omega and 35SBP (Figure 3). Based on ANOVA and student's t-tests, there were statistically significant differences between NGUS and the other constructs in leaf expression ($P < 0.01$) (Figure 3). The mean expression level of NGUS was five-fold stronger than PGUS, and 9- and 14-fold stronger than OGUS and SGUS, respectively. Based on student's t-tests, there were also significant differences between the PGUS and the other two weak promoter constructs. Mean expression level of PGUS was 1.8-fold higher than OGUS and three-fold higher than SGUS (two-sided t-tests, $P = 0.01$ and 0.00, respectively). No significant differences were detected between OGUS and SGUS (two-sided t-test, $P = 0.17$).

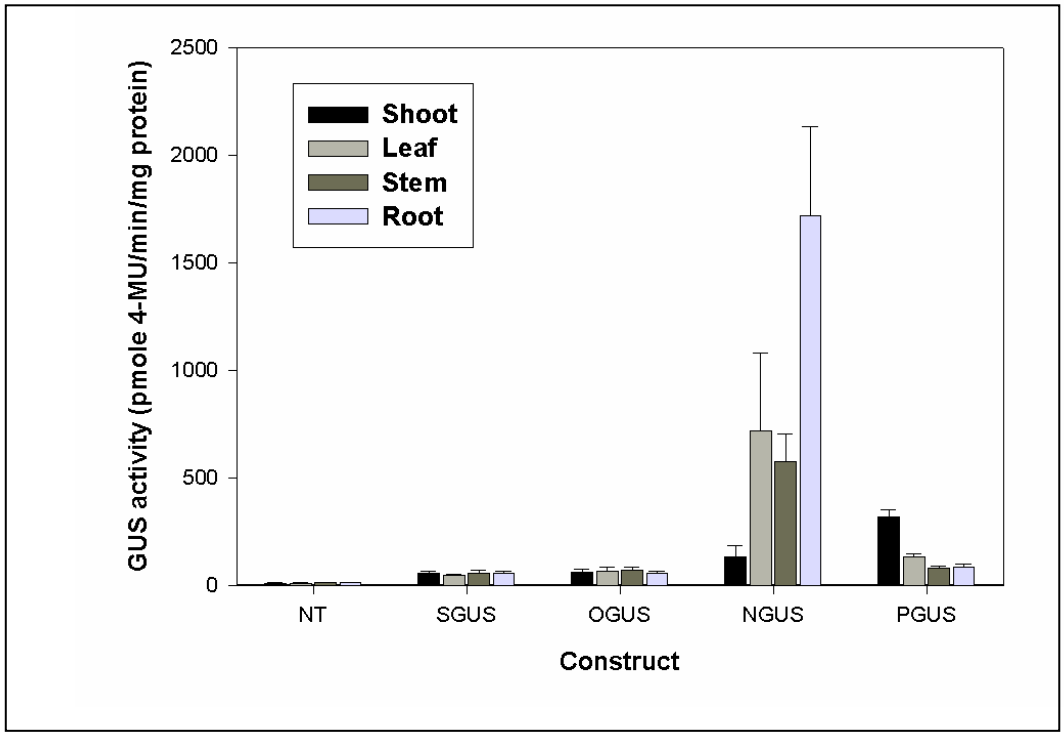

Figure 4. Variation in GUS expression among tissues and constructs. Bars show one standard error over line means. Construct abbreviations are given in Figure 1.

The constructs varied widely in their patterns of tissue-specific expression (Figure 4, Table 1). There were no significant differences in expression levels among the four tissue-types in SGUS and OGUS transformants (one way ANOVA, P = 0.90 and 0.81, respectively). In SGUS transformants, the highest mean expression level was in the stem, which was only 1.3-fold higher than the leaves, which had the lowest mean level. In OGUS transformants, the highest (stem) and lowest (root) expression levels also only differed 1.3-fold. In contrast, the NGUS transformants exhibited strong tissue-specific expression. The strongest expression occurred in roots, with a mean expression level 2.4-fold higher than in leaves. The weakest expression occurred in shoots, where mean expression level was 5.0-fold lower than in leaves. The difference in mean expression between the highest (root) and the lowest tissues (shoot) was 12.7-fold. The *PTLF* promoter also showed statistically significant (P <

0.01) differential expression among tissues. The difference between the strongest (shoot) and weakest (stem) expressing tissues was 4.0-fold; shoot expression was 2.3-fold higher than in leaves. There were no statistically significant differences between roots and stems (two-sided t-test, P = 0.63); their mean GUS expression was 1.7-fold lower than in leaves.

Discussion

We found that only the *NOS* and *PTLF* promoters imparted visually detectable GUS expression. No expression was observed in any plants among transformants of SGUS and OGUS, and non-transgenic controls. The *NOS* promoter conveyed stronger expression than the *PTLF* promoter in most tissues. The *NOS* promoter may therefore be most effective at driving a cytotoxin inhibitor for full attenuation of cytotoxin expression in vegetative tissues, especially considering the high expression of *PTLF* in shoots (Figure 4).

Intensities of staining varied widely among tissues in both PGUS and NGUS transformants. In PGUS transformants staining was strongest in leaf promodia and newly emerging leaves, and weaker staining occurred in veins, edges of older leaves, and stems. This suggests that the *PTLF* promoter was most active in young and newly differentiating tissues. The *NOS* promoter exhibited much less intensive staining than *PTLF* in shoot tips, and much more expression in mature leaves, stems, and especially roots.

The results of quantitative fluorometric GUS assays showed that the *NOS* promoter had several-fold higher activity than the *PTLF* promoter in most vegetative tissues, making it a good candidate to direct a cytotoxin inhibitor. In contrast, the 35SBP and 35SBP omega promoters had 3.0- and 1.8-fold lower expression than the

PTLF promoter. The highly uniform expression levels in different tissues imparted by the 35SBP and 35SBP omega promoters shows that all of major tissue-specific and quantitative regulatory regions from the parent CaMV 35S promoter were removed in SGUS and OGUS constructs. Despite the existence of the TMV omega element, the 35SBP omega promoter showed a small (1.5-fold) but not statistically significant increase in expression compared to 35SBP. The apparent failure of the omega element to substantially elevate translational efficiency of GUS mRNA may be due to the very low number of mRNA molecules transcribed with this basal promoter, or due to a lack of compatibility with the cellular environment of poplar.

Our results suggest that the *PTLF* and *NOS* promoters could be an effective match in an attenuation system, whereas 35SBP and 35SBP omega might not be expressed high enough to rescue vegetative growth when *PTLF* directs cytotoxin expression. However, we have not characterized cell-specific expression, and cell-types could vary widely within complex differentiated tissues like leaves and shoots. Moreover, selection during transformation could give rise to events with a sufficiently high ratio of attenuation to cytotoxin expression, and complete cytotoxin elimination may not be reached to enable normal vegetative growth. Functional attenuation studies using the barnase cytotoxin and barstar attenuation protein are reported in Chapter 3.

CHAPTER 3

Attenuation of barnase cytoxicity in transgenic poplar via vegetative expression of barstar

Abstract

We developed an attenuation system to avoid the deleterious effects of floral promoter::cytotoxin genes on vegetative growth of transgenic sterile plants. The system utilizes a floral predominant promoter to drive the ribonuclease cytotoxin barnase, and a weak to moderate constitutive promoter to drive barstar, an inhibitor of barnase. Combinations of the barnase gene directed by the floral promoter of the poplar *LEAFY* gene *PTLF* with barstar driven by either the CaMV 35S basal promoter +5 to –72 fragment (35SBP), 35SBP fused to the TMV omega element (35SBP omega), or the *NOS* promoter, were evaluated by analyzing transformation efficiencies and growth rates of transgenic plants.

The unattenuated *PTLF*::barnase construct failed to give rise to any transgenic lines, whereas the attenuated constructs had transformation efficiencies above four percent, still significantly below that of transgenic constructs lacking barnase (mean transformation efficiency of 6.1%). There were no statistically significant differences in the absolute or relative growth rates of plants with attenuated constructs *vs.* transgenic controls and non-transgenic plants. However, four independent attenuation lines (7% of attenuation lines) from two attenuation constructs were identified that had very poor growth and significantly lower barstar:barnase RNA ratios than the other attenuated lines. Although the *NOS*::barstar containing attenuation lines had significantly higher barstar:barnase RNA ratios than the 35SBP::barstar or 35SBP Omega::barstar containing attenuation lines, there were no significant differences

between them in relative growth rates. A statistically significant ($P < 0.01$) positive linear association was found between relative growth rate and barstar:barnase ratio in the attenuated lines, and graphical analysis suggested a threshold for barstar attenuation of barnase, above which additional levels of barstar did not provide further attenuation. By enabling regeneration and vegetative propagation of transgenic plants with normal growth, the attenuation system may be an important means of producing healthy, sterile trees where containment of exotic organisms or genes are desirable.

Introduction

Genetic engineering can import novel traits that may elevate tree productivity in diverse ways (Tzfira et al. 1998; Peña and Seguin 2001; Rishi et al. 2001; Strauss et al. 2001). However, transgenes from weakly domesticated tree crops can disperse long distances via pollen and seed, potentially causing undesired ecological effects (Strauss et al 2001). Environmental concerns over transgene dispersal, and attendant regulatory barriers, are substantial impediments to deployment of genetically engineered plantations (Strauss et al. 1995). One way to greatly diminish the environmental impacts caused by gene dispersal is to employ transgenic sterile trees, which can be produced via several genetic mechanisms (Skinner et al. 2000).

