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Field trial detects incomplete *barstar* attenuation of vegetative cytotoxicity in *Populus* trees containing a poplar *LEAFY* promoter::*barnase* sterility transgene

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Abstract We tested the efficacy of an attenuation system developed to preclude the deleterious effects of floral promoter::cytotoxin genes on vegetative growth of transgenic sterile plants. We tested the promoter (2.6 kb 5′ region) of the poplar *LEAFY* gene *PTLF* driving *barstar*, combined on the same T-DNA 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. The unattenuated *pPTLF::barnase* construct failed to give rise to any transgenic events, suggesting substantial non-reproductive expression from this promoter. The *barstar*-attenuated constructs enabled transformation, but the rate was reduced by nearly one-third. Four events (7% of attenuated events) had highly abnormal morphology, and were identified during the early phases of propagation; these events had significantly higher *barnase:barstar* expression ratios based on quantitative RT-PCR. A greenhouse study showed that phenotypically normal attenuated plants grew at the same rate as wild-type and *barnase*-lacking transgenic plants. A statistically significant positive linear association was found between relative growth rate (RGR) and *barstar:barnase* ratio in the attenuated events, and graphical analysis suggested a threshold for *barstar* attenuation of *barnase*, above which additional levels of *barstar* did not provide further attenuation. Surprisingly, the appearance and growth rate of the nearly all of the attenuated events were substantially reduced after one or two growing seasons in the field, and the extent of growth reduction was associated with *barstar:barnase* expression ratio. These results demonstrate the importance of field testing during early phases of research to identify pleiotropic effects of transgenic sterility genes in trees.

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Introduction

Genetic engineering can import novel traits that may elevate tree productivity in diverse ways (Peña and Séguin 2001; Boerjan et al. 2005). However, transgenes from weakly domesticated tree crops can disperse long distances via pollen and seed, potentially causing undesired ecological effects (James et al. 1998; Strauss et al. 2001; Walter and Fenning 2004). Environmental concerns over transgene dispersal, and attendant regulatory barriers, are substantial impediments to deployment of genetically engineered plantations (Strauss et al. 1995; Merkle and Dean 2000; Bhalero et al. 2003). One way to greatly diminish the environmental impacts caused by transgene dispersal is to employ sterile trees, which can be produced via several genetic mechanisms, including cell ablation (Skinner et al. 2000; Lemmetyinen and Sopanen 2004a; Meilan et al. 2004; Brunner et al. 2006).

Genetic cell ablation methods employ promoters active in specific cell types or under certain conditions to control the expression of a gene encoding a lethal product, usually a cytotoxin (Leuchtenberger et al. 2001; Bi et al. 2001; Burgess et al. 2002; Skinner et al. 2003). For engineering plant sterility, a floral-predominant promoter has been used to control the expression of a cytotoxin, often *barnase* (Goldman et al. 1994). *Barnase* is a small protein consisting of 110 amino acids (Paddon and Hartley 1986, 1987; Hartley 1988) that is an extracellular ribonuclease of *Bacillus amyloliquefaciens*. By fusion to a floral promoter, it has been used to successfully engineer male and female plant sterility (Mariani et al. 1990, 1992; Block and Debrouwer 1993; Goldman et al. 1994; Block et al. 1997). *Barstar*, a specific inhibitor of *barnase*, has been used to counteract *barnase* activity and restore male-fertility for crop production (Mariani et al. 1990; Mariani et al. 1992; Beals and Goldberg 1997).

Ideally, expression of cytotoxin such as *barnase* should be confined to floral cells; however, it appears that the majority of promoters derived from genes regulating floral development are not expressed exclusively in floral tissues (Kyozuka et al. 1997; Southerton et al. 1998a, b; Brunner et al. 2000; Rottmann et al. 2000; Skinner et al. 2003), and even low levels of unintended cytotoxin expression may impair plant growth (Skinner et al. 2000). A high rate of aberrant vegetative development was also reported in transgenic birch (*Betula pendula*) when *barnase* was driven by the floral-predominant promoter derived from the *BpMADS1* gene (homologous to *SEPALLATA3* or *AGL9*: Lemmetyinen and Sopanen 2004b).

We have been developing a general attenuation system that can insure normal vegetative growth when sterility is caused by a variety of floral ablation genes. The system has two major components. First, sterility is caused by the cytotoxin *barnase* under the control of a promoter from a floral homeotic gene that is predominantly active in male and female floral primordia. Second, *barstar* is expressed under the control of a weakly or moderately expressed constitutive promoter that is primarily active in vegetative tissues. Theoretically, 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 and Goldberg 1997).

PTLF, the *Populus trichocarpa* ortholog of *LEAFY* (*LFY*) and *FLORICAULA*, shows strong expression in developing male and female inflorescences and, therefore, is an excellent candidate for the complete male and female sterility desired in undomesticated, vegetatively propagated trees. The *Arabidopsis LEAFY* promoter was used to ablate flowers in transgenic *Arabidopsis* (Nilsson et al. 1998), suggesting that use of the *LEAFY* ortholog *PTLF* could also be successful. *PTLF* also has detectable expression in leaf primordia, young leaves, and other vegetative tissues (Rottmann et al. 2000; Wei et al. 2006), thus providing a stringent test of the effectiveness of an attenuation system. Three weakly to moderately expressed, variably constitutive promoters were

selected as candidates to counter the expected vegetative expression of *barnase* from the *PTLF* promoter (*pPTLF)*. These included 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 *pPTLF::barnase* element was co-introduced on the same T-DNA with the promoter:*barstar* cassette. In all constructs, flanking tobacco matrix attachment regions (MARs) were included in the T-DNA region of the binary vectors to enhance and stabilize expression level (Allen et al. 2000). The MAR employed was previously suggested to elevate transgene expression in poplar (Han et al. 1997). We show that this attenuation system enabled successful transformation and normal vegetative growth during early stages of development. However, the attenuation system was not sufficiently robust under field conditions. Results from studies of flowering and sterility will be reported when the majority of the trees have reached reproductive maturity, which may be several years away.

Material and methods

Construct assembly

pPTLF::GUS assembly was described in Wei et al. (2006). The binary vector pG3M was derived from pGreen II (Hellens 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 derived by inserting the blunt-ended bacterial barstar operon obtained by digestion of pMT416 (Hartley 1988) with *Xba*I and *Hin*dIII (Klenow blunted) into the pG3M *Dra*III site (T4 polymerase blunted).

