# High rate of mutagenesis in gene-edited poplars and eucalypts

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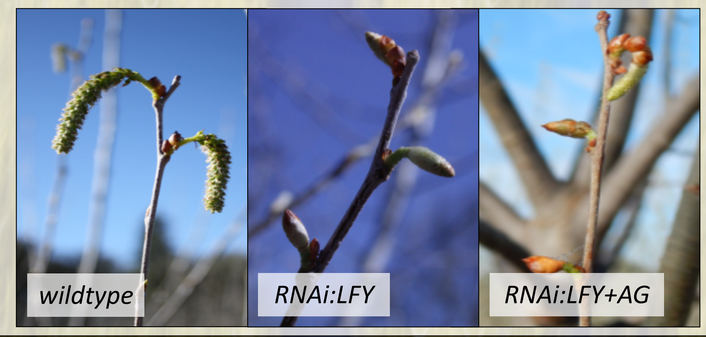
## Abstract

The Strauss laboratory has long been interested in developing robust means for complete sexual containment of GE and exotic plantation trees. We believe this tool will facilitate public and regulatory acceptance, and mitigate unwanted agronomic or ecological impacts. Although a wide variety of methods for inducing sexual sterility have been developed, the reliability of these methods when used on a large scale in the field are uncertain, and complete male and female sexual sterility in the absence of detrimental vegetative effects has not been demonstrated. Gene editing via the CRISPR-Cas system, by permanent mutation of genes essential for male and female reproductive development, has the potential to overcome these limitations. We report high rates of knock-out mutations in the floral genes *LEAFY* and *AGAMOUS* in *Populus* and *Eucalyptus*. Additional experiments are underway to study knockouts of three novel Eucalyptus genes, *TAPETAL DEVELOPMENT AND FUNCTION 1, SYNAPTIC 1 and EMBRYO DEVELOPMENT ARREST 33.* CRISPR-Cas is a powerful means for specific mutation of selected target genes in these widespread and economically important plantation genera.

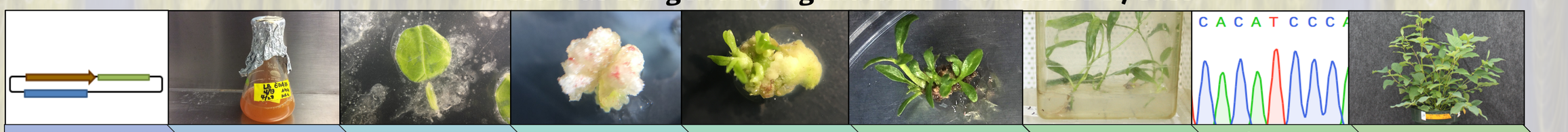
## RNA silencing of *LEAFY* and *AGAMOUS* shows promising phenotypes in plantation-grown poplars

- In *Populus*, silencing of *LEAFY* and *AGAMOUS* by RNAi produces phenotypes with abnormal floral development and infertility, but normal vegetative development.
- RNAi can suppress most gene expression, but genome editing tools such as CRISPR may be needed to disable the gene altogether.

***Flowering poplar in RNAi field trial***



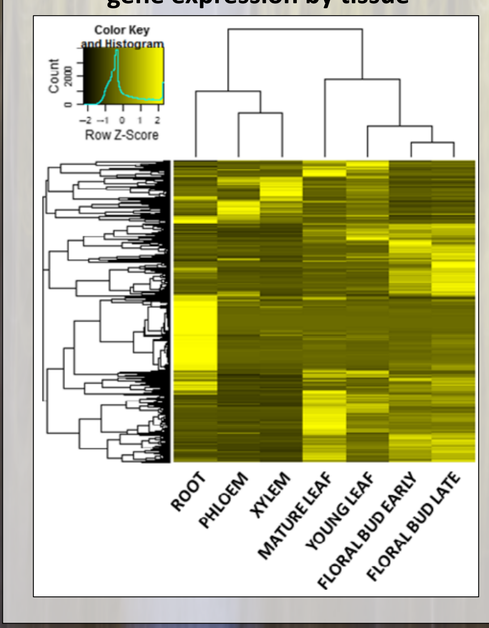

## Methods for generating mutants with CRISPR/Cas9




## *Eucalyptus* transcriptome facilitates target gene identification

- Strauss Laboratory previously published the floral transcriptome of *Eucalyptus grandis (New Phytologist 2014).*
- We compared gene expression data from various tissues and stages of vegetative and floral development and selected candidate genes.
- After building shortlists of gene targets based on expression data, we filtered down to the selected targets by literature review and bioinformatics approaches.

