RNAi suppression of *AGAMOUS* causes sterility without impacts on vegetative development in field-grown poplars

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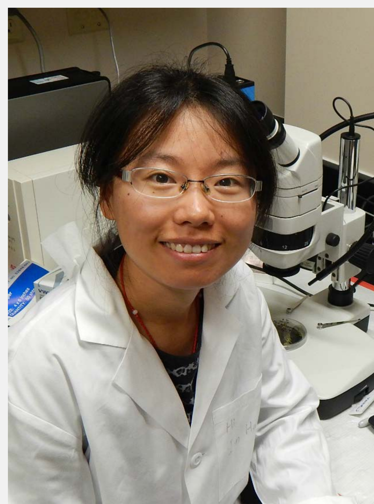


Abstract

Concerns over transgene dispersal have greatly limited field studies and commercial use of genetically engineered (GE) trees. We seek to mitigate such concerns by producing sexual sterility in poplar. Based on gene sequences from *Populus trichocarpa*, we created two RNA interference (RNAi) constructs, PTG and its matrix-attachment-region flanked version MPG, to suppress expression of the two duplicate *AGAMOUS* (*AG*) orthologs in *P. alba* genotype 6K10, an early flowering female clone. A total of 35 transformed events, together with 24 wild-type (WT) control trees, were planted as part of a larger field trial in 2011. We started screening the resulting floral phenotypes in 2014 when the trees initiated flowering; by 2017, 34 transformed events and 19 WT trees had flowered. Six out of 22 PTG events and 11 out of 13 MPG events showed a modified floral phenotype; their floral buds flushed early in the field and the capsules on each catkin often had "carpel-inside-carpel" phenotypes as expected from impairment of AG activity. A complete disruption of ovule and seed production was observed in a number of gene insertion events within both constructs. In all cases, trees appeared normal in their vegetative morphology and growth, and alterations in floral phenotypes were stable over multiple years. RNAi suppression of *AG* appears to be a safe and effective means of genetic containment in poplar.

Two RNAi constructs targeted the duplicate *AG* genes in poplars

Two RNAi constructs, namely PTG and MPG, were created for poplar transformation. Both constructs contained an inverted repeat (IR) of 393 bp of the *P. trichocarpa AG* genes (*PtAG*), which was homologous to several small exons (Fig. 1). The IR was cloned into the pART27 vector under control of the constitutive 35S promoter and terminated by the octapine synthase 3' untranslated region (tOCS) (Fig. 2). The neomycin phosphotransferase gene (nptII), controlled by the constitutive nopaline synthase promoter (pNOS) and terminated by the nopaline synthase 3' untranslated region (tNOS), was also cloned into the vector. Additionally, two matrix attachment regions (MARs) were added to the vector just within the T-DNA borders when creating the MPG construct.

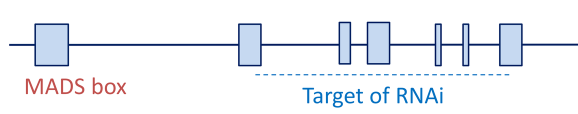


Fig.1. Transcript of the *PtAG* genes. Exons are indicated by boxes. The portion of the cDNA sequence used to make the RNAi constructs is indicated by the blue dashed line. The location of the MADS box is indicated in red.

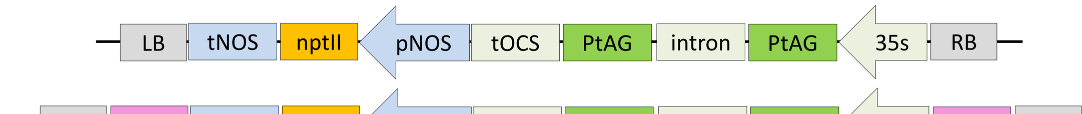


Fig. 2. Schematic diagrams of the two AG-RNAi constructs used, PTG (above) and MPG (bottom). Arrows indicate the direction of transcription. The left- and right-hand T-DNA borders are indicated by LB and RB, respectively.