To make the unattenuated *pPTLF::barnase* construct, the *pPTLF::barnase* cassette and kanamycin resistance marker (*NPT II*) 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′-AG CCGCGGTACTAAATAAATATATAAAC-3′ and 5′-T GC *GGCCGC*GATCTTTCACAGGTGCATGTC-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 *Sac*II and *Not*I sites of the intermediate construct. The *barnase* gene was PCR-amplified with the primers: 5′- TGGAT *CC*ATGGTACCGGTTATCAACAC-3′ and 5′-C GAT *ATC*TTATCTGATTTTTGTAAAGG-3′ with *Bam*HI and *Eco*RV sites (underlined) incorporated. The *barnase* amplicon was then digested with *Bam*HI and *Eco*RV and inserted into the intermediate construct's *Bam*HI/*Eco*RV sites. The 3′ untranslated region of gene 7 from *Agrobacterium tumefaciens* (G7 3′) (Velten and Schell 1985) was PCR-amplified using the primers: 5′-C GA *TATC*GAGCTAAGCTAGCTATATCA-3′ and 5′-GA AAGCTTATCTTGAAAGAAATATAGTT-3′, into which *Eco*RV and *Hin*dIII sites (underlined) were incorporated. The G7 3′ fragment was subcloned into *Eco*RV/*Hin*dIII sites of the intermediate construct. 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 *pPTLF*::barnase 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 digestion with *Sma*I and *Sac*II.

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* (Wei et al. 2006). The *barstar* gene was amplified with primers: 5′-GGGTACCATGAAAAAAGCAGTCATTAA-3′ and 5′-GGGATCCTTAAGAAAGTATGATGGTGA-3′ with *Kpn*I and *Bam*HI sites (underlined) incorporated. The *GUS* gene was released from *CGUS*, *OGUS*, and *NGUS* via *Kpn*I and *Bam*HI digestion. The *barstar* amplicon digested with *Kpn*I and *Bam*HI was inserted into *CGUS*,

OGUS, and *NGUS*, producing *C35S*, *COmega*, and *CNOS*, respectively.

Attenuated constructs were assembled via subcloning the *pPTLF::barnase::G7 3′* cassette into *C35S, COmega*, and *CNOS*. The *pPTLF::barnase::G7 3′* cassette was excised from an intermediate construct by *Hin*dIII digestion (Klenow blunted) followed by *Sac*II digestion and then inserted into the *Sma*I/*Sac*II sites of *C35S, COmega*, and *CNOS*, generating *Att35S, AttOmega*, and *AttNOS*, respectively. All the PCR-amplified fragments were confirmed by sequencing. Construct names and elements are summarized in Fig. 1.

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 (*P. tremula* × *P. alba*; INRA 717-1B4) as described by Filichkin et al. (2006). All transgenic events were verified by PCR using primers specific for *barnase* (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 events (lines) were regenerated, and each line was propagated in vitro to produce five ramets.

Growth measurements

Measurements of plant vegetative growth were carried out twice at two different stages of plant development. Growth of transgenic plants was measured over several months when plants were maintained in greenhouse, and then in a field trial, which was established to further analyze vegetative growth and to evaluate floral sterility.

In greenhouse

Two-month-old plants were transferred into soil and maintained in a lighted growth room for one month, before transfer to a greenhouse maintained under ambient day-night cycles (Corvallis, Oregon, USA) during spring and summer of 2003. To analyze growth, we employed a randomized block design, which was maintained after the plants were transferred to soil. Height and basal diameter (2 cm above soil) were measured using a ruler and digital calipers, respectively. The first set of growth measurements were begun when the mean height of plants was 59 cm, 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)].

In field trial

Subsets of trees from the greenhouse study, totaling 390 trees, were planted in Benton County, Oregon (central Willamette Valley) in September of 2003 at a spacing of 6 × 6 feet (1.8 × 1.8 m). Two two-tree plots of each transgenic event or control transgenic event were randomly planted. The entire planting was surrounded by one or two rows of unmeasured border trees. For the *pPTLF::GUS* and *pPTLF::barstar*-constructs (*PGUS, C35S*, *COmega*, and *CNOS)* there were six events, and for the three attenuation constructs (*Att35S*, *AttOmega*, and *AttNOS)* there were by 21, 17, and 19 events, respectively. The non-transgenic controls (NTCs) were planted in 9 two-tree plots. The plants were irrigated as needed throughout two growing seasons and weeds were controlled manually or by spraying herbicides (active ingredients; Oryzalin, Oxyfluorfen, Metsulfuron methyl, Sulfometuron methyl, or Glyphosate). Heights and basal diameters (both in cm) were taken on all trees near the end of each growing season (September 2004 and November 2005). Height and diameter were assessed in both years, and leaf chlorosis was assessed only at the very end of the growing season when visually apparent (November 2005). A visual index was used to evaluate chlorosis, where 1 = virtually all leaves on the tree were green; 2 = a substantial portion

