Ending Project / Final Report

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

CAFS/TBGRC Research Personnel 2016-17

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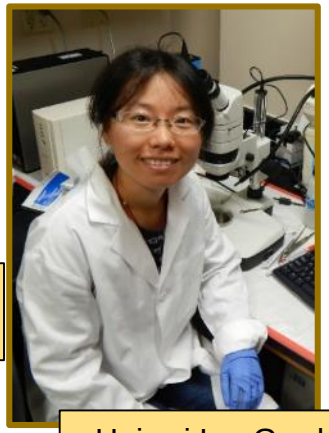
Anna Magnuson, Program & Field Manager

Emily Helliwell, Post-Doc, Genomics and Bioinformatics

Amy Klocko, Post-doc, Cloning, Gene Expression, Flowering

Estefania Elorriaga, Grad Student, CRISPRs

Michael Nagle, Grad Student, Gene Targets



Haiwei Lu, Grad Student, ZFNs

Sarah Higgins, Technician, Floral Analysis

Jeremy Jacobson, Undergraduate Research

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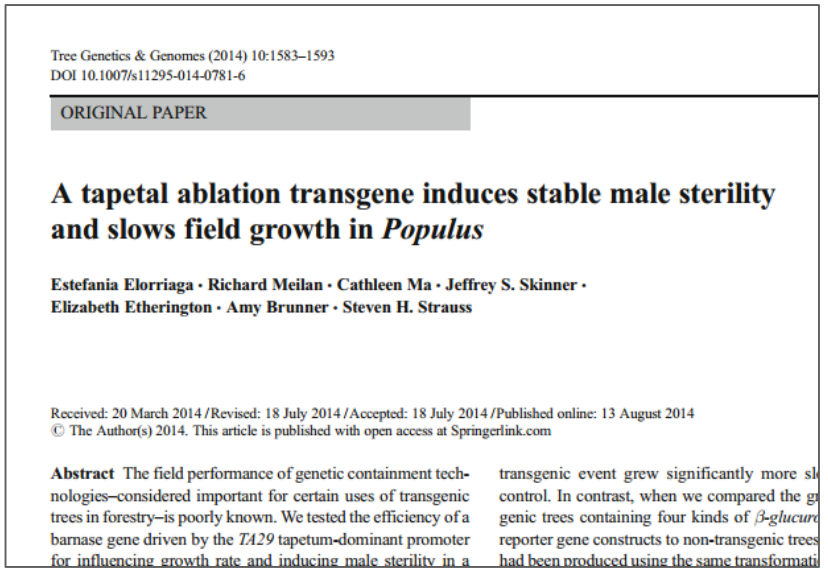
Main objective is to identify approaches and gene targets which lead to sterility in Eucalyptus

- 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



Tree Genetics & Genomes (2014) 10:1583–1593
DOI 10.1007/s11295-014-0781-6

ORIGINAL PAPER

A tapetal ablation transgene induces stable male sterility and slows field growth in *Populus*

Estefania Elorriaga · Richard Meilan · Cathleen Ma · Jeffrey S. Skinner · Elizabeth Etherington · Amy Brunner · Steven H. Strauss

Received: 20 March 2014 / Revised: 18 July 2014 / Accepted: 18 July 2014 / Published online: 13 August 2014

Abstract The field performance of genetic containment technologies–considered important for certain uses of transgenic trees in forestry–is poorly known. We tested the efficiency of a barnase gene driven by the *TA29* tapetum-dominant promoter for influencing growth rate and inducing male sterility in a transgenic event grew significantly more sl control. In contrast, when we compared the g genic trees containing four kinds of *β-glucur* reporter gene constructs to non-transgenic trees had been produced using the same transformati