Cell ablation has been extensively used to impart plant sterility. Genetic ablation methods employ promoters active in specific cell types or conditions to control the expression of a lethal gene, usually encoding a cytotoxin (Leuchtenberger et al. 2001; Bi et al. 2001; Burgess et al. 2002). For engineering plant sterility, a floral predominant promoter is used to control the expression of a cytotoxin such as the ribonuclease barnase (Mariani et al. 1990; Mariani et al. 1992; Goldman et al. 1994).

Ideally, cytotoxin expression will be confined to floral cells, however, it appears that the majority of floral promoters are not expressed exclusively in floral tissues (Kyozuka et al. 1997; Southerton et al. 1998a; Southerton et al. 1998b; Brunner et al. 2000; Rottmann et al. 2000), and even low levels of unintended expression may impair plant growth (Skinner et al. 2000).

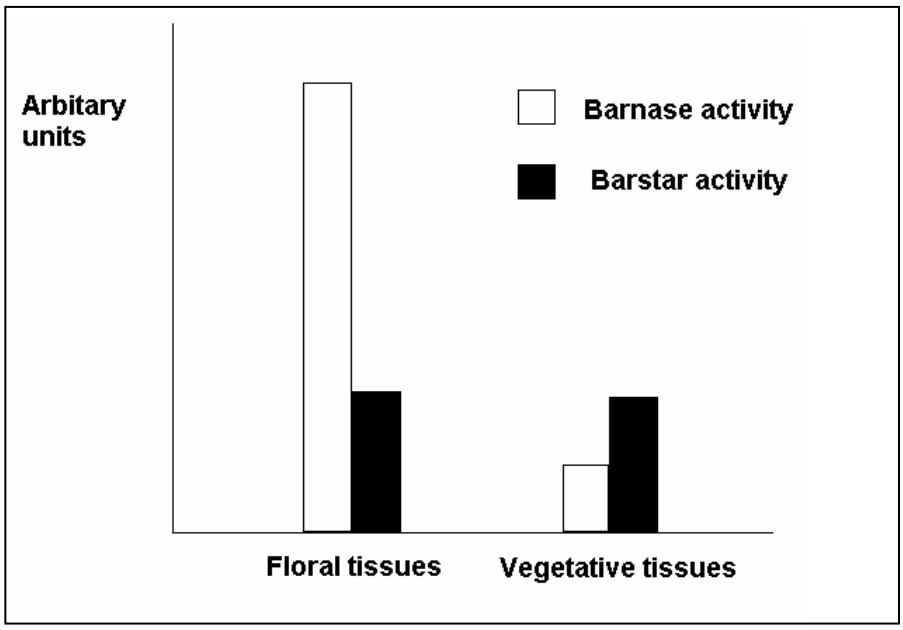

Figure 1. A model of activities of barnase (white bars) and its inhibitor barstar (dark bars) in plant floral and vegetative tissues. Under the control of a floral promoter, barnase shows high activity in floral tissues and low activity in vegetative tissues. Controlled by a "constitutive" weak promoter, barstar displays low activity in both floral tissues and vegetative tissues. In most transgenic regenerants, barstar activity is expected to be high enough to completely attenuate barnase in vegetative tissues, but not in floral tissues.

We are developing an attenuation system to insure normal vegetative growth when sterility is caused by floral ablation genes. The system has two major components (Figure 1). First, sterility is caused by the cytotoxin barnase under the control of a floral homeotic gene promoter active in male and female floral primordia. Second, barstar, a specific inhibitor of barnase, is expressed under the control of a weak or moderate constitutive promoter. Only in floral tissues where barnase expression far exceeds that of barstar should cell ablation occur. In vegetative tissues,

where barnase levels are equal or below that of barstar, cells should be protected from ablation or dysfunction (Beals et al. 1997).

Barnase, the extracellular ribonuclease of *Bacillus amyloliquefaciens,* is a frequently used cytotoxin for cell ablation. It is a small protein consisting of 110 amino acids (Paddon and Hartley 1986; 1987; Hartley 1988; Mariani et al. 1990). Barstar is the intracellular inhibitor of barnase, and specifically inhibits barnase by combining with it in a 1:1 complex. By fusion to a floral promoter, barnase has been used to successfully engineer male and female plant sterility (Mariani et al. 1990; Mariani et al. 1992; Block et al. 1993; Goldman et al. 1994; Block et al. 1997), and barstar has been used to counteract barnase activity and restore male-fertility for crop production (Mariani et al. 1992; Mariani et al. 1997; Beals et al. 1997).

PTLF, the *Populus trichocarpa* ortholog of *LEAFY* (*LFY*) and *FLORICAULA,* shows strong expression in developing male and female inflorescences, but also detectable expression in leaf primordia, young leaves, and other vegetative tissues (Rottmann et al. 2000). It was therefore selected to test the effectiveness of the attenuation system. Three weakly to moderately expressed, putatively constitutive promoters were selected as candidates to counter the expected vegetative expression of barnase from *PTLF*. They were the cauliflower mosaic virus (CaMV) 35S basal promoter +5 to -72 fragment (35SBP), 35SBP fused to the tobacco mosaic virus (TMV) omega element (35SBP omega), and the nopaline synthase (*NOS*) promoter.

The effectiveness of the attenuation system was studied by monitoring transformation rate and vegetative growth of transgenic poplars in which the *PTLF*::barnase element was co-transformed with the promoter::barstar genes. In all constructs, flanking tobacco matrix attachment regions (MARs) were included into T-

DNA region of the binary vectors to enhance expression level and stability (Allen et al. 2000). The MAR employed was previously shown to elevate transgene expression 10-fold in poplar (Han et al. 1997). We show that this attenuation system successfully enabled transformation, and maintained normal vegetative growth in the large majority of transgenic trees. Studies of flowering and sterility are underway, and will be reported elsewhere.

Materials and methods

Construct assembly

PTLF::GUS assembly was described in Chapter 2. The binary vector pG3M was derived from pGreen II (Helens et al. 2000) by first inserting two *Asc*I linkers at *Hpa*I and *Stu*I sites, and then inserting two 1.16-kb tobacco RB7 MARs (Allen et al. 1996) as direct repeats at *Fsp*I and *Sap*I (blunted) sites that flank the polylinker. pG3MB was made by inserting the blunt ended bacterial barstar operon obtained by digestion of pMT416 (Hartley 1988) with *Xba*I and *Hind*III (*Klenow* blunted) into the pG3M *Dra*III site (T4 polymerase blunted).

To make the *PTLF*::barnase unattenuated construct, a *PTLF*::barnase cassette and *kanamycin* selectable marker were first assembled in an intermediate construct based on *pBluescript II SK (+)* as follows: The 2.6-kb *PTLF* promoter was amplified from the genomic clone using the primers: 5’-AGCCGCGGTACTAAATAAATATA TAAAC-3’ and 5’-TGCGGCCGCGATCTTTCACAGGTGCATGTC-3’ with *Sac*II and *Not*I sites (underlined) incorporated at the 5’ and 3’ ends, respectively. The *PTLF* ampilcon was digested with *Sac*II and *Not*I and subcloned into intermediate construct’s *Sac*II / *Not*I sites. The barnase gene was PCR amplified with the primers: 5’-TGGATCCATGGTACCGGTTATCAACAC-3’ and 5’-CGATATCTTATCTG

ATTTTTGTAAAGG-3’ with *BamH*I and *EcoR*V sites (underlined) incorporated. The barnase amplicon was then digested with *BamH*I and *EcoR*V and inserted into the intermediate construct’s *BamH*I / *EcoR*V sites. The 3’ untranslated region of gene 7 from *Agrobacterium tumefaciens* (G7 3’) (Velten and Schell 1985) was PCR amplified using the primers: 5’-CGATATCGAGCTAAGCTAGCTA TATCA-3’ and 5’-GAAAGCTTATCTTGAAAGAAATATAGTT-3’, with *EcoR*V and *Hind*III sites (underlined) incorporated. The G7 3’ fragment was subcloned into the intermediate construct’s *EcoR*V / *Hind*III sites. An *Xho*I fragment containing a *kanamycin* resistance element (CamV 35S::*NPT II*::*NOS* terminator) was inserted into the intermediate construct’s *Sal*I site. The *PTLF*::barnse cassette and kanamycin resistant element were then excised from the intermediate construct using *Xho*I (*Klenow* blunted) and *Sac*II, and subcloned into pG3MB after *Sma*I / *Sac*II digestion.

The barstar control constructs (lacking barnase) were assembled via replacing the GUS reporter gene with the barstar gene in the previously assembled GUS constructs: CGUS, OGUS and NGUS (Chapter 2). The barstar gene was amplified with primers: 5’-GGGTACCATGAAAAAAGCAGTCATTAA-3’ and 5’-GGGATCC TTAAGAAAGTATGATGGTGA-3’ with *Kpn*I and *BamH*I sites (underlined) incorporated. The GUS gene was released from CGUS, OGUS, and NGUS via *Kpn*I and *BamH*I digestion. The barstar amplicon digested with *Kpn*I and *BamH*I was inserted into CGUS, OGUS, and NGUS, producing C35S, COmega, and CNOS, respectively.