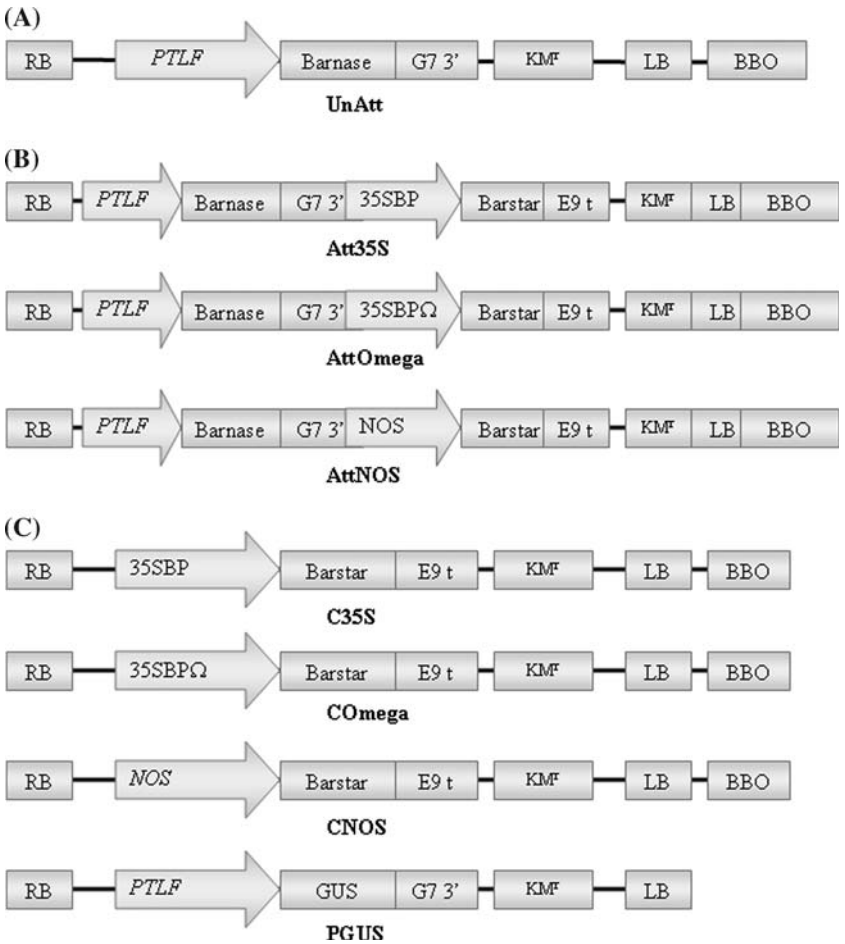

Fig. 1 Constructs used for transformation. (**A**) Unattenuated *barnase* construct. (**B**) Attenuated *barnase* constructs. (**C**) *Barstar* constructs. Arrows show direction of transcription. Abbreviations: *35SBP*: CaMV 35S basal promoter (+5 to –72 fragment), *35SBP* Ω: *35SBP* fused to TMV omega 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 the floral gene *PTLF*, GUS: GUS reporter gene, *BBO*: Bacteria barstar operon

of the leaves on the tree were chlorotic; and 3 = virtually all leaves on the tree were chlorotic.

Plant RNA extraction and real time RT-PCR

Young leaves of actively growing plants (one to two nodes below the apex) were excised and frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), quantified spectrophotometrically at OD_{260}, and purified from DNA using the DNA-Free Kit (Ambion, Austin, TX, USA). First-strand cDNA was synthesized using 2 μg of total RNA and the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen, 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 et al. 2004). PCR primers were designed using Primer Express (Applied Biosystems, Foster City, CA, USA) for: *barnase* (GenBank accession # E31988) 5′-GGCTGGGTGGCATCAAAA-3′ and 5′-GATGTCTCCGCCGATGCTT-3′; *barstar* (GenBank # AY283058) 5′-TGGACGCTTTATGGGATTGTC-3′ and 5′-ACTGCCTCCATTCCAAAACG-3′; and *ACT2* (GenBank accession # BU879695) 5′-CCCATTGAGCACGGTATTGT-3′ and 5′-TACGACCACTGGCATACAGG-3′. Real-time RT-PCR was performed in a total volume of 25 μl, consisting of SYBR Green PCR Master mix (Applied Biosystems) and cDNA using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The reactions were carried out in 96-well plates; triplicate PCR reactions were run for each sample.

To verify that the *barstar*:*barnase* ratio measured 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 (*Att35S* containing one copy of *barnase* and two copies of *barstar*). We found no significant difference in affinity between the *barnase* and *barstar* primers; the average ratio of *barstar* to *barnase* was nearly two (mean of 1.83, $n = 7$; two-sided, paired *t*-test, $P = 0.16$).

Statistical analysis

Transformation efficiency x was calculated as:$x = X/N$ where X = Number of independent (one shoot per explant) transgenic events produced and N = Number of explants co-cultivated; standard error = $\sqrt{[x(1-x)/N]}$. For the greenhouse trial, a randomized block design was employed to reduce error variance resulting from position in the growth chamber and greenhouse, as well as from initial size due to time of propagation. Biomass index and RGR computed from growth measurements were subjected to mixed model ANOVA to test effects from constructs, events within constructs, and blocks. This analysis treated constructs as fixed factors, and blocks, events within constructs, and interactions between blocks and constructs as random factors. Unless specified otherwise, comparisons of means among construct types [i.e., NTC, transgenic controls (TCs) (no barnase), and attenuated], or between constructs, employed two-tailed Student's *t*-tests using event means. Differences among construct types and groups for chlorosis were approximated using Student's *t*-tests, and via Fisher's exact test of frequencies of events in the different classes.

Results

Transformation efficiency

Of the eight plasmids used to transform poplar, only the unattenuated construct (*UnAtt*) failed to give rise to any transgenic plants (Table 1). In contrast, the attenuated constructs and the TC constructs all showed transformation efficiencies above 4%. The presence of *barnase* in the attenuated constructs appeared to significantly depress transformation efficiency. The TC constructs (*C35S*, *COmega*, *CNOS*, and *PGUS*) resulted in a mean efficiency of 6.1%, compared to 4.2% for the three attenuated constructs, a difference that was statistically significant (χ^2 test, one degree of freedom, $P = 0.02$).

Table 1 Transformation efficiencies for the control and attenuated constructs

Construct	Explants co-cultivated	Independent events 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

Transformation efficiency was calculated as the percentage of co-cultivated explants that produced at least one PCR-confirmed transgenic shoot. *Barnase*-containing attenuated constructs are the lower three constructs. SE = standard error

Greenhouse trial

ANOVA carried out on biomass index (BI) of plants in the greenhouse trial (Table 2) showed that although block and event within construct were highly significant sources of variation, construct effects were not. The mean early growth of plants from all constructs was very similar (Fig. 2), and a large majority of plants were very similar in appearance (Fig. 3A). The high level of significance for events within constructs ($P = 0.00$) was partly due to four visibly abnormal events (Fig. 3B), two transformed with *Att35S* and two with *AttNOS*, both of which are attenuation constructs. These four events had considerably lower biomass when compared to the rest of the events 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 (data not shown). By excluding these events, the mean square of event-within-construct in ANOVA was reduced by 53%, from 0.77 to 0.36, though it still

Table 2 Mixed-model analysis of variance of biomass index of plants in greenhouse. Model was Y_{ijk} = u + Construct$_i$ + Block$_j$ + Event$_k$ (Construct$_i$) + Error$_{ijk}$

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

DF = degree of freedom; MS = Mean square; EMS = Expected mean square; Var = Variance

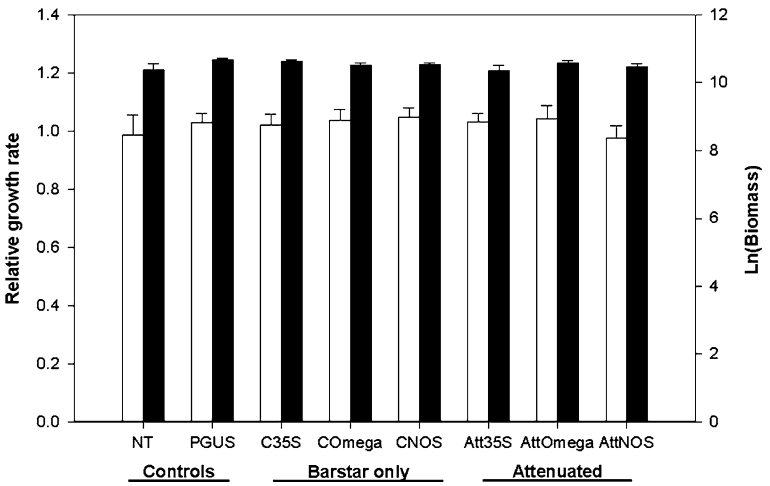

Fig. 2 Biomass index (BI, black bars) and relative growth rate (RGR, white bars) of transgenic plants harboring attenuated, *barstar* only, and control constructs in the greenhouse trial. Bars show one standard error over event means. Abbreviations are given in Fig. 1

remained statistically significant ($P = 0.02$). These four events were pooled into a ''low vigor'' class for comparison to normally growing, attenuated events in subsequent analyses.

