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A tapetal ablation transgene induces stable male-sterility and slows field growth in *Populus*

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Abstract The field performance of genetic containment technologies–considered important for certain uses of transgenic trees in forestry–are poorly known. We tested the efficiency of a barnase gene driven by the *TA29* tapetum-dominant promoter for influencing growth rate and inducing male-sterility in a field trial of transgenic hybrid poplar (*Populus tremula* x *P. tremuloides*). When the growth of 18 transgenic insertion events with the sterility transgene were compared to non-transgenic controls after two growing seasons, they grew 40 % more slowly in stem volume, and all but one transgenic event grew significantly more slowly than the control. In contrast, when we compared the growth of transgenic trees containing four kinds of GUS reporter-gene constructs to non-transgenic trees—all of which had been produced using the same transformation method and poplar clone and grown at the same field site—there were no statistically significant differences in growth after three growing seasons. In two years where gross pollen release from catkins was monitored and found to be abundant in the control, no pollen was visible in the transgenic trees; microscopy suggested the cause was tapetal collapse, and revealed the presence of a very few normal sized pollen grains of unknown viability. In two additional years when viable, well-formed pollen was microscopically documented in controls, no pollen could be observed in any transgenic trees. We conclude that this construct resulted in robust and possibly complete male sterility that was stable over four years in the field.

Keywords *Populus***,** barnase, TA29 promoter, pollen, risk assessment, forest biotechnology, genetic containment, genetic engineering

Introduction

Genetically engineered (GE) trees in field trials have shown improved wood quality; faster growth; and markedly improved insect, disease, herbicide, and abiotic-stress resistance (Harfouche et al. 2011; Hinchee et al. 2011). However, regulations and substantial market barriers hinder research progress and commercial applications. A major obstacle to application of GE trees is concern over transgene dispersal in the environment (Strauss et al. 2009a, b). Although most pollen from wind-pollinated woody plants falls close to its point of release, a minority can travel from hundreds of meters to several kilometers. For example, paternity analyses done in two northwestern *Populus trichocarpa p*opulations, one in western Oregon and another in eastern Oregon, revealed that one-third to one-half of the fertilizing pollen originated from beyond 1km and 10 km , respectively (DiFazio et al. 2012; Slavov et al. 2009). When sexually compatible, pollen can fertilize the abundant wild and feral populations of poplars that are common in many temperate-zone regions (James et al. 1998). A minority of seed—abetted by animal-, water-, or storm-associated dispersal—can also travel large distances and establish in the wild. Due to this potential for wide dispersal, and because the possible ecological effects of novel genes in the wild are difficult to predict with confidence, there has been long-term interest in the development of containment methods to prevent or strongly mitigate transgene dispersal.

There are several major genetic containment strategies that have been discussed for forest trees (reviewed in Brunner et al. 2007, Vining et al. 2012). These include fitness reduction, ablation, transgene excision, and floral gene suppression at the RNA or protein levels. Ablation methods have been most widely studied, and in the case of floral sterility rely on cell- or tissue-dominant promoters to drive expression of a cytotoxin gene to destroy tissues essential for gamete development. Previous studies using the pTA29::*BARNASE* construct in alfalfa (*Medicago sativa*, Rosellini et al. 2001), oilseed mustard (*Brassica juncea*, Jagannath et al. 2001), oilseed rape (*Brassica napus*, Mariani et al. 1990), tobacco (*Nicotiana tabacum,* Mariani et al. 1990), and wheat (*Triticum aestivum*, De Block et al. 1997) demonstrated this construct is effective in inducing male-sterility by disrupting the development of tapetal cells. Jagannath et al. (2001) also found that the *Arabidopsis* tapetal promoter A9 was highly effective; 94 % of the transformants with the tobacco TA29 promoter and 87 % of the transformants with the A9 promoter showed stable male sterility, and none of the plants produced seed by selfing (i.e., reverted to being fertile). Mariani et al. (1990) found that 106 out of 115 transformed tobacco events showed stable male sterility. The 14 transgenic lines of wheat studied by De Block et al. (1997) had one to three copies of the barnase gene and all but one showed stable and complete male sterility that was inherited in offspring of a varietal hybrid. The constructs BpMADS1::*BARNASE* (Lemmetyinen et al. 2001, 2004) and BpFULL1::*BARNASE* (Lännenpää et al. 2005) produced male sterility in *Arabidopsis*, tobacco (*Nicotiana tabacum*) (Lännenpää et al. 2005; Lemmetyinen et al. 2001), and silver birch (*Betula pendula*) (Lännenpää et al. 2005; Lemmetyinen et al. 2004). The employed promoters were derived from the birch *BpMADS1*