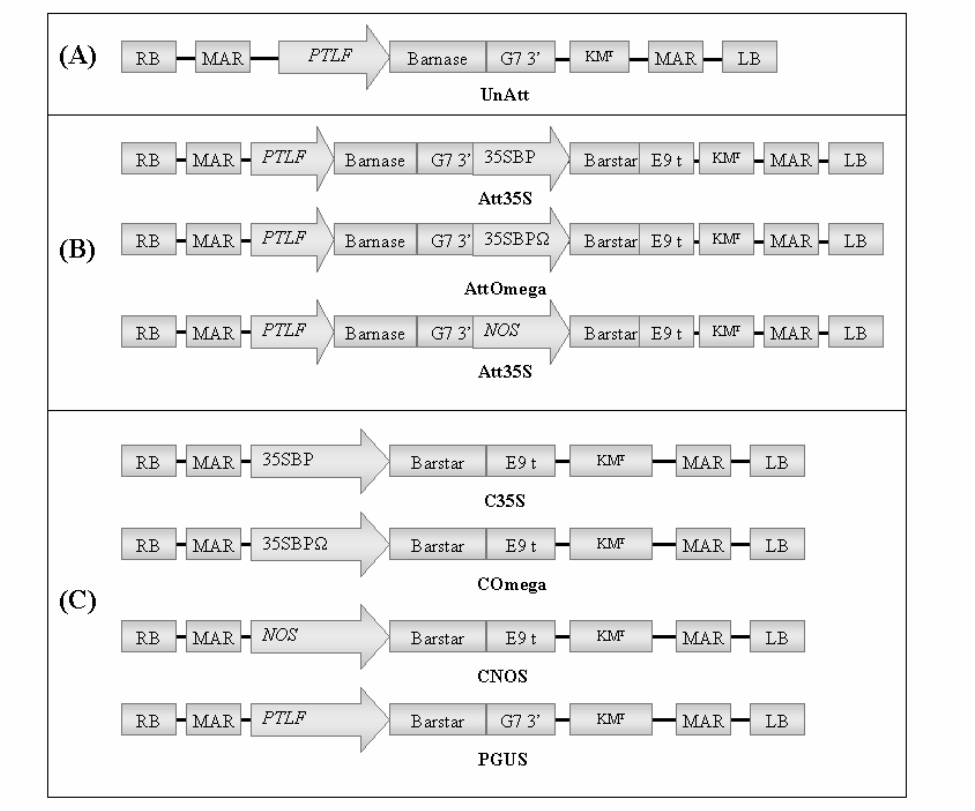

Figure 2. Constructs used in transformation experiments. Abbreviations are given in Chapter 2.

Attenuated constructs were assembled via subcloning the *PTLF*::barnase::G7 3' cassette into C35S, COmega, and CNOS. The *PTLF*::barnase::G7 3' cassette was excised from an intermediate construct by *Hind*III digestion (*Klenow* blunted) followed by *Sac*II digestion and then inserted into the C35S, COmega, and CNOS *Sma*I / *Sac*II sites, generating Att35S, AttOmega, and AttNOS respectively. All the PCR amplified fragments were sequence confirmed. Construct names and elements are summarized in Figure 2.

Plant transformation

All plasmids were transformed into *Agrobacterium tumefaciens* strain C58 harboring the pSoup plasmid using the freeze-thaw method (Holsters et al. 1978). All

constructs were transformed into hybrid poplar (*Populus tremula* × *P. alba: INRA-France 717-1B4)* essentially as described by Han et al. (2000). All transgenic lines were verified by PCR using primers specific for the barnase gene (5'-TGGATCCATGGTACCGGTTATCAACAC-3' and 5'-CGATATCTTATCTGATTTTTGTAAAGG-3') or the barstar gene (5'-GGGTACCATGAAAAAAGCAGTCATTAA-3' and 5'-GGGATCCTTAAGAAAGTATGATGGTGA-3'). For each construct at least 17 independent transgenic lines were regenerated, and then each transgenic line was propagated *in vitro* to produce five ramets.

Growth measurements

Two-month-old plants were then transferred into soil and maintained in a lighted growth room in Corvallis, Oregon, USA for one month, and then moved into a greenhouse under an ambient day-night cycle during spring and summer of 2003.

For analyzing growth, we employed a randomized block design, begining when plants were transferred into soil and continuing through growth analysis. Height and basal diameter (2 cm above soil) were measured using a ruler and a digital caliper, respectively. When the mean height of plants was 59 cm, the first set of growth measurements were began and finished within two days. A second set of measurements was completed 21 days later, when the plants had reached an average height of 94 cm. These two sets of growth measurements were used to compute biomass index (BI: height × diameter^2) and relative growth rate [RGR: Ln(BI2/BI1)].

Plant RNA extraction and real time RT-PCR

Table 1. Primers used for real-time RT-PCR.

Gene	GenBank accession number	Primer sequence
Barnase	E31988	5'- GGCTGGGTGGCATCAAAA-3' 5'-GATGTCTCCGCCGATGCTT-3'
Barstar	AY283058	5'-TGGACGCTTTATGGGATTGTC-3' 5'-ACTGCCTCCATTCCAAAACG-3'
ACT2	BU879695	5'-CCCATTGAGCACGGTATTGT-3' 5'-TACGACCACTGGCATACAGG-3'

Young leaves of actively growing plants (from nodes one or two below the apex) were excised and frozen in liquid nitrogen. Total RNA was extracted from leaf tissues using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). RNA was quantified via spectrophotometry at OD_{260}, and total RNA was purified from DNA using the DNA-Free Kit (Ambion, Austin, TX, USA). First-strand cDNA was synthesized using 2 µg of total RNA with the SuperScript First-Strand Synthesis system for RT-PCR (Invetrogen, Carlsbad, CA, USA). Real-time RT-PCR was performed with an actin gene (ACT2) as an internal control because of its relatively stable expression across different tissue types and plant development stages in poplar (Brunner pers. comm.). PCR primers (Table 1) were designed using Primer Express (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed in 25ul of reaction mixture composed of SYBR Green PCR Master mix (Applied Biosystems) and cDNA using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The reaction was carried out in 96 wells plates, with triplicate PCR reactions run for each sample.

To verify that the barstar:barnase ratio measured the relative quantities of barstar and barnase molecules, and was not substantially influenced by different affinities of PCR primers to their target genes, we performed real-time RT-PCR using plasmid DNA as a template. The plasmid DNA used was Att35S, which contained one copy of barnase and two copies of barstar. We found no significant difference in affinity between barnase and barstar primers; the average ratio of barstar to barnase was close to two (mean of 1.83, n = 7; two-sided, paired t-test, P = 0.16).

Statistical analysis

The randomized block design was employed to reduce error variance as a result of position in the growth chamber and greenhouse, as well as from initial size due to time of propagation. Biomass index and relative growth rate computed from growth measurements were subjected to mixed model ANOVA to test effects from constructs, lines within constructs, and blocks. The mixed model we employed treated constructs as fixed factors, and blocks, lines within constructs, and interactions between blocks and constructs as random factors.

Results

Transformation efficiency

Table 2. Transformation efficiencies of constructs studied. Transformation efficiency was calculated as the percentage of explants co-cultivated that produced at least one PCR-confirmed transgenic shoot. Barnase-containing attenuated constructs are shown below the lower dashed line and the unattenuated barnase construct above the upper dashed line. Abbreviations are given in Figure 1. # Transformation efficiency: x = X / N where X = Number of transgenic lines produced and N = Number of explants co-cultivated. SE: Standard error = $\sqrt{[x(1-x)/N]}$.

Construct	Explants co-cultivated	Independent lines produced	Transformation # efficiency (%) ± SE
UnAtt	735	0	0.0 ± 0.00
PGUS	388	20	5.1 ± 1.12
C35S	341	27	7.9 ± 1.46
COmega	339	20	5.9 ± 1.28
CNOS	355	20	5.6 ± 1.22
Att35S	456	21	4.6 ± 0.98
AttOmega	435	17	3.9 ± 0.93
AttNOS	463	19	4.1 ± 0.92

All of the eight plasmids were used to transform poplar. Only the unattenuated construct (UnAtt) failed to give rise to any transgenic lines (Table 2). In contrast, the attenuated constructs and the transgenic control constructs all showed transformation efficiencies above four percent. The presence of barnase in the attenuated constructs appeared to significantly depress transformation efficiency. The transgenic control constructs (C35S, COmega, CNOS, and PGUS) had a mean efficiency of 6.1%, compared 4.2% for the three attenuated constructs. This is a reduced efficiency of 31%, and was statistically significant (χ^2 test, one degree of freedom, P = 0.02).