Transformed poplar trees are in their seventh growing season

The RNAi constructs were transformed into a female clone of white poplar, *P. alba* genotype 6K10 (Fig. 3). Sequence analysis of the *AG* cDNAs from *P. alba and P. trichocarpa* showed very high sequence identity among both of the *AG* paralogs. *PaAG1* and *PtAG1* were 99% identical across their coding sequences and across the region used for the RNAi IR; *PaAG2* and *PtAG2* were 98% identical across their coding sequences and across the region used for the RNAi IR.

A total of 35 transformed events, including 22 PTG events and 13 MPG events (Table 1), were selected for field testing. For each event, four ramets were planted in Corvallis, Oregon in 2011 as part of a nine-acre field trial (Fig. 3c). This field trial included 24 wild-type (WT) 6K10 control trees and employed a randomized block design (RBD) with two blocks. Apart from two PTG trees, all the experimental trees are currently alive and growing well .

We started screening for altered floral phenotypes in 2014 when most of the trees initiated flowering. By 2017, 22 PTG events, 12 MPG events, and 19 WT trees had flowered. Six PTG events and 11 MPG events had modified floral morphology.

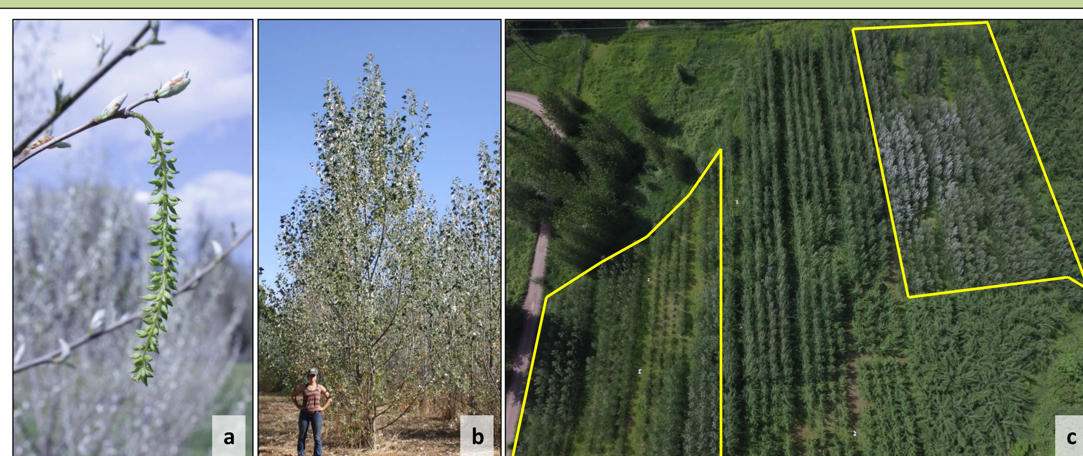


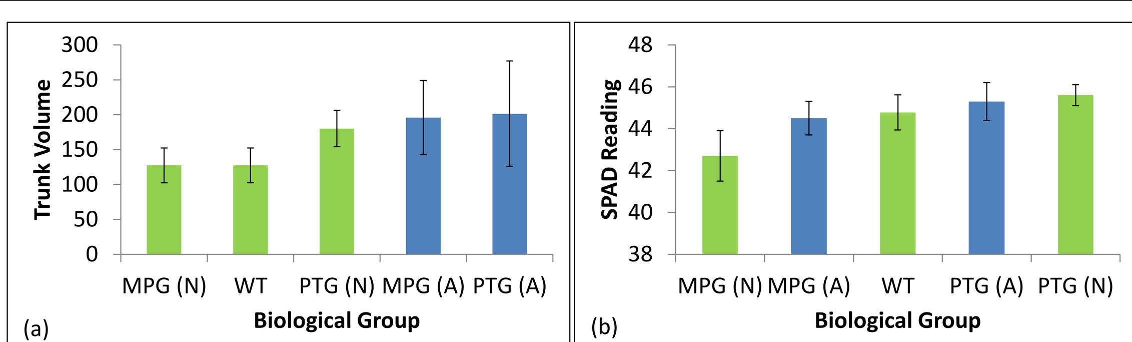
Fig. 3. The clone 6K10 trees in field. (a) Catkin with fully enlarged carpels and emerging leaves (April 2014). (b) Trees in the field (August 2016). (c) Aerial view of the field site and the whole plantation. Two blocks of the clone 6K10 trees are indicated by yellow lines (May 2017).