The mean RGRof the low-vigor class was 0.63, whereas the rest of the attenuated transgenic events had a mean RGR of 1.18 approximately twice that of the low-vigor class (Table 3). RNA from young leaves were collected from four transgenic events of the low-vigor class and from four high-vigor transgenic events sampled from each attenuated construct, using two ramets per event. In total, 12 high-vigor events (4 events × 3 attenuation constructs) and four low-vigor events were compared. The two vigor classes had significantly different *barstar:barnase* RNA ratios (Table 3). 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.

We also compared *barstar:barnase* RNA ratios among the different attenuation constructs (Table 4). Because the tobacco omega element acts post-transcriptionally, similar levels of *barstar* mRNA should have been observed in attenuated plants containing *Att35S* and *AttOmega*. Our results supported 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* plants were compared to *35SBP*-P plants, a highly significant difference in *barstar*:*barnase* RNA ratios were observed ($P = 0.00$, Table 4), with a 2.5-fold higher *barstar*:*barnase* RNA ratio seen in *AttNOS* plants than in *35SBP*-P; however, the RGRs of the two groups were not significantly different (Table 4: two-sided *t*-test, $P = 0.91$).

Barstar:barnase RNA ratios and RGR data of plants in the greenhouse trial were plotted to

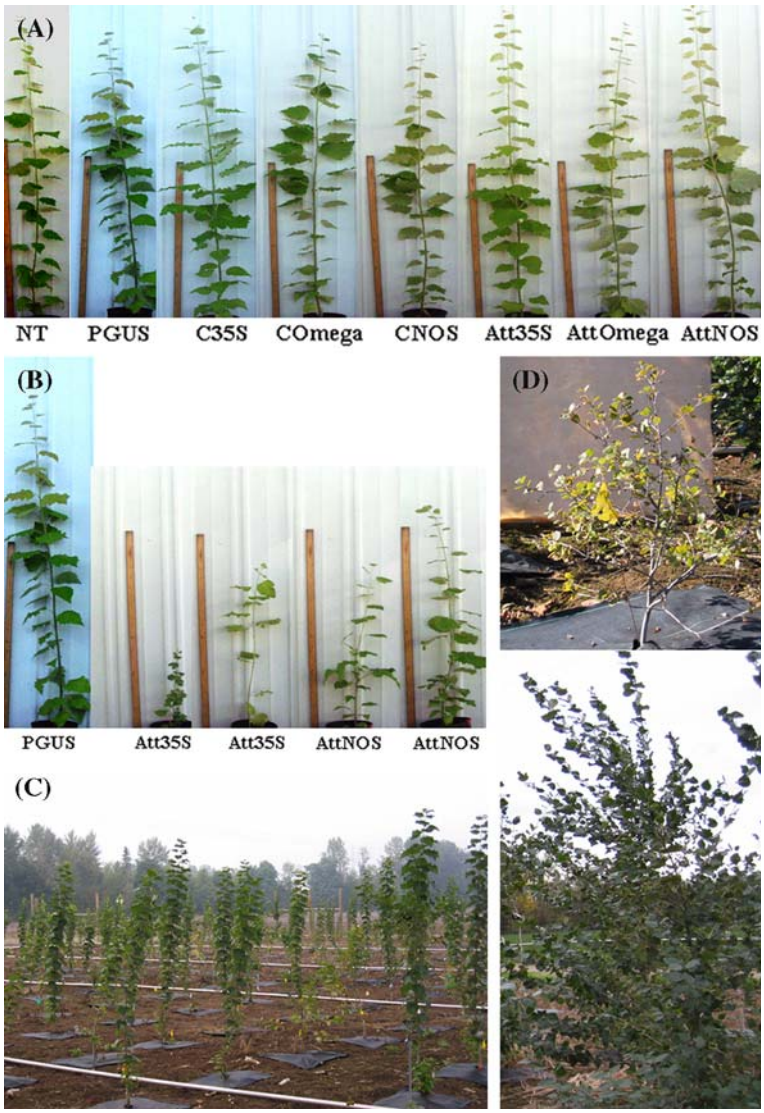

Fig. 3 Examples of plants in field and greenhouse environments. (**A**) Representative (near to mean) non-transgenic control (NTC) plants and transgenic plants from the different constructs in the greenhouse trial and (**B**) low-vigor, attenuated transgenic plants compared to a high-vigor transgenic control (TC) plant (*GUS*) from the greenhouse trial. (**C**) View of field trial after the first growing season. (**D**) Example of poorly growing, chlorotic (index value 3) transgenic tree compared to normal tree (index value 1) in field trial

Table 3 Student's *t*-tests of gene expression and relative growth rate (RGR) of plants in the greenhouse trial between the low and high vigor classes

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

BBRR: *Barstar:barnase* RNA ratio

explore the association between them. There was a statistically significant ($P = 0.00$, $R^2 = 0.28$) positive linear relationship between early RGR and *barstar:barnase* RNA ratio (Fig. 4A). Because the trend appeared to plateau above a ratio of 1.4 (dashed line in Fig. 4A), 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 early RGR best ($P < 0.00$), giving an R^2 of 42%. Above a ln(*barstar:barnase* RNA ratio) of about 0.3 (dashed line in Fig. 4B), all trees appeared to grow normally; for this sub-population *barstar:barnase* RNA ratio and early growth rate appeared to be uncorrelated ($R^2 = 0.00$, $P = 0.75$).

