Efficacy of RNAi and CRISPR Containment Technologies in Poplar

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Project Summary

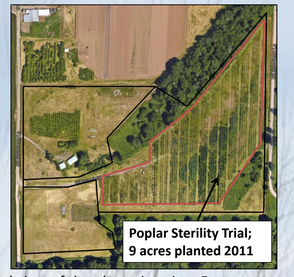
The dispersal of transgenes from genetically engineered poplars presents substantial challenges to biotechnology regulatory bodies. This is because they are weakly domesticated, have wild relatives, and pollen or seeds can spread widely. However, plantation trees are vegetatively propagated, making fertile flowers unnecessary for commercial use. Thus, genes that induce complete sterility could provide strong and simple mitigation of dispersal, simplifying regulatory decisions. Sterility may also lead to increased biomass productivity. We are studying the efficacy, stability, and ecological impacts of floral developmental genes as tools for mitigating or preventing transgene spread. We have two study populations of transgenic poplar trees. The first is a field plantation used to test floral modification by RNA interference **(RNAi)** of conserved floral genes under natural flowering conditions. The second is a laboratory study of the efficiency of direct modification of selected floral genes by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated Cas system (**CRISPR-Cas**) mutagenesis. The field plantation of transgenic *Populus* includes 19 different constructs in three poplar genotypes (2 female, one male) that modify the expression of poplar orthologs of conserved floral development genes, including *LEAFY (LFY)*, *AGAMOUS (AG),* and *APETALA1 (AP1)*. Some constructs are designed to target two to three genes simultaneously. 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* often led to highly modified flowers that appeared sterile, and were reproduced over three growing seasons. Detailed analysis of trees with RNAi targeting *LFY* (Pt-LFY:RNAi) showed that the flowers from some of these events were sterile, and lacked stigmas or ovules. Despite this severe reduction in reproductive growth, Pt-LFY:RNAi trees had robust vegetative growth, and were morphologically similar to control trees. A paper presenting this work is about to be published in Nature Biotechnology (September issue). Based on the results of our field study, we selected the *AG* and *LFY* genes as targets for direct modification by CRISPR-Cas nucleases. Compared to other sterility methods, the mutations that the CRISPR Cas system induces should be highly predictable and permanent, as reversion will be extremely rare or impossible (e.g., with deletions of essential parts of coding regions). We are testing the mutation efficiency of CRISPR-Cas on the single-copy *LFY* gene and the duplicated *AG* gene. We produced six CRISPR and one empty vector control (Cas9 only) construct and produced approximately 120 transgenic shoots per construct. Analysis of 252 *LFY* events showed an overall mutation rate of 88%, with 12% of events showing homozygous loss-of-function mutations. Of 248 events analyzed for changes in the *AG1* gene, 60% had mutations, and 6% of events had homozygous mutations. These high rates of gene targeting means that CRISPR-Cas technology is a very efficient means for altering floral development genes in poplar. Overall, our work demonstrates that suppression of floral development genes is an effective means for genetic containment of poplar, and that these same genes can be efficiently targeted using CRISPR-Cas technology. Pending continued funding of this research, the effects of these mutations on vegetative growth and flowering in male and female trees will be studied in the field in upcoming years, hopefully leading to a ready-to-deploy containment technology.

Field Test of Floral Modification by RNAi of Floral Development Genes

We selected a variety of poplar genes from the floral development pathway (see Table 1). We created 22 constructs targeting floral development genes, both singly and in combination. These were transformed into three poplar clones, one male and two female. We planted 4–25 independent transformation events per construct per clone. On average, we planted 4 ramets (trees) per event. Pairs of ramets were randomized into two blocks per clone. OvExp = over expression, DNM = dominant negative mutation, RNAi = RNA interference.

Table 1: Genes targeted for suppression or modified expression in transgenic poplar trees

Gene name(s)	Location(s) in floral pathway	Poplar gene(s) from Phytozome	Construct Type(s)
FPF1 (FPFL1, FPFL2)	Input from GA pathway	Potri.006G276100, Potri.018G005200	RNAi
AGL20 (SOC1)	Signal integration	Potri.014G074200	RNAi
FT (FT1, FT2)	Signal integration	Potri.010G179700, Potri.008077700	RNAi
AGL24	Signal integration Meristem determination	Potri.002G105600	OvExp, RNAi
LFY	Meristem determination	Potri.015G106900	RNAi
SVP	Meristem determination	Potri.007G010800	OvExp
AP1 (AP1-1, AP1-2)	Meristem determination Floral organ determination	Potri.008G098500, Potri.010G154100	DNM, RNAi
AP3	Floral organ determination	Potri.005G118000	RNAi
AG (AG-1, AG-2)	Floral organ determination	Potri.004G064300, Potri.011G075800	DNM, RNAi



Arial view of the plantation site. Fences are indicated by red and black lines.