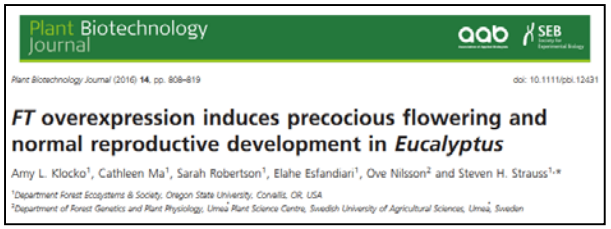
Experimental Plan

- 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

Control flower

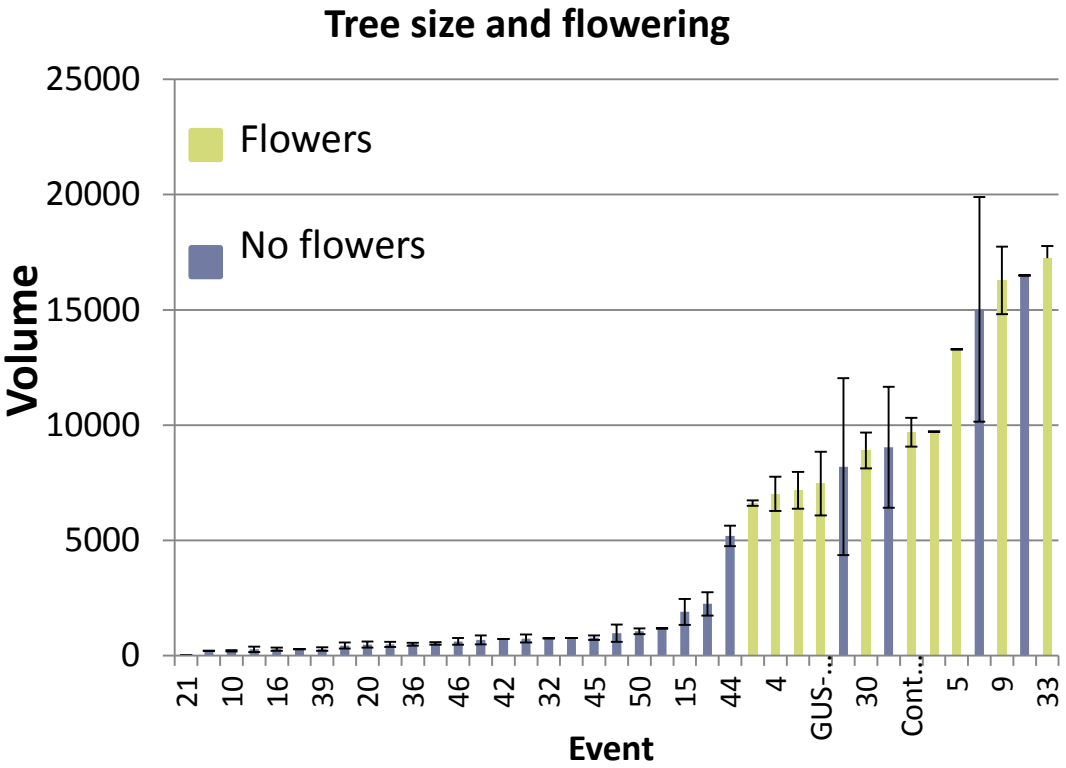
RNAi-LFY flower

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



However, field planted *LFY* RNAi SP7-urograndis eucalypts in Israel (Futuragene) growing well