Vegetative growth analysis

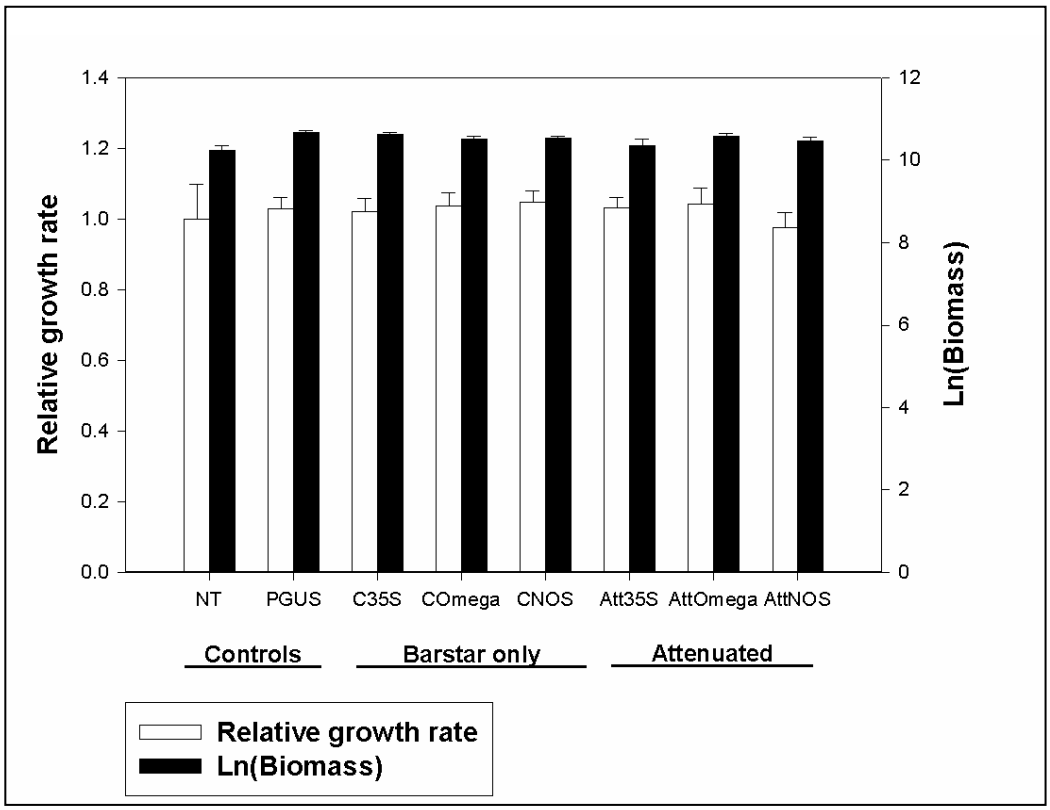

Figure 3. Biomass and relative growth rate of transgenic plants harboring attenuated, barstar only, and control constructs. Bars show one standard error over line means. Abbreviations are given in Figure 2.

Table 3. Mixed model analysis of variance of biomass index. DF = degree of freedom**,** MS = Mean square**,** EMS = Expected mean square**,** Var = Variance. Model: $Y_{ijk} = u + Construct_i + Block_{j+} Line_k (Construct_i) + Error_{ijk.}$

Source	DF	MS	Components of EMS	F	P-value
Construct	7	1.14	Var(Residual) + 6.42 Var(Line(Construct)) + Q(Construct)	1.18	0.319
Block	4	1.62	Var(Residual) + 122.33 Var(Block)	5.85	<0.001
Line(Construct)	123	0.77	Var(Residual) + 4.65Var(Line(Construct))	2.79	<0.001
Residual	488	0.28	Var(Residual)		

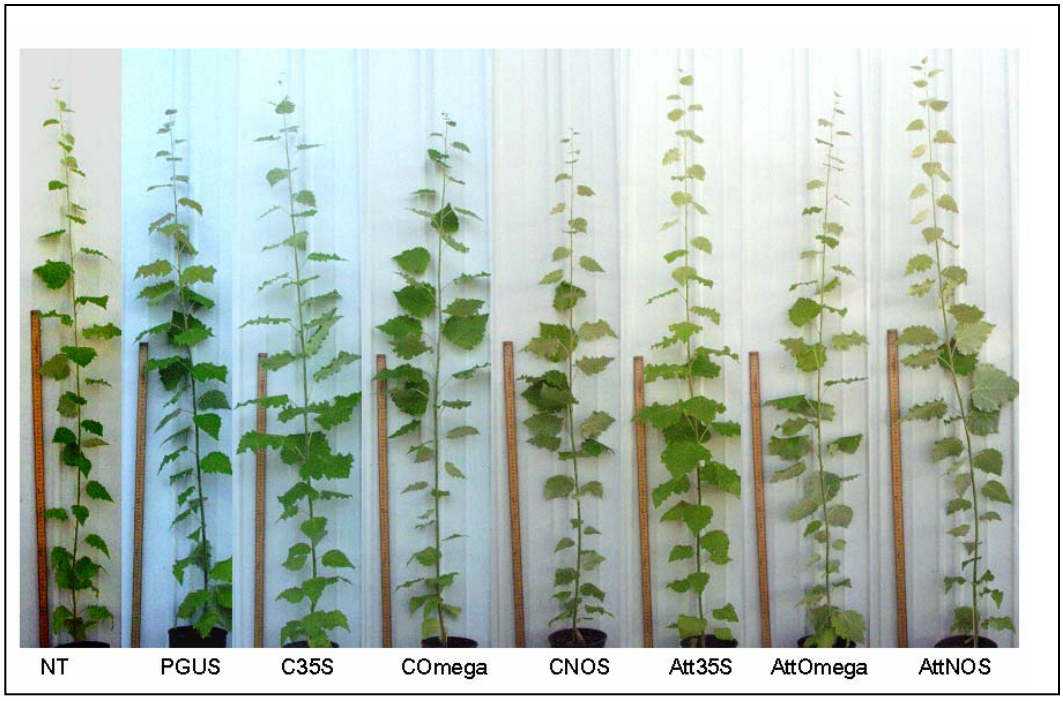

Figure 4. Examples of representative (near to mean) non-transgenic control plants and transgenic plants from the different constructs after final measurement.

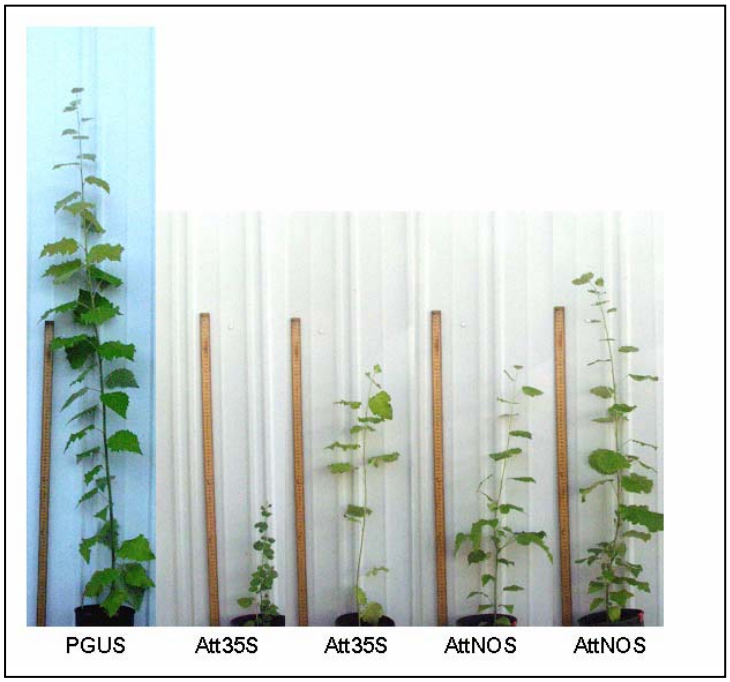

Figure 5. Examples of low vigor attenuated transgenic plants compared to a high vigor transgenic control plant (PGUS).