gene (homologous to the *Arabidopsis* gene *SEPALLATA3,* previously known as *AGL19*), and the birch *BpFRUITFULL-LIKE1* gene. Zhang et al. (2012) showed that a male cone-dominant promoter from Monterey pine (*Pinus radiata*), *PrMC2*, fused to an attenuated version of barnase gave complete and stable male sterility in multi-year field trials of a pine hybrid (*Pinus rigida* x *P. taeda*) and a eucalypt hybrid (*Eucalyptus grandis* x *E. urophylla*).

There have been several reports of vegetative abnormalities when the barnase gene was employed for sterility. In the greenhouse studies of silver birch (Lännenpää et al. 2005; Lemmetyinen et al. 2004), transformants were bushy, short, and grew more weakly than the control. Jagannath et al. (2001), studying *Brassica*, found that constructs with the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter driving the selectable-marker gene showed more vegetative abnormalities than transformants with a weaker promoter driving the selectable-marker gene. They inferred this to be a result of 35S enhancer effects causing vegetative expression of barnase. Thus, it is important to carefully evaluate vegetative growth in transgenic plants containing barnase-based ablation transgenes.

We report that a TA29::*BARNASE* transgene was highly effective at inducing male sterility in poplar, and did so in the field over several years, but it also caused significant growth retardation. These results show that sterility transgenes can be highly effective at mitigating transgene dispersal in poplar, but that further technology development and testing is required to establish methods for imparting female sterility and reducing undesired effects on growth.

Materials and methods

Gene constructs

Sterility: Hybrid poplars were transformed with the binary plasmid pTTM8 provided by Plant Genetic Systems (Gent, Belgium). The vector (described in Li et al. 2007) contained three transgenes within its T-DNA, including *BARNASE*; neomycin phosphotransferase II (*NPTII*), which provides resistance to kanamycin; and *bar* (selectable marker for glufosinate ammonium herbicide resistance). The *BARNASE* gene was derived from *Bacillus amyloliquefaciens* (Mariani et al. 1990) and encodes a ribonuclease.

Reporter: To evaluate whether transformation affected growth rate, we produced a number of transgenic trees that lacked the *BARNASE* gene, and contained only the *β-glucuronidase* (GUS) reporter gene and the selectable marker gene for kanamycin resistance. Between 1998 and 1999, 10 transgenic events were produced via the same *Agrobacterium tumefaciens* transformation methods for each of the four GUS transgene constructs: PTD::*GUS* (abbreviated 3PG), EnACT11::*GUS* (3A11G), EnACT2::*GUS* (3A2G), and En35S::*GUS* (3SG) (Electronic

Supplementary Material (ESM), Table S1). The promoter from the *PTD* gene is expressed in a floral predominant manner (Sheppard et al. 2000; Skinner et al. 2003), while the other three promoters (EnACT11, EnACT2, and En35S) allow for approximately constitutive expression (An et al. 1996, Huang et al. 1997). In brief, constructs En35S::GUS::E9, EnACT11::GUS::E9, and EnACT2::GUS::E9 were generated using pMON10547 as the binary vector backbone (ESM, Table S1). The vector pMON10547 contains two copies of the 35S promoter enhancer region, the 35S promoter basal region, a multicloning site (MCS), and the E9 transcriptional terminator. Construct En35S::GUS::E9 was generated by adding the GUS reporter gene behind the constitutive 35S promoter. Both actin promoters, EnACT11 and EnACT2, were amplified from *Arabidopsis thaliana* ecotype Columbia based on the corresponding actin2 (AT3G18780) and actin11 (AT3G12110) gene sequences, using primers with flanking restriction enzyme adaptor sites. The ACT2 promoter region was amplified using primers ACT2.001 (ESM, Table S2) and ACT2.002 (ESM, Table S2), and the ACT11 promoter region using primers ACT11.001 (ESM, Table S2) and ACT11.002 (ESM, Table S2). Amplicons were subcloned into the PCR cloning vector pCR-TOPO2.1 and confirmed by Sanger sequencing. The cloned promoters were excised and subcloned into the binary vector to replace the 35S basal promoter region (i.e. the -90 fragment of Benfey and Chua 1990) while retaining the upstream double 35S promoter enhancer regions to generate constructs EnACT2::GUS::E9 and EnACT11::GUS::E9. The tandem 35S promoter enhancer regions were thus fused upstream of the respective actin promoter to augment the endogenous constitutive actin regulatory and basal promoter regions. Sanger sequencing was performed to confirm the correct assembly of the respective promoter GUS reporter gene fusion constructs EnACT2::GUS::E9 and EnACT11::GUS::E9.