Wild type

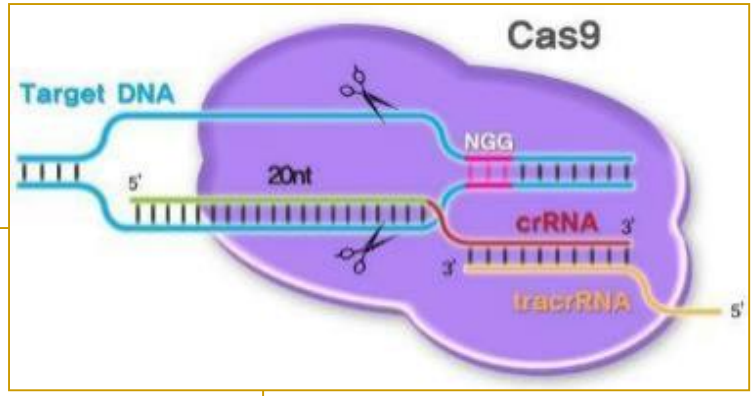
LFY RNAi event 8

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

Gene editing technology

A big deal throughout biology



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

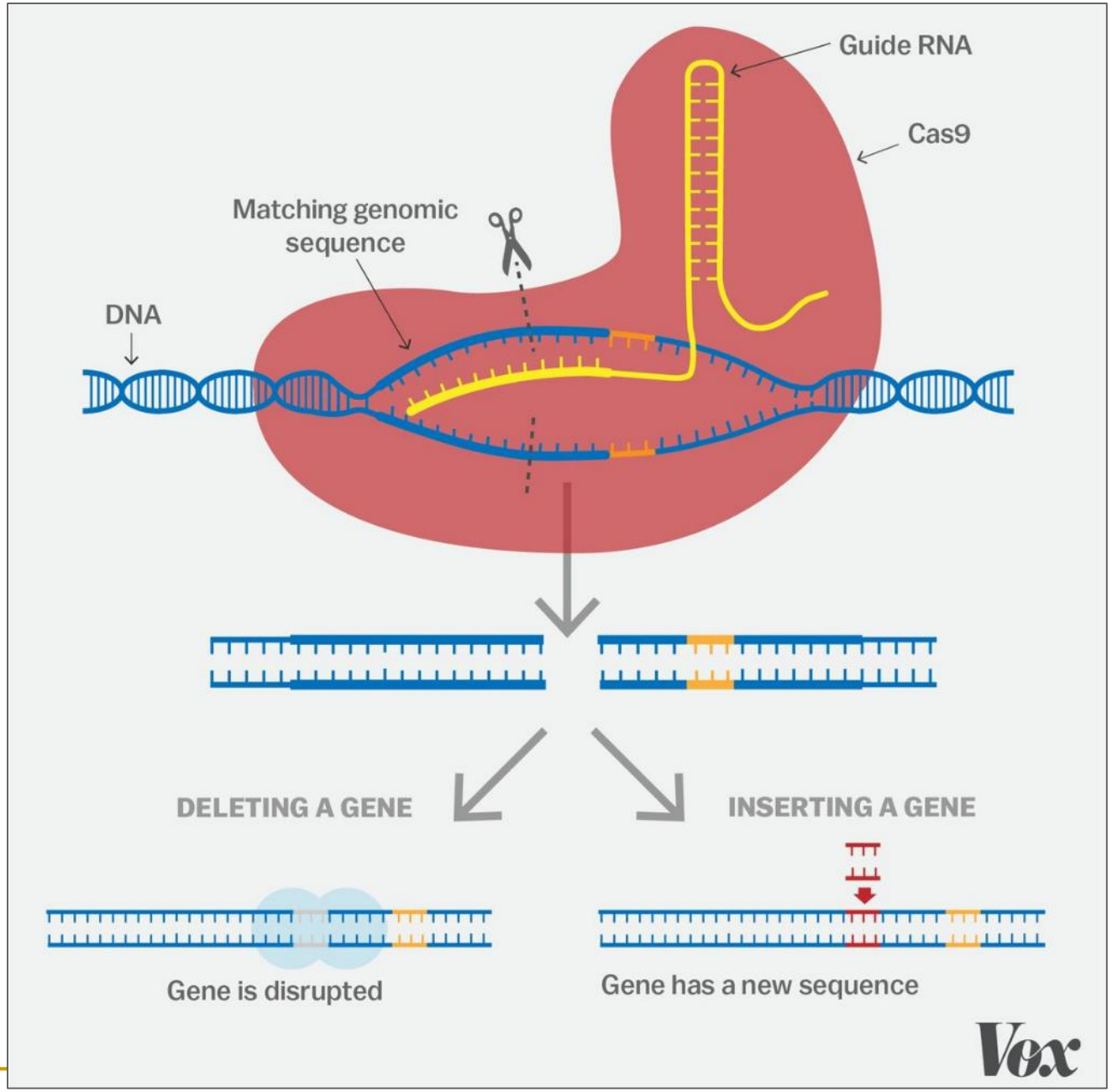
CRISPR-Cas9 targeting of floral genes for genetic containment

- **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

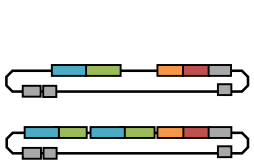
Sandman CRISPR !

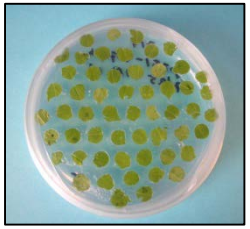
Summary of CRISPR Cas-gene editing mechanism

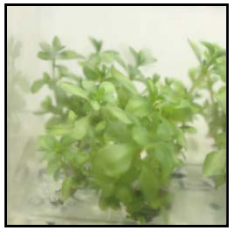
Two major types of edits

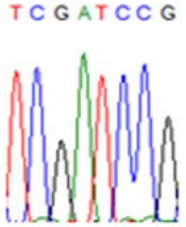


Overview of CRISPR methods









Build CRISPR constructs

Transform plant tissue with *Agrobacterium*

Grow transformed plantlets

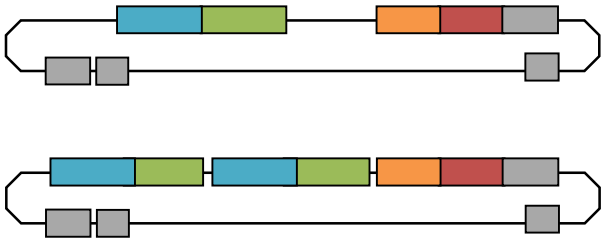
Extract DNA and PCR amplify target sites

