Efficacy and Ecological Impacts of Transgenic Containment Technologies in Poplar

Estefania Elorriaga, Emily Helliwell, Haiwei Lu, Anna Magnuson, Amy L. Klocko, Kristin Jones, Cathleen Ma,
Matt Betts, and Steven H. Strauss
Department of Forest Ecosystems and Society, Oregon State University, Corvallis OR
Steve.Strauss@oregonstate.edu

Abstract

The dispersal of transgenes from genetically engineered plants presents substantial challenges to biotechnology regulatory bodies. Because forest trees are weakly domesticated, have wild relatives, and pollen or seeds can spread widely, they are especially problematic. However, plantation trees are often vegetatively propagated, making fertile flowers unnecessary for commercial use. Thus, genes that induce complete sterility could provide strong and simple mitigation of dispersal, simplifying regulatory decisions. We are studying the efficacy, stability, and ecological impacts of modified floral developmental genes as tools for mitigating or preventing transgene spread. We established a plantation with three clones of transgenic poplar containing 22 different constructs to modify the expression of poplar orthologs of conserved floral pathway genes, including *LEAFY* (*LFY*), and *AGAMOUS* (*AG*). Some constructs are designed to target two to three genes simultaneously. The overarching hypothesis that we are testing is that suppression or overexpression of selected floral development genes from poplar are useful tools for development of effective, stable containment technologies, and some of them can be modified without adverse effects on vegetative growth.

We screened all trees for the presence of floral buds, as well as alterations in floral morphology. We found that RNAi constructs targeting *LFY* and/or *AG* gave rise to some insertion events with strongly modified flowers, sterile floral phenotypes, and normal vegetative growth. Events with strong phenotypes lacked ovule and seed formation. Growth analysis of the plantation showed that, in general, trees for all constructs, including those targeting *LFY* and *AG*, are growing well, very similar to wild type or transgenic trees without floral modification. Constructs that caused overexpression of the gene for *SHORT VEGETATIVE PHASE* (*SVP*), a known floral suppressor, and dominant negative mutant forms of the *APETALA1* (*AP1*) MADS-box protein, resulted in large (normally reproductively active) trees that didn't flower at all, or had extremely few flowers, indicative of a delay or permanent postponement of floral onset. We will continue to monitor floral morphology and floral abundance for a fifth year.

Based on field results to date, we have selected *LFY* and *AG* for targeting for preliminary studies of CRISPR Cas gene editing. We found CRISPR Cas leads to efficient gene targeting, with an average biallelic knockout rate of 66% in *LFY* and 84% in *AG*. We plan to continue this work through to greenhouse trials and field trials in upcoming years. Finally, we worked with wildlife ecologists and sociologists to conduct a literature review of the impacts of floral modification on biodiversity and public perception in four of the major genera of planted forest trees. The manuscript was published in New Phytologist.

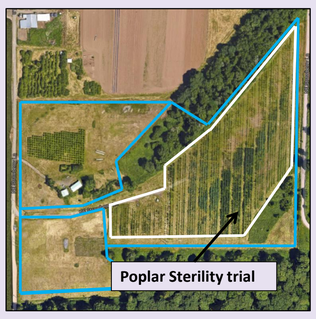
Objectives

1. Analyze the spectrum of vegetative, floral, and capsule morphology
2. Identify constructs which induce modified floral development in at least two events
3. Analyze literature on ecological impacts of containment technologies on biological diversity in tree plantations, and propose needed ecological research and the most desirable forms of genetic containment technology

For constructs of interest, also study:

4. Stability of floral modification and sterility, and associated transgene expression/suppression over years
5. Association of floral expression with vegetative expression within and over years
6. Association of gene expression/suppression with vegetative morphology and rate of biomass growth