Micropropagation

Sterile *in vitro* young micro-cuttings (shoot tips and micro-nodes) of male hybrid aspen genotype (INRA 353-38, *P. tremula* × *P. tremuloides*) were propagated on hormone-free, half-strength Murashige and Skoog medium (MS). Plants grew on these media for 4-6 weeks and then micropropagation was repeated three to five times until enough plants for field tests were produced. They were then were transplanted to soil and acclimated in the greenhouse and field prior to planting in field trials.

Transformation

Sterile *in vitro* cultures of genotype 353-38 were used for all transformations. Internodes and leaf discs, in admixture, were used as explants for cocultivation. All transformation and regeneration was essentially as described in Filichkin et al. (2006), using *Agrobacterium tumefaciens* strain C58/pMP90 (GV3101). For the sterility construct, 18 independent transgenic events were generated and each transgenic event was micropropagated to produce three ramets for field testing (except for event 2, which had four). There were nine non-transgenic wild-type controls propagated in the same way. For the reporter gene field study, 10 events were produced

using each construct and multiplied and readied for the field essentially the same as for the sterility field study.

Transgene confirmation

Genomic DNA was isolated from leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. All transgenic plants were confirmed by polymerase chain reaction (PCR) against one or more target genes within the T-DNA. We used non-transgenic, *in vitro* grown trees produced at the same time as negative PCR controls. The *NPTII* and *BARNASE* genes were amplified from the male-sterile trees (ESM, Table S2) and the GUS gene was amplified in the reporter trees (ESM, Table S2); negative controls, using template from a non-transgenic control, were also included during all PCR analyses. For simplicity, genotype designations have been recorded in this manuscript as follows (format is: "original code/manuscript code"): 7/1, 43/2, 15/3, 102/4, 2/5, P/6, 58/7, 29/8, 39/9, 8/10, 38/11, 28/12, 16/13, 40/14, 9/15, 30/16, 41/17, and 63/18.

Field layout

The reporter and sterility field trials were planted adjacent to one another in a field site near the Willamette River in Benton County, OR. The sterility trial was planted in September 1995 and the reporter trial in September 2000; both trials were irrigated in the first and second growing season after planting.

The sterility trial was planted in a randomized block design with three blocks; each block contained a single ramet of each transgenic event (except for block 1 which had two ramets for event 2), and each block had three ramets for each control type. The reporter trial was planted in a completely randomized design with 10 transgenic events for each construct (except 3PG, which had 3 ramets) and two wild-type controls for each construct (except for the 3PG construct, which had 4), for a total 10 control trees. The spacing was 3 m between rows and 1.5 m within rows. As described above, for the reporter trial all transgenic events started with two ramets (except for event 3PG96, which had three). However, by 2003, one event each in 3A2G, 3PG, and 3SG, and one control ramet (out of the 10), was lost due to animal damage or unknown causes. We conducted a one-way ANOVA with constructs (including control as a "construct") as the main effect to test if mean volume index varied among constructs for the years 2001 and 2003. The volume index of the different construct-associated control groups was not significantly different (ESM, Table S3 and Table S4), so all controls were pooled for analysis.

Tree growth measurements

Tree height and/or stem diameter at 1.5 m above ground were measured for both trials. Where height and diameter were both available, they were used to calculate a volume index (height * diameter^2) for each tree. Volume index data for the sterility trial is presented after two growing seasons, approximately at the onset of visible inter-tree competition. Data for the reporter trial is presented for growth after one and three growing seasons; the latter was at the onset of inter-tree competition.