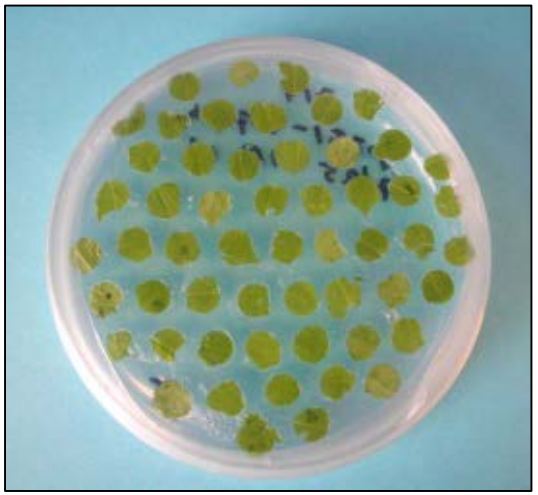
Sequence target sites

Determine mutation types and frequencies

Select events for rapid flowering and field studies

Overview of CRISPR methods





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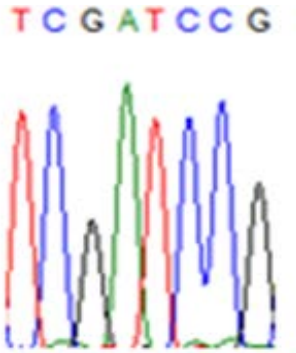


Extract DNA and PCR amplify target sites

Sequence target sites

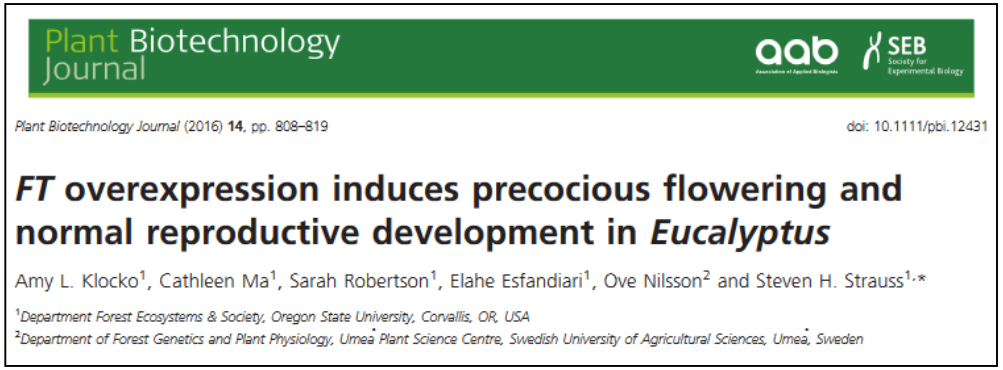
Determine mutation types and frequencies

Select events for rapid flowering and field studies

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


Plant Biotechnology Journal

Plant Biotechnology Journal (2016) **14**, pp. 808–819 doi: 10.1111/pbi.12431

FT* overexpression induces precocious flowering and normal reproductive development in *Eucalyptus

Amy L. Klocko[1], Cathleen Ma[1], Sarah Robertson[1], Elahe Esfandiari[1], Ove Nilsson[2] and Steven H. Strauss[1,*]

