Genetic containment of forest trees by RNAi suppression of *LEAFY*

Amy L. Klocko[1], Amy M. Brunner[1], Jian Huang[2], Rick Meilan[1], Haiwei Lu[1], Cathleen Ma[1], Alice Morel[1], Dazhong Zhao[2], Kori Ault[1], Michael Dow[1], Glenn Howe[1], Olga Shevchenko[1], and Steven H. Strauss[1]

[1] Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR 97331, USA Amy.Klocko@oregonstate.edu

[2] Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI 53211, USA

Frozen scientist and lead author conducts fieldwork

Abstract

Gene flow into wild and feral populations of forest trees present a significant barrier to field studies and commercial use of exotic and recombinant DNA-modified trees. Both male and female sexual reproduction are significant concerns in most species. To provide bisexual containment, we used RNA-interference (RNAi) to suppress the poplar *LEAFY* (*PoLFY*) gene, which is essential for development of floral primordia in male and female sexual organs. We transformed this RNAi-*PoLFY* construct into male and female clones of poplar; here we present results from early-flowering female clone 6K10 (*Populus alba*). We obtained 15 independent transformed events in clone 6K10 and examined their floral and vegetative traits over 4 seasons of growth in an APHIS-approved field trial. Trees were planted in 2011 and began flowering in 2014. Floral phenotypes were initially assessed through indoor flushing of dormant floral buds followed by observation of field-opened buds. We found that suppression of *PoLFY* gave rise to complete and stable sexual sterility in the field. Of the 15 RNAi-*PoLFY* events, 2 had extremely small inflorescences that lacked functional sexual organs and had reduced expression of *PoLFY* in developing floral buds. The floral phenotype was repeated over two growing seasons, and the trees showed normal survival, seasonal dormancy, vegetative morphology, and growth rate. Suppression or mutation of the *LFY* gene should greatly facilitate field research, regulatory approval, and public acceptance of exotic and recombinant DNA modified forest trees.

Methods

***RNAi-PoLFY* construct**

We created an RNAi construct based on the *Populus trichocarpa LEAFY* (*PoLFY*) gene. This construct was used to transform an early flowering female clone of *Populus alba* 6K10 (RNAi-*PoLFY*). Analysis of the *LFY* coding sequences from both clones showed that they are 98.15% identical across their coding sequences; and 98.62% across the 285 bp region used to generate the inverted repeat.

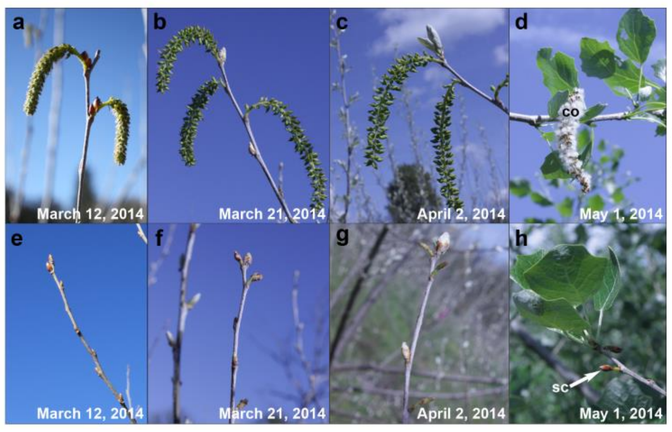
The 6K10 RNAi-*PoLFY* trees were planted in 2011 as a part of a larger field trial including 3 total poplar clones and 23 total sterility constructs. We planted 15 independent 6K10 RNAi-PoLFY events, each represented by 4 genetically identical ramets (trees), along with 24 non-transgenic 6K10 control trees. Trees were screened yearly for the presence of dormant floral buds, which were larger than vegetative buds.

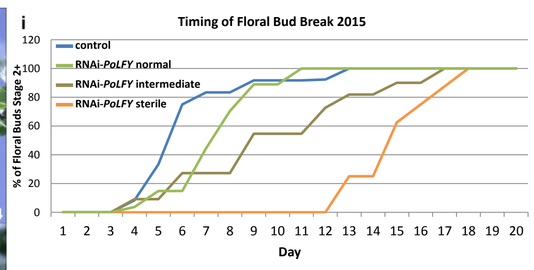
(**a**) Dormant floral buds on clone 6K10, (**b**) 6K10 trees in January 2015, flagging indicates trees with dormant floral buds.

Indoor bud flush identified two events with small catkins

Dormant buds were collected and screened to identify events with altered catkin phenotypes. Samples were imaged at the start of indoor incubation (**a-c**) and after full catkin emergence (**d-f**). (**a**) Buds from control trees, (**b**) buds from RNAi-PoLFY event 194, (**c**) buds from event 139-1. Catkins from (**d**) control and (**e**) 13 of the RNAi-PoLFY events fully flushed in 3 days (event 194 shown). (**f**) Catkins from events 17 and 139-1 fully flushed in 7 days (event 139-1 shown). Scale bar, 5 cm.