Trees growing well in 9-acre plantation



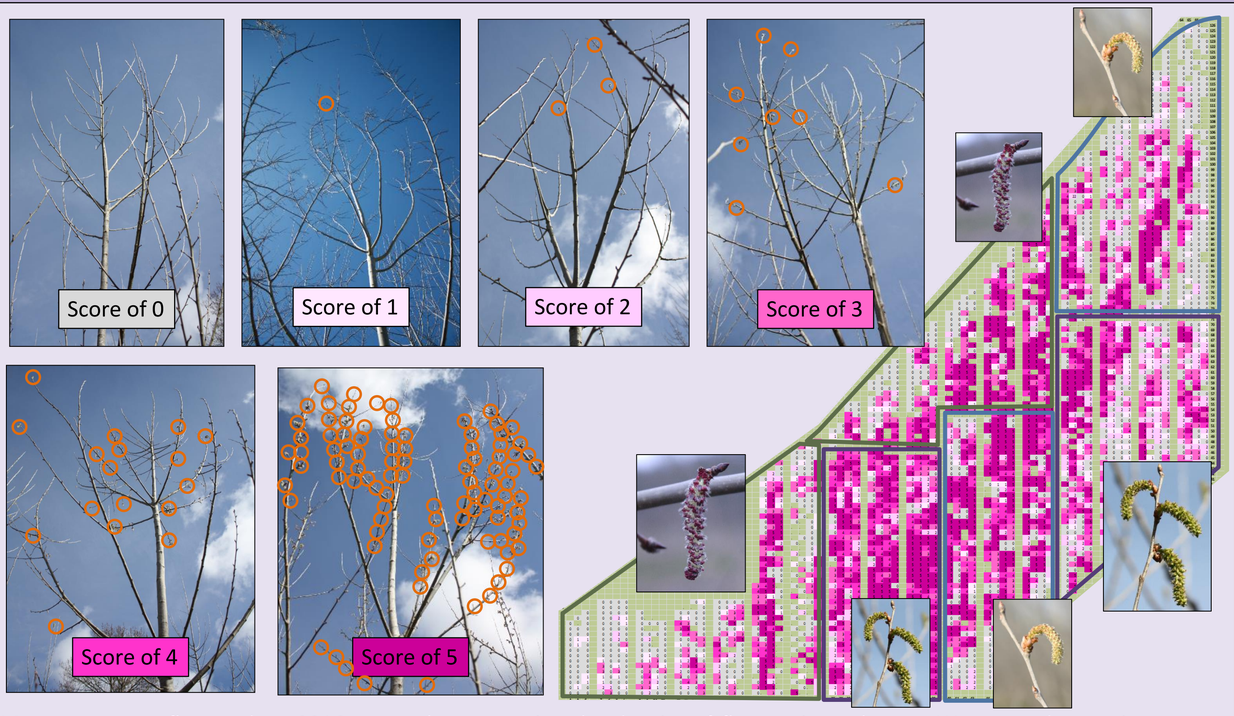
Arial view of the plantation. Fences are indicated by white and blue lines. Tree photos taken on 08/2016.

Male clone 353
P. tremula x tremuloides
1,039 trees

Female clone 717
P. tremula x alba
1,137 trees

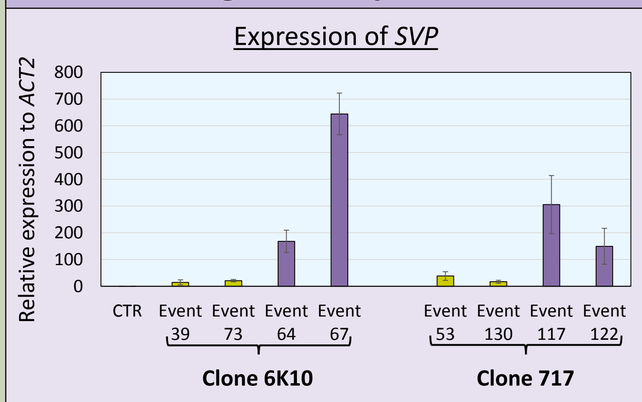
Female clone 6K10
P. Alba
1,238 trees

Flowering abundance varies widely by location



Most constructs flowered on 2016, so we began scoring the density of flowers in each tree. We created a scoring system according to flowering abundance and crown floral arrangement. Over 63% of the trees flowered during 2017, which equated to an increase of almost 6% from the year 2016 (206 trees more). Compared to 2016, we saw an increase in heavy set flowering (score of 5) of 7%, in intermediate flowering (score of 3) of 1%, and a decrease in low-to-moderate (score of 2) of 3%. About 7% (266 trees) were dead in both 2016 and 2017. SVP and AP1 had the most effect on floral abundance.

