CRISPR/Cas9 Efficiency and Biological Impacts in Transgenic Poplars and Eucalypts
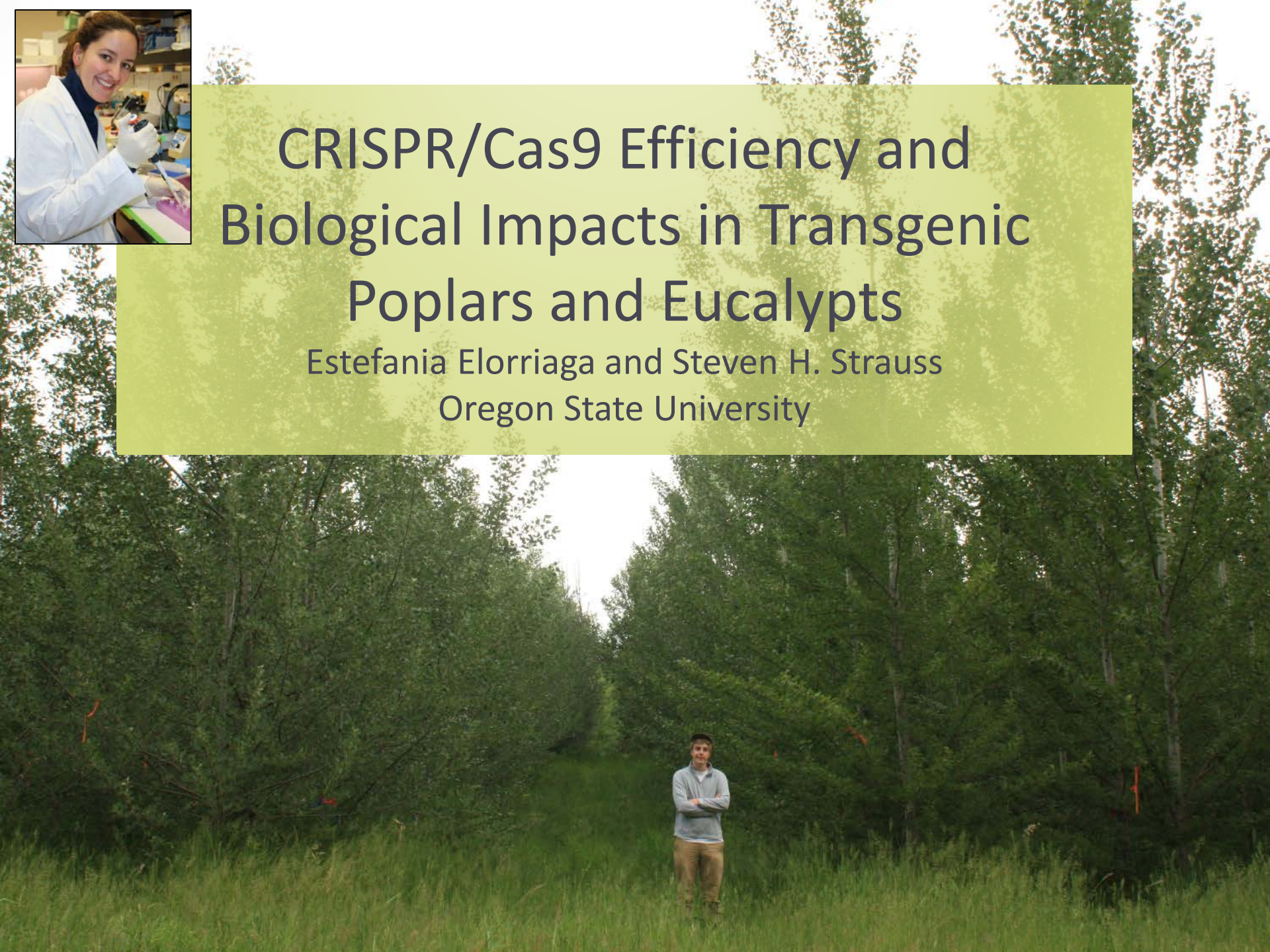
Estefania Elorriaga and Steven H. Strauss

Oregon State University

Outline

- Background
  - CRISPR, goals, target genes
- Mutagenesis results
  - Poplars and eucalypts
- RNAi vs. CRISPR
  - Vegetative function of eucalypt LFY?

What are CRISPR/Cas systems?

- CRISPR stands for clustered, regularly interspaced, short palindromic repeats
- The CRISPR/Cas system is an adaptive defense system in prokaryotes to fight against alien nucleic acids
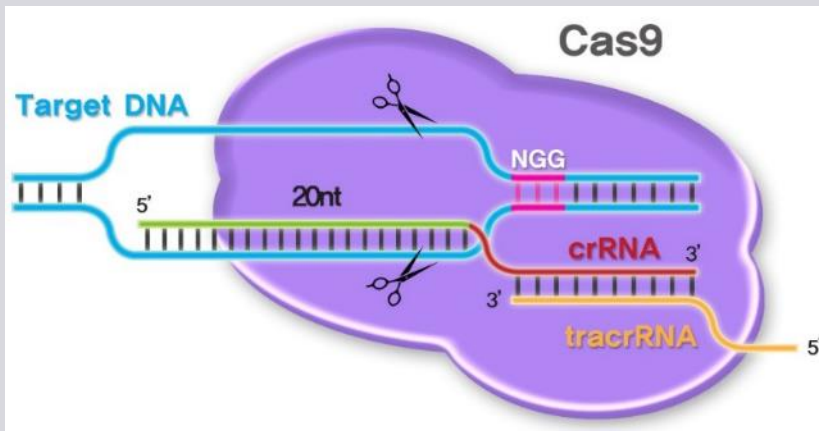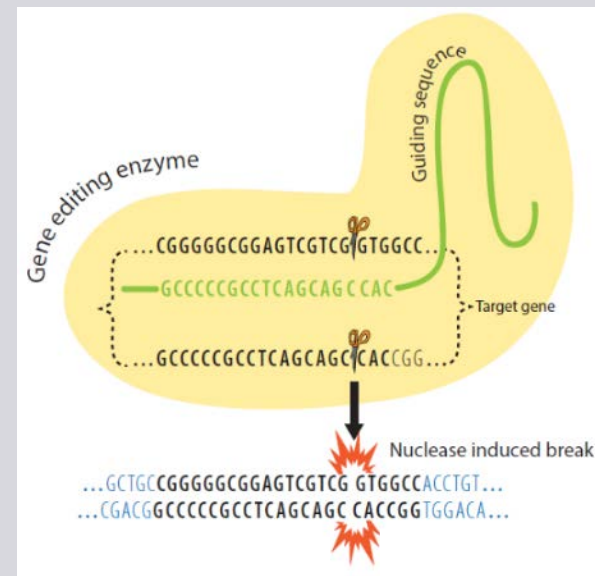
Defense system in nature

Synthetic nuclease system

Image credit: http://pnabio.com/products/RGEN.htm

Value of CRISPR-Cas nucleases

- Engineered CRISPR systems <u>nice</u>
  - Affordable
  - Easy to implement
  - Efficient
  - Insensitive to methylation
- Used successfully in many plant species with high mutation rates, frequent biallelic mutation
  - Transient assays, 1 to 38 %
  - Stable assays, 4 to 100 %

CRISPR-Cas9 targeting of floral genes might provide robust containment

- **Goal:** To develop robust <u>male and female</u> containment technologies for <u>vegetatively propagated</u> forest trees

- **Why:** Regulatory, market, and public acceptance with exotic and native trees can be costly or impossible – even for <u>field research</u>