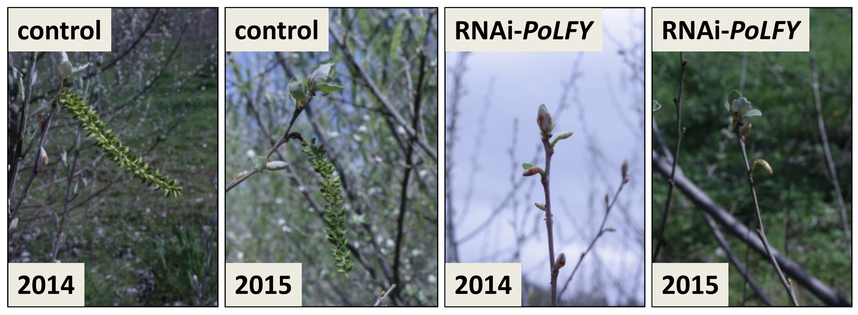
Field-grown RNAi-*PoLFY* events had small, late-opening catkins





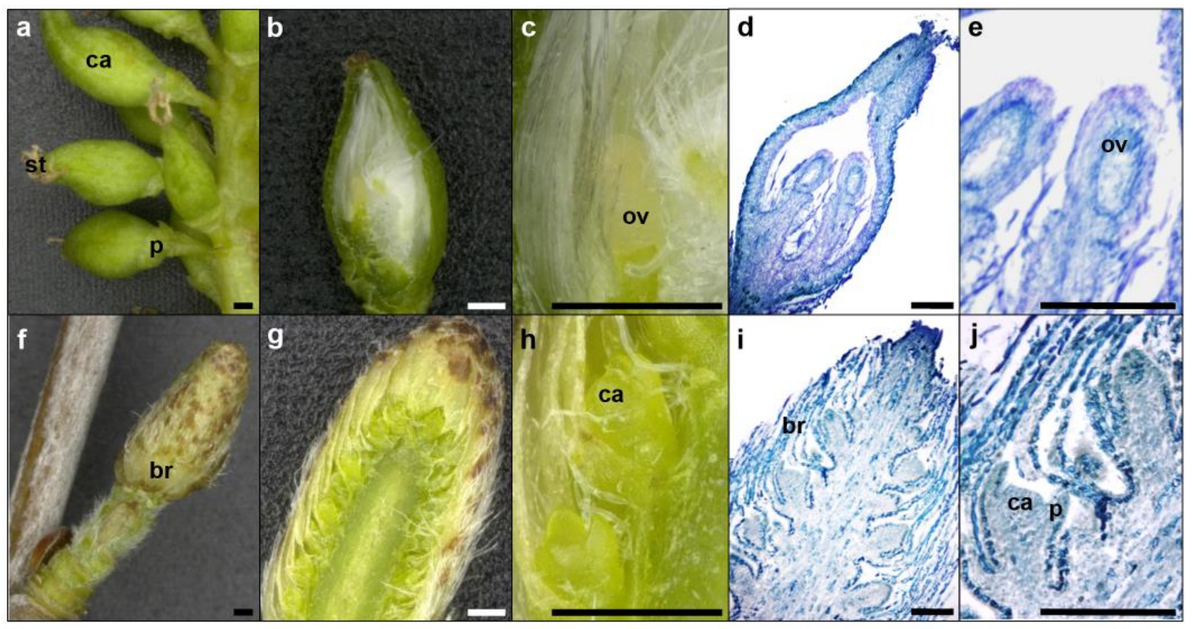
Catkins from (**a-d**) control trees and (**e-h**) RNAi-*PoLFY* trees with sterile catkins were imaged over time in 2014 until control flowers fully matured and shed cotton (co). Small RNAi-*PoLFY* catkins observed on the same date were still encased in bud scales (sc). Catkins were photographed on the dates indicated in the lower right corner. (**i**) Scoring of floral bud opening over time beginning January 28, 2015 (day 0).

Floral phenotypes were stable across two growing seasons



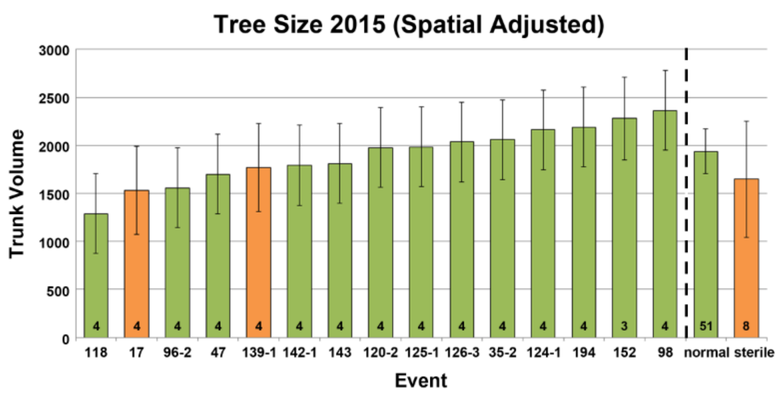
Non-transgenic control trees formed large catkins with prominent carpels in both 2014 and 2015. RNAi-*PoLFY* events had sterile catkins with no externally visible carpels in both 2014 and 2015. Images were taken March 26, 2014, and March 26, 2015. Data show that the floral phenotype was stable across flowering seasons.