**Heat map of *Eucalyptus* gene expression by tissue**




*Eucalyptus* floral transcriptome
↓
Limit to 87 genes only expressed in flowers
↓
BLAST to find *Arabidopsis* homologs

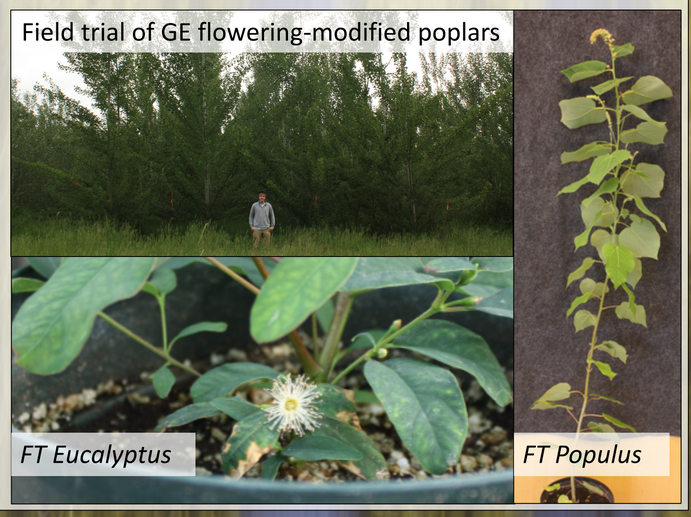
*Arabidopsis* floral transcriptome
↓
Limit to 15 annotated meiosis genes
↓
BLAST to find *Eucalyptus* homologs

↓
Review literature on floral and vegetative effects of mutations in *Arabidopsis*
↓
Compare functional domains of *Arabidopsis* and *Eucalyptus* homologs and predict functional conservation using SMART
↓
BLAST and limit to genes that do not appear to be redundant in *Eucalyptus*

**Selected three new eucalypt gene targets for transformation**

## *FT*-accelerated flowering speeds floral assessment in poplars and eucalypts, non-FT field trials beginning

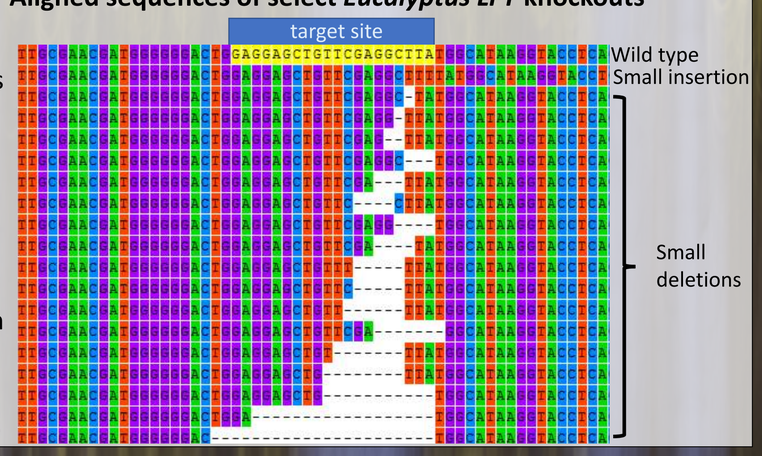
- Ectopic expression of *FLOWERING LOCUS T* can reduce the time needed for plants for flower from years to months.
- Useful for studying mutants of flowering genes in tree species that take years to reach sexual maturity
- We have also transformed non-FT backgrounds for field trials to assess vegetative impacts and infertility under normal development.