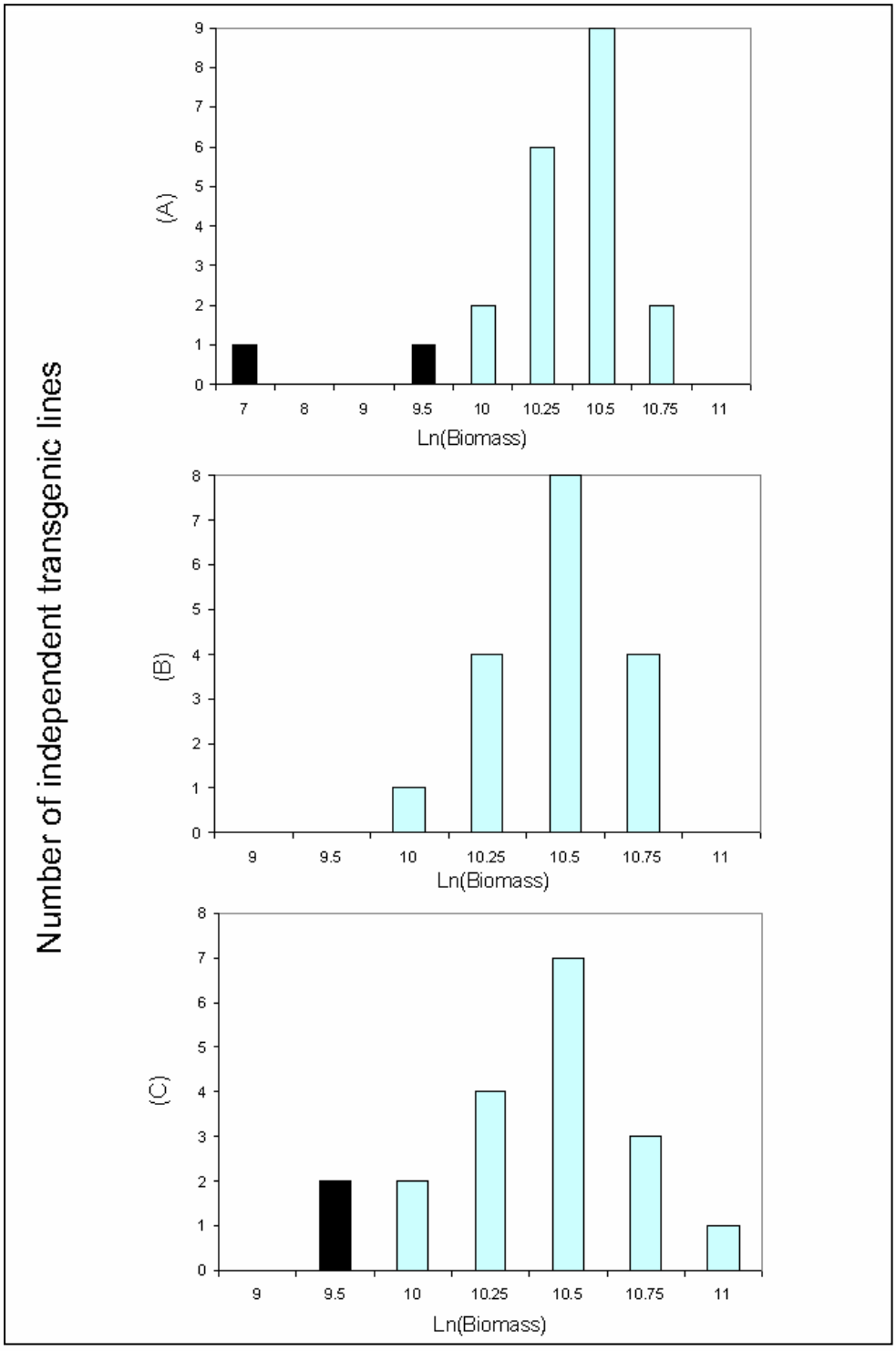

Figure 6. Distributions of biomass in high vigor (light bars) and low vigor (dark bars) attenuated transgenic lines. A) Att35S had two low vigor lines. B) All lines of AttOmega had high vigor. C) AttNOS had two low vigor lines.

ANOVA carried out on biomass index (Table 3) showed that although block and line within construct were highly significant sources of variance, construct effects

were not. The mean growth of plants from all constructs was very similar (Figure 3), and the appearance of the large majority of plants was the same (Figure 4). The high level of significance of lines within constructs (P = 0.00) was partly due to four visibly abnormal lines (Figure 5), two within Att35S and two within AttNOS, both attenuation constructs. These four lines had considerably lower biomass compared to the rest lines from the attenuated constructs (one-sided t-test P = 0.00); they also formed a tail at the low end of the biomass size distribution (Figure 6). By excluding these lines, the mean square of line within construct in ANOVA reduced by 53.2% from 0.77 to 0.36, though it still remained statistically significant (P = 0.02). These four lines were pooled into a "low vigor" class for comparison to normally growing attenuated lines in subsequent analyses.

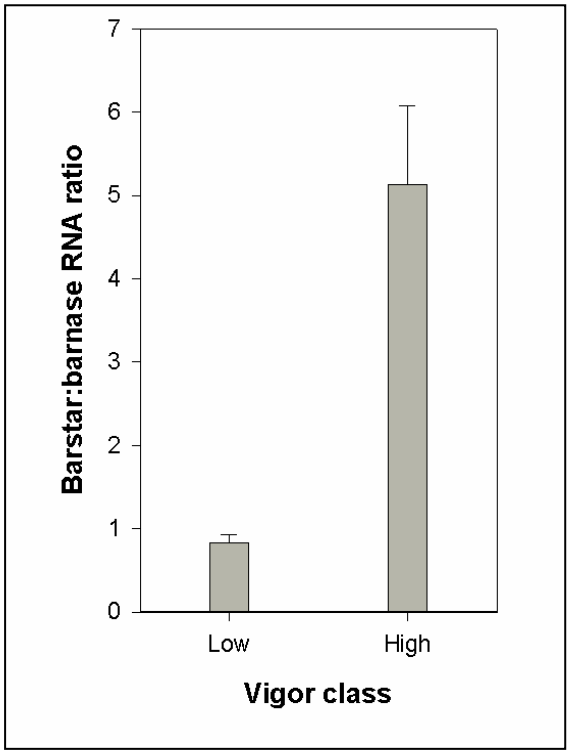

Figure 7. Barstar:barnase RNA ratio in two vigor classes of attenuated transgenic lines (n = 4 lines in low vigor class, n = 12 lines in high vigor class). Bars show one standard error over line means.

Table 4. T-tests of gene expression and relative growth rate between two vigor classes. BBRR: Barstar:barnase RNA ratio, RGR: Relative growth rate.

Data	Mean of low vigor class	Mean of high vigor class	Type	t	P-value
BBRR	0.83	5.13	One-sided	3.19	0.01
RGR	0.63	1.18	One-sided	7.50	0.00

Table 5. T-tests of gene expression and relative growth rates between different promoters. BBRR: Barstar:barnase RNA ratio, RGR: Relative growth rate.

Data	Promoter (Mean)	Promoter (Mean)	Type	t	P-value
BBRR	NOS (8.59)	35SBP-P (3.40)	One-sided	3.04	0.00
BBRR	35SBP (3.28)	35SBP Omega (3.52)	Two-sided	0.33	0.76
RGR	35SBP (1.22)	35SBP Omega (1.14)	Two-sided	-0.80	0.49
RGR	NOS (1.17)	35SBP-P (1.18)	Two-sided	-0.11	0.91

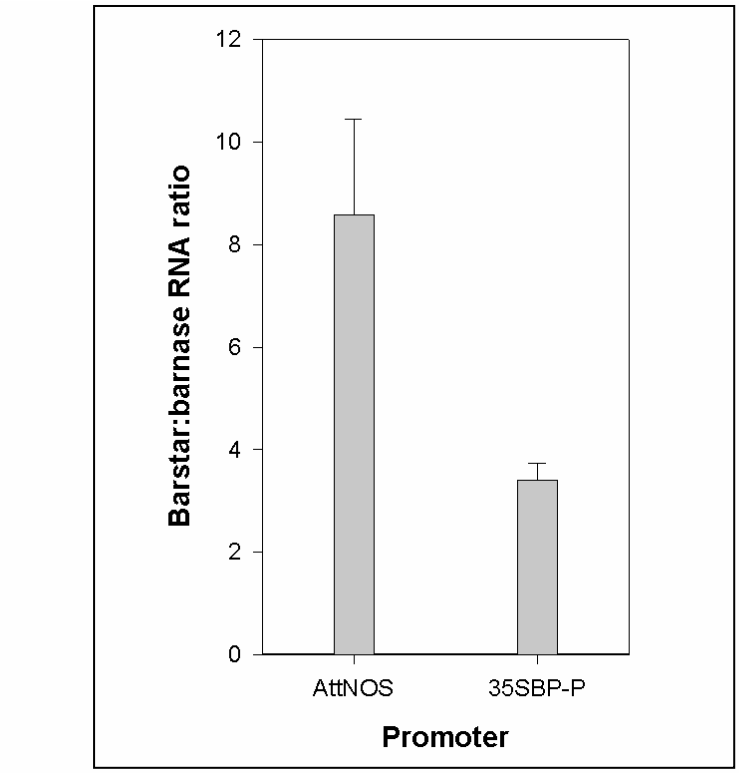

Figure 8. Barstar:barnase RNA ratios in attenuated plants harboring different heterologous promoters. 35SBP-P includes both Att35S and AttOmega constructs. Bars show one standard error over line means.

The mean relative growth rate of the low vigor class was 0.63, whereas the rest of the attenuated transgenic lines had a mean relative growth rate of 1.18, approximately twice that of the low vigorclass (Table 4). RNA from young leaf materials were collected from four transgenic lines of the low vigor class, using two ramets per line, and from four high vigor transgenic lines sampled from each attenuated construct in the same way; in total, 12 high vigor lines and four low vigor lines were compared. The two vigor classes had significantly different barstar:barnase RNA ratios (Table 4). In the low vigor class, the mean barstar:barnase RNA ratio was less than unity (0.83), whereas the high vigor class had a mean barstar:barnase RNA ratio of 5.1, a 6-fold difference (Figure 7). We also compared barstar:barnase RNA ratios among the different attenuation constructs (Table 5). Because the tobacco omega element acts as post-transcriptional factor, similar levels of barstar mRNA should be observed in attenuated plants containing Att35S and AttOmega. Our results agreed this expectation (two sided t-test, P = 0.76). The barstar:barnase RNA ratios of Att35S and AttOmega were therefore pooled to form a new group, “35SBP-pooled” (35SBP-P). When AttNOS was compared to 35SBP-P, a highly statistically significant difference in barstar:barnase RNA ratios were observed (P = 0.00, Table 5), with a barstar:barnase RNA ratio 2.5-fold higher in AttNOS than in 35SBP-P (Figure 8), however, the relative growth rates of the two groups were nearly identical and not significantly different (Table 5: two sided t-test, P = 0.91).