Table 4 Student's *t*-tests of gene expression and relative growth rates (RGR) of plants in the greenhouse trial between different promoters

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	*NOS* (1.17)	*35SBP*-P (1.18)	Two-sided	–0.11	0.91
RGR	*35SBP* (1.22)	*35SBP omega* (1.14)	Two-sided	–0.80	0.49

BBRR: *Barstar:barnase* RNA ratio

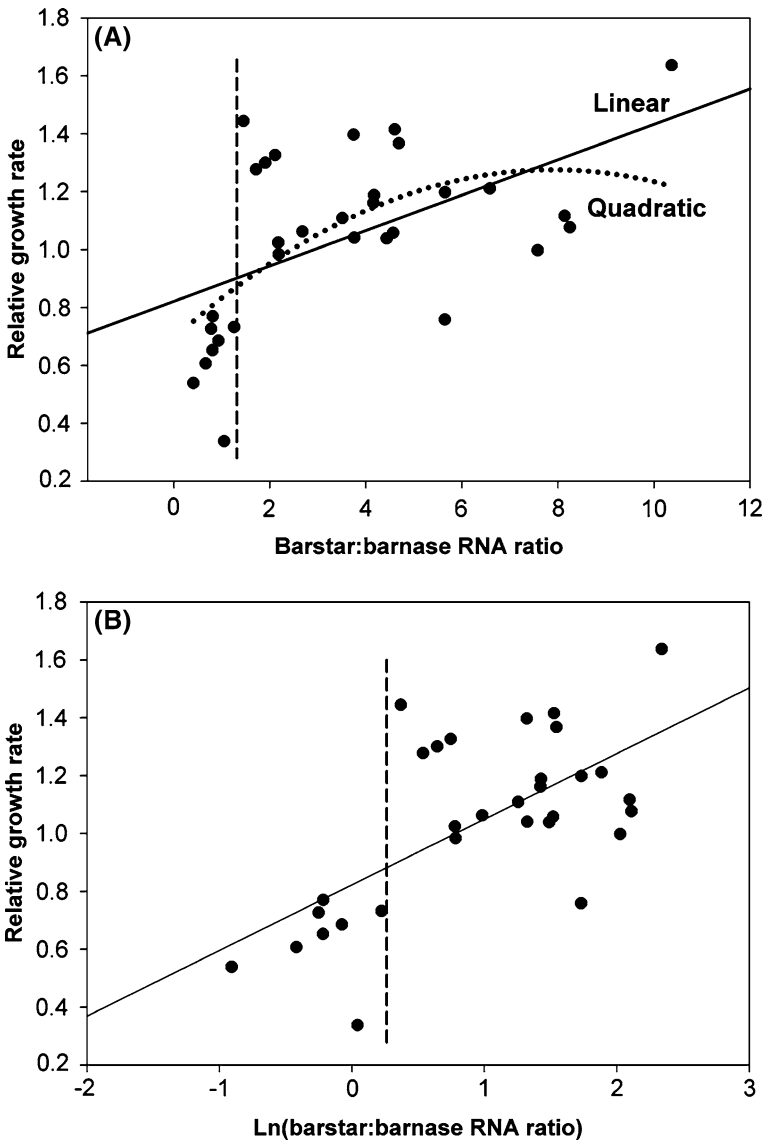

Fig. 4 Linear regression of relative growth rate (RGR) of plants from greenhouse trial on *barstar:barnase* RNA ratio. (**A**) Linear and quadratic regressions of RGR on *barstar:barnase* ratio. Linear: $y = 0.06x + 0.82$, $R^2 = 0.28$. Quadratic: $y = -0.01x^2+0.15x+0.70$, $R^2 = 0.33$. (**B**) Linear regression of RGR on ln(*barstar:barnase* ratio). $y = 0.23x + 0.82$, $R^2 = 0.42$. The dashed lines delineate the start of an apparent plateau in the response

In the greenhouse, there were no statistically significant differences in growth between plants containing any of the constructs and the NTCs, or between the attenuated and other transgenic genotypes. When averaged over all event means (after excluding the morphologically distinct,

low-vigor class), BI and RGR for the TC and attenuated events was nearly identical in the greenhouse trial (Fig. 5, Tables 5, 6). For BI, the means for the TC and attenuated events 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% (Table 6). ANOVA on BI of NTC, TC, and attenuated event means revealed no statistically significant differences among the three groups ($P = 0.29$).

Field trial

After only a single year in the field (Fig. 3C), a pattern emerged that was markedly different than seen in the greenhouse, and which remained consistent after a second year of growth. Although the NTC and TC trees continued to perform similarly within and between groups (year 1, $P = 0.99$; year 2, $P = 0.32$), the attenuated trees had markedly reduced performance. Plants containing the attenuation constructs had significantly ($P < 5\%$) lower mean BI than did the TC and NTC plants in both years (Fig. 6). When the TC and NTCs were pooled into a single group and compared to a group composed of plants containing attenuation constructs, the difference in BI was statistically significant below 3×10^{-8} level in both years; the growth of the attenuated trees was approximately 50% that of the control means. When comparing the three attenuation constructs, only the difference between *AttNOS* vs. *Att35S* was statistically significant at the 5% level, but only in year one ($P = 0.02$). However, the ranking of growth for plants containing the three attenuation constructs was consistent between both years, and correlated with the strength of the promoter used to drive *barstar* expression (Wei et al. 2006). Plants containing *pNOS*, by far the strongest promoter, accumulated the most biomass, and the promoter with intermediate levels of expression, omega-enhanced 35S, resulted in intermediate growth.

Table 5 Pooled biomass index (BI) and relative growth rates (RGR) of plants in the greenhouse trial

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)

NTC: Non-transgenic controls; TC: Transgenic controls; ATT: Attenuated events

The lack of full attenuation was also expressed in leaf coloration. At the end of the growing season but prior to leaf senescence, a number of plants showed signs of chlorosis (Fig. 3D), and the attenuated plants had obviously lighter foliage than did the pooled TC and NTCs ($P < 0.001$ via *t*-test and Fisher's Exact Test; Fig. 7 and Table 7). There was no difference between the TC and NTCs (P~0.87). The *Att35S* and the *AttOmega* plants had the most chlorosis, whereas the *AttNOS* plants had little, and were significantly less chlorotic than plants containing the other two attenuation constructs ($P < 1 \times 10^{-5}$ via *t*-test