## Small and large indels are common mutations

- CRISPR targets were analyzed in transgenic plants by Sanger sequencing of both gene alleles.
- A wide variety of large and small deletions, as well as insertions and inversions, were observed.
- Cleavage by dual sgRNAs can produce large deletions that span tandem targets, improving odds of knockout.

**Aligned sequences of select *Eucalyptus LFY* knockouts**




## Bioinformatics tools aid design of sgRNAs

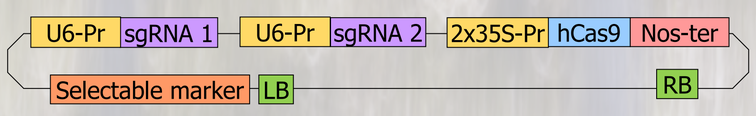
**CRISPR-Direct**
- Generate list of sgRNAs without off-target matches
- Spots thymine tetramers, sequences with poor mutagenesis

**sgRNA scorer 2.0**
- Rank sgRNAs by predicted on-target mutation efficiencies

**BLAST**
- Basic Local Alignment Search Tool finds off-target sites
- Partially redundant with CRISPR-Direct

## Dual target CRISPR/Cas9 transformation constructs cloned

- For each target gene, most transformation constructs were built with two target guide RNAs (sgRNAs) aimed at different sequences.




## High mutation rates in poplars, eucalypts

- Allele-specific natural SNPs were utilized to ensure both alleles amplified.
- Biallelic knock-outs (KOs) of *LFY* and *AG* were determined by PCR amplification followed by bacterial cloning and sequencing, or by using allele-specific PCR primers followed by sequencing.
- Biallelic KO rates varied from 65 to nearly 100%.
- Field and greenhouse trials to begin in 2017

| Population | | Total events | Mutation | # events | frequency |
|---|---|---|---|---|---|
| ***Populus*** | LFY-CRISPR 717 | 256 | Biallelic KO | 168 | 0.65 |
| | | | WT | 88 | 0.35 |
| | LFY-CRISPR 353 | 38 | Biallelic KO | 27 | 0.71 |
| | | | WT | 11 | 0.29 |
| | AG-CRISPR 717 | 159 | Biallelic KO | 133 | 0.84 |
| | | | WT | 26 | 0.16 |
| | AG-CRISPR 353 | 35 | Biallelic KO | 29 | 0.83 |
| | | | WT | 6 | 0.17 |
| ***Eucalyptus*** | FT LFY-CRISPR | 60 | Biallelic KO | 58 | 0.97 |
| | | | WT | 2 | 0.03 |
| | SP7 LFY-CRISPR | 10 | Biallelic KO | 10 | 1.00 |
| | | | WT | 0 | 0 |

## Knockouts of target genes are expected to be infertile

| Ortholog of gene target | Expected phenotype |
|---|---|
| *AGAMOUS* | Indeterminate floral meristem, carpels, stamens |
| *LEAFY* | Indeterminate floral meristem |
| *TAPETAL DEVELOPMENT AND FUNCTION 1* | Indeterminate tapetum, inability to provide nutrients to pollen; starvation of pollen cells |
| *SYNAPTIC 1* | Failure of homologous chromatids to align on metaphase plate; anaphase unable to begin |
| *EMBRYO DEVELOPMENT AND ARREST 33* | Failure of valve margin of ovule to develop |

## Acknowledgements

We thank the members of the Tree Biosafety and Genomics Research Cooperative (TBGRC) at OSU, the United States Department of Agriculture (USDA award 2011-68005-30407, System For Advanced Biofuels Production From Woody Biomass In The Pacific Northwest), the USDA Biotechnology Risk Assessment (grants 2011-68005-30407 and 2010-33522-21736, the NSF I/UCRC Center for Advanced Forestry (grant 0736283), the USDA-IFAS (grant OREZ-FS-671-R), and the J. Frank Schmidt Charitable Foundation. We thank Dr. Jian-Kang Zhu at Purdue University and Dr. Yanfei Mao at Shanghai Center for Plant Stress Biology for providing CRISPR-Cas9 backbones. We thank Sarah Robertson, Clark Embleton, and Analeslie Martinez and Melissa Meyhoff for their work in the laboratory.