Barstar:barnase RNA ratios and relative growth rate data were plotted to more closely investigate their association. There was a statistically significant (P = 0.00, R^2 = 0.28) positive linear association between relative growth rate and barstar:barnase

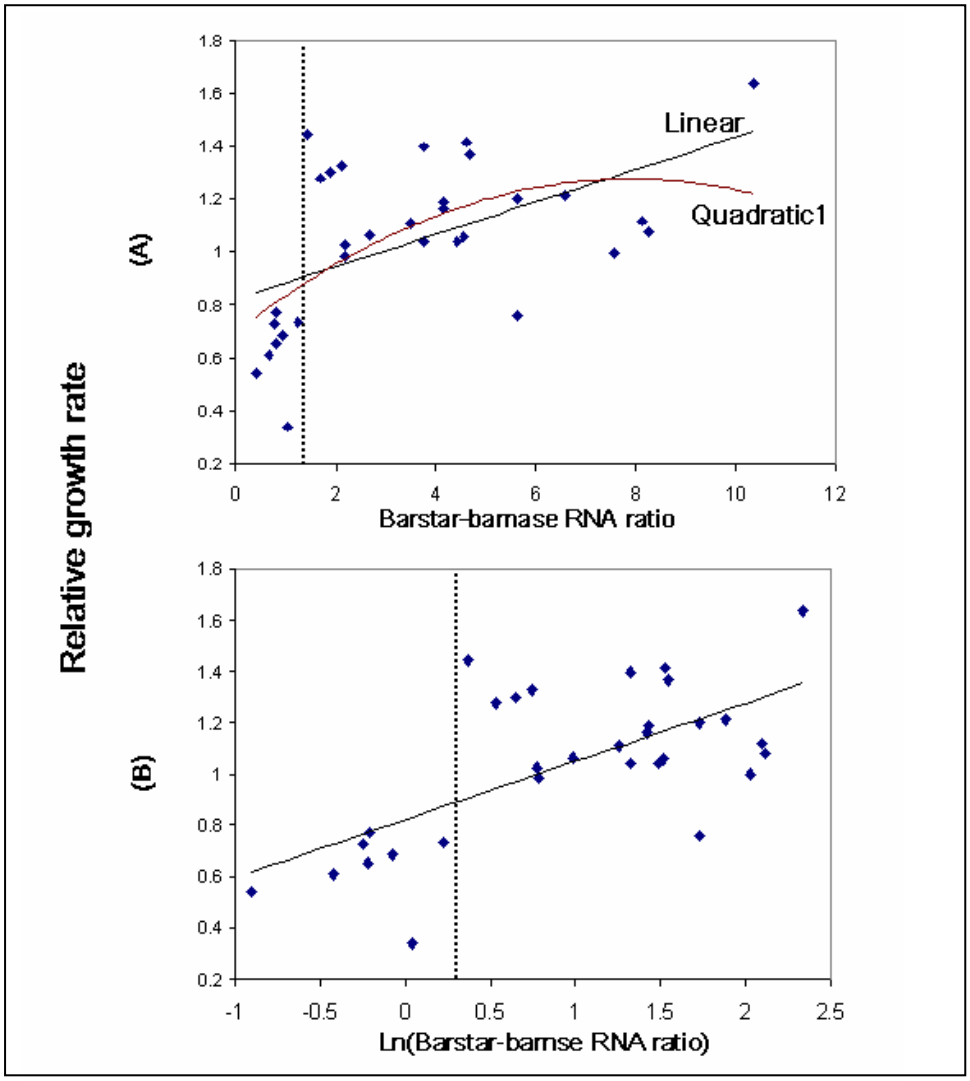

Figure 9. Linear regression of relative growth rate on barstar:barnase RNA ratio. A) Linear and quadratic regressions of RGR on barstar:barnase ratio. Linear: $y = 0.06 x + 0.82$, $R^2 = 0.28$. Quadratic: $y = -0.01 x^2 + 0.15 x + 0.70$, $R^2 = 0.33$. B) Linear regression of RGR on ln(Barstar:barnase ratio). $y = 0.23 x + 0.82$, $R^2 = 0.42$. The dashed lines delineate the start of an apparent plateau in response.

RNA ratio (Figure 9 A). Because the trend appeared to plateau above a ratio of 1.4 (dashed line in Figure 9 A), we tested whether a quadratic model fit the data better. The quadratic term was statistically significant ($P = 0.01$), but adding it to the linear model raised the R^2 value only 0.05. In contrast, the logarithm of barstar:barnase RNA ratio explained variation in relative growth rate best ($P < 0.00$), giving an R^2 of 42%. Above a ln(barstar:barnase RNA ratio) of about 0.3 (dashed line in Figure 9 B),

all trees appeared to grow normally; for this subpopulation barstar:barnase RNA ratio and growth rate were unrelated ($R^2 = 0.00$, $P = 0.75$).

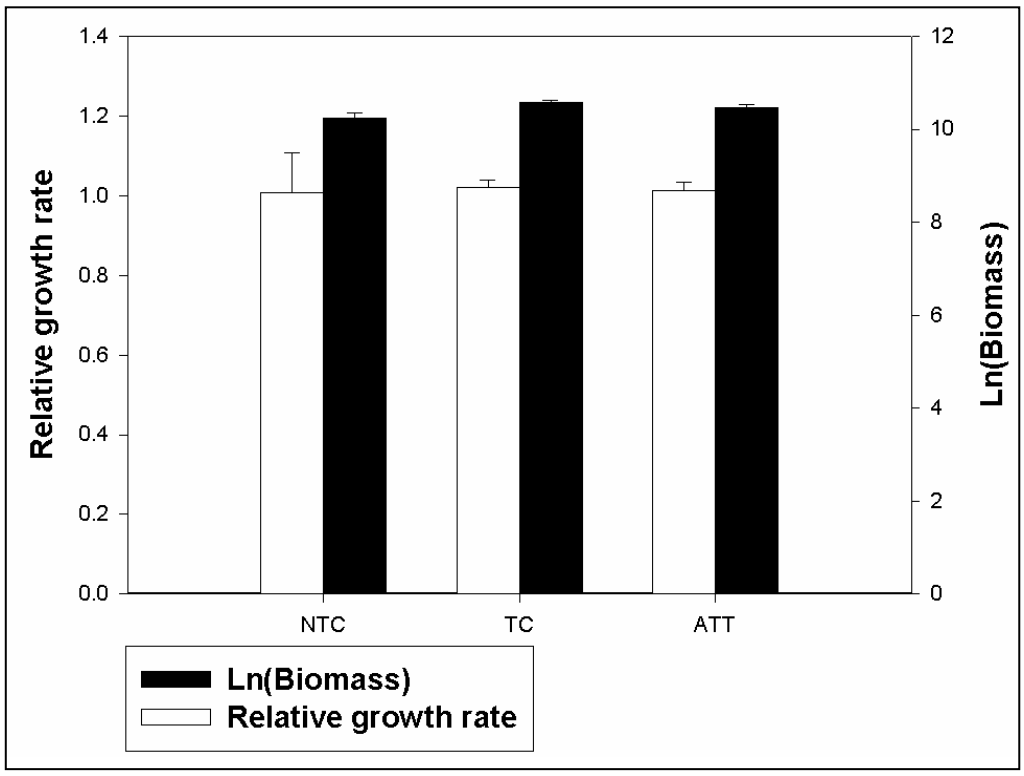

Figure 10. Mean growth of pooled non-transgenic control (NTC), transgenic control (TC, n = 73 lines), and attenuated plants (ATT, n=53 lines). Standard errors calculated over line means.

Table 6. Pooled biomass index and relative growth rates. NTC: Non-transgenic controls, TC: Transgenic controls, ATT: Attenuated lines.

Group	BI Mean	RGR Mean
NTC	10.24 (±0.12)	1.00 (±0.10)
TC	10.58 (±0.03)	1.03 (±0.02)
ATT	10.46 (±0.06)	1.01 (±0.02)

There were no large or statistically significant differences in growth between any of the constructs and the non-transgenic controls, nor between the attenuated and other transgenic genotypes. When averaged over all line means (after excluding the morphologically distinct low vigor class), BI and RGR for the transgenic control and attenuated lines was nearly identical (Figure 10, Table 6). For BI, the means for the

transgenic control and attenuated lines differed by 1.1% and the standard errors were less than 0.6% of the means. Likewise, for RGR the means differed by 1.9% and the standard errors were less than 1.9%. ANOVA on BI of non-transgenic control, transgenic control, and attenuated line means revealed no statistically significant differences among the three groups ($P = 0.29$).