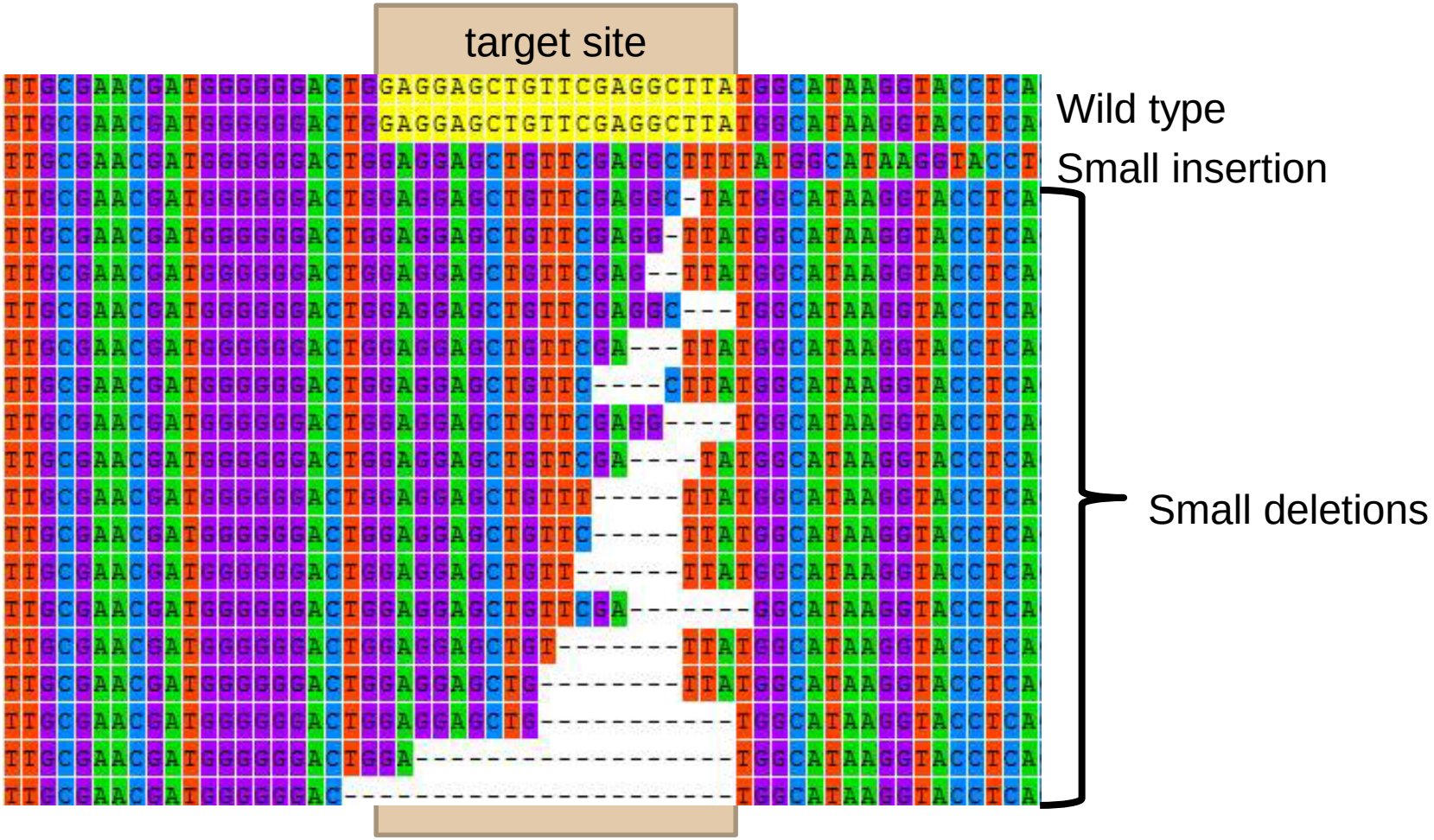
[1]*Department Forest Ecosystems & Society, Oregon State University, Corvallis, OR, USA*
[2]*Department of Forest Genetics and Plant Physiology, Umeå Plant Science Centre, Swedish University of Agricultural Sciences, Umeå, Sweden*

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

Population	Total events	Mutation	# events	frequency
FT LFY-CRISPR	60	**Biallelic**	**58**	**97%**
		WT	2	3%
FT Cas9 control	10	Biallelic	0	0%
		WT	10	100%
SP7 LFY-CRISPR	10	Biallelic	**10**	**100%**
		WT	0	0%
SP7 Cas9 control	2	Biallelic	0	0%
		WT	2	100%

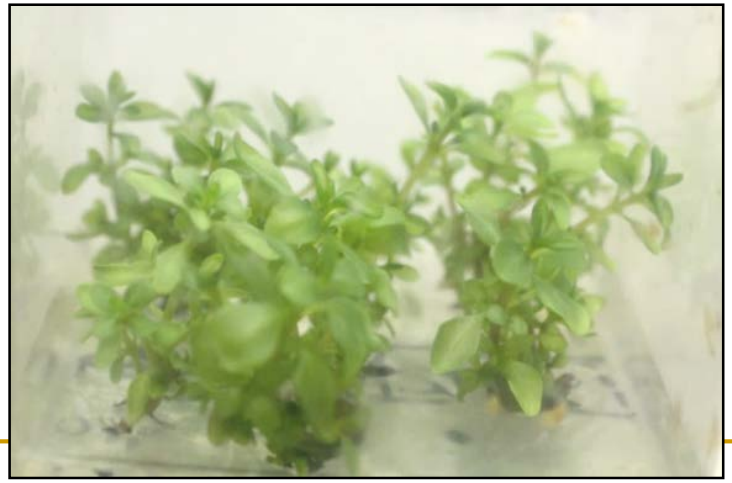
Examples mutations at one *LFY* target site