Trees with high SVP expression have few or no flowers



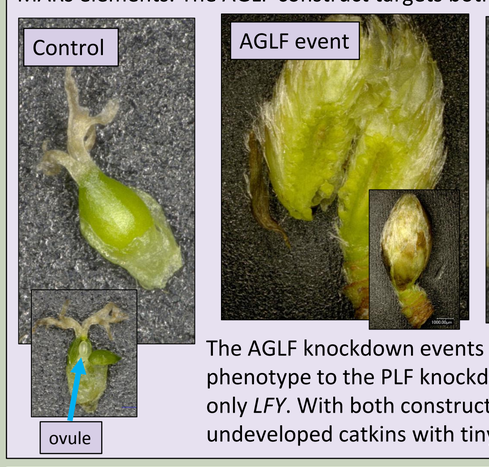
One of the constructs caused overexpression of the *SVP* gene. In wildtype, this gene works to suppress flowering under non-inductive conditions (e.g. short days) by controlling gibberellin expression at the shoot apex. None of the trees containing this construct flowered on 2015. However, they did flower in 2016 but with a range of flowering abundance scores. We hypothesized that the range of floral abundance must be due to the level of overexpression experienced by each particular event. So, we selected four events, two with high floral abundance scores and two with low scores in 6K10 and 717 and we used qPCR to measure the expression of *SVP* in each event. As expected, events with high floral abundance (e.g. 39 in 6K10 and 53 in 717) had low expression of *SVP.* Meanwhile, events with little floral abundance (e.g. 67 in 6K10 and 117 in 717) had high expression of *SVP.*

Photo of a 6K10 SVP event with a flowering abundance score of 0 like that of events 64 and 67. The neighboring tree (unlikely an SVP event) has an abundance score of 5 comparable to SVP events 39 and 73.

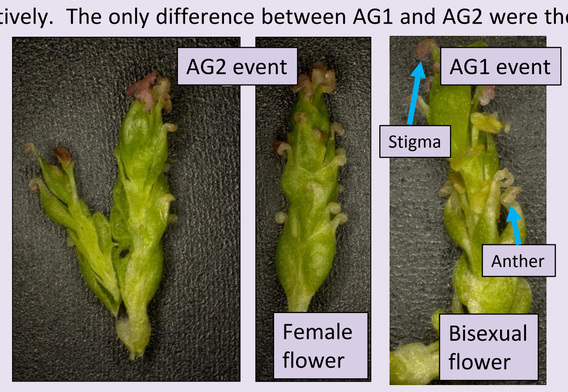
Photo of a 717 SVP event with a flowering abundance score of 0 like SVP events 117 and 122. The neighboring tree (not SVP) has an abundance score of 5 like that of SVP events 53 and 130.

Complete sterility seen in *AG* and *LFY* RNAi knockdowns

There are three constructs targeting both *AG* paralogs using RNAi inverted repeats (IDs: AG1, AG2, and AGLF). The AG2 construct had MARs (matrix attachment regions) elements at the 5' and 3' regions of the inverted repeat. This construct had the highest frequency of altered flowers at 92% (11 of 12 flowering events). The other two constructs had frequencies of 27% (6 of 22) and 10% (1 of 10) corresponding to AG1 and AGLF respectively. The only difference between AG1 and AG2 were the MARs elements. The AGLF construct targets both *AG* and *LFY*.