Discussion

To determine whether attenuation with barstar could ameliorate the cytotoxic effects of the unintended expression of barnase in vegetative tissues, we compared transformation efficiencies between attenuated and unattenuated constructs. The attenuated constructs exhibited a mean efficiency of 4.2%, while the unattenuated construct failed to give rise to any transgenic lines. For this ablation construct, therefore production of transgenic plants requires an attenuation system. In addition, the higher mean efficiencies of the control constructs compared to the attenuated constructs suggests that barnase attenuation was incomplete during the unorganized growth of callogensis and shoot differentiation that part of the transformation process. This may indicate that gene expression is more “promiscuous” during unorganized growth, or that cells are particularly susceptible to barnase during re-differentiation, or both. In other studies we have observed that transgenic poplars with a *TA29*:barnase gene are much more difficult to re-transform than other poplars, suggesting a similar lethal effect of barnase “leakage” at this stage (J. Li, Strauss, pers. comm.). Finally, the presence of MAR elements in the constructs may have increased the base expression of barnase sufficiently to have made attenuation essential.

In the growth analysis, the highly significant difference among blocks found in ANOVA was partly a result of variation in size of plants during propagation. Trees were allocated among blocks based on early vigor during propagation. When plants were transferred from *in vitro* growth to soil, the weakest ramets were always assigned to block 5, which then had the lowest final biomass. When block 5 was removed from the ANOVA, the mean square for blocks in ANOVA decreased by 34.6% from 1.62 to 1.06, though still remained statistically significant. The remaining variance among blocks might from variation in soil (commercial sources varied) and greenhouse microclimate.

The lack of statistically significant effects of constructs on vegetative growth was surprising. From GUS analysis (Chapter 2), we found that the *PTLF* promoter directed higher mean expression than the 35SBP and 35SBP omega promoters by 3- and 1.8- fold, respectively. Therefore an excess of barnase over barstar, and thus reduced vegetative growth in many lines was expected. Because of the one-to-one binding inhibition of barnase by barstar (Hartley 1988; Mariani et al. 1992), a barstar:barnase ratio below unity is expected to cause incomplete attenuation, possibly leading to deleterious effects on vegetative tissues. This is in agreement with the association of poor growth of the low vigor class with a mean barstar:barnase RNA ratio less than unity (0.83). Presumably, barstar expression was inadequate to fully attenuate barnase expression. In contrast, the high vigor class showed a mean barstar:barnase RNA ratio of 5.1, and all lines in that class had a ratio above 1.4, suggesting that barnase was fully attenuated in all lines. A mean tissue ratio above

1.4, rather than above unity, may be required to ensure full attenuation in all cell types required for normal growth in this environment and at this stage of the life cycle.

Surprisingly, in the 35SBP-P group, which was formed by pooling Att35S and AttOmega, the mean barstar:barnase RNA ratio was above unity (3.4), even though barstar was driven by a weaker promoter than the *PTLF* promoter that is driving barnase (GUS analysis in Chapter 2). Moreover, they appeared to be as effective as AttNOS at enabling normal vegetative growth. This could be due to higher stability of barstar versus barnase mRNA, or a result of selection during transformation and regeneration removing those lines with high barnase compared to barstar activity. The similar capability for enabling normal vegetative growth in 35SBP-P and AttNOS, despite a mean barstar:barnase RNA ratio that was 4-fold higher in AttNOS than in 35SBP-P, suggests that there is a threshold above which additional barstar molecules do not provide further attenuation, nor deleteriously impact cellular metabolism.

The statistically significant regression of growth rate on barstar:barnase ratio suggests that measured RNA levels are indeed correlated with active protein levels, and further demonstrates that plant vigor is affected by the barstar-barnase attenuation. Regression analysis also identified a threshold beyond which early plant growth and barstar-barnase ratio were uncorrelated. This further supports the existence of a critical threshold for complete attenuation.

Table 7. Plants used to establish a field trial in 2003.

Construct	Number of transgenic lines	Ramets per line	Total plants
SGUS	8	2	16
OGUS	8	2	16
NGUS	8	2	16
PGUS	6	4	24
C35S	6	4	24
COmega	6	4	24
CNOS	6	4	24
Att35S	21	4	84
AttOmega	17	4	68
AttNOS	19	4	76
Non-transgenic	-	-	18

The inability to produce transgenic plants with the *PTLF*::barnase floral ablation gene alone, and the large majority of attenuated transgenic plants that showed normal growth, suggests that this attenuation system could be a valuable tool for producing a wide variety of ablation-based transgenic plants. A long-term trial has been established (Table 7) to further analyze vegetative growth, and to evaluate floral sterility, under field conditions.

CHAPTER 4

Conclusions

Summary of key results

Promoter::GUS results

1. Transgenic poplars harboring PGUS and NGUS constructs displayed histochemical GUS expression. PGUS imparted stronger GUS staining in the shoot tip region than lower leaves, whereas NGUS conveyed stronger GUS expression in lower leaves than shoot tips.
2. Among the four GUS constructs (PGUS, SGUS, OGUS, and NGUS), NGUS showed highest mean GUS expression level, which was five-fold stronger than PGUS, and 9- and 14-fold stronger than OGUS and SGUS, respectively. Mean expression level of the PGUS was 1.8-fold higher than the OGUS and three-fold higher than the SGUS. No significant differences were detected between the OGUS and SGUS.
3. SGUS and OGUS did not specify different expression levels among four tissue-types (shoot, leaf, stem, root).
4. NGUS and PGUS exhibited differential expression among tissue-types. NGUS showed strongest expression in the root, with a mean expression level 2.4-fold higher than in leaves. Its weakest expression was in the shoot, where its mean expression level was five-fold lower than in leaves. PGUS conveyed the strongest expression in shoots, where it was 2.3-fold higher than in leaves. It

did not show a significant difference between roots and stems, for which GUS expression was 1.7-fold lower than in leaves.

Attenuation / growth results

5. Among the eight constructs (one unattenuated ablation construct, three attenuated ablation constructs, and four control constructs), the unattenuated construct failed to give rise to any transgenic lines. The attenuated constructs and the transgenic control constructs showed transformation efficiencies above four percent.
6. Four transgenic control constructs had higher transformation efficiencies (mean of 6.1%) compared to the three attenuated constructs (mean of 4.2%).
7. In ANOVA of vegetative growth, no significant construct effects were found for biomass index or relative growth rate.
8. Two vigor classes were found in attenuated transgenic plants. Four lines formed a low vigor class; its mean relative growth rate was 0.63. The rest of the attenuated transgenic lines formed the high vigor class; its mean relative growth rate was 1.18.
9. The two vigor classes had significantly different barstar:barnase RNA ratios. The mean barstar:barnase RNA ratio of the low vigor class was 0.83, whereas the high vigor class had a mean barstar:barnase RNA ratio of 5.1.
10. Similar levels of barstar mRNA were observed in attenuated plants containing Att35S and AttOmega.
11. There was a significant difference in the barstar:barnase RNA ratio between the AttNOS and 35SBP-P. The mean barstar:barnase RNA ratio of AttNOS

was 4-fold higher than 35SBP-P, however, the relative growth rate of the two groups did not differ significantly.

12. There were statistically significant linear and quadratic associations between relative growth rate and barstar:barnase RNA ratio.

Major conclusion

1. The TMV omega element failed to notably elevate the translational efficiency of target mRNA possibly due to the very low amount of mRNA molecules to initiate translation, or perhaps due to the lack of compatibility of the tobacco omega element with the cellular environment of poplar.
2. The reduced vegetative growth of the low vigor attenuated plants is due to its less than unity barstar:barnase RNA ratio (0.83), which implies that the barstar was inadequate to attenuate barnase activity completely. The higher mean barstar:barnase RNA ratio of the high vigor attenuated plants (5.1) may ensure that barnase activity was fully attenuated in all cell types essential for growth.
3. The AttNOS and 35SBPP transgenic plants had significantly different barstar:barnase RNA ratios but similar relative growth rates, indicating that there is a threshold above which additional barstar molecules do not provide further attenuation.
4. A statistically significant linear and quadratic association between relative growth rate and barstar:barnase RNA ratio confirms that barstar-barnase attenuation substantially affects plant vigor, and confirms the existence of a critical threshold for barstar attenuation of barnase activity.

Recommendation for further research

Growth analysis of trees in mature stage

In this study, the proposed attenuation ablation system was investigated for its ability to confer normal early vegetative growth under greenhouse conditions. Future work should include collecting growth measurements from older trees grown in a variety of field conditions.

Floral ablation

In this thesis, only one aspect of the attenuation ablation system was studied: the capability of the attenuation system to maintain normal vegetative growth. No results on floral ablation were available due to the several year period before poplar flowering begins. To confirm the effectiveness of the attenuation system, the efficiency of sterility induction with the different constructs requires careful study.

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APPENDICES

Appendix A Confirmation study on cytotoxic effects on vegetative growth imparted by direct floral ablation

To confirm the deleterious effects of unintended cytotoxin expression on poplar growth reported earlier (Skinner et al. 1999), we performed biomass analysis and real-time RT-PCR on transgenic trees in a field trial. Skinner et al. (1999) demonstrated that trees with a heterologous tobacco tapetum-specific *TA29* promoter (Koltunow et al. 1999) or a tobacco transmitting tissue specific (*TTS*) promoter (Wang et al. 1993) driving barnase grew poorly, though had normal morphology. Similar poor growth was observed by skinner et al. (1999) in transgenic poplars harboring the diphtheria toxin A chain (*DTA*) (Greenfield et al. 1983) driven by the *TA29* promoter, the *TTS* promoter, or *SLG* promoter (Hackett et al. 1992).