Table 1 Summary of tree flowering and floral phenotypes by construct

Construct ID	No. of Events Planted/Survived	No. of Events Flowering in 2017	No. of Events with Altered Floral Morphology
PTG	22/22	22	6 (27%)
MPG	13/13	12	11 (92%)
WT	24/24	19	0 (0%)

Transformed poplar trees have normal vegetative growth

To monitor vegetative growth, we measured tree height and DBH (diameter breast height) after each growing season, and calculated trunk volume index (height x diameter^2). We also examined leaf morphology, including chlorophyll content (SPAD meter), dry weight, petiole length and petiole width. In no cases was there statistically significant differences ($p < 0.05$) between altered vs. non-altered events, or between transformed vs. WT trees (Fig. 4).



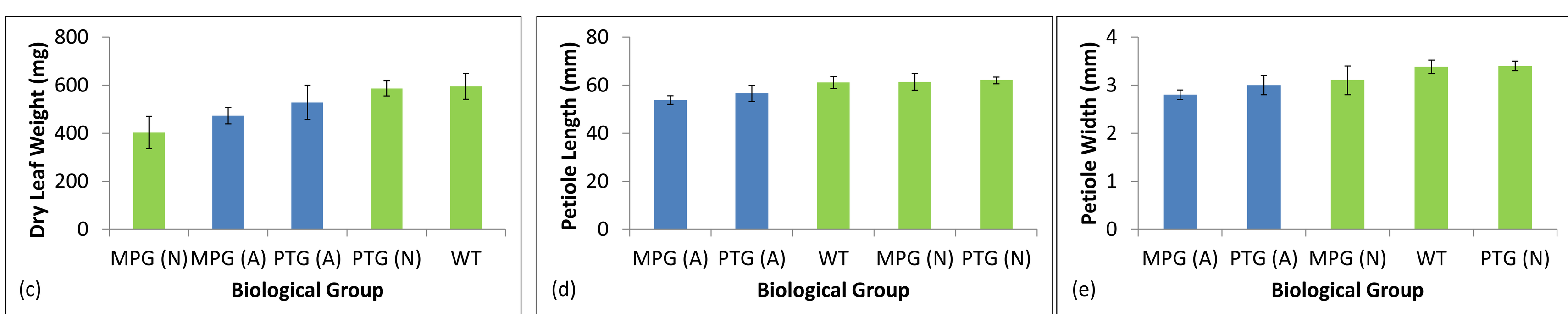


Fig. 4. Vegetative parameters by biological group in 2015. (a) Trunk volume index (calculated as height x diameter^2). (b) Chlorophyll content (measured as SPAD reading). (c) Dry leaf weight. (d) Petiole length. (e) Petiole width. A=altered flowers, N=normal flowers, bars show one standard error of the mean.