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

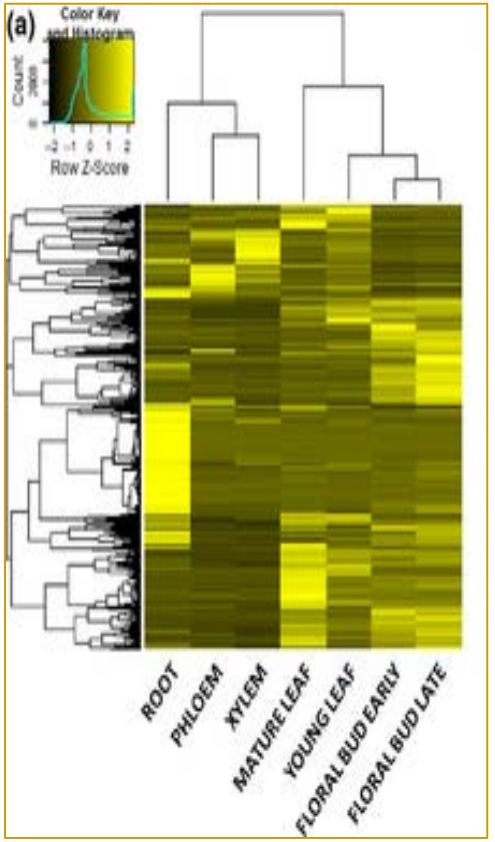
- In collaboration with Sappi, obtained staged *E. grandis* floral samples to generate transcriptome datasets to inform future gene target identification
- Staged floral samples were collected and photographed
- All relevant shipping permits were obtained and materials were received at OSU in March