- **Advantage of gene editing:** Expected to be more <u>predictable and stable</u> than alternative genetic containment methods that depend on gene expression
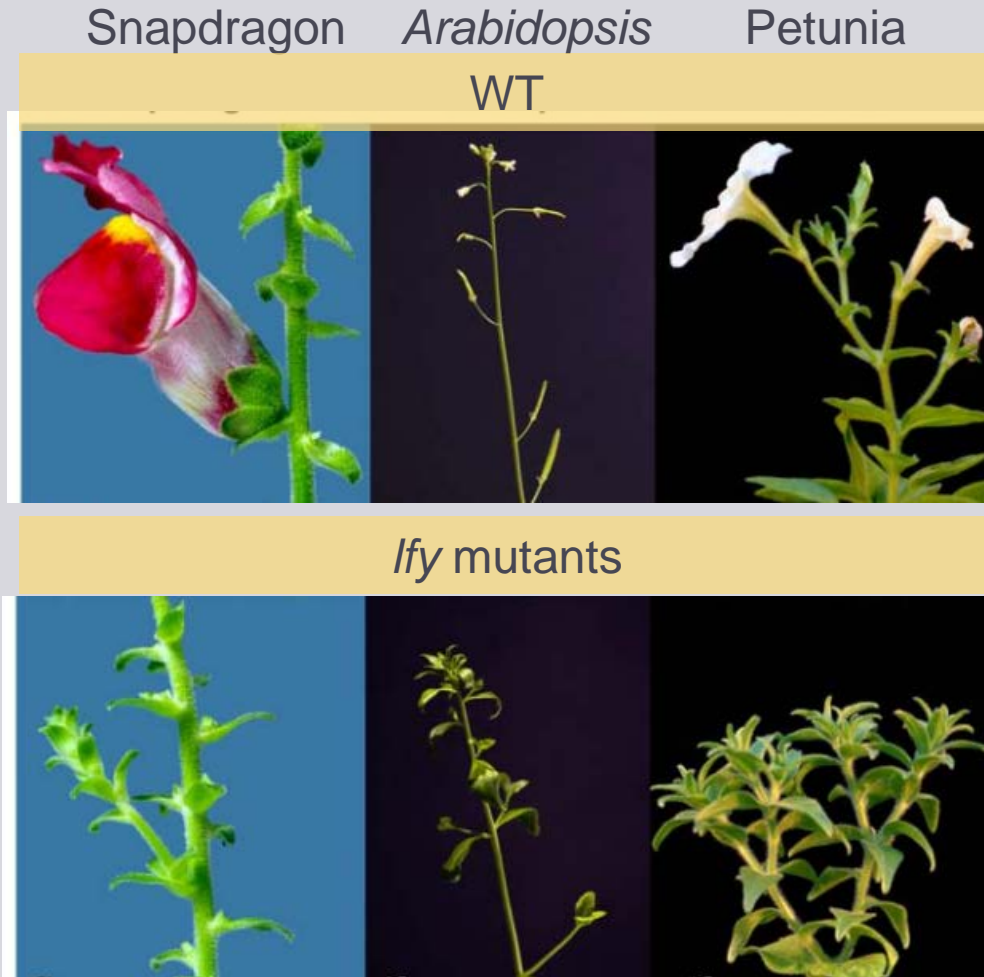
Target genes for bisexual sterility

- *LEAFY* – floral meristem prior to organ differentiation
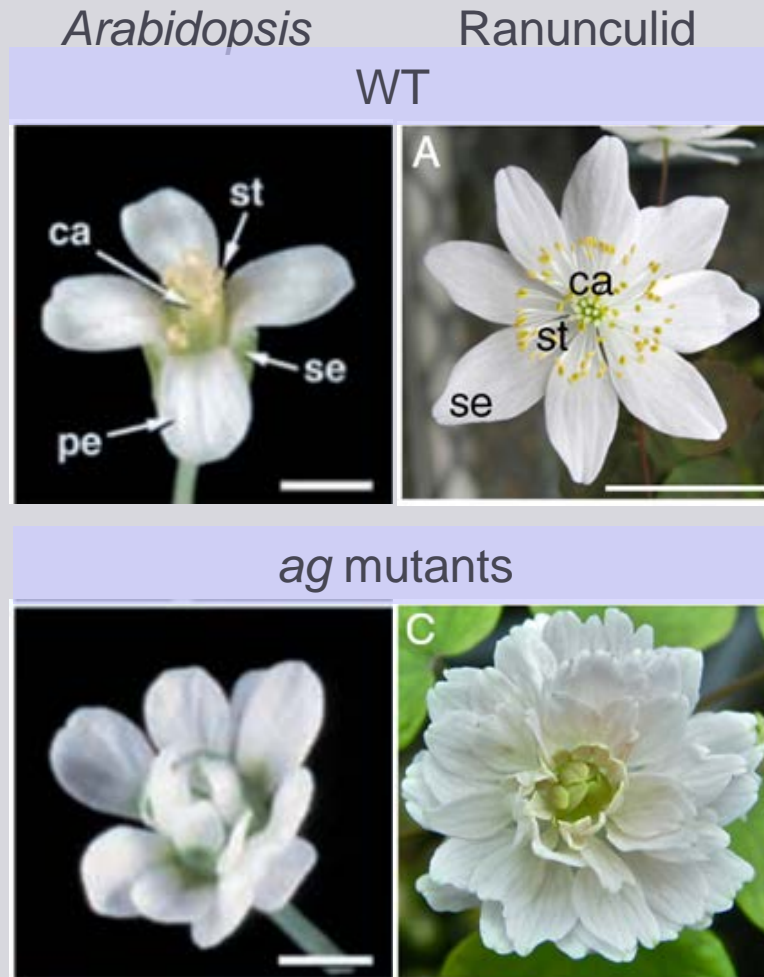- *AGAMOUS* – Male and female organ development and floral determinacy
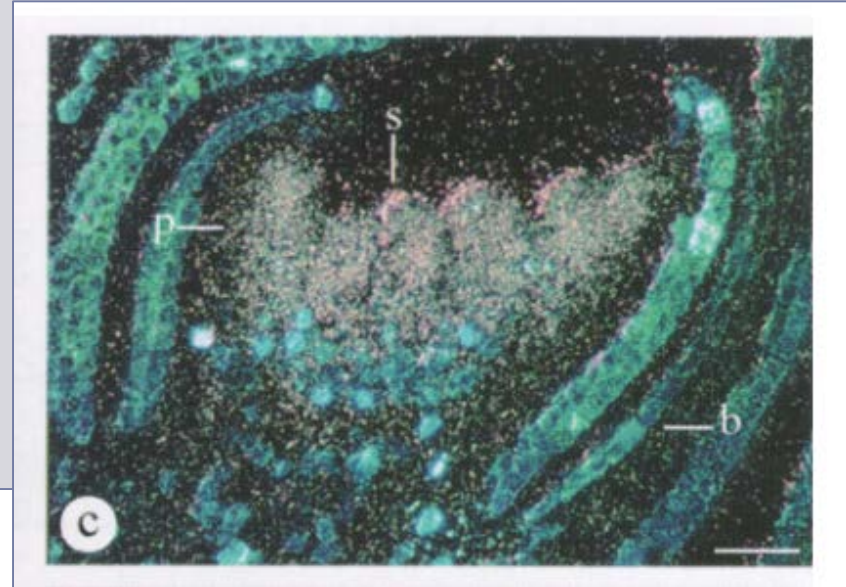
Strong *lfy* mutants appear to have no flowers