Floral buds on PTG and MPG events flushed earlier than control trees

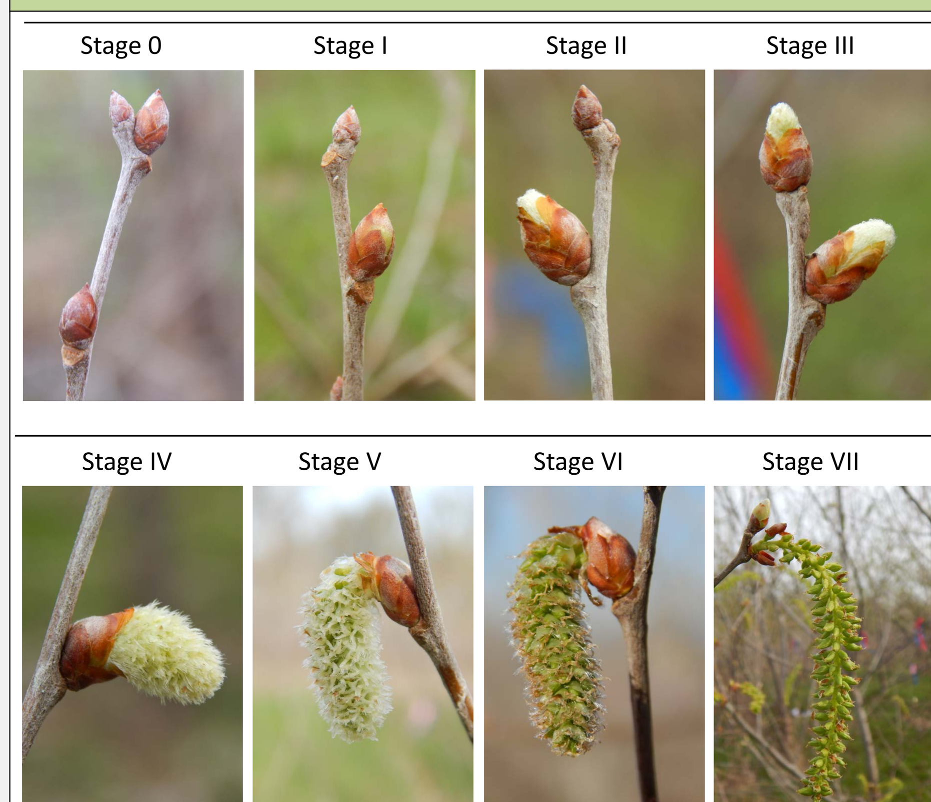


Fig. 5 Scoring system for quantifying floral bud-break. Stage 0, bud is quiescent; Stage I, bud is swelling but still closed; Stage II, catkin starts to emerge bud and is shorter than one bud scale; Stage III, catkin is about equal to the bud scale in length; Stage IV, catkin is longer than the bud scale; Stage V, catkin is three times longer than the bud scale; Stage VI, carpels (if externally visible) start to enlarge and turn green; Stage VII, carpels (if externally visible) are fully enlarged. (Adopted from Klocko et al, 2016, Nature Biotechnology 34:918-922)

We monitored floral bud opening for three consecutive years, from 2015 to 2017, based on an eight-category scoring system (Fig. 5; Klocko et al, 2016). PTG and MPG events with altered floral morphology tended to open earlier than control (Fig. 6).