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



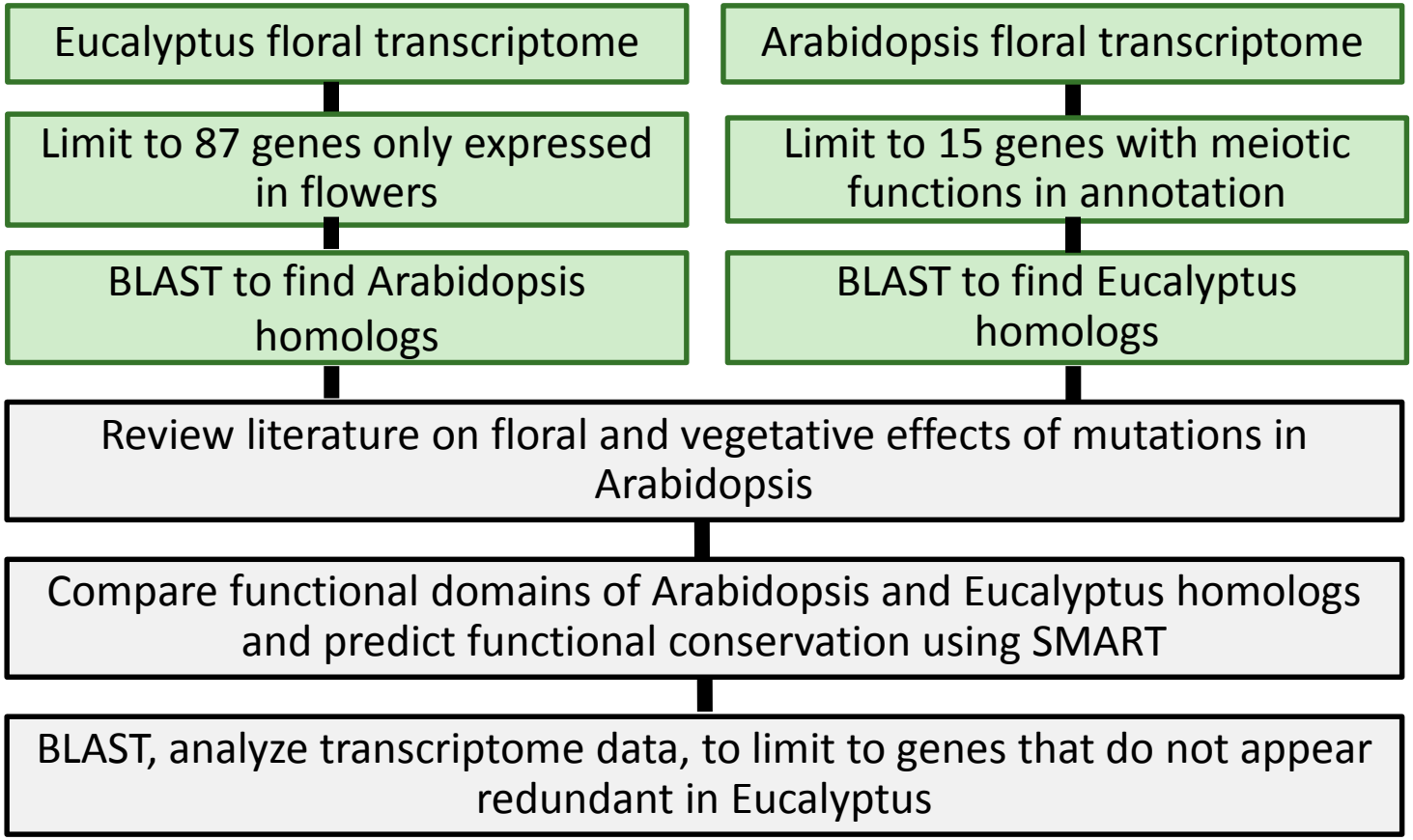
Research

New Phytologist

The floral transcriptome of *Eucalyptus grandis*

Kelly J. Vining[1], Elisson Romanel[2], Rebecca C. Jones[3], Amy Klocko[1], Marcio Alves-Ferreira[4], Charles A. Hefer[5], Vindhya Amarasinghe[1,6], Palitha Dharmawardhana[6], Sushma Naithani[6], Martin Ranik[7], James Wesley-Smith[8], Luke Solomon[9], Pankaj Jaiswal[6], Alexander A. Myburg[7] and Steven H. Strauss[10]

Overview of gene target results



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

the plant journal

The Plant Journal (2008) 55, 266–277 doi: 10.1111/j.1365-313X.2008.03500.x

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*

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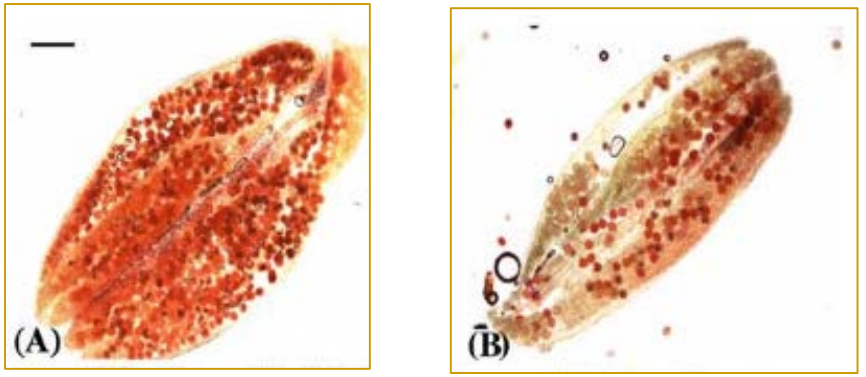
Selected target genes: *SYNAPTIC 1*

- *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



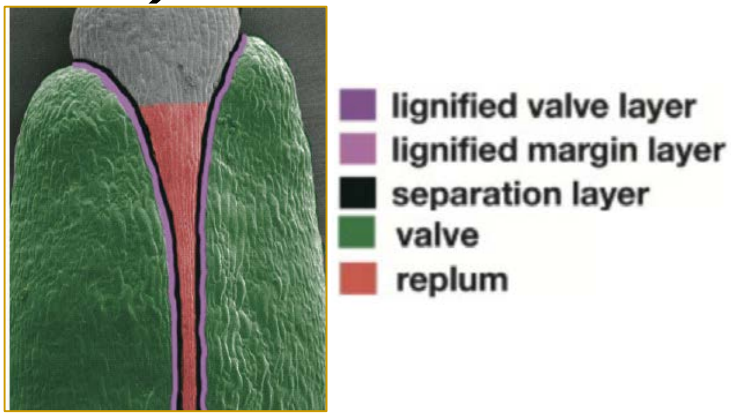
Rice anthers are shown with pollen dyed red. When *OsRad21-4* is suppressed (B), little viable pollen is produced compared to control (A)
(Zhang 2006, Plant Mol Bio)