Catkin collection and measurements

Flowering was studied only in the sterility trial, where catkins were collected every year from 2006 to 2009 starting just prior to the onset of pollen release, and then additional catkins were collected for approximately one month or until no new catkins could be seen opening on trees. Prior to the onset of flowering, two out of the three original blocks had to be removed due to other demands for that land. Catkins were placed in coolers with ice immediately after collection. Because the branches bearing catkins were approximately 10 to 30 m above the ground, pole pruners and hydraulic lifts were used to facilitate sampling (Fig. 1). During the collection period in 2009, we also took data on catkin morphology (length, weight, and curvature). To quantify a visible difference in catkin curvature, we measured six visibly representative catkins, one from the control and one from five different transgenic events, during the March 17th collection, using ImageJ freeware (Schneider et al. 2012). The angle made by a line projected from the catkin base with that from the catkin tip was used to approximate curvature.

Pollen dehiscence and assessment

After catkin length and weight were measured, they were placed in a refrigerator until they completed development and began to release pollen. Full pollen release was observed after transfer to Petri dishes kept at room temperature (~20 °C). Catkins were shaken to aid in release of pollen, and the pollen then diluted in water for microscopic quantification. The numbers of well-formed (i.e., normal and uniform size and shape) grains were counted under a dissecting microscope with a hemocytometer (ESM, Figs. S1 and S2). For each sample, seven hemocytometer squares were counted. To estimate pollen viability in 2008 and 2009, we used 2,2,5–triphenyl tetrazolium chloride (TTC) (Cook and Stanley 1960); only control pollen was stained because the transgenic trees did not produce any detectable pollen. Photos of catkins in Petri dishes were taken with an Axio camera model AxioCam ICc 1 and a ZEISS Stemi SV11 Apo stereomicroscope, and the photos were examined using Carl Zeiss Vision/Axiovision software (release 4.8.1, November 2009). Images of catkins from transgenic events and controls releasing pollen in Petri dishes were taken for all the years of study (2006-2009). Images of some catkins were previously published in review papers by Brunner et al. (2007) and Vining et al. (2012), and thus not shown here.

Anther microscopy

To observe anther structure, catkins were placed in a formalin–acetic acid–alcohol (FAA) fixative solution and vacuum infiltrated for 1-2 h, then stored at 4 °C in the dark. For the histological images taken for anthers collected in 2006 and 2009, samples were fixed, dehydrated, embedded in glycol GMA methacrylate plastic, sectioned, and mounted on slides. Sections were stained in 0.5 % Toluidine Blue O in citrate buffer. Photographs were taken using a DFC 290 camera with a Leica DM5000 microscope at both 10X and 25X magnifications, and analyzed using the Leica Application Suite software on the camera. Histology images from 2006 were published previously and not presented here (Brunner et al. 2007; Dalton et al. 2013).

Quantitative analyses

Statistical analyses were conducted using the R statistical computer language and environment version 3.0.1 (R Core Team, 2013) using the R packages lsmeans (Lenth 2013), multcomp (Hothorn et al. 2008), and nlme (Pinheiro et al. 2013). Because of heteroscedasticity observed after inspection of residuals, all models were fit using generalized least squares (Hothorn et al. 2008; Lenth 2013; Pinheiro et al. 2012) with variances allowed to differ among constructs or events. Graphical checks of the residuals then showed that variances were approximately uniform and normal in distribution. For the sterility trial, we conducted a two-way ANOVA with blocks and events (including controls) as main effects to test if mean volume index varied significantly. Following ANOVA, a Dunnett's multiple comparison test was used to compare the mean volume index of each transgenic event against the control. For the reporter trial, we carried out a one-way ANOVA to test if mean volume index varied among constructs or controls for the years 2001 and 2003 separately. We also conducted a one-way ANOVA for each construct to test if mean volume index varied among events within a construct. Following ANOVA, a Dunnett's multiple-comparison test was used to compare the mean volume index of lines from each construct against the control.

In 2009, catkins were collected from events 7, 9, 12, 14, 17, and control trees on March 10th and March 17th to measure their length and weight (no catkins were collected from event 17 on March 10th). During the first collection, the number of catkins collected were 47, 37, 54, 36, and 60, corresponding to events 7, 9, 12, 14, and control, respectively. On the second collection, the number collected were 6, 31, 14, 13, 9, and 35, corresponding to events 7, 9, 12, 14, 17, and control, respectively. Mean length was calculated by averaging the length of all the catkins from a single event or control. A Dunnett's multiple-comparison test was used to compare the mean catkin length of each transgenic event against that of the control for each collection date. Mean weight was determined by averaging the total weight of all the catkins belonging to an event by

the number of catkins. A single-sample Student's t-test was used to compare the mean catkin weight of each transgenic event against that of the control for each collection date.