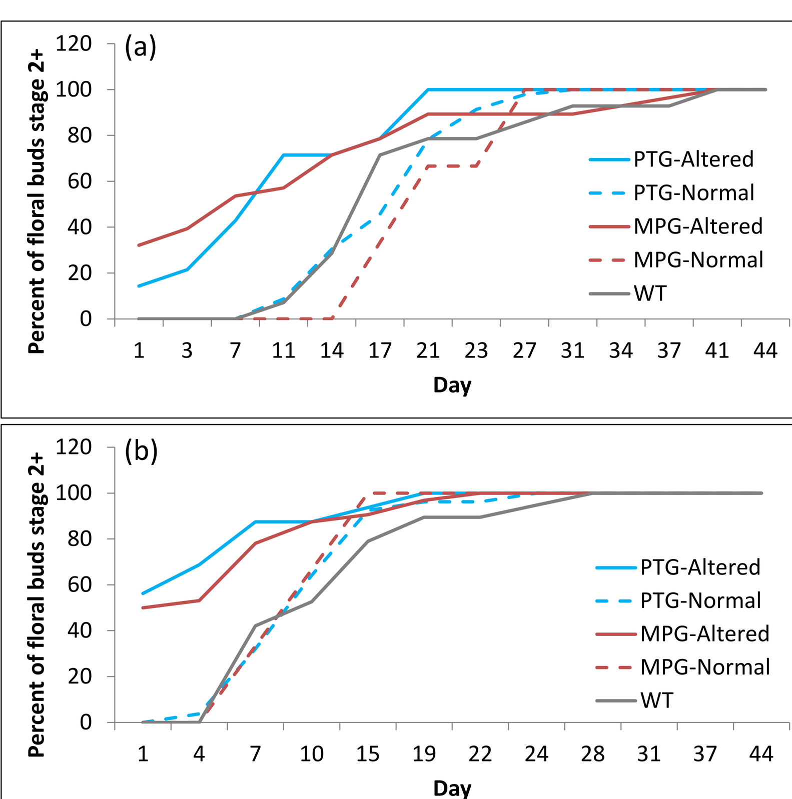


Fig. 6 Floral bud opening over time. (a) 2015 data, collected beginning January 28 (day 1). (a) 2016 data, collected beginning February 2 (day 1).

PTG and MPG constructs produced altered floral phenotypes

Catkins from the WT clone 6K10 usually bear 20 to 40 individual carpels (*i.e.*, female flowers), which has four stigmas (st) and several ovules (ov) (Fig. 6a to 6d) . White "cotton" develops upon carpel/seed maturation. Carpels from the PTG and MPG altered events often had more than four stigmas and showed a "carpel-inside-carpel" (or layered) phenotype (Fig. 6e to 6l); some flowers had anther-looking structures (anth) (Fig. 6j to 6l). All the MPG and PTG flowers dissected had no ovules or pollen in sight.