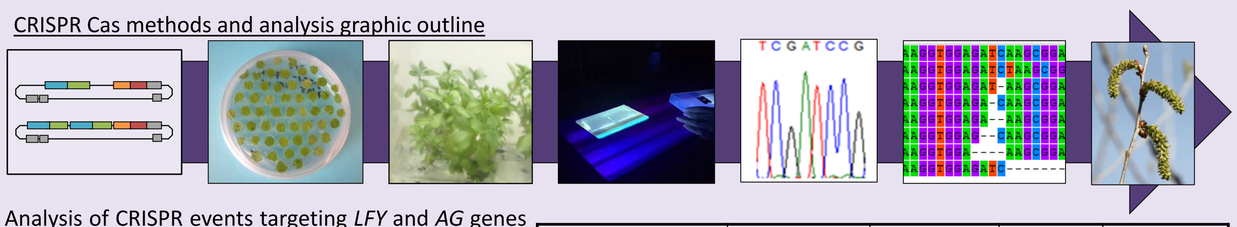
The AGLF knockdown events had a similar phenotype to the PLF knockdowns. PLF targeted only *LFY*. With both constructs we see undeveloped catkins with tiny flower primordia.



Both AG1 and AG2 knockdowns showed this distinct "capsule-inside-capsule" phenotype. Many events had both female and bisexual flowers. Most events with this phenotype had no ovules or pollen in sight.

CRISPR Cas9 nucleases are highly active in two poplar genotypes

CRISPR Cas methods and analysis graphic outline



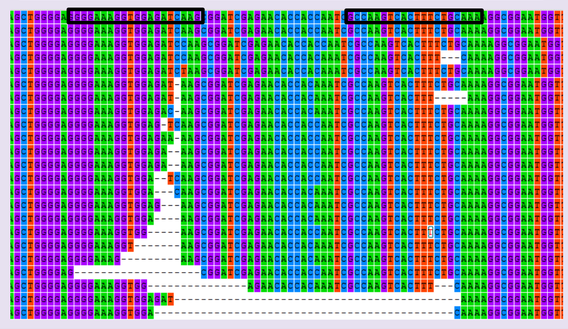
Analysis of CRISPR events targeting *LFY* and *AG* genes in clones 717 and 353 show high frequency rates (average 73%) of biallelic knockouts. The differences in rates are due to the activity of the specific sgRNAs. The ones selected for *AG* were very active. After determining that our sgRNAs were highly active in female clone 717 when targeting *LFY* and both *AG* genes, we chose to transform male clone 353 only with the constructs containing two sgRNAs. In 717, we noted that when both sgRNAs were highly active, the large majority of mutants had large deletions (inversions or small mutations in both sites or only one site were less common).

The alignment and graph below are both from the 353 population containing both sgRNAs matching *AG*. For this population, we sequenced individual alleles for both *AG1* and *AG2* allowing us to analyze mutation frequency and type for each allele of each gene. Black boxes highlight the location of the target sites.

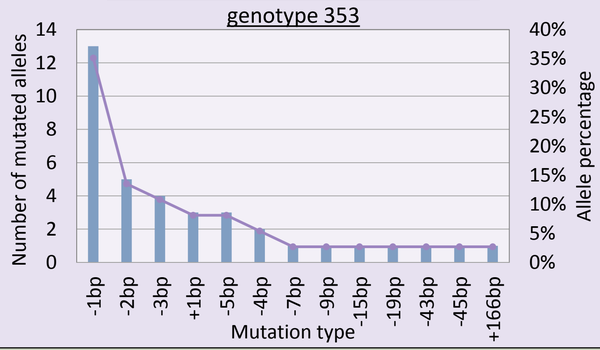
Population	Total events	Mutation	# events	Frequency
LFY-CRISPR 717	256	Biallelic KO	168	65%
		WT	88	35%
LFY-CRISPR 353	38	Biallelic KO	27	71%
		WT	11	29%
AG-CRISPR 717	159	Biallelic KO	133	84%
		WT	26	16%
AG-CRISPR 353	35	Biallelic KO	29	83%
		WT	6	17%
Cas9 control 717	33	Biallelic KO	0	0%
		WT	33	100%
Cas9 control 353	17	Biallelic KO	0	0%
		WT	17	100%
All poplar	**488**	**Biallelic KO**	**357**	**73%**
		WT	**131**	**27%**

Selected 717 and 353 events underwent re-transformation for early floral induction as well as propagation for natural flowering under field conditions. Floral and vegetative morphology will be studied for the selected events. Note the absence of mutation in the Cas9-only control.