Results

Slowed early growth of transformants with a barnase transgene

Statistical analysis of early growth in the sterility trial showed that blocks and events were significant sources of variance ($F_{2,46} = 52$, $p < 0.0001$; ESM, Table S5). All of the transgenic events showed lower mean volume indexes than the control (Fig. 2), and all but one of the 18 events was statistically different (smaller) than the control based on Dunnett's test (Fig. 2; ESM, Table S6). On average, the sterility transgenic events cumulatively grew 40 % more slowly than the control trees over the two seasons of the growth trial.

Absence of evidence for growth impairment due to transformation

Because expression of the GUS reporter gene in transgenic plants is widely known to be essentially free of pleiotropic effects on plant phenotypes (e.g., Gilissen et al. 1998), we employed a population of these plants for helping to assess whether the growth loss we observed in our sterility trial could have been due to transformation itself. In the reporter trial, the mean volume indexes of the transgenic constructs were not significantly different from each other or the controls in 2001 (one year of growth) ($F_{4,36} = 0.212$, $p = 0.930$; Fig. 3; ESM, Table S7) or in 2003 (three years of growth) ($F_{4,36} = 0.263$, $p = 0.900$; ESM, Table S8). The lack of differences among constructs was also revealed by Dunnett's test for both 2001 and 2003 (ESM, Tables S9 and S10). Nonetheless, events within construct type were a statistically significantly source of variance (ESM, Tables S11 and S12), likely due to an unusually fast- or slow-growing event (data not shown).

Transgenic catkins showed distinct size and morphology

Measurements of catkin length from two collections in 2009 showed that transgenic catkins were often significantly shorter than control catkins (ESM, Fig. S3; Tables S13 and S14). Measurements of weight on the same catkins showed that, for the first collection, weight was similar between transgenic and control catkins ($p = 0.116$); however, for the second collection, when catkins were more fully mature, the weight of the control catkins was significantly lower than that of the transgenic catkins ($p = 0.009$) (ESM, Fig. S4). When we measured the angle projected from the two tips of the catkin toward one another, the transgenic catkins were distinctly curved ($91° \pm 10.3$), whereas control catkins all appeared perfectly straight (180°) (ESM, Fig. S5; Table S15).

Absence of pollen during visual inspection of transgenic catkins

Based on visual inspection of whole Petri dishes after manual agitation, we found that none of the transgenic trees released significant amounts of pollen during any of the years of study (Fig. 4). After microscopic inspection, control trees released an average of 73,000 and 85,000 pollen grains per catkin in years 2006 and 2007, respectively, whereas only a few possible pollen grains (based on similarity in size and shape to wild type pollen) were observed from transgenic trees in 2006 (Brunner et al. 2007) and 2007 (ESM, Fig. S1). In 2008 and 2009, no pollen, viable or otherwise, was macro- or microscopically detected from the transgenic trees, though it was abundantly produced by the control trees (ESM, Fig. S2). For the years 2008 and 2009, the control trees released approximately 775,000 and 2,700,000 pollen grains per catkin, respectively. The abundant pollen that was produced on the control catkins were also highly visible when whole catkins with mature anthers were examined; the control catkins had swollen anthers, but the anthers of transgenics were shrunken (Fig. 5).

Transgenic anthers showed a collapsed tapetum and absence of pollen

In 2009, we selected catkins from one of the sterile lines (event 12) to study in further detail the structure and morphology of the anther sacs, including the tapetum. Microscopic analyses showed that transgenic anthers had significant developmental abnormalities compared to control anthers (Fig. 6). The tapetum of the transgenic anthers appears to have collapsed and no pollen grains were observed inside the pollen sacs. It was difficult to differentiate the tapetum from the endothecium, but it appears that the tapetum was completely ablated and the endothecium appeared thicker than in wildtype.