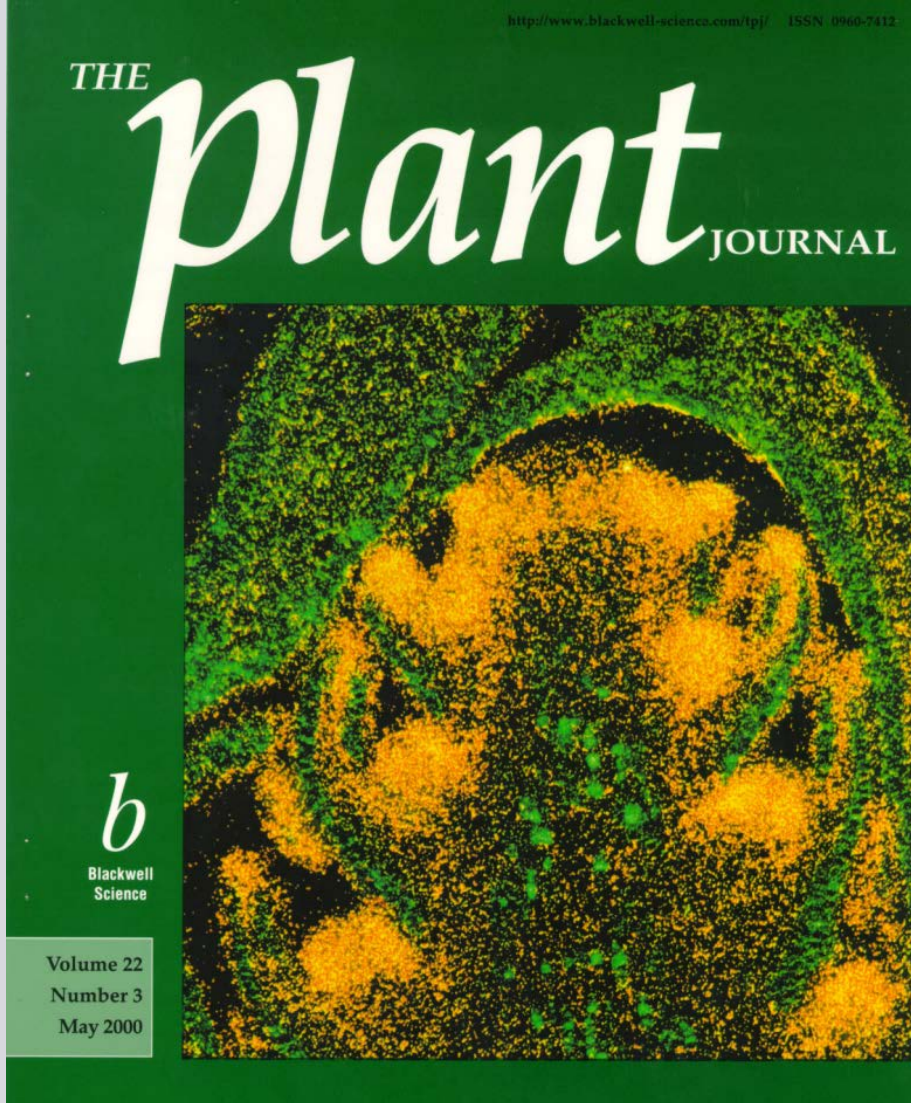Snapdragon   *Arabidopsis*   Petunia

WT

lfy mutants

Parcy et al. 2002; Moyroud et al. 2010

Flowers in strong *ag* mutants are missing both stamens and carpels

Arabidopsis   Ranunculid

WT

ag mutants

Parcy et al. 2002; Galimba et al. 2012

LEAFY and *AGAMOUS* homologs in poplar studied in prior work

Structure and expression of duplicate AGAMOUS orthologues in poplar

Amy M. Brunner, William H. Rottmann[1], Lorraine A. Sheppard[2], Konstantin Krutovskii, Stephen P. DiFazio,
Stefano Leonardi[3] and Steven H. Strauss*

*Department of Forest Science, Oregon State University, Corvallis, OR 97331, USA (*author for correspondence; e-mail:
strauss@fsl.orst.edu); present addresses: 1 Westvaco Forest Science and Technology, P.O. Box 1950, Summerville, SC 29484, USA; 2Institute
of Forest Genetics, USDA Forest Service c/o Department of Environmental Horticulture, One Shields Ave., University of California, Davis,
CA, 95616, USA; 3Department of Environmental Science, University of Parma, Parco Area delle Scienze 33a, 43100 Parma, Italy*

**Both with veg expression,
two active *AG* paralogs**

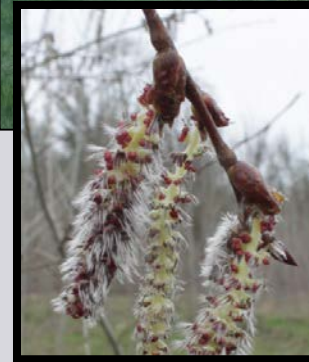# Poplar: Dioecy, field trials, *FT* acceleration

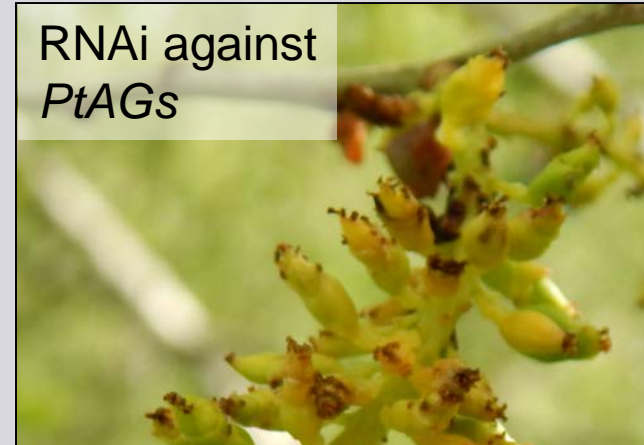**Female clone 6K10**
Populus alba

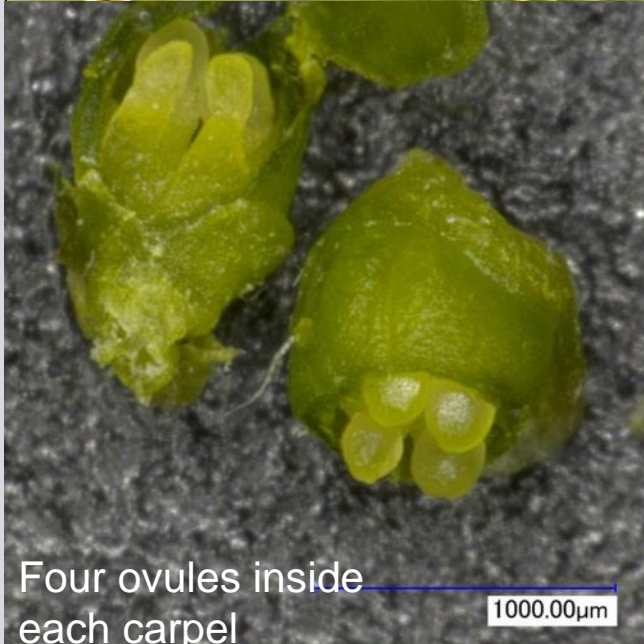**Female clone 717**
P. tremula x P. alba

Male clone 353
P. tremula x P. tremuloides

FT

Strong poplar *AG-RNAi* events in the field with similar mutant flowers

WT

Four ovules inside each carpel

1000.00μm

RNAi against *PtAGs*
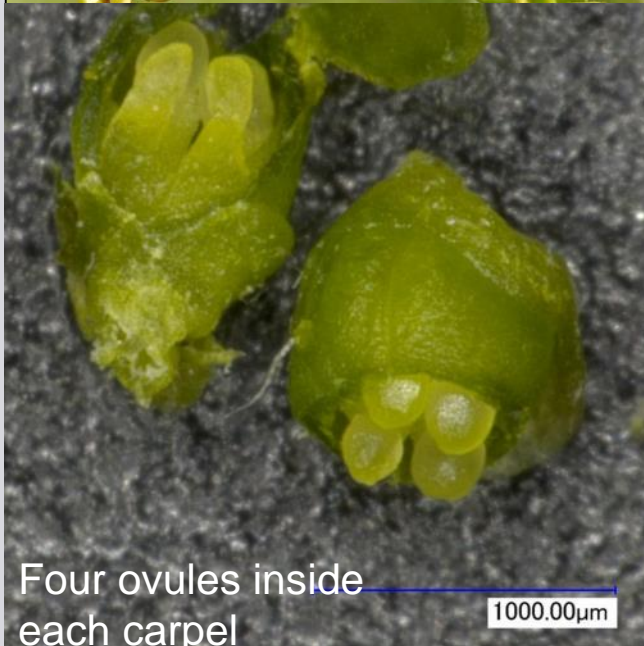
Replicated carpel
No ovules
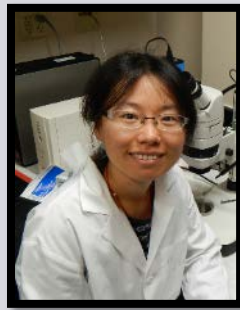
1000.00μm

Strong poplar *LFY-RNAi* events in the field with similar mutant flowers

WT
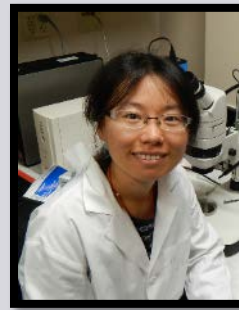
Four ovules inside each carpel

1000.00μm

RNAi against *PtLFY*

Immature carpel
No ovules

Eucalypt *LFY* is a single gene, expressed in leaf primordia as well as floral organs

EgLFY, the *Eucalyptus grandis* homolog of the *Arabidopsis* gene *LEAFY* is expressed in reproductive and vegetative tissues

Marcelo Carnier Dornelas[1]*, Weber A. Neves do Amaral[2] and Adriana Pinheiro Martinelli Rodriguez[1]