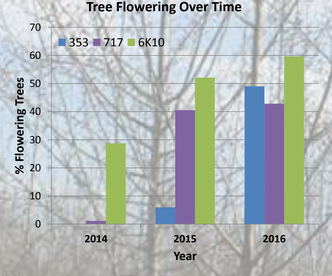
All Poplar Clones Flowered Well in 2016 and Are Growing Well Across Constructs and Clones

The field plantation was established in 2011 as small rooted ramets. All trees were scored for flowering every spring; the first flowers were observed in 2014. Examples of representative flowers for each clone are shown below. In spring 2016 all three clones had flowering rates of 42.8-59.6%.

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

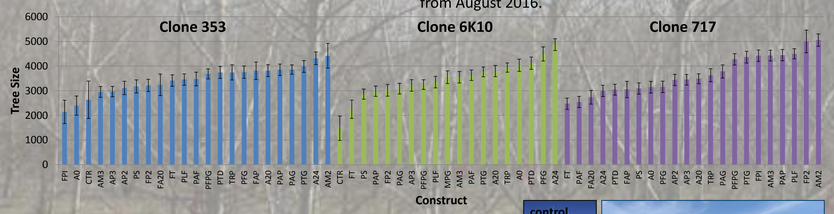
Female clone 717
P. tremula x alba
1,137 trees

Female clone 6K10
P. alba
1,238 trees

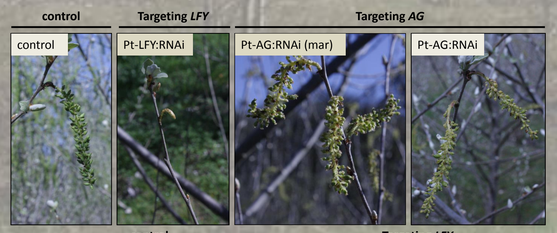


Overall survival for all trees to date was nearly 98%. All three clones are growing vigorously in most parts of the field. Image from August 2016.

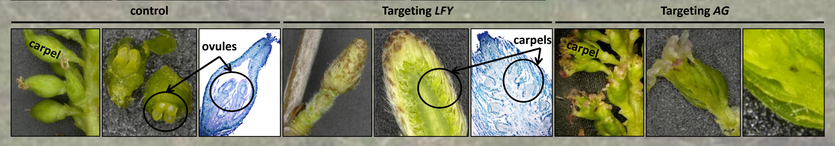
Tree size was measured yearly for all trees in the plantation. Bars show the least square means of trunk diameter after spatial analysis to account for plantation site variability, standard error of the means is shown. Control plants for 6K10 were small at initial planting. Only the RNAi-*FT* construct appears to significantly inhibit growth based on preliminary analyses and field observations.



RNAi of *LFY* and/or *AG* Led to Reductions in Female Fertility



Seven of our RNAi constructs had trees with altered floral morphology (three are shown here). All of these constructs were designed to target *LFY* and/or *AG*, sometimes in combination with other floral genes. Constructs targeting just *LFY* led to catkins with no externally visible carpels. Constructs targeting *AG* led to catkins with replicated carpels within carpels, often missing ovules.



Microscopic analysis of control catkins showed that the carpels contained well-formed ovules, which later developed into seeds. By contrast, catkins with targeted *LFY* were very small, and lacked externally visible carpels; no ovules were detected in these carpels. Catkins with targeted *AG* had carpels formed inside of other carpels, giving them a distinctive replicated appearance. Sectioning of these carpels showed that some events lacked ovules entirely, thus will be fully sterile.

Female–sterile RNAi-*LFY* trees grew as well as control trees (left panels). A new field study will examine if *LFY*-RNAi might affect growth rate. Planting crew in July 2016.

CRISPR-Cas Targeting of *LFY* and *AG* Genes in Poplar

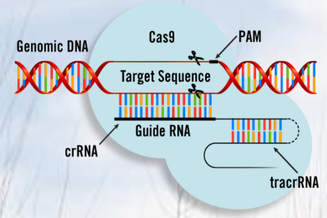
We are utilizing CRISPR-Cas mutagenesis to target the *LFY* and *AG* genes in poplar. *This approach should lead to very strong and non-reversible changes to targeted genes as the physical gene sequence is disrupted, thus giving stable containment*.

This method allows for very precise gene editing as it relies on an exact match between the guide RNA and the target DNA sequence. Matched sequences are cleaved by the Cas9 protein. Repair of the cut DNA can lead to permanent changes in the targeted sequence.