Discussion

Based on macroscopic inspection of dehiscing catkins, all of the flowering transgenic trees were male-sterile in all four years of study. In the two years where pollen viability was also determined and analyzed microscopically, only the control produced viable pollen; no pollen was detected from the transgenics. In the review paper by Brunner et al. (2007), preliminary data based on hemocytometer counts were presented that showed extremely little pollen production in 2006; of six events studied, two produced no detectable pollen, and the other four events produced a mean of six pollen grains per catkin, consistent with the very rare grains we observed in 2007 (ESM, Fig. S2). However, viability was not determined in either 2006 or 2007. Thus, it remains unclear if transgenic trees in this study could produce any viable pollen.

Our results showed strong and consistent, but possibly not absolute, male sterility. This finding is similar to several other studies. Lemmetyinen et al. (2004) saw no pollen in transgenic birch. Similarly, no pollen was observed on any transformed tobacco plants (Mariani et al. 1990) nor on transgenic wheat (De Block et al. 1997). Moreover, none of the wheat produced seed from selfing; seed was only produced when cross-pollination was performed. Studying *Brasssica*, Jagannath et al. (2001) found that all of the transformed lines lacked pollen, and none of the plants produced seed by selfing or reverted to being fertile. However, Jagannath et al. (2001) also produced semi-sterile plants and they noted that many of them had both sterile and fertile anthers, the former of which eventually reverted to being fully fertile. Rosellini et al. (2001) noted "traces" of pollen in three out of five transformed alfalfa lines, while two lines showed no pollen at all. The three transgenic pollinating lines were selfed and produced just a few seeds; however, the control produced greater than six-fold more seed than the transgenic lines.

In addition to pollen sterility, we found alterations in catkin morphology in the transgenic trees. The catkins from the transformants were smaller and curved when compared to the control catkins (ESM, Figs. S3 and S5). In one collection, we were surprised to find that the transgenic catkins were heavier than the control catkins (ESM, Fig. S4). We hypothesize that this is because the control catkins were weighed after most of their pollen had been released. The control catkins were significantly longer than the transgenic catkins (ESM, Fig. S3); it is unlikely that pre-dehiscence catkins would also be lighter. Male-sterile *Brassica* transformants also had smaller flowers than controls (Jagannath et al. 2001).

The pollen sacs in our transformants were collapsed, with the tapetum and pollen grains absent. Similar results were reported by Mariani et al. (1990), De Block et al. (1997), and Rosselini et al. (2001) in tobacco, wheat, and alfalfa, respectively. In transgenic alfalfa deterioration of the tapetum was evident during premiotic development (Rosselini et al. 2001).

Because of its potent and nearly indiscriminant degradation of cellular RNA, barnase is toxic in both prokaryotes and eukaryotes (Ulyanova et al. 2011). All of the transgenic trees we studied had inferior growth to the control trees. This growth difference had also been briefly reported earlier in a preliminary report from our laboratory (Skinner et al. 2000). The GUS enzyme is one of the most widely used reporter genes in transgenic plants, partly because it is not toxic to transformed cells (Gilissen et al. 1998, Miki and McHugh 2004). Transgenic GUS plants have shown no growth effects (Gilissen et al. 1998) or pleiotropic changes in gene expression (Ouakfaoui and Miki 2005) when compared to non-transgenic control plants. Moreover, in a randomized greenhouse study Lemmetyinen et al. (2001) saw no significant difference in growth between their non-transgenic control line and a transgenic line containing BpMADS5::*GUS*.

Based on the lack of evidence for growth effects of GUS reporter constructs in the literature, and the current results where GUS transgenic trees did not differ from non-transgenic trees in