[1]Universidade de São Paulo, Centro de Energia Nuclear na Agricultura. Lab
Piracicaba, SP, Brasil; [2]Universidade de São Paulo, Depto. De Ciências Fl
Brasil; *Corresponding author: mcdornel@cena.usp.br
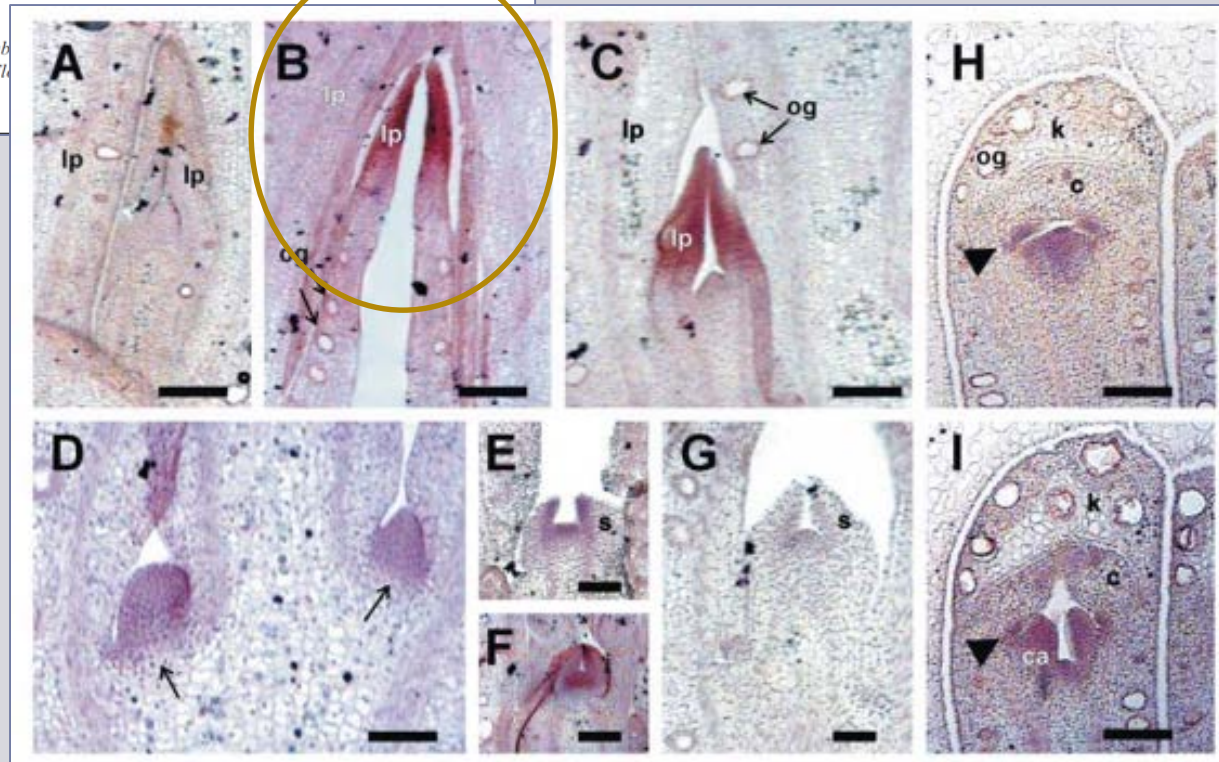
Eucalypt: *FT-* and *E. occidentalis* early-flowering, collaborator field trials

Flowering *E. occidentalis*

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],*

[...]partment Forest Ecosystems & Society, Oregon State University, Corvallis, OR, USA
[...]partment of Forest Genetics and Plant Physiology, Umeá Plant Science Centre, Swedish University of Agricultural Sciences, Umeá, Sweden

Summary

Eucalyptus trees are among the most important species for industrial forestry worldwide. However, as with most forest trees, flowering does not begin for one to several years after planting which can limit the rate of conventional and molecular breeding. To speed flowering, we transformed a *Eucalyptus grandis* × *urophylla* hybrid (SP7) with a variety of constructs that enable overexpression of *FLOWERING LOCUS T* (*FT*). We found that *FT* expression led to very early flowering, with events showing floral buds within 1–5 months of transplanting to the glasshouse. The most rapid flowering was observed when the cauliflower mosaic virus 35S promoter was used to drive the *Arabidopsis thaliana FT* gene (*AtFT*). Early flowering was also observed with *AtFT* overexpression from a 409S ubiquitin promoter and under heat induction conditions with *Populus trichocarpa FT1* (*PtFT1*) under control of a heat-shock promoter. Early flowering trees grew robustly, but exhibited a highly branched phenotype compared to the strong apical dominance of nonflowering transgenic and control trees. *AtFT*-induced flowers were morphologically normal and produced viable pollen grains and viable self- and cross-pollinated seeds. Many self-seedlings inherited *AtFT* and flowered early. *FT* overexpression-induced flowering in *Eucalyptus* [...]g bre[...] studies as the transgene can be [...]ng r[...] form.
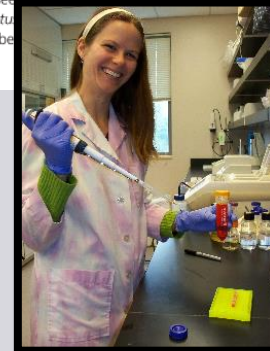
Outline

- Background
  - CRISPR, goals, target genes
- Mutagenesis results
  - Poplars and eucalypts
- RNAi vs. CRISPR
  - Vegetative function of eucalypt LFY?

Overview of experimental methods

| Build constructs | Transform poplar tissue with *Agrobacterium* | Grow transformed plantlets | Extract DNA and gel-purify gene amplicons | Sequence amplicons across target sites | Identify mutation types and determine frequency |

- Stable transformation, no removal of CRISPR locus at this time
- Mutation rates on a per transgenic event basis

Experimental constructs – single and double targets per gene
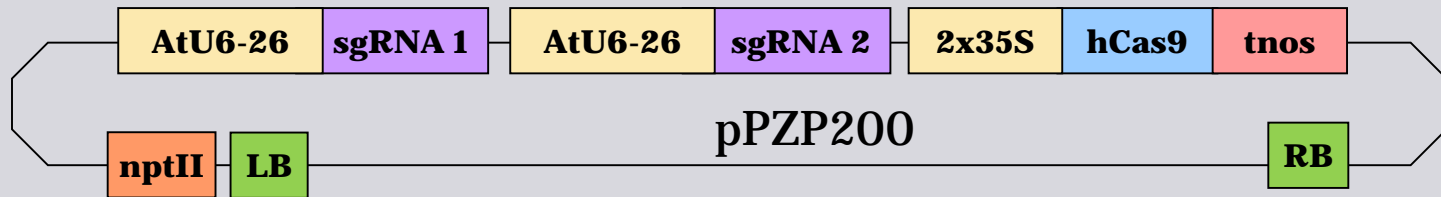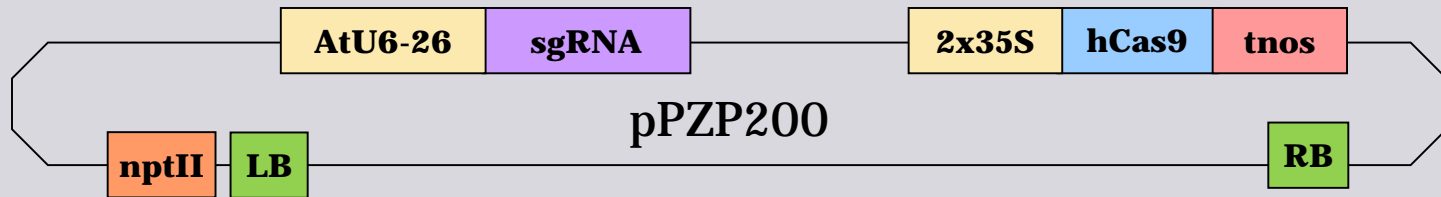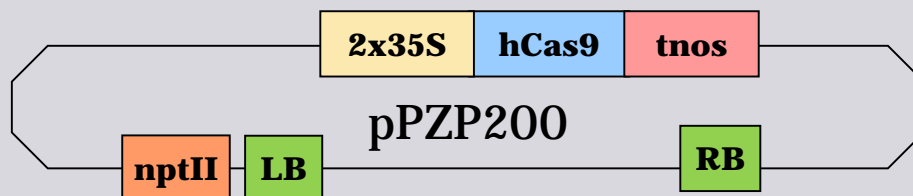
Targeting two near-identical sites in the two paralogous *AG* genes in poplar

>PtAG1

...GGATCAGCTAGCTAGACTGCAGCT**ATG**GAATATCAAAATGAATCCCTTGAGAGCTCCCCCCTGAGGAAGC
TAGGAA**GGGGAAAGGTGGAGATCAAG**CGGATCGAGAACACCACCAATC**GCCAAGTCACTTTCTGCAAA**
AGGCGCAGTGGTTTGCTCAAGAAAGCCTACGAATTATCTGTTCTTTGCGATGCTGAGGTTGCACTCATCG...

Target site for AGsg2   Target site for AGsg1

>PtAG2

...GATCAGCTAGCTAGGCAGCAGCT**ATG**GCATACCAAAATGAATCCCAAGAGAGCTCCCCCCTGAGGAAGC
TGGGRA**GGGGAAAGGTGGAGATCAAG**CGGATCGAGAACACCACAAATC**GYCAAGTCACTTTCTGCAAA**
AGGCGGAATGGTTTGCTCAAGAAAGCCTATGAATTATCTGTTCTTTGCGATGCTGAGGTTGCACTCATCG...