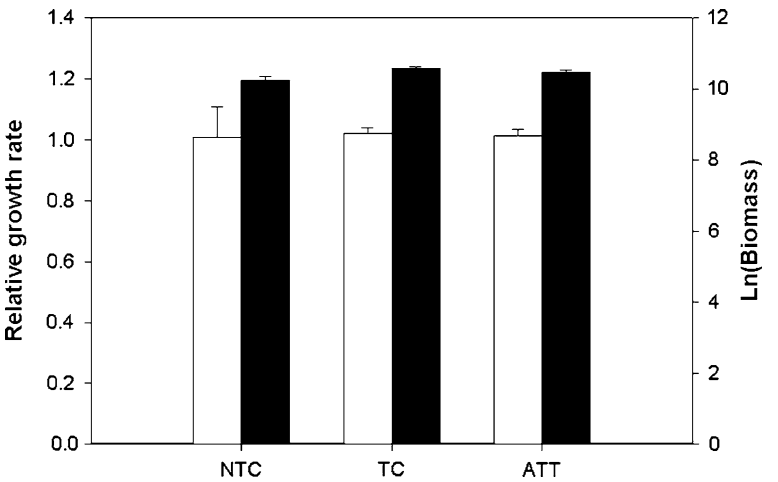

Fig. 5 Mean growth from greenhouse trial of pooled non-transgenic control (NTC), transgenic control (TC, $n = 73$ events), and attenuated plants (ATT, $n = 53$ events). Standard errors calculated over event means. White and black bars indicate RGR and BI, respectively

Table 6 Student's *t*-tests of relative growth rates (RGRs) of plants in the greenhouse trial, among different promoters

Data	Promoter (Mean)	Promoter (Mean)	Type	*t*	*P*-value
RGR	*35SBP* (1.22)	*35SBP omega* (1.14)	Two-sided	–0.80	0.49
RGR	*NOS* (1.22)	*35SBP omega* (1.14)	Two-sided	–0.80	0.49
RGR	*NOS* (1.17)	*35SBP* (1.18)	Two-sided	–0.11	0.91

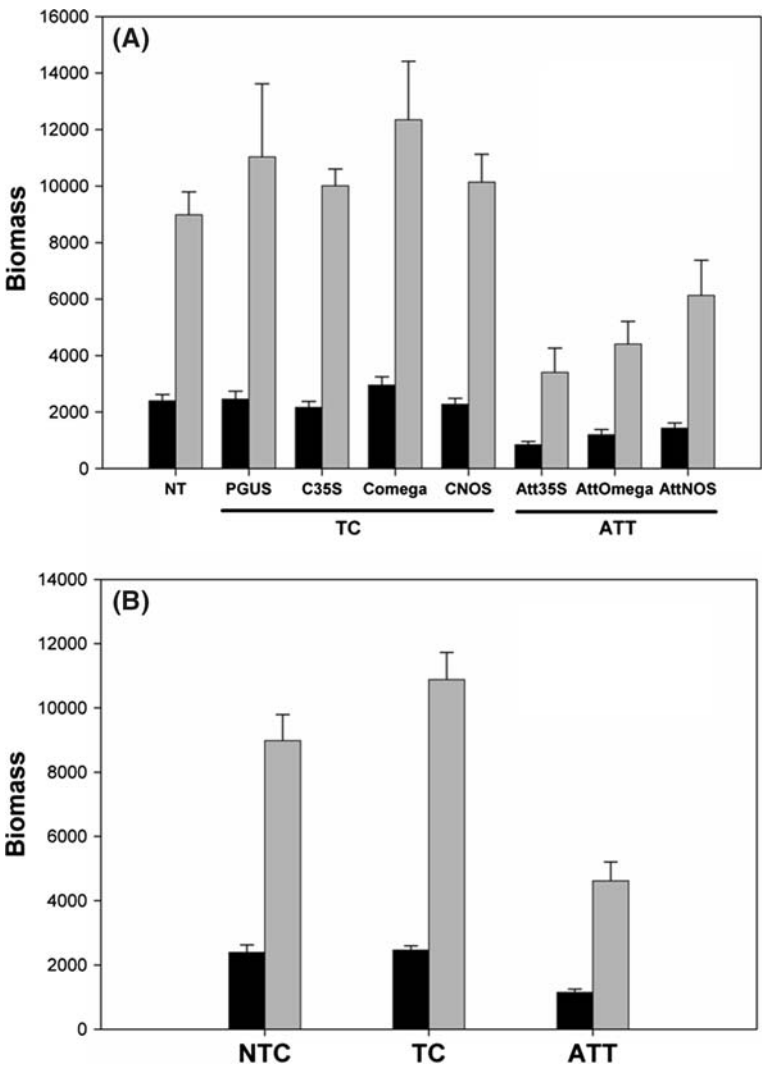

Fig. 6 Biomass index (height × diameter^2) of transgenic plants in field trial. (**A**) Biomass of plants harboring attenuated, *barstar* only, and control constructs in field trial. (**B**) Mean biomass of pooled non-transgenic control (NTC), transgenic control (TC, n = 24 events), and attenuated plants (ATT, n = 59 events) in field trial. Bars show one standard error over event means. Black and gray color indicate the biomass data collected in years one and two, respectively

and $P < 0.001$ via Fisher's Exact Test, Table 7). Again, *barstar* promoter strength was correlated with extent of chlorosis, and the *pNOS* plants showed the lowest degree of chlorosis.

Within constructs, some events accumulated biomass to the same extent as control plants (Fig. 8), suggesting that some transgenic events may have sufficient *barstar* expression for full attenuation. As in the greenhouse study, there was a very strong association between growth and transgene expression (P~ 0.001): the best growing events tended to have the highest *barstar:barnase* ratio (Fig. 9). However, a threshold, where full attenuation might be occurring, was more difficult