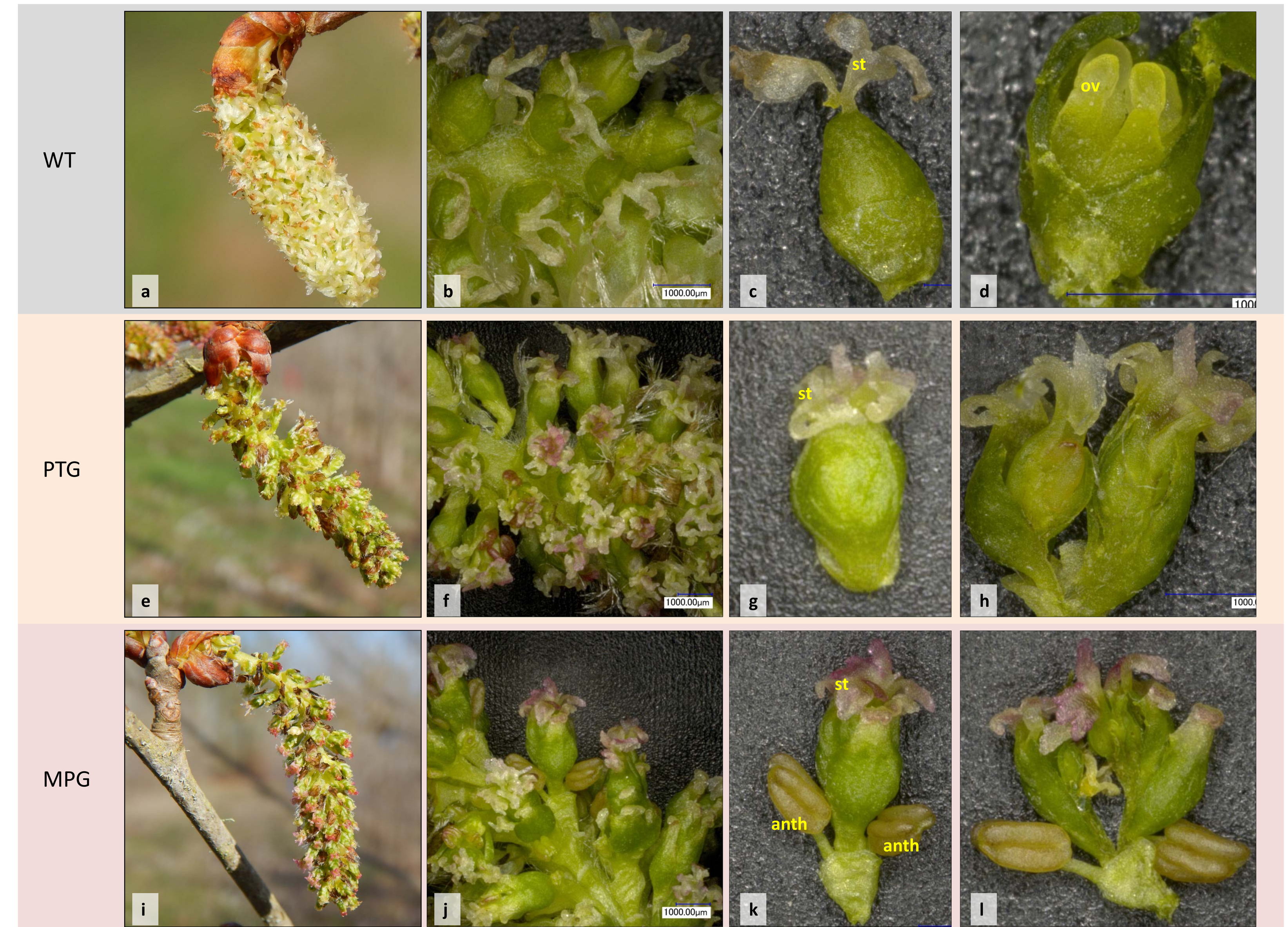


Fig. 6 Catkins and individual capsules for WT and altered events. (a) WT catkin in field. (b) WT catkin under microscope. (c) WT carpel. (d) WT carpel, dissected. (e) PTG catkin in field. (f) PTG catkin under microscope. (g) PTG carpel. (h) PTG carpel, dissected. (i) MPG catkin in field. (j) MPG catkin under microscope. (k) MPG carpel. (l) MPG carpel, dissected. (Images taken in February 2015)

Complete sterility was observed from both constructs

To determine seed viability, we collected mature catkins in early April in 2015, when seed pods just opened and seeds were about to be released. For each event, we dissected 15 to 50 individual seed pods to check for seed production. After recording the number of seeds, we placed seeds on 1% agar where seeds usually germinated within two days. On average, we found 1.2 germinated seeds per seed pod in WT and PTG and MPG events that had normal floral morphology (Fig. 7). Although viable seeds were found in several events (with a rate lower than 0.2 seed/seed pod), most altered PTG and MPG events did not produce viable seeds at all.

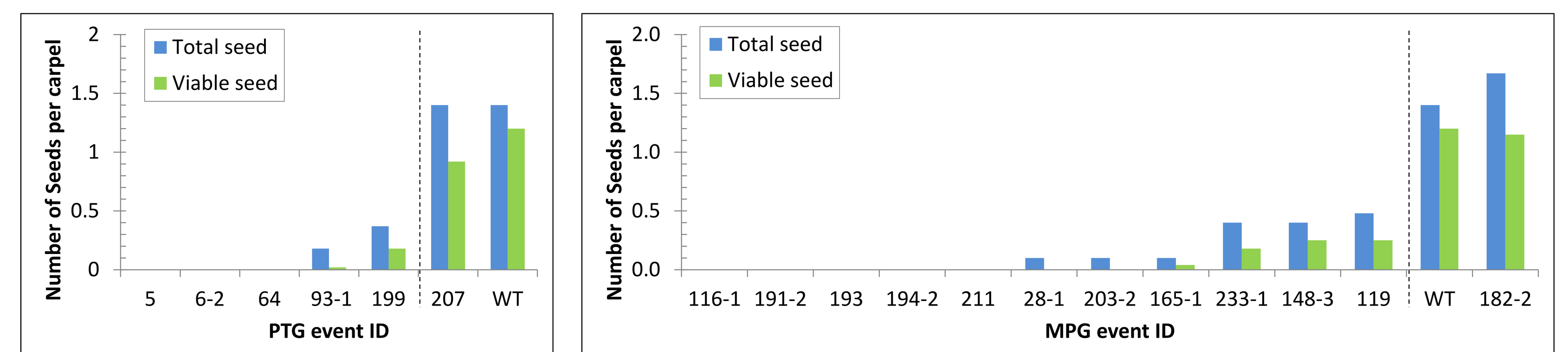


Fig. 7 Seed production and germination by event. Apart from PTG event 207 and MPG event 182-2, all PTG and MPG events had abnormal floral morphology.