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

Liangran Zhang[1,2], Jiayi Tao[1,2], Shunxin Wang[1], Kang Chong[1] and Tai Wang[1,*]
[1]*Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Research Center of Molecular & Developmental Biology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China;* [2]*Graduate School of the Chinese Academy of Sciences, Beijing 100049, China (*author for correspondence; e-mail twang@ibcas.ac.cn)*

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



Arabidopsis siliques are shown, with GUS staining demonstrating localization of *EDA33* to valve margins (Liljegren 2004, Cell)

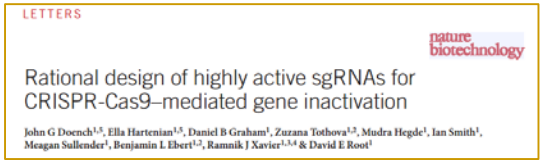
Cell, Vol. 116, 843–853, March 19, 2004, Copyright ©2004 by Cell Press

Control of Fruit Patterning in *Arabidopsis* by INDEHISCENT

Sarah J. Liljegren,[1,2,3] Adrienne H. K. Roeder,[1,2] Sherry A. Kempin,[1] Kristina Gremski,[1] Lars Østergaard,[1] Sonia Guimil,[1] Daengnoy K. Reyes,[1] and Martin F. Yanofsky[1,*]

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)

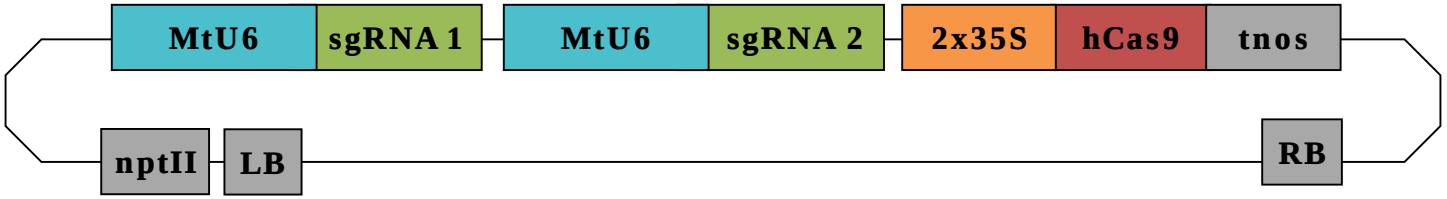

LETTERS

nature biotechnology

Rational design of highly active sgRNAs for CRISPR-Cas9–mediated gene inactivation

John G Doench[1,5], Ella Hartenian[1,5], Daniel B Graham[1], Zuzana Tothova[1,2], Mudra Hegde[1], Ian Smith[1], Meagan Sullender[1], Benjamin L Ebert[1,2], Ramnik J Xavier[1,3,4] & David E Root[1]

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

Oregon State University

Amy Klocko – Transgenic plant and molecular analysis

Estefania Elorriaga – *LFY* CRISPR construction, sequencing of CRISPR events

Michael Nagle – Novel target selection and CRISPR construction

Xinmin An – Sequencing of *LFY* CRISPR events

Visiting scholar from Beijing Forestry University

Cathleen Ma – Transformation and propagation

Elahe Esfandiari – Analysis of transgene expression (RNAi)

Futuragene – Transformation method for SP7, field testing SP7

SAPPI – Collection and shipment of *E. grandis* samples

Arborgen – Transformation of *E. occidentalis*

All members of the Tree Biosafety and Genomics Research Cooperative for financial support

CAFS/TBGRC Research Personnel 2016-17

Cathleen Ma, Transformation & Greenhouse Experiments

Steve Strauss, Director TBGRC, Professor

Anna Magnuson, Program & Field Manager

Emily Helliwell, Post-Doc, Genomics and Bioinformatics

Amy Klocko, Post-doc, Cloning, Gene Expression, Flowering

Estefania Elorriaga, Grad Student, CRISPRs

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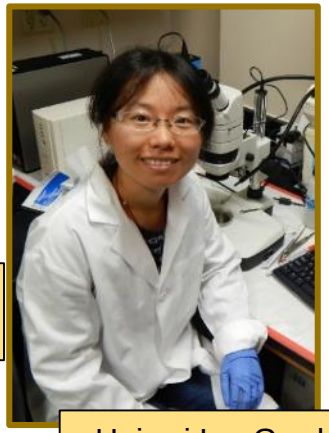

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Jeremy Jacobson, Undergraduate Research

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