Targeting two sites in the single-copy *LFY* gene of poplar

Target site for LFYsg2 (in promoter region)

>*PtLFY*
…CATGCACCAGTGAAA**GATCACAGAGAGAGAGACAA**GGGGGCAGATAGAT**ATG**GATCCGGAGGCTTT
CACGGCGAGTTTGTTCAAATGGGACACGAGAGCAATGGTGCCACATCCTAACCGTCTGCTTGAAATGGT
GCCCCCGCCTCAGCAGCCACCGGCTGCGGCGTTTGCTGTAAGGCCAAGGGAGCTATGTGGGCTAGAGG
AGTTGTTTCAAGCTTATGGTATTAGGTACTACACGGCAGCGAAAATAGCTGAACTCGGGTTCACAGTGA…

Target site for LFYsg1 (in exon 1)

Two conserved target sites in *euc LFY*

Target site for EgLFYsg1

>*EgLFY*
ATGGATCCAGAAGCATTTGCGGTTGTGGGGGTTGCGAACGATGGGGGGGACTG**GAGGAGCTGTTCGAGGCTTA**
TGGCATAAGGTACCTCACGGCCTCCAGGATAGCGGAAATGGGGTTTACGGCCAACACCCTCCTCGACATGAAG
GAGGAGGAGCTCGACGACATGATGAACTCCCTCTCCCACATCTTCCGCTGGGACCTCCTCGTCGGCGAGCGCT
ACGGCATCAAGGCCGCCATCCGCGCCGAGCGCCGACGCCTCCTCGAAGCCGATGACCGCCGCCACCACCTCC
A**CTCCACCGACCATGCCCTCC**TCGATGCTCTCTCCCACAAA...

Target site for EgLFYsg2

PCR amplicons are large to capture most mutations in both alleles

Forward primer   Reverse primer   PCR product ~700 bps

Exon 1

PtAG1 and Pt*AG2* loci

Forward primer   Reverse primer   PCR product ~270 bps *(PtAG1)* and ~400 bps *(PtAG2)*

Exon 1   Exon 2   Exon 3

PtLFY locus

Forward primer   Reverse primer   PCR product ~500 bps

Exon 1   Exon 2   Exon 3

EgLFY locus
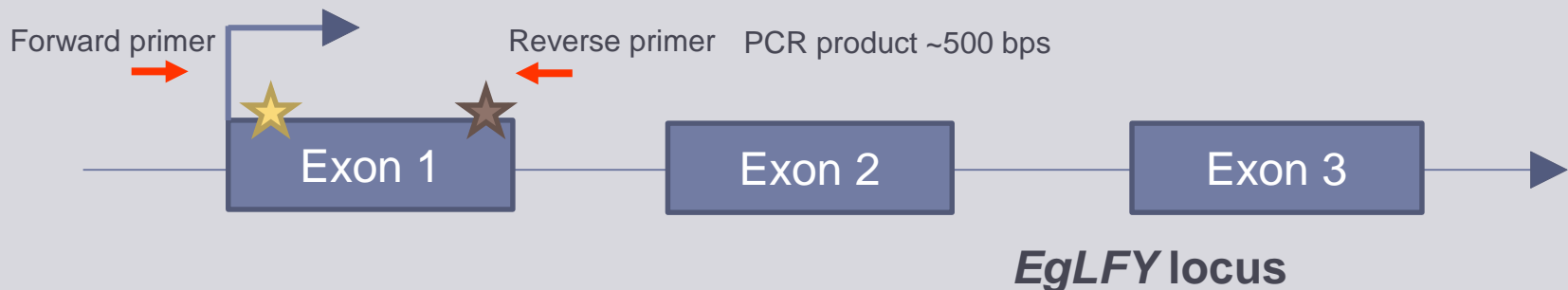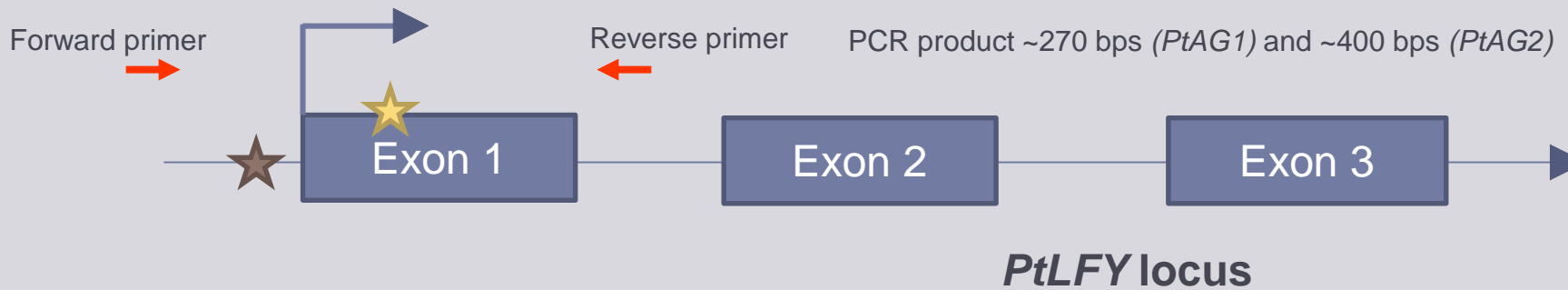
PCR amplicons have more than two SNPs to identify separate alleles

- *PtLFY:*
  - C...T...T...G
  - T...C...G...A

- *PtAG1:*
  - A...G
  - G...A

- *PtAG2:*
  - A...A...T...G...C...C...C...T
  - G...C...C...A...T...T...T...C

- *EgLFY:*
  - C...T...T...G
  - T...C...G...A

Homozygous mutants have missing peaks in trace files
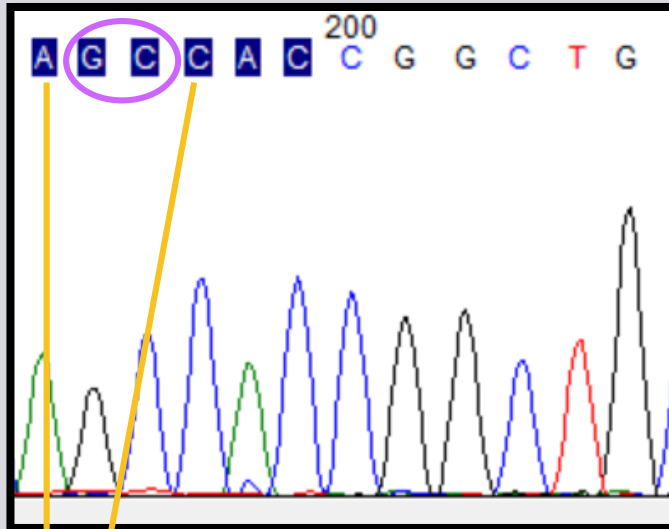
WT sequence

2 base deletion

Heterozygous mutants have double peaks in trace files

WT sequence
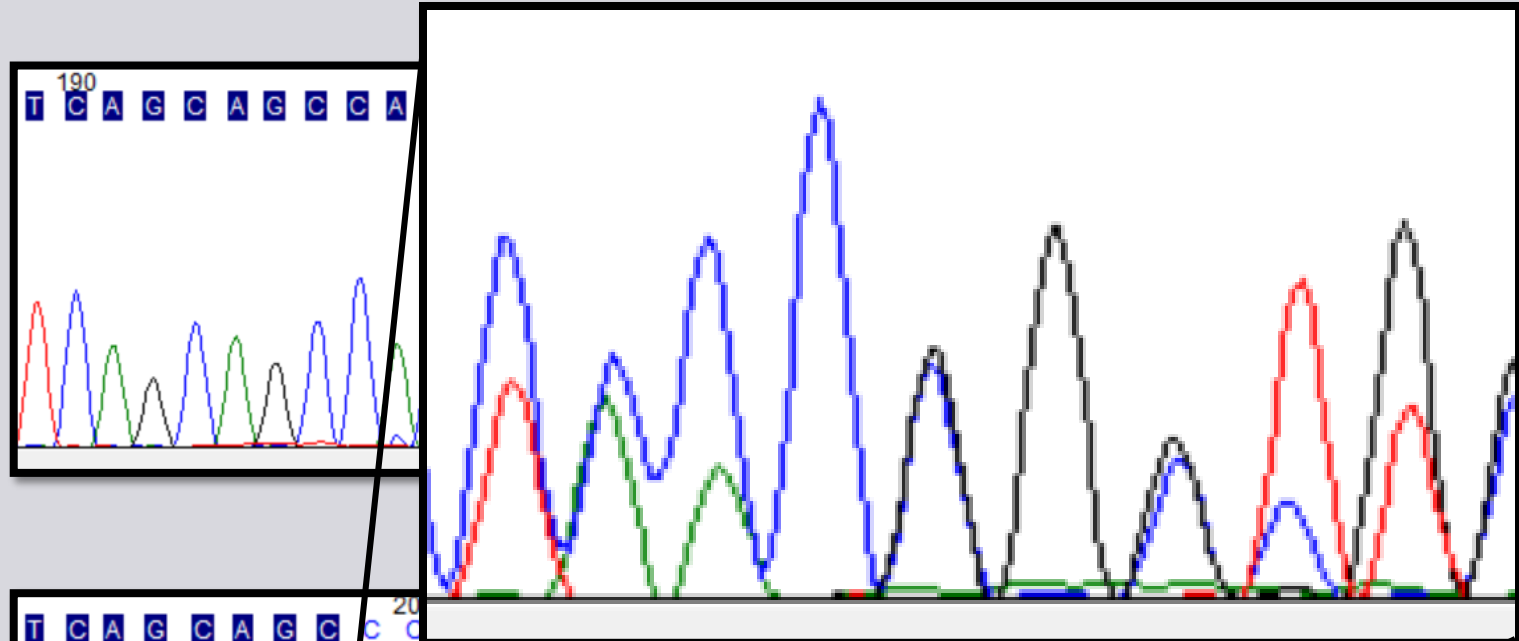
Heterozygous mutant

AGsg1 has low mutagenic activity: low GC and SNP

>PtAG1

…GGATCAGCTAGCTAGACTGCAGCT**ATG**GAATATCAAAATGAATCCCTTGAGAGCTCCCCCCTGAGGAAGCTAGGAAGGGGAAAGGTGGAGAT
CAAGCGGATCGAGAACACCACCAATC**GCCAAGTCACTTTCTGCAAA**AGGCGCAGTGGTTTGCTCAAGAAAGCCTACGAATTATCTGTTCTTTGC
GATGCTGAGGTTGCACTCATCG…

Target site for AGsg1
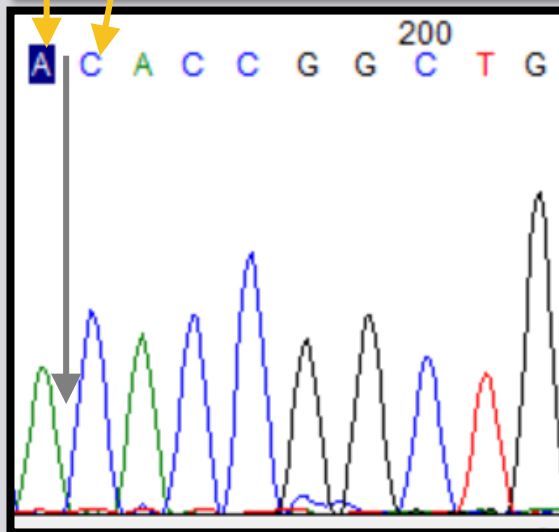
>PtAG2

…GATCAGCTAGCTAGGCAGCAGCT**ATG**GCATACCAAAATGAATCCCAAGAGAGCTCCCCCCTGAGGAAGCTGGGRAGGGGAAAGGTGGAGAT
CAAGCGGATCGAGAACACCACAAATC**GYCAAGTCACTTTCTGCAAA**AGGCGGAATGGTTTGCTCAAGAAAGCCTATGAATTATCTGTTCTTTGC
GATGCTGAGGTTGCACTCATCG…

Construct	Gene	GE events sequenced	Mutant events	Type of mutation	# of events (%)
AGsg1	AG1	64	7 (11%)	Homozygous	0 (0%)
				Heterozygous	7 (11%)
				None	57 (89%)
	AG2	11	0 (0%)	Homozygous	0 (0%)
				Heterozygous	0 (0%)
				None	11 (100%)

High mutation rate in poplar *AG1* gene, despite AGsg1

Construct	Gene	GE events sequenced	Mutant events	Type of mutation	# of events (%)
AGsg1	AG1	64	7 (11%)	Homozygous	0 (0%)
				Heterozygous	7 (11%)
				None	57 (89%)
AGsg2	AG1	41	33 (80%)	Homozygous	3 (7%)
				Heterozygous	30 (73%)
				None	8 (20%)
AGsg1-AGsg2	AG1	143	108 (76%)	Homozygous	10 (7%)
				Heterozygous	98 (69%)
				None	35 (25%)
Total	**AG1**	**248**	**148 (60%)**	**Homozygous**	**13 (6%)**
				Heterozygous	**135 (54%)**
				None	**100 (40%)**
Total (w/out AGsg1)	**AG1**	**184**	**141 (77%)**	**Homozygous**	**13 (7%)**
				Heterozygous	**128 (70%)**
				None	**43 (23%)**

High homozygous mutation rates in poplar *LFY* gene

Construct	GE events sequenced	Mutant events	Type of mutation	# of events (%)
LFYsg1 (exon 1)	131	109 (83%)	Homozygous	16 (12%)
			Heterozygous	93 (71%)
			None	22 (17%)
LFYsg2 (promoter)	46	43 (93%)	Homozygous	10 (22%)
			Heterozygous	33 (71%)
			None	3 (7%)
LFYsg1-LFYsg2	75	70 (93%)	Homozygous	4 (5%)
			Heterozygous	66 (88%)
			None	5 (7%)
Cas (empty vector)	14	0 (0%)	None	14 (100%)
Total (w/out control)	**252**	**222 (88%)**	**Homozygous**	**30 (12%)**
			Heterozygous	**192 (76%)**
			None	**30 (12%)**

High mutation rates in eucalypt *LFY* gene, but rare homozygous mutants

Construct	GE events sequenced	Mutant events	Type of mutation	# of events (%)
EgLFYsg1	20	17 (85%)	Homozygous	0 (0%)
			Heterozygous	17 (85%)
			None	3 (15%)
EgLFYsg2	11	10 (91%)	Homozygous	1 (9%)
			Heterozygous	9 (82%)
			None	1 (9%)
EgLFYsg1-EgLFYsg2	7	7 (100%)	Homozygous	0 (0%)
			Heterozygous	7 (100%)
			None	0 (0%)
Cas (empty vector)	10	0 (0%)	None	10 (100%)
Total (w/out control)	38	34 (90%)	Homozygous	1 (3%)
			Heterozygous	33 (87%)
			None	4 (10%)

Rarity of homozygous knock-outs in eucalypt *LFY* compared to poplar

Total mutations and rates	*PtAG1*	*PtAG2*	*PtLFY*	*EgLFY*
Homozygous	13 (7%)	5 (14%)	30 (12%)	1 (3%)
Heterozygous	128 (70%)	27 (77%)	192 (76%)	33 (87%)
None	43 (23%)	3 (9%)	30 (12%)	4 (10%)
TOTAL	184 (100%)	35 (100%)	252 (100%)	38 (100%)

Homozygous mutation rate in *PtLFY* below that in *EgLFY* (p=0.014, Fisher's Exact Test)

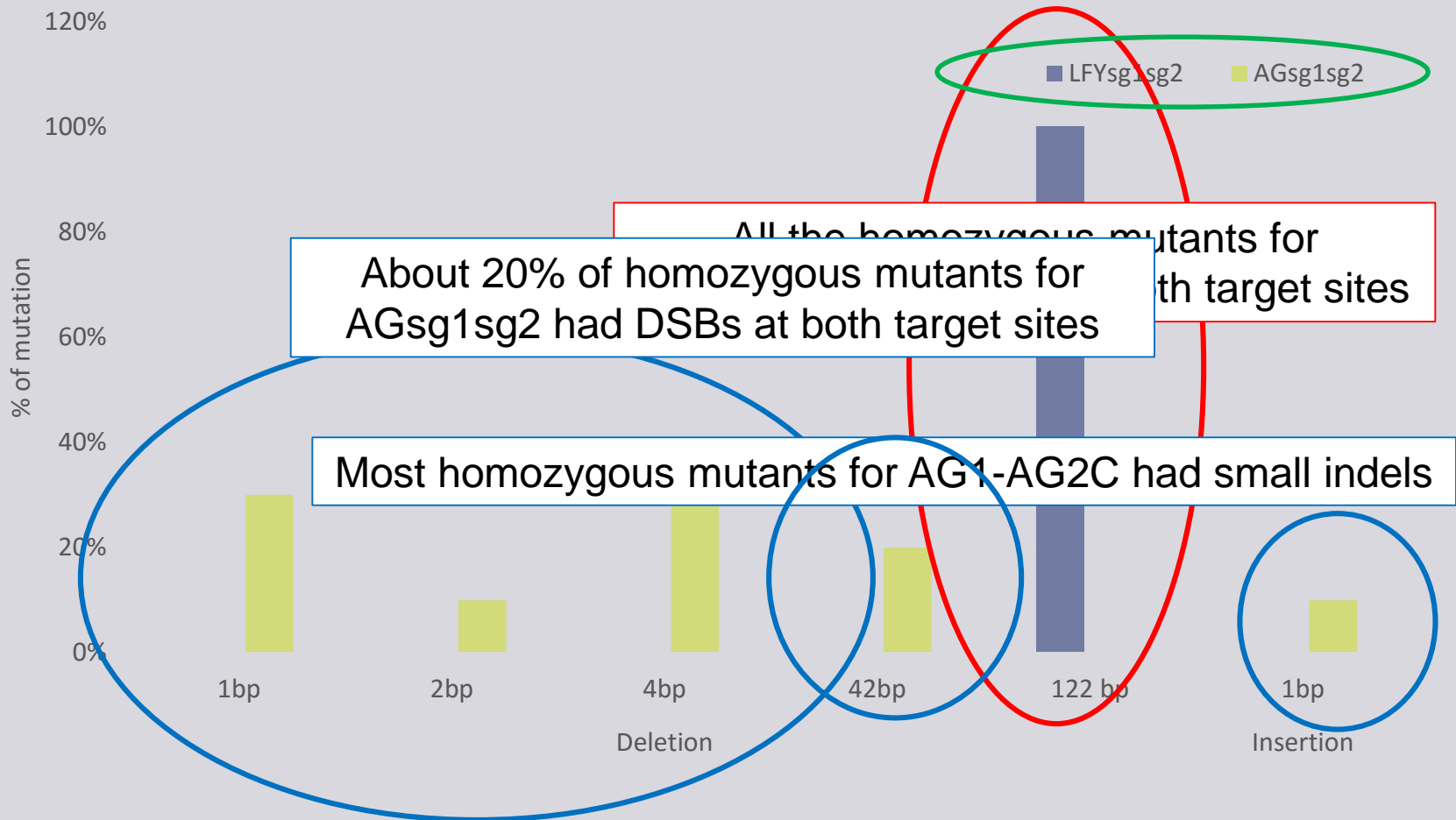# Small indels were frequent for single target nucleases
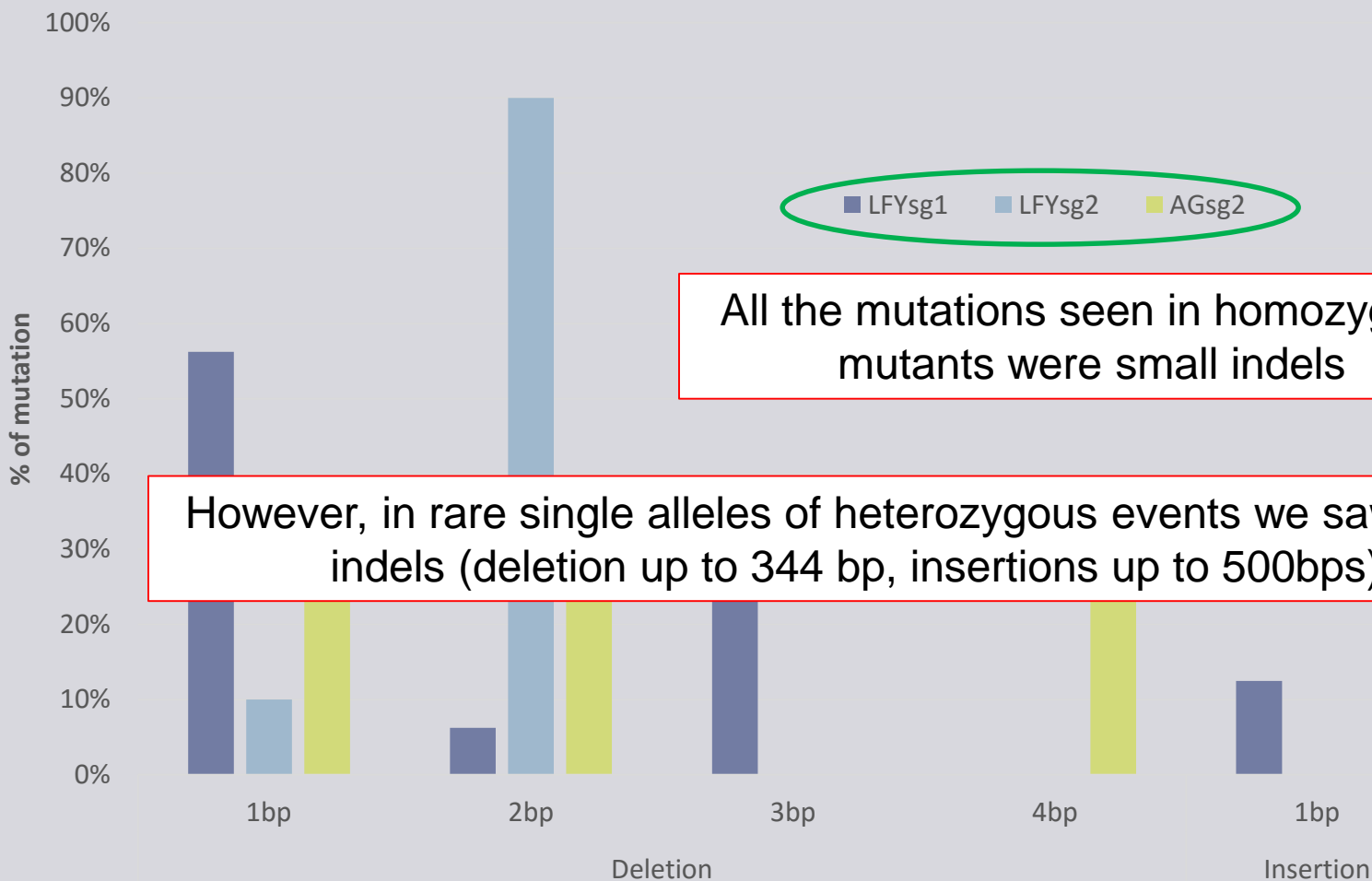
All the mutations seen in homozygous mutants were small indels

However, in rare single alleles of heterozygous events we saw larger indels (deletion up to 344 bp, insertions up to 500bps)
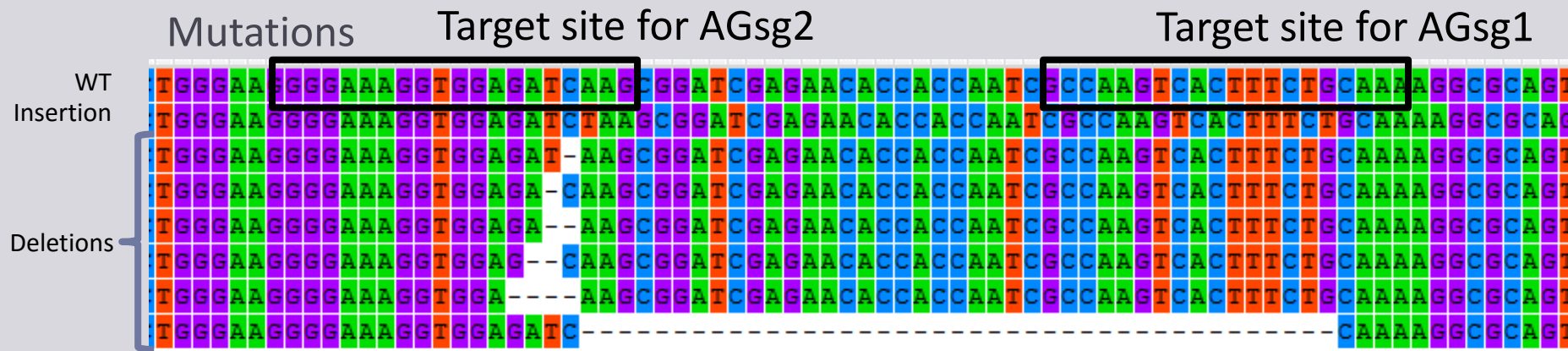
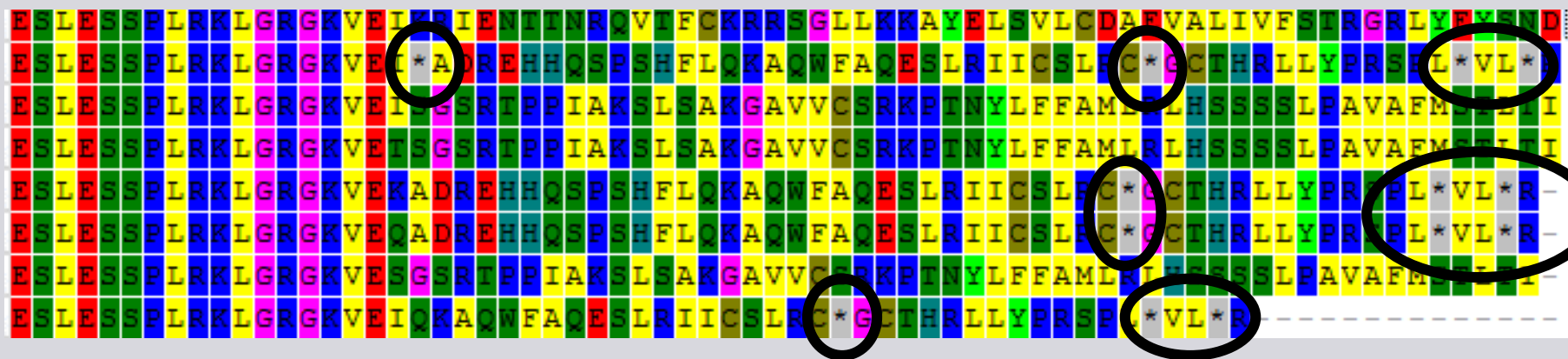# Large mutations were common among active double nucleases

Homozygous events expected to have non-functioning PtAG1

Mutations

Target site for AGsg2

Target site for AGsg1

WT
Insertion

Deletions

Partial peptide sequence

Early stop codons

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- RNAi vs. CRISPR
  – Vegetative function of eucalypt LFY?

Rarity of homozygous knock-outs in eucalypt *LFY* compared to poplar

Total mutations and rates	*PtAG1*	*PtAG2*	*PtLFY*	*EgLFY*
Homozygous	13 (7%)	5 (14%)	30 (12%)	1 (3%)
Heterozygous	128 (70%)	27 (77%)	192 (76%)	33 (87%)
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TOTAL	184 (100%)	35 (100%)	252 (100%)	38 (100%)

Homozygous mutation rate in *PtLFY* below that in *EgLFY* (p=0.014, Fisher's Exact Test)

Normal growth of *LEAFY*-RNAi poplars

control

LFY

Control

LFY

3-12-14
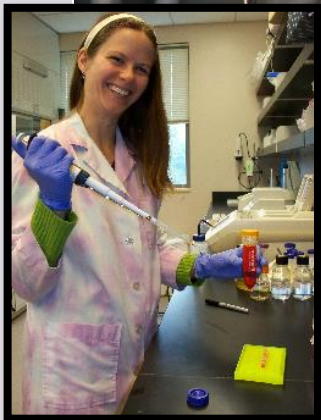
Klocko et al. 2016, In press,
Nature Biotechnology

Euc-LFY RNAi plants have very poor growth

Control

Control, GUSPLUS, LFY 37, 38, & 39
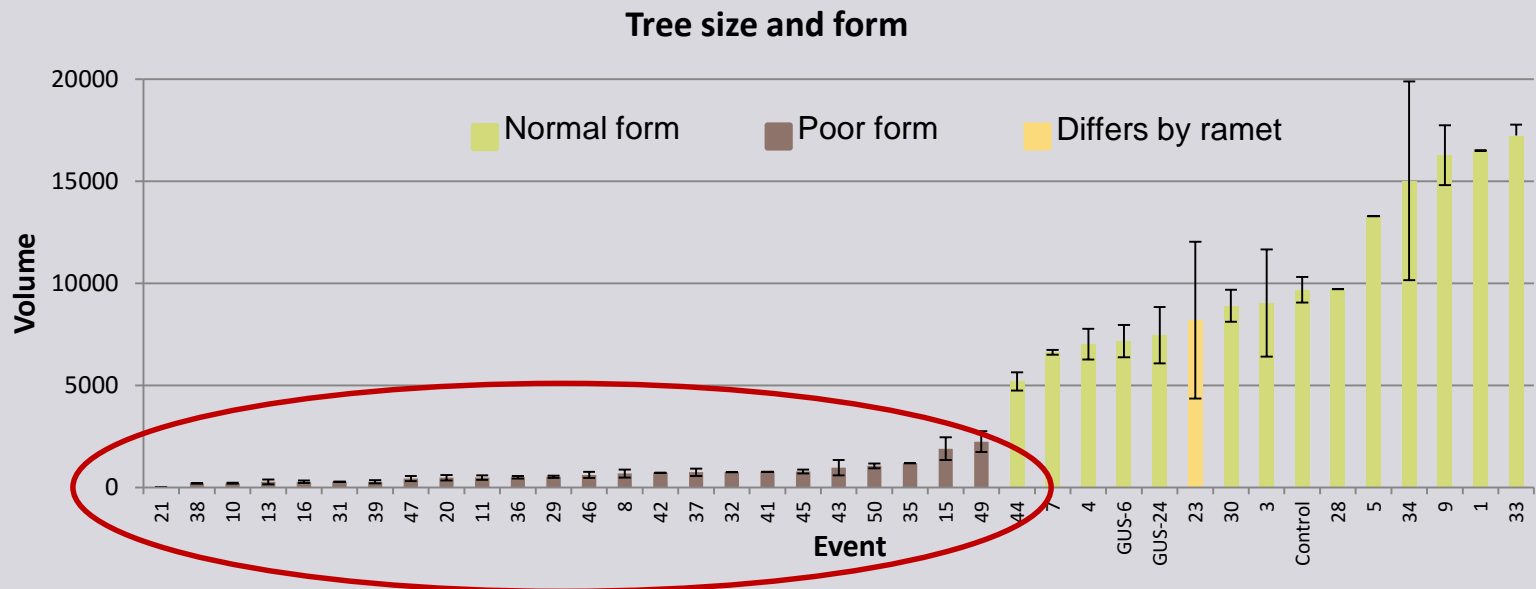
Control, GUSPLUS, LFY 7

LFY 7-1

LFY 7-2

Control, GUSPLUS, LFY 45 & 46

Euc-RNAi in early flowering *E. occidentalis* suggests *LFY* may have vegetative function in eucalypts

**qPCR studies of gene suppression underway**

Summary

- CRISPR/Cas9 nucleases are highly efficient at mutation of poplar and eucalypt flowering genes
- Large mutations are frequent with multiple active sgRNAs
- *LFY* appears to have an important vegetative function in eucalypts
  - Knock-out mutants rare, RNAi severe phenotypes
- Future work
  - Determine knock-out rate among heterozygotes
  - Impact of knock-outs on field grown trees (poplar)
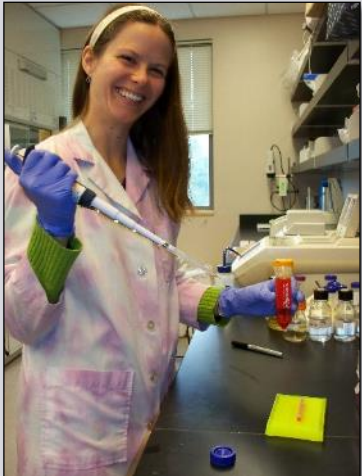 - Search for new CRISPR targets for eucalypt containment

Acknowledgments

Cathleen Ma
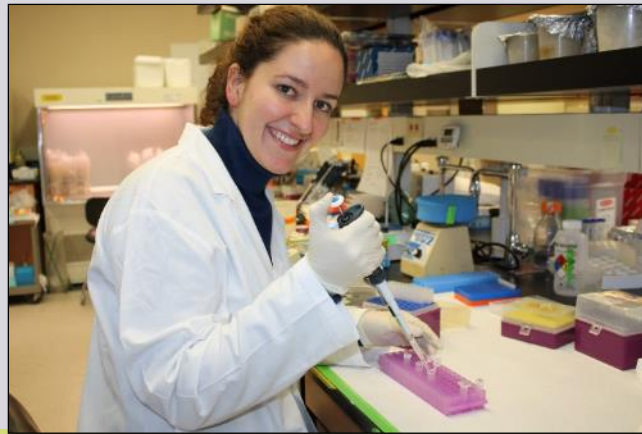
Amy Klocko

Stef Elorriaga
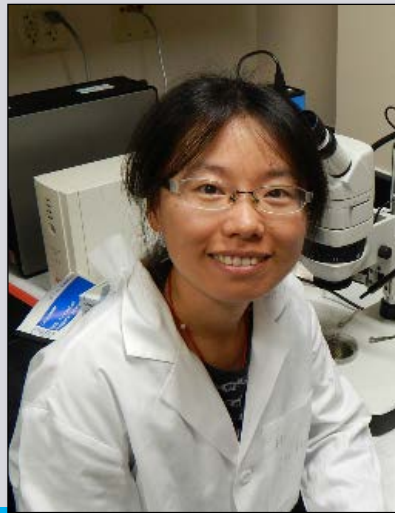

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Clark Embleton,
High School intern