CRISPR/Cas9 mutagenesis for genetic containment of forest trees

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Background

- Introduction of transgenic forest trees for field research or commercial use is a challenge due to potential outcrossing to wild and feral populations
- These challenges could be ameliorated through genetic containment of transgenic trees by induction of bisexual sterility in these asexually propagated perennial crops
- Gene editing technologies such as CRISPR/ Cas9 have the potential to rapidly generate novel genotypes which are unable to form floral organs, viable pollen, or embryos

Objectives

- Investigate efficacy and stability of modified floral developmental genes as tools for mitigating or preventing transgene spread using CRISPR/Cas9
- Study the frequency of off-target mutagenesis in CRISPR/ Cas9 transgenics
- Develop site-specific excision systems for somatic removal of CRISPR/Cas9

Research Status

- Selected five genes that are essential to normal reproductive development (*AG*, *LFY*, *EDA33*, *REC8*, *TDF1, and HAP2*)
- Re-transformed *FT* overexpressing eucalypts with CRISPR/Cas9 to speed fertility analysis
- Performed greenhouse studies to assess fertility (*FT* events) and growth/morphology
- Established poplar field trials near Corvallis, OR; planted in the fall of 2017, with WT, edited predicted KOs/Hets, Cas9-only transgenics, and unedited but transgenic trees in 353 (male *P. tremula x tremuloides*) and 717 (female *P. tremula x alba*) backgrounds, with 80 total trees per genotype
- Developing methods for somatic removal of gene editing components using site-specific recombinases: we are building vectors compatible with existing gene editing tools and are still in the cloning phase and testing suitable promoters
- Will be detecting if any off-target mutations were generated during the editing process; we are using a targeted (bait-based) sequencing approach that will begin in July 2019

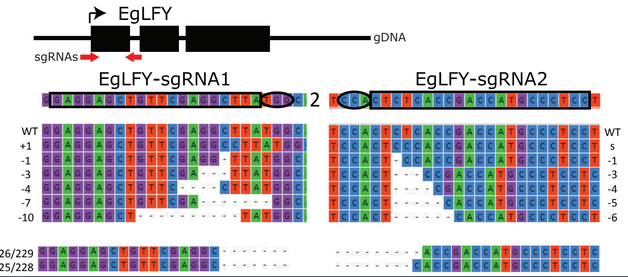
Successful generation of flowering gene knockouts in poplar and eucalypts

Homologs of characterized genes involved in sexual development were targeted for editing

Gene	Function	Predicted Phenotype
AG	Stamen and carpel development	Bisexual sterility
LFY	Transition to flowering	Bisexual sterility
REC8	Chromosome structure during meiosis	Bisexual sterility
EDA33	Seed pod valve development	Female sterility
TDF1	Tapetal development	Male sterility
HAP2	Pollen tube guidance	Male sterility

Leaf and stem explants from poplar and eucalypts were transformed with constructs targeting the above loci, to obtain 396 and 198 number of transgenic events, respectively. Transformation rates averaged 16.2% in poplars and 2.23% in eucalypts. High rates of editing amongst the transgenic events were obtained. The lowest rate was 66% and the highest rates were at 100%.

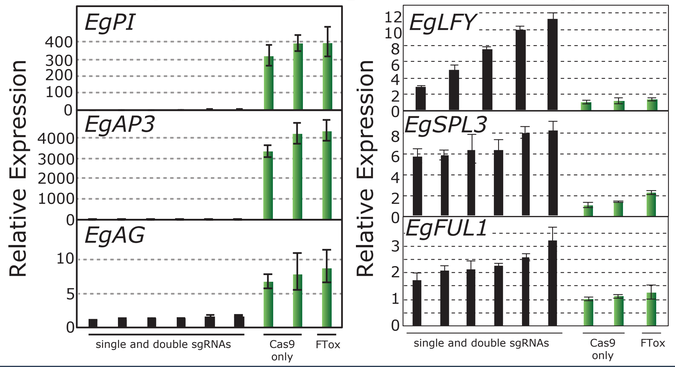
Typical mutations observed are short indels and large deletions



leafy mutants in *Eucalyptus* fail to form floral meristems

Developmental sequence of flower formation in *Eucalyptus*

Downstream regulators failed to express in *lfy* mutants, and *lfy* and upstream regulators were overexpressed



