Production and analysis of flowering-modified eucalypts

CAFS.14.51

Amy Klocko, Estefania Elorriaga, Cathleen Ma, Michael Nagle and Steve Strauss, Oregon State University

Presented by Steve Strauss
Main objective is to identify approaches and gene targets which lead to sterility in Eucalyptus

- A tool for reducing regulatory, ecological, market, and public acceptance risks of using exotic and GE trees
- Male sterility has been achieved in a variety of trees
  - Pine, poplar and Eucalyptus
  - But employed barnase that can be deleterious and unstable
- No reports of female or bisexual sterility in any tree species
Experimental Plan

- Identify candidate genes for creating novel sterility constructs from transcriptome analysis
- Create CRISPR-Cas9 genome editing constructs to target selected floral genes
- Transform novel vectors into early flowering eucalypts
- Identify events of interest by screens for gene mutagenesis
- Study fertility and vegetative growth of both CRISPR- and previously produced RNAi-transformed trees
Agenda for talk

- RNAi results – brief look
- Gene editing
  - Overview
  - Results from targeting LFY
  - New constructs
Targeting of *LFY* by RNAi in early-flowering SP7 gave rare floral alterations

- Analyzed floral morphologies of 53 events
- Identified 1 event of RNAi-LFY with altered floral morphology
- Use of *FT* may be increasing expression of *LFY*, making sufficient suppression via RNAi rare
- CRISPR-based targeting should overcome this difficulty
With help from Arborgen, produced RNAi transgenics in a naturally rapid flowering species

- Used naturally early flowering, though hard to transform and propagate, species: *Eucalyptus occidentalis*
- Targeted *LFY, AGAMOUS, NOZZLE* genes
  - *LFY* - Floral primordium
  - *AGAMOUS* – Stamen and Carpel
  - *NOZZLE* - meiosis
- No mutant phenotypes observed with *NOZZLE* or *AGAMOUS* despite analysis of several dozen insertion events
- *LFY* gave a complex story....
RNAi of *LFY* in *E. occidentalis* resulted in many poor growing events

- Analyzed 38 events, 25 were small and non-flowering
- No floral alterations observed in normal growing plants

![Tree size and flowering graph](image)

- **Flowers**
- **No flowers**

*CAFS*
However, field planted LFY RNAi SP7-urograndis eucalypts in Israel (Futuragene) growing well
RNAi and barnase findings highlight the need for other sterility options

- Inefficient targeting of LFY in the early-flowering FT background
- Poor vegetative performance in the *E. occidentalis* background
- Uncertain effects in field trial – will take some years to determine
Science magazine names CRISPR ‘Breakthrough of the Year’

By Robert Sanders  |  DECEMBER 18, 2015

In its year-end issue, the journal *Science* chose the CRISPR genome-editing technology invented at UC Berkeley 2015’s Breakthrough of the Year.

A runner-up in 2012 and 2013, the technology now revolutionizing genetic research and gene therapy “broke away from the pack, revealing its true power in a series of spectacular achievements,” wrote *Science* correspondent John Travis in the Dec. 18 issue. These included “the creation of a long-sought ‘gene drive’ that could be used to control the spread of malaria, and the development of CRISPR–Cas9, a cut-and-paste system that allows researchers to rewrite genetic code.”
CRISPR-Cas9 targeting of floral genes for genetic containment

- **Advantage of gene editing**: Expected to be more predictable from juvenile tissues, and more stable, than alternative genetic containment methods that depend on modified gene expression.

- **High efficiency**: Biallelic knock-outs needed in one or more genes.
Sandman CRISPR!
Summary of CRISPR Cas-gene editing mechanism

Two major types of edits

- Deleting a gene: Gene is disrupted
- Inserting a gene: Gene has a new sequence
Overview of CRISPR methods

Experimental Plan

Build CRISPR constructs
Transform plant tissue with Agrobacterium
Grow transformed plantlets
Extract DNA and PCR amplify target sites
Sequence target sites
Determine mutation types and frequencies
Select events for rapid flowering and field studies
Overview of CRISPR methods

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Use of the *FT*-accelerated background allows for fast analysis of floral phenotypes in eucalypts

- *LFY* and novel constructs are being studied in previously made transgenic *FT* early-flowering genotypes under earlier CAFS project - **CAFS.13.42 FT accelerated flowering**

- Plants flower quickly, allowing for rapid phenotyping
CRISPR is an effective means of altering the LFY gene

- Both *FT* early-flowering and SP-7 WT plants are undergoing analysis
- Plants are growing well in tissue culture
- Events have been selected for greenhouse evaluation

<table>
<thead>
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<th>Population</th>
<th>Total events</th>
<th>Mutation</th>
<th># events</th>
<th>frequency</th>
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<td>FT LFY-CRISPR</td>
<td>60</td>
<td>Biallelic</td>
<td>58</td>
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<tr>
<td>SP7 LFY-CRISPR</td>
<td>10</td>
<td>Biallelic</td>
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</table>
Examples mutations at one *LFY* target site

Larger deletions and inversions were also observed
Progress on 2016 Deliverables

- Identify biallelic CRISPR-LFY events of interest for future floral analysis in the greenhouse.
- We have analyzed 60 events in the early-flowering background and identified 58 bi-allelic events (97%).
- No mutations were observed in 10 events with just the Cas in the absence of guide RNAs.