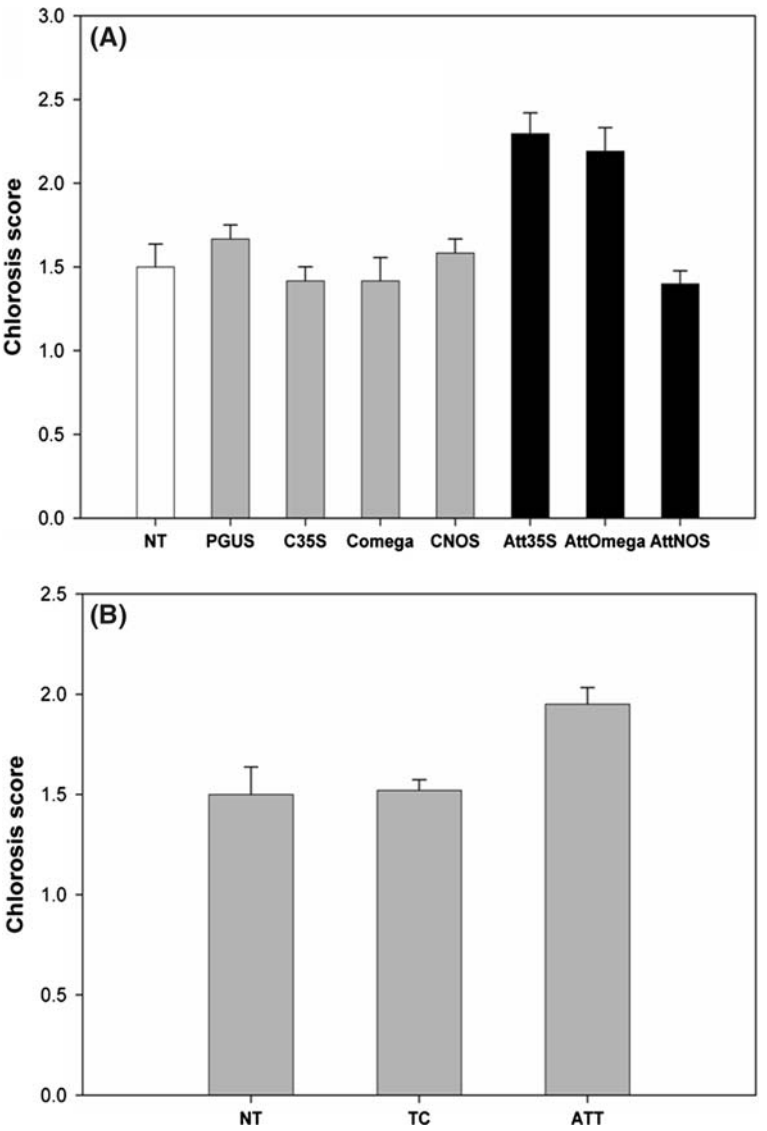

Fig. 7 Chlorosis score of transgenic plants in field trial, where higher scores indicate greater chlorosis. (**A**) Mean chlorosis score of plants harboring attenuated, *barstar* only, and control constructs in field trial. (**B**) Mean chlorosis score of pooled non-transgenic control (NTC), transgenic control (TC, $n = 24$ events), and attenuated plants (ATT, $n = 59$ events) in the field trial. Chlorosis scores were collected only in year two. Bars show one standard error over event means

to identify, and may require a *barstar:barnase* ratio of 8.0 or more.

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 using attenuated and unattenuated constructs. The former exhibited a mean efficiency of 4.2%, while the unattenuated construct failed to give rise to any transgenic events. When the *PTLF* promoter is used to drive *barnase* expression, recovery of transgenic plants *requires* an attenuation system. In addition, the higher mean efficiencies of the control constructs, when compared to the attenuation constructs, suggests that *barnase* inactivation was incomplete during the unorganized growth of callogenesis and shoot differentiation, both of which are part of the regeneration 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

Table 7 Numbers of transgenic events with chlorotic leaves in various groups and construct types

Group	Construct type	No. of events	Chlorosis class		
			1	2	3
NTC[a]	Control	5	1	4	0
TC	PGUS	6	0	6	0
TC	C35S	6	3	3	0
TC	COMEGA	6	3	3	0
TC	CNOS	6	1	5	0
Subtotal (TC)[a]		24	7	17	0
ATT	ATT35S[y]	23	1	11	11
ATT	ATTOMEGA[y]	17	2	7	8
ATT	ATTNOS[x]	20	10	10	0
Subtotal (ATT)[b]		60	13	28	19
	Total	89	21	49	19

Numbers given are the number of events where its trees had the average score type shown. NTC = non-transgenic controls; TC = transgenic controls (*barnase* only or *PTLF::GUS*); ATT = attenuated constructs (*barnase* plus *barstar*). 1 = no chlorosis, 2 = intermediate, 3 = extensive chlorosis (see methods for full description)

[a, b, y, x] Groups or transgenic types with different superscripts were significantly different using Fisher's exact test (P-value < 0.001)

both. In other studies, we have observed that transgenic poplars with a *pTA29*::*barnase* insert were much more difficult to re-transform than other poplars, suggesting a similar lethal effect of *barnase* "leakage" at this stage (J. Li and S.H. Strauss, pers. comm.). Finally, the presence of MAR elements, or the use of the 35S promoter/enhancers that drove the *NPTII* selectable marker gene (and which can affect gene expression up to several kb in both directions: Weigel et al. 2000; Yoo et al. 2005), may have increased the base expression of *barnase* sufficiently to necessitate attenuation. Nevertheless, these results show that our attenuation system can permit a promoter such as that of *PTLF* with a very "leaky," but otherwise desirable, expression pattern to give rise to transgenic plants.

The lack of statistically significant effects of constructs on early vegetative growth was surprising. From analyses using promoter::*GUS* fusions (Wei et al. 2006), 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*, leading to reduced vegetative growth in many events, 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 in the low-vigor class, which had 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 events in that class had a ratio above 1.4, suggesting that *barnase* expression might have been fully atten-

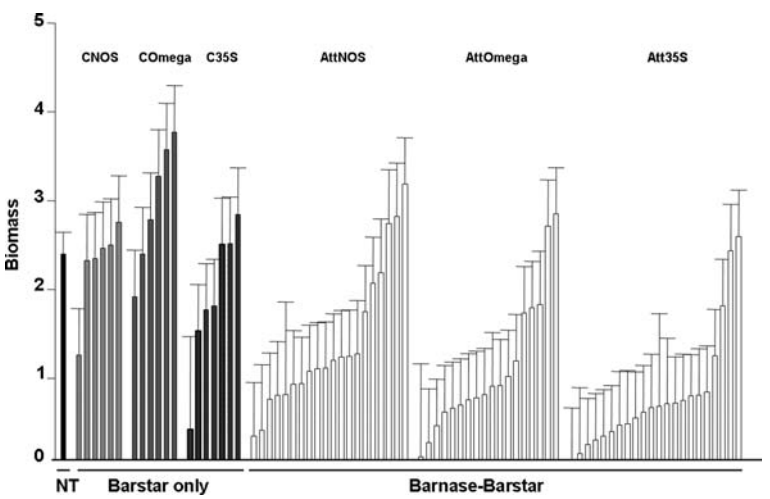

Fig. 8 Mean first-year growth (biomass index, height × diameter^2) by event in field trial. The units on the y axis are biomass indices in cm^3 multiplied by 10^3