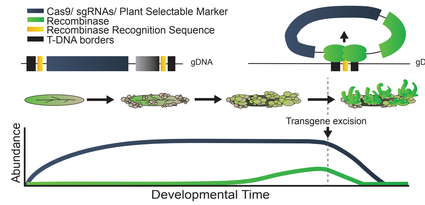
Internal structures underdeveloped

Floral KOs effective for bisexual sterility

- Mutations in floral specification genes have the intended sterility effect predicted from model systems
- *lfy* mutants in *Eucalyptus* form indeterminate flowers with bisexual sterility
- *ag* mutants and *lfy* mutants in poplar fast flowering lines are ready to be tested soon in the greenhouse
- *Eucalytpus* lines currently being tested in South Africa to confirm similar phenotypes

Site-specific excision for removal of CRISPR/Cas9

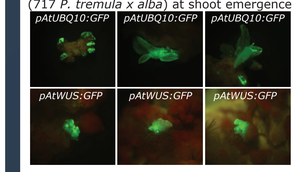
Using development to time excision



Testing performance using GFP-Cas9

We are validating promoters to drive recombinase expression

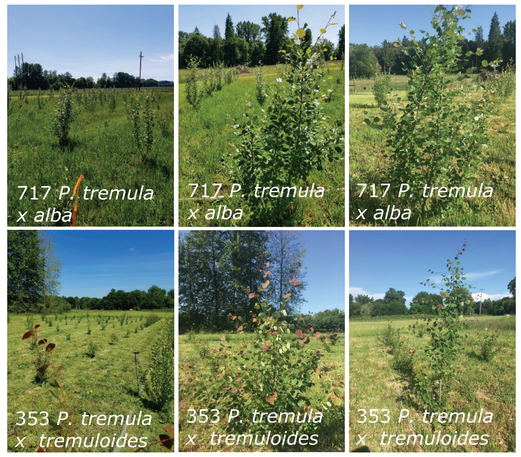
Regenerating explants (717 *P. tremula x alba*) at shoot emergence



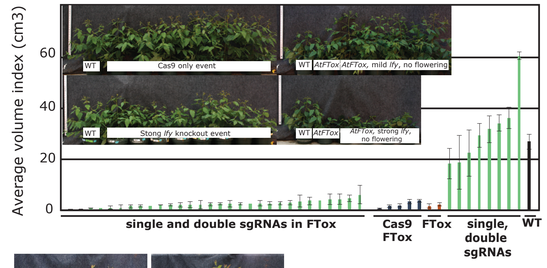
- An ideal promoter to drive recombinase is strongly expressed at the SAM but weakly expressed during callus formation
- We transformed explants with a constitutive *promoter:GFP* fusion to observe stable transgenic expression during regeneration, and the *Arabidopsis WUS* promoter to test if it was expressed in the SAM
- Our initial results suggest that promoter expression will be easy to visualize in regenerating explants, and *WUS* may have a good promoter to drive recombinase
- We are also testing other SAM promoters such as *STM*, *ER*, and *YAO*

Field and greenhouse-grown edited trees show similar growth phenotypes to controls

Poplar field tests ongoing for *lfy* and *ag*



Eucalyptus lfy KOs grow without detectable vegetative modification



- *FT* overexpression strongly effects branching
- Eucalypt CRISPR/Cas9 are similar in greenhouse experiments to their control genetic backgrounds
- Survival in the field is 100%

Summary

- CRISPR/ Cas9 generated knockouts are an effective tool for the induction of sexual sterility in forest trees
- Floral gene loss-of-function mutants in poplar and eucalypts behave similarly to analogous mutants in other plant species
- CRISPR/Cas9 trees are not phenotypically aberrant; we will test for off-target mutations that may have been generated during the gene editing process
- Somatic removal of editing components could potentially decrease off-target mutations and enable multiple rounds of editing; we are currently developing this system

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