Shoots from LFY CRISPR eucalypts
Progress on 2016 Deliverables

- In collaboration with Futuragene, began to analyze field plantings of RNAi SP7 eucalypts with respect to floral and vegetative phenotypes in the absence of accelerated flowering
- Field plantings of RNAi SP7 trees were established and tree size measured
- Photos taken in March 2017 show healthy trees with no major alterations in vegetative form
Progress on 2016 Deliverables

- In collaboration with Sappi, obtained staged *E. grandis* floral samples to generate transcriptome datasets to inform future gene target identification.
- Staged floral samples were collected and photographed.
- All relevant shipping permits were obtained and materials were received at OSU in March.

Stage 2 floral buds

Newly opened flowers
New CRISPR constructs: Strategy for selection of new target genes

- Began with list of floral-specific genes from Vining et al. 2014 floral transcriptome paper
- Ran BLAST and examined function of homologs in Arabidopsis databases
- Selected targets critical for reproduction, but not vegetative development
- Also examined Arabidopsis meiotic genes directly

The floral transcriptome of *Eucalyptus grandis*

Kelly J. Vining¹, Elisson Romanel², Rebecca C. Jones³, Amy Klocko¹, Marcio Alves-Ferreira⁴, Charles A. Hefer⁵, Vindhya Amarasinghe⁶, Palitha Dharmawardhana⁶, Sushma Naithani⁶, Martin Ranik⁷, James Wesley-Smith⁸, Luke Solomon⁹, Pankaj Jaiswal⁶, Alexander A. Myburg⁷ and Steven H. Strauss¹⁰
Overview of gene target results

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

Arabidopsis floral transcriptome
- Limit to 15 genes with meiotic functions in annotation
- 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, analyze transcriptome data, to limit to genes that do not appear redundant in Eucalyptus

Selected three new eucalypt gene targets
Selected target genes: **TAPETAL DEVELOPMENT AND FUNCTION 1 (TDF1)**

- Tapetum: specialized cells in the anther deliver nutrients to growing spores
- Loss-of-function mutant *tdf1* Arabidopsis is male sterile due to an inability to nurture spores

Arabidopsis anthers are shown. No viable pollen (dyed red) is produced in *tdf1* mutant

Siliques in *tdf1* mutant are small and contain no seeds

*Defective in Tapetal Development and Function 1 is essential for anther development and tapetal function for microspore maturation in Arabidopsis*

Jun Zhu, Hui Chen, Hui Li, Ju-Fang Gao, Hua Jiang, Chen Wang, Yue-Feng Guan and Zhong-Nan Yang
Selected target genes: **SYNAPTIC 1**

- **SYNAPTIC 1 / REC8** is an essential gene for chromosome division in sex cells.
- If the gene is non-functional in rice and Arabidopsis, plants are infertile but have normal vegetative growth.

Rice anthers are shown with pollen dyed red. When *OsRad21-4* is suppressed (B), little viable pollen is produced compared to control (A) (Zhang 2006, Plant Mol Bio)

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The rice *OsRad21-4*, an orthologue of yeast Rec8 protein, is required for efficient meiosis

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Selected target genes: *EMBRYO SAC DEVELOPMENT ARREST 33 (EDA33)*

- *EDA33 / INDEHISCENT* encodes a protein that is necessary for normal development of the valve margin.
- Valve margins separate valve from replum.
- Arabidopsis mutants of *eda33* have reduced fertility due to inability to properly develop, release fruits.

Control of Fruit Patterning in Arabidopsis by INDEHISCENT

Sarah J. Liljegren,1,23 Adrienne H. K. Roeder,1,2 Sherry A. Kempin,1 Kristina Gremski,1 Lars Østergaard,1 Sonia Guimil,1 Daengnay K. Reyes,1 and Martin F. Yanofsky1,2,*

Arabidopsis siliques are shown, with GUS staining demonstrating localization of *EDA33* to valve margins (Liljegren 2004, Cell).
Considerations for designing CRISPR/Cas9 constructs

- sgRNAs must match the gene at a position where a frame shift mutation or deletion would lead to a non-functional protein (usually upstream, conserved exon)
- Have a high expected mutation rate (“sgRNA Scorer”)
- Target both alleles of the target gene in our *E. grandis* x *urophylla* test hybrid
- Cause no off-target mutations (at similar loci)

All constructs have dual targets to have two chances, induce deletions
Major Findings

- Identified three novel candidate genes for achieving male, female, or bisexually sterile eucalypts
- Successfully created CRISPR constructs to target these genes, transformation is underway
- CRISPR-Cas was an efficient means for targeting the LFY gene in eucalypts
  - Identified bi-allelic knock-out events for future analysis
- Field planted RNAi-transgenic eucalypts are growing well
Ongoing work and future plans

- Generate transcriptome datasets from staged E. grandis floral samples for identification of additional novel gene targets
- Identify events of interest for our novel CRISPR constructs for future analysis of floral and vegetative morphology
- Analyze floral and vegetative phenotypes of bi-allelic LFY CRISPR events
  * Rapid flowering in greenhouse
  * Wild-type backgrounds in the field
- Collect and analyze vegetative and floral data from RNAi eucalypts undergoing natural flowering in field conditions
Company Benefits

- Proven, tested genetic containment tools to facilitate use of exotic and GE varieties
- Aid in regulatory and public acceptance, facilitating the adoption of many other kinds of transgenically improved varieties
  - Faster growth
  - Higher wood quality
  - Pest or abiotic stress resistance
  - High value co-products
Acknowledgments

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Xinmin An – Sequencing of LFY CRISPR events

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Elahe Esfandiari – Analysis of transgene expression (RNAi)

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SAPPI – Collection and shipment of E. grandis samples
Arborgen – Transformation of E. occidentalis

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