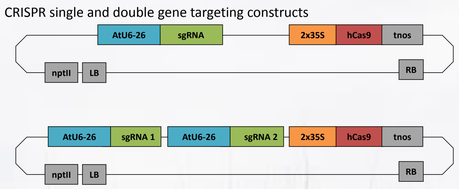
We created CRISPR constructs with one or two guide RNA sequences for each of our targets. A construct with just Cas9 serves as a negative control as we do not expect any DNA changes in the absence of the guide RNAs.

We are targeting two regions of the *LFY* gene (partial gene sequence shown to the right, CRISPR target sites shown in green). As these sites flank the start codon (ATG, in bold), we predict that deletion of this region will lead to a complete loss of LFY function

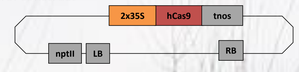
Cas9 (blue shape) interacting with the matched DNA target and guide RNA sequences



CRISPR single and double gene targeting constructs



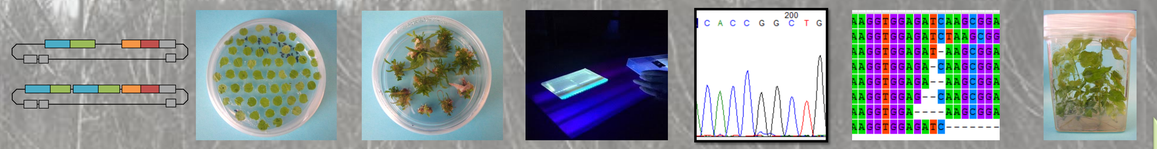
Cas9 control construct



***LFY* target sequences:**

…GACATGCACCAGTGAAA**GATCACAGAGAGAGAGACAA**GGGGGCAGATAGAT**ATG**GATCCGGAGGCTTTCACGGCGAGTTT
GTTCAAATGGGACACGAGAGCAATGGTGCCACATCCTAACCGTCTGCTTGAAATGGT**GCCCCGGCCTCAGCAGCCAG**CGGCTG
CGGCGTTTGCTGTAAGGCCAAGGGAGCTATG…

CRISPR-Cas Mutations Were Detected by DNA Sequencing of Transgenic Plants



Build CRISPR Constructs → Transform Poplar Explants → Grow Transformed Plantlets → Extract DNA, PCR Amplify Targeted DNA Regions → Sequence Target Sites → Determine Mutation Types and Frequencies → Select Events for Rapid Flowering and Field Studies

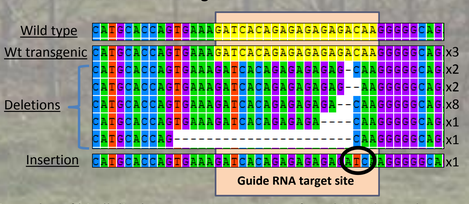
CRISPR-Cas Mutagenesis Efficiently Targeted Poplar Floral Genes

Analysis of CRISPR events targeting *LFY* in clone 717 shows a high rate (12%) of bi-allelic, homozygous gene mutation. Similar results were obtained for targeting of *AG* genes. Selected events are undergoing re-transformation for early floral induction as well as propagation for natural flowering under field conditions. Note the absence of mutation in the Cas9-only control. Transformation of constructs into clones 6K10 and 353 is underway.

Table 2: Results of CRISPR targeting of LFY in clone 717

Construct/target	# events sequenced	Type of mutation	# of events (%)
Single LFY1C	131	Bi-allelic	16 (12%)
		Heterozygous	93 (71%)
		None	22 (17%)
Single LFY3C	46	Bi-allelic	10 (22%)
		Heterozygous	33 (71%)
		None	3 (7%)
Double LFY1C-LFY3C	75	Bi-allelic	4 (5%)
		Heterozygous	66 (88%)
		None	5 (7%)
Cas (empty vector)	14	None	14 (100%)
Total (w/out control)	252	Bi-allelic	30 (12%)
		Heterozygous	192 (76%)
		None	30 (12%)

Biallelic mutations of the *LFY* gene



Examples of bi-allelic, homozygous mutations found in independent events targeted by a single guide RNA in *LFY*. Dashes indicate deleted bases, numbers on the right indicate how many events had a given mutation. Large mutations (not shown) were observed in events transformed with the double guide RNA construct.

Acknowledgements

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Summary

- Trees for all constructs are growing well in our 9 acre experimental plantation, and nearly all constructs should have a majority of trees flowering in 2017.
- RNAi targeting of *LFY* and/or *AG* strongly decreases fertility in two female clones studied to date., and does not appear to negative affect vegetative growth or morphology.
- CRISPR-Cas modification of floral development genes is efficient and should lead to strong and stable disruption of gene function, and thus reliable genetic containment. A field test of growth rate and flowering is planned to begin in 2017.