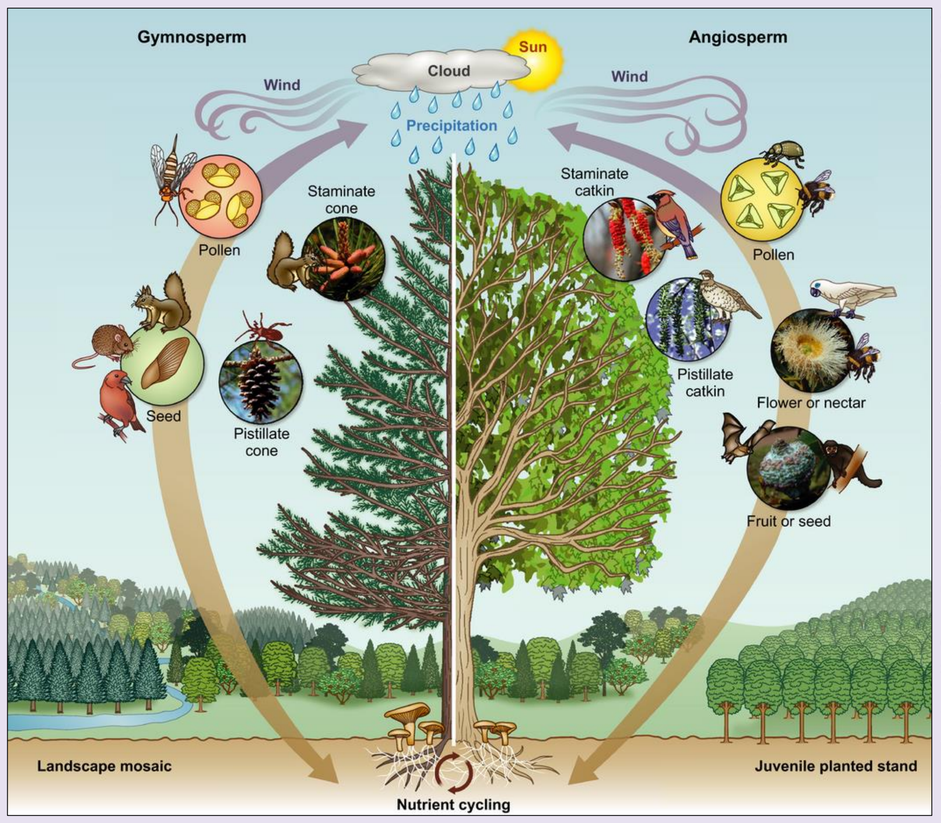
Examples of CRISPR mutations in poplar genotype 353



Frequency of mutated alleles in poplar genotype 353



Transdisciplinary review of reproductive tissue-biodiversity-society interactions



We wrote an invited review evaluating the possible ecological impacts of tree reproductive modifications on biodiversity. We also examined how social factors are expected to affect public, interest group, and government responses to reproductive modifications. We identified the need for field sampling of perturbed and non-perturbed forest tree plantations to understand the use and dependence of local biota on reproductive organs. This knowledge will help inform the design of hypothesis-driven stand and landscape-level experiments, and models of what landscape impacts might be and how they could be mitigated. We also need to understand better what drives public attitudes and perceptions of benefits and risks. Last, field studies of GE reproduction-modified trees are needed that include products of gene editing technologies to assess their impacts and stability. doi:10.1111/nph.14374

Summary

- Trees for all constructs are growing well in our 9 acre experimental plantation.
- RNAi targeting of *LFY* and/or *AG* strongly decreases fertility in the two female clones studied.
- Repressor overexpression and dominant negative protein overexpression delays floral onset.
- CRISPR Cas gene editing is an efficient means for targeting endogenous genes in poplar.
- CRISPR Cas modification of genes is underway and should lead to strong, stable disruption of gene function.
- Literature review of potential impacts to floral modification on biodiversity identified many areas of needed research.

Acknowledgements

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