volumetric growth, the data implicate the barnase cytotoxin transgene as the probable cause of the slowed early tree growth seen in the male-sterile transgenic poplars. Reduced growth associated with barnase expression was also seen in greenhouse studies of silver birch (Lännenpää et al. 2005; Lemmetyinen et al. 2004). In the study with the *BpFULL1* promoter, one third of their 12 non-flowering lines showed severely reduced growth and small narrow leaves (Lännenpää et al. 2005), and in the study with the *BpMADS1* promoter 38 of the 45 non-flowering transformants studied were weaker, shorter, and showed abnormal dichotomous branching. Vegetative impairment was seen in all *Brassica* transformants (Jagannath et al. 2001) where the CaMV 35S promoter drove expression of the *bar* selectable marker gene in a construct containing the TA29 promoter driving expression of the *barnase* gene. The authors attributed the morphological abnormalities in their transformants to the unintended expression of barnase in vegetative tissues associated with 35S enhancers, as has been reported in other studies (Yoo et al. 2005). Interestingly, in the Jagannath et al. (2001) study no morphological abnormalities were seen in transformants with a 5-kb spacer between the 35S promoter and the *barnase* gene driven by the TA29 promoter. Our barnase construct, however, did not include the 35S promoter. Instead, the nopaline synthase gene promoter (NOSp) drove the *NPTII* gene and the promoter from the ribulose-1,5-bisphosphate carboxylase small subunit (*rbc*S) gene from *Arabidopsis*, atS1A, drove the *bar* gene. Both of these promoters are generally considered to be expressed in numerous tissue types, however, the NOS promoter has shown significantly less expression (at least 30-fold) of marker genes compared to the 35S promoter in both petunia and moss (Horstmann et al. 2004; Sander et al. 1987). The alfalfa *rbc*S promoter also showed much weaker activity than the 35S promoter in young leaves, old leaves, shoot tips, and nodules of alfalfa (Samac et al. 2004). Nonetheless, we believe that the NOSp, together with the effects of "random" transgene integration, were a likely cause of unintended barnase expression in vegetative tissues, and thus growth inhibition. It is also likely that the TA29 promoter has imperfect tissue fidelity, especially in the taxonomically distant dicot *Populus* (it was isolated from tobacco).

The deleterious effects of barnase observed in this study may have been exacerbated by growth in a field environment. No adverse effects on growth were observed in the greenhouse studies of oilseed rape (Mariani et al. 1990), tobacco (Mariani et al. 1990), or wheat (De Block et al. 1997). These plants showed normal height, leaf size, tillering, and/or high vigor based on causal observation. They also found no abnormalities in any of the flowering organs except for the tapetum. In the randomized greenhouse study on *Arabidopsis* and tobacco by Lemmetyinen et al. (2001), there were no differences in growth of transgenic vs. control lines until flowering began. Likewise, Wei et al. (2006) reported normal growth and morphology of greenhouse-grown trees expressing *barnase* under the poplar *LEAFY* promoter, whereas their field-grown transgenic trees had highly abnormal morphology and reduced growth. Surprisingly, this occurred in spite of co-expression of the barnase inhibitor *barstar* (Wei et al. 2006). In contrast, Zhang et al. (2012) did not observe any growth impairment in their field-grown, male-sterile

pines and eucalypts, possibly because of the reduced toxicity of the barnase variant employed. They did not, however, present any data or statistical analysis in support of this observation. These results suggest that barnase toxicity can vary widely depending on species and growth environment, and the need for steps to reduce barnase toxicity due to mis-expression. These could include the use of spacers (Jagannath et al. 2001), separation of subunits among plants following crossing (Burgess et al. 2002; Bihao et al. 2012), or attenuated versions of the protein (Zhang et al. 2012).

The male sterility we observed was expressed over four years in the field. Similarly, Zhang et al. (2012) reported complete male sterility over four years in pine and over two years in eucalypts. Tobacco and silver birch transformed with the BpMADS1::*BARNASE* and the BpFULL1::*BARNASE* constructs were highly sterile for three and two consecutive years, respectively (Lännenpää et al. 2005). Commercial male-sterile *Brassica* has been authorized for use since 1996 in the USA and Canada (CERA 2013). Thus, it appears that barnase expression can be a highly reliable means for generating male sterility.

There remain a number of research needs if barnase technology is to become a general tool for transgene containment in forest trees. These include statistically robust and long-term field tests evaluating the level of sterility and impacts on vegetative growth, and the development of new promoters that are more specific in their expression patterns and/or employ less toxic forms of cytotoxin genes. Female sterility will also be needed for many forest trees due to animal, wind, and water dispersal of seeds. This is particularly true for poplars, which have seeds that are very small and can "float" in air and on water over large distances. To our knowledge, no genes that cause bisexual or female sterility appear to have been field-tested in any plant species.

Field tests are essential for determining the efficacy and stability of transgenic sterility. Unfortunately, the stringent regulation of all forms of direct genetic modification that are in place around the world make even small field trials very difficult, and in many cases impossible, to carry out (Viswanath et al. 2012). The development of robust containment technology would therefore benefit not only from additional laboratory research, but from more discriminating regulatory systems that are based on trait risks and benefits, not on a presumed hazard due to use of recombinant DNA methods (Strauss et al. 2010; Meilan et al. 2012).