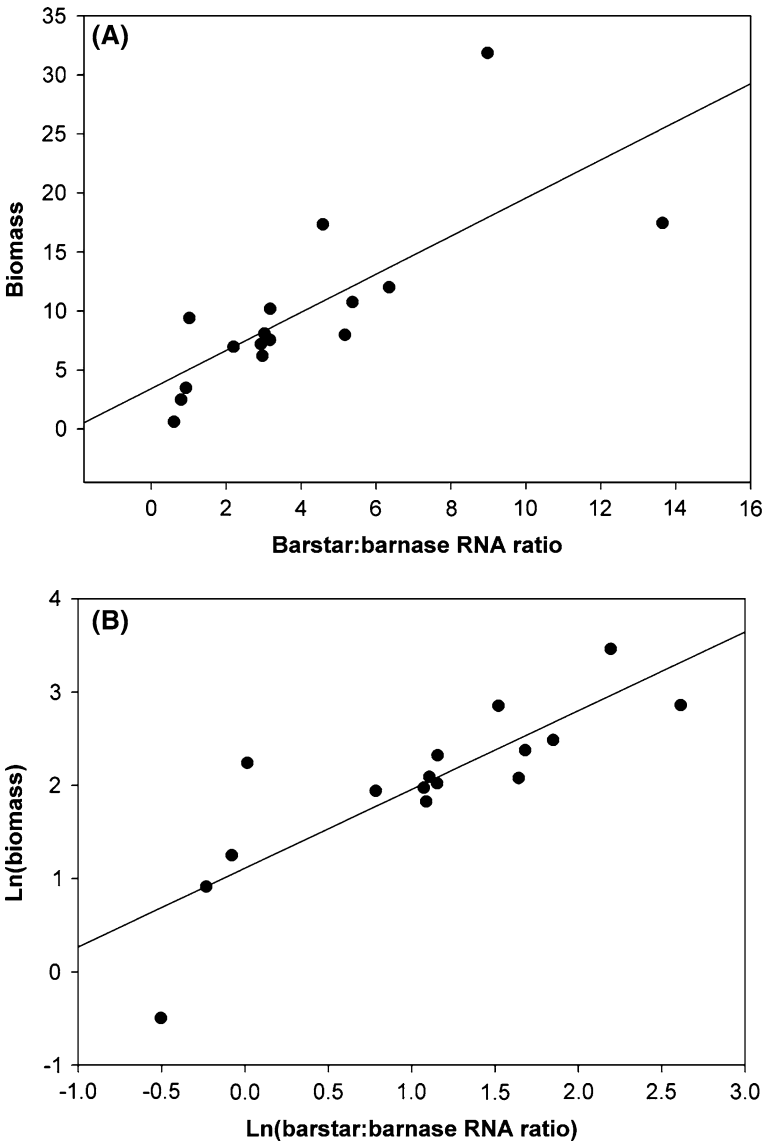

Fig. 9 Linear regressions of biomass index for plants in the field trial after the first year of growth on *barstar:barnase* RNA ratio. (**A**) $y = 1.61x + 3.43$, $R^2 = 0.56$, $P = 0.001$. (**B**) ln(biomass) on ln(*barstar:barnase* RNA ratio): $y = 0.84x + 1.11$, $R^2 = 0.69$, $P < 0.0001$

uated in all events. 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/or at this stage of development.

Surprisingly, in the *35SBP*-P group, which was formed by pooling *Att35S* and *AttOmega*, the mean *barstar:barnase* RNA ratio was well above unity (3.4), even though *barstar* was driven by a weaker promoter than that of *PTLF*, which was used to drive *barnase* (Wei et al. 2006). Moreover, they appeared to be as effective as *AttNOS* at enabling normal early vegetative growth. This could be due to higher stability of *barstar* vs. *barnase* mRNA, or a result of selecting against high *barnase* activity during transformation and regeneration. Another possibility is that the deleterious effects of *barnase* may require time to accumulate to detectable levels. The similar capability for enabling early, 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* activity does not provide further protection in greenhouse-grown plants, nor does it have a deleterious impact on 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 suggested a threshold above which early plant growth and *barstar–barnase* ratio were not correlated. This further supports the existence of a critical threshold for complete attenuation. However, the threshold appeared to be less clear, though obviously several-fold higher, in the field compared to the greenhouse.

The poor attenuation in the field might result from the larger size, greater maturation, and longer time frame of the field study compared to the greenhouse study. Promoter selection is especially difficult for trees where complex developmental phase changes, and increasing size and tissue complexity across years, makes it difficult to comprehensively study promoter activity. Many genes that show floral-predominant expression in poplar show some degree of vegetative expression, and this can vary widely in intensity across an annual cycle of growth and dormancy (O. Shevchenko, S. Strauss, and A.M. Brunner, unpubl. data). Furthermore, field-grown trees are exposed to highly variable abiotic and biotic conditions over their years of growth that can markedly affect gene expression. This could include interactions of transgenes with weed management regimes; a number of trees in the study appeared to show some damage from spot applications of the herbicides used (unpubl. observations)—which may have caused the strong genetic differences in chlorosis we observed. This study reinforces the conventional wisdom in molecular plant breeding that field evaluation of transgenic plants is essential to determine their true value and risk(s). By field testing at this early stage of technology development, we were able to show that new coding or regulatory elements are likely to be needed to establish a robust, reliable system for trees. Had moratoriums on field tests (as have been proposed under the United Nations Convention on Biodiversity), or excessively strict or costly regulations, prevented field evaluations until very late in technology development, many years and hundreds and thousands of dollars would been wasted. Unfortunately, the strict regulations in place in many parts of the world are likely to have produced this very consequence, greatly slowing the development of the biosafety technologies that regulatory guidelines for strong confinement often seek, if they did not prevent technology development entirely (Valenzuela and Strauss 2005).

We chose the *PTLF* promoter, which we knew had significant vegetative expression, and the *barnase* protein, that we knew was highly toxic to plant tissues, to provide a stringent test of the attenuation system concept. There are other cytotoxin genes, as well as other construct promoters, that might prove more effective and have fewer side effects. The stilbene synthase gene provides one possible alternative for pollen sterility (Höfig et al. 2006). However, there is also the risk that less potent cytotoxins would lead to less effective ablation under the variable environments faced by trees, and thus less reliable sterility. Moreover, obtaining regulatory approval may be more difficult if the proteins have not been previously approved for food and/or environmental use, as have *barnase* and *barstar*. Given the level of controversy surrounding transgenic trees in many places, an extremely high level of stable containment and familiarity may be essential for public acceptance. Though not available to us at the time of construct design, there are now a number of genomic-scale databases, including of microarrays, ESTs, and gene traps, to help in selection of new promoters for sterility and attenuation genes from poplar or other organisms. The partial attenuation we obtained in our first generation system suggests that by careful design and field testing, an effective ablation-based sterility system can be developed for trees.

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