RNAi-*PoLFY* catkins were small and lacked stigmas or ovules



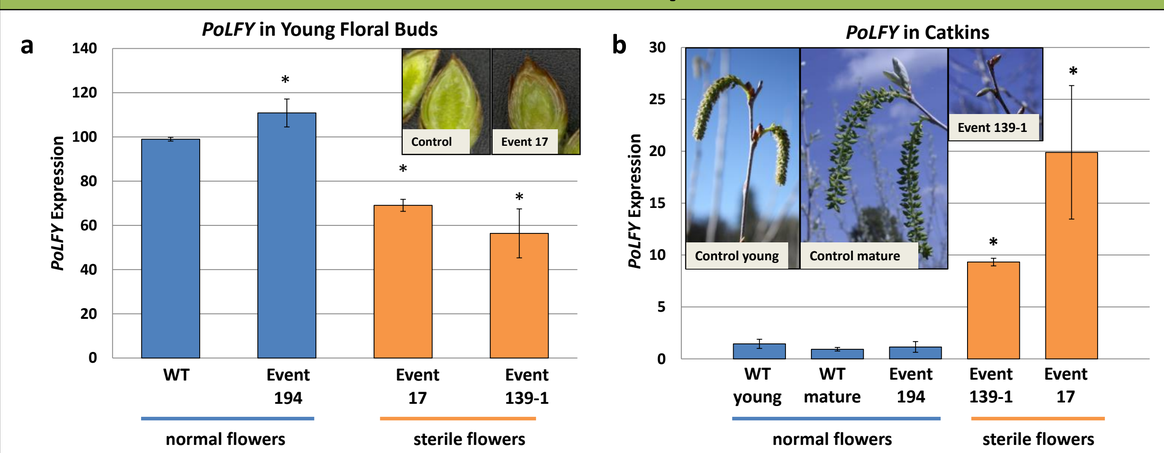
We used microscopy and sectioning to obtain detailed views of catkin morphology for normal and tiny catkins. (**a**) Non-transgenic control catkins with carpels (ca) nested inside perianth cups (p) and topped with stigmas (st). (**b,c**) Hand-sectioned control carpel with cotton fibers and ovules (ov). (**d,e**) Wax sectioning of control carpels with ovules. (**f**) RNAi-*PoLFY* catkins covered by bracts (br). (**g,h**) Hand-sectioned RNAi-*PoLFY* catkins with carpels (ca). (**i,j**) Wax-embedded sections of RNAi-*PoLFY* catkins with carpels and perianth cups (p). Catkins were collected for light microscopy April 9, 2014, control and RNAi-*PoLFY* catkins were collected for sectioning March 14, 2014. Scale bar, 500 µm

RNAi-*PoLFY* trees had normal vegetative growth



We measured tree size at planting and every dormant season. Due to environmental variation across the plantation, tree growth was analysed with and without spatial adjustment. Adjustment decreased the errors of the measurements. Bars show the adjusted average tree size across events of normal flowering RNAi-*PoLFY* (green bars) and RNAi-*PoLFY* trees with sterile catkins (orange bars). Numbers at the bases of bars show the number of trees; error bars show error of the means. Sterile trees were not significantly different in size from normal-flowering trees.

RNAi-*PoLFY* trees had reduced *LFY* expression in floral buds



We used quantitative real-time PCR to measure relative *PoLFY* expression in *P. alba.* (**a**) Analysis of relative *PoLFY* transcript levels in developing floral buds collected October 3, 2014 showed a significantly lower level of *PoLFY* in sterile events than control events. (**b**) Analysis of relative *PoLFY* levels in young catkins collected March 21, 2014 and mature catkins collected April 2, 2014. Sterile RNAi-*PoLFY* catkins were collected March 21, 2014. In these samples, sterile catkins has significantly higher levels of *PoLFY* than control samples. Typically, *LFY* expression is high in developing floral buds and decreases as flowers mature. Perhaps the high level of *LFY* in sterile catkins indicates they were stuck at an earlier developmental stage. Representative images of sampled tissues are shown. Bars show standard error, asterisks indicate significant differences from non-transgenic, control samples (P <0 .05).

Summary

- RNAi against *LFY* was effective for achieving female-sterile flowers
- Floral phenotypes were stable across growing seasons
- RNAi-*PoLFY* trees had normal vegetative phenotypes
- Gene expression levels were associated with floral morphology but were dependent on tissue type and age
- Our results suggest that disruption of *LFY* is a powerful tool for genetic containment of trees

Acknowledgements

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