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DATA ARCHIVING STATEMENT

Data used in this manuscript will be made publicly available through DRYAD (http://datadryad.org/depositing).

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Figure captions

Fig. 1. Field trial during early growth and catkin collection. a, Photographed in July 1997, two growing seasons after planting. Note the person (~1.8 m) just visible within the trees (center bottom). **b**, the trees in November 1998, after the third growing season after planting. **c**, collecting catkins with a lift while using a pole pruner during March 2009. **d**, collecting catkins by hand with a Swedish ladder and a pole pruner in February 2007.

Fig. 2. Transgenic trees showed reduced growth when compared to non-transgenic control. The heights and diameters of all the trees were measured in fall 1997, two growing seasons after planting. Each bar identifies an individual gene insertion event or control. The brackets represent 95 % confidence intervals. The asterisks indicate whether the volume index of the specific event was significantly different than the control based on a Dunnett's test (three asterisks: $P<0.001$, two asterisks: $P<0.01$, and one asterisk: $P<0.05$; all rounded up). The events with darker bars were also studied for their pollen sterility; cc, cubic centimeters.

Fig. 3. Reporter and non-transgenic trees grew at similar rate. The volume index of each transgenic construct was not significantly different from the controls (all $P > 0.60$, see Tables S7-S8). Brackets represent 95 % confidence intervals. Darker bars show data from 2001 and lighter bars show data from 2003; cc, cubic centimeters.

Fig. 4. Absence of visible pollen release from transgenic catkins. a and **c**, show control catkins with released pollen. **b** and **d**, show transgenic catkins without visible released pollen. **b**, catkins correspond to event 17. **d**, catkins correspond to event 14. **a** and **b**, catkins were collected in 2007. **c** and **d**, catkins were collected in 2009. Images of catkins from 2006 and 2008 were previously published in Brunner et al. (2007) and Vining et al. (2012).

Fig. 5. Transgenic catkins lacked visible pollen. a and **b**, catkin and stamens are from a control tree. **c** and **d**, catkin and stamens are from transgenic event 12. **e** and **f**, event 9. **g** and **h**, event 14. **i** and **j**, transgenic event 7. The black bar in the catkin image **i** corresponds to 1 cm and the black bar in the stamen image **j** corresponds to 1 mm. Photos were taken during March 2009. Histology photos from 2006 can be found in Brunner et al. 2007 and Dalton et al. 2013.

Fig. 6. Collapsed tapetum and absence of pollen in transgenic anther sacs. a and **c**, show control anther sac with pollen. **b** and **d**, anther sac with collapsed tapetum belonging to transgenic event 12. For both control and transgenic, the black scale bar in **b** corresponds to 2.5 mm and the black scale bar in **d** to 1 mm. All images were from collections made during February 2009. E, epidermis; En, endothecium; T, tapetum; PG, pollen grain; PS, pollen sac.

Supplemental figure captions

Fig. S1. Putative pollen observed in 2007. a Although pollen production was extremely low, some of the debris associated with transgenic pollen from transgenic event 12 appeared to have normal shape and size. Two examples of normal size pollen are circled. The bar **a** corresponds to 1 mm and that in **b** (an enlargement of A) corresponds to 0.5 mm.

Fig. S2. Abundant viable pollen from the non-transgenic control in 2009. TTC-stained pollen from the control is shown (see methods). The bar in **a** corresponds to 1 mm and the bar in **b,** an enlargement of **a**, corresponds to 0.5 mm.

Fig. S3. Non-transgenic control trees had longer catkins than transgenic trees in 2009. The brackets represent 95 % confidence intervals. The asterisks indicate whether the event was significantly different than the control for the specific collection date based on a Dunnett's test (three asterisks: $P<0.001$, two asterisks: $P<0.01$, and one asterisk: $P<0.05$; all rounded up).

Fig. S4. Transgenic catkins were heavier than control catkins. A single sample t–test over all transgenic lines compared to the control value showed that catkin weight of the transgenics was significantly higher than that of the control ($P = 0.009$) for the May 17 collection date. The difference was not statistically significant for the March 10 collection date.

Fig. S5. Transgenic catkins were curved and dark in tone (less red). Shown are single representative catkins collected March 17th 2009. Event number is shown below each transgenic catkin.