Reproduction control tools for *Eucalyptus*

Knockout of flowering and meiosis genes using CRISPR/Cas9 -- Effects on male-sterility and vegetative development

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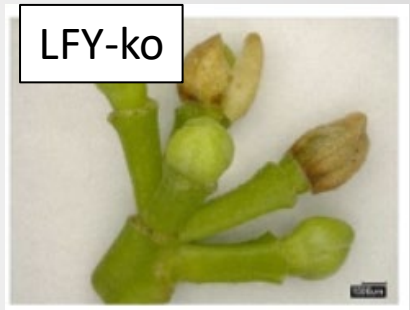
Rationale

- *Eucalyptus* of global significance
  - Uses include fiber, energy, wood, ornamentals
- Spread of *Eucalyptus* often raises social/environmental concerns about invasiveness
- Transgenic traits tend to increase pubic and regulatory concerns from dispersal - within and outside native range
- Hybrids in common use: Male-sterility may facilitate seed production
- General study goal: Use CRISPR to knock-out of selected floral genes in both early-flowering and wild-type genetic backgrounds: male-reproduction and vegetative growth/form

Floral modification a major emphasis of work in lab over many years

- Knockout of *Eucalyptus* ortholog *LEAFY (LFY)* using CRISPR/Cas
- Studied in rapid-flowering FT-overexpression lines
- High biallelic mutation rates, near 100% when transformation successful
- Floral disruption – nearly complete lack of flowers



Estefania Elorriaga, Ph.D.

Plant Biotechnology Journal

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Genetic containment in vegetatively propagated forest trees: CRISPR disruption of *LEAFY* function in *Eucalyptus* gives sterile indeterminate inflorescences and normal juvenile development

Estefania Elorriaga[1,a], Amy L. Klocko[2], Cathleen Ma[1], Marc du Plessis[3], Xinmin An[