Table A1 T-tests of barnase transgene expression and growth rate between fast and slow growing trees in an experimental plantation. Barnase expression was calculated as relative quantity = barnase / ACT2.

Data	Mean of slow growing class (sample size)	Mean of fast growing class (sample size)	Type	t	p-value
Plant biomass (cm^3)	728 (4)	3,833 (5)	Two-sided	-3.1	0.00
Barnase expression	0.092 (4)	0.030 (5)	Two-sided	5.1	0.02

To determine whether growth inhibition in poplar truly resulted from unintended cytotoxin expression, we sampled clonal replicates of several transgenic lines for real-time RT-PCR analysis of barnase expression. Plant growth was indexed as: Biomass index = height × $diameter^2$ (cm^3), taken at 8 months of age (mean tree height was 78 cm). Growing shoot tips (average length of 2.0cm) were collected from sample trees and subjected to RNA extraction. Six slow growing trees (mean biomass index of 577 cm^3) were compared to 6 trees with normal growth (mean biomass index

of 3605 cm^3). In total, 12 genetically independent trees were sampled for RNA extraction and subsequent real-time RT-PCR. The a*ctin* 2 gene was used as an internal control to calculate the relative quantity of barnase RNA. Primers of a*ctin* 2 used in real-time RT-PCR were ACT2-forward: 5'-CCCATTGAGCACGGTATTGT-3', and ACT2-reverse: 5'-TACGACCACTGGCATACAGG-3'. Primers used for the barnase gene were BARNASE-forward: 5'- GGCTGGGTGGCATCAAAA-3' and BARNASE-reverse: 5'-GATGTCTCCGCCGATGCTT-3'.

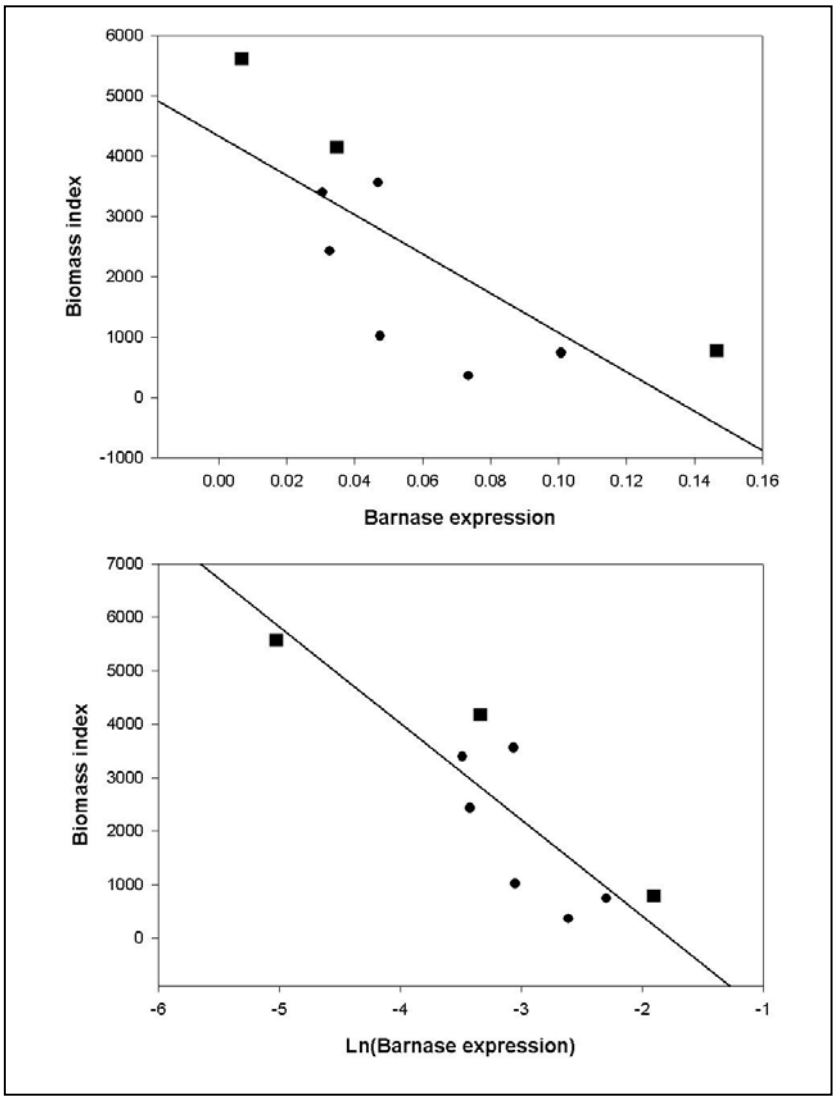

Figure A1 Relationship of biomass index of 8-month-old poplar to barnase expression conferred by the *TA29* or *TTS* promoter. A) Linear regressions of plant biomass on barnase expression. y = -32549 x + 4332.1, R^2 = 0.57. B) Linear regression of plant

biomass on ln(Barnase expression). $y = -1803.9 x – 3199.9$, $R^2 = 0.74$. Square: *TTS* promoter, round: *TA29* promoter.

Based on the barnase RNA levels, we found three samples that displayed extremely high levels of barnase mRNA (mean of 30.6 in relative units), 530-fold higher than the mean of the other 9 samples. One of these three trees was from the fast-growing set and the other two were from the slow-growing set. The other 9 trees showed much lower barnase expression (mean of 0.06 in relative units). Five of the nine samples were from the fast-growing set, and the other four were from the slow-growing set. The three samples exhibited that excessive barnase expression were treated as an outlier population and thus excluded from subsequent analyses.

When the two groups of slow and fast growing trees were compared, we found a statistically significant difference in barnase RNA levels (two-sided test, $P = 0.02$) (Table A 1), suggesting that the plant growth was affected by barnase RNA level. Not surprisingly, growth also differed significantly ($P = 0.0$) between the selected groups of trees. We also tested the association between barnase RNA level and plant biomass by regression analysis. A statistically significant negative linear correlation was found between barnase RNA level and plant biomass index ($P = 0.02$) (Figure A 1). However, beyond the barnase expression level of 0.06 (relative units) the association appears to be lost, suggesting that a logarithmic transformation of barnase RNA level may fit the data better. By introducing the log transformed barnase RNA level into the linear model, the R^2 was raised from 0.57 to 0.74, and displayed much higher statistical significance ($P = 0.00$) (Figure A 1). This suggests that barnase under the control of the promoters studied has a limited effects on growth, perhaps due to the complete debilitation of specific cell types at moderate expression levels.

In the two populations that showed extremely different barnase mRNA levels, the unexpectedly high barnase expression of the first population was possibly due to

the confinement of “leaky” expression of the cytotoxin barnase in cells that are dispensable or highly tolerant, thus no highly deleterious effects were observed. With this outlier population segregated, the statistically significant negative linear correlation observed between barnase and plant biomass index suggests that unintended expression of barnase does affect plant vigor, however, the associations are complex. Within this subpopulation there was a saturation point for barnase, above which no further reductions in biomass occurred. More detailed analysis is needed to understand these relationships.

Appendix B Construct names used in research

Table A2 Names of constructs used in research. Each construct had different names during different stages of our study.

Final construct name	**Functional elements**	**Laboratory identifier**
PGUS	*PTLF*::GUS	pGPG
SGUS	35SBP::GUS	pGWGS
OGUS	35SBPΩ::GUS	pGWGO
NGUS	*NOS*::GUS	pGWGN
C35S	35SBP::Barstar	pGSB
COmega	35SBPΩ::Barstar	pGOB
CNOS	*NOS*::Barstar	pGNB
UnAtt	*PTLF*::Barnase	pGPB
Att35S	*PTLF*::Barnase::35SBP::Barstar	pGBSB
AttOmega	*PTLF*::Barnase::35SBPΩ::Barstar	pGBOB
AttNOS	*PTLF*::Barnase::*NOS*::Barstar	pGBNB

Appendix C *Ecoli*. strain glycerol stock storage

Glycerol stocks of *Ecoli*. DH5α strain harboring DNA constructs PGSB, PGOB, PGNB, PGBSB, PGBOB, PGBNB, PGPB, and NGUS (labeled as PGWGN) are stored in -80°C freezer in Strauss Lab, box labeled “TTT”. Glycerol stocks of *Ecoli*. DH5α strain harboring DNA constructs PGUS (labeled as PGPG), SGUS (labeled as PGWGS), and OGUS (labeled as PGWGO) are stored in -80°C freezer in Strauss Lab, box labeled “KKK”.

Appendix D Location of Electronic Files

Data files

The data files used for this study are stored in the DATA subdirectory of the T:\group\TGERC\THESIS\Wei\Wu directory of the Oregon State University College of Forestry computer network. There are three sub-subdirectories under DATA subdirectory: Chapter2, Chapter3, and Confirmation study. Under the three directories, data files include GUS staining pictures, quantitative GUS data, barstar barnase RNA expression data, biomass and relative growth data, and pictures of transgenic trees.

Thesis

An electronic copy of the entire thesis including all text, tables, and figures is stored in the THESIS subdirectory of the T:\group\TGERC\THESIS\Wei directory of the Oregon State University College of Forestry computer network as the file "thesis.doc".