Suppression of *AG* expression was found in altered events

We used quantitative real-time PCR (qPCR) to measure the relative expression levels of the *AG* genes in floral buds of each event as compared to two reference genes, *eIF-5A* (eukaryotic initiation factor 5A gene) and *EF1-beta* (elongation factor-1 beta). As predicted, all MPG events with altered floral morphology had reduced expression in both *AG1* and *AG2* (Fig. 8), ranging from 19% to 43% for *AG1* and 24% to 75% for *AG2*, compared with WT controls. Analysis of PTG events is underway.

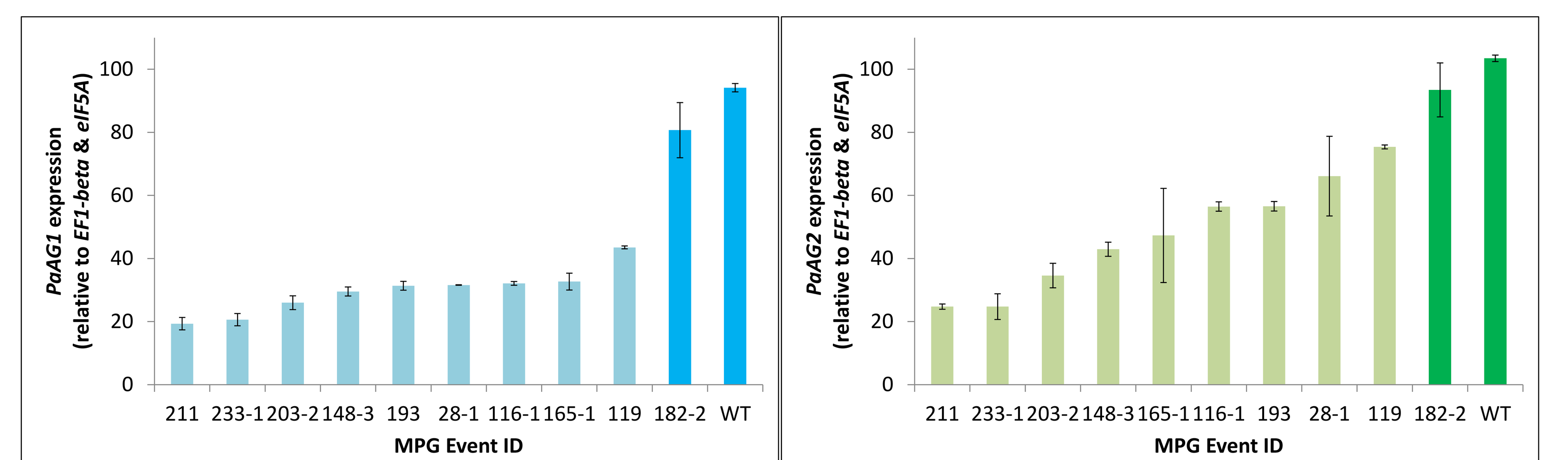


Fig. 8 Relative *AG1* (left) and *AG2* (right) transcript levels in developing floral buds collected November, 2016. Apart from event 182-2, all MPG events had altered floral morphology. Bars denote standard error between biological replicates.

Summary and future work

Summary

- Suppression of *AG* expression can lead to severe alteration of floral morphology in *Populus*
- Suppression of *AG* expression does not appear to affect vegetative growth in *Populus*
- Despite some minor phenotypic variation observed within events and single trees, RNAi-induced phenotypic changes were stable over several years (i.e., strongly altered events were uniformly altered in all branches and trees in each year of study)
- Floral buds on events with altered floral morphology tend to flush early, which might be due to suppression of dormancy-related genes (examinations underway)
- The floral homeotic gene *AG* appears to be a good target for producing sterile trees, at least in female clones

Future work

- Compare gene suppression in the two tested constructs
- Evaluate off-target effects on expression of AG-related MADS-box genes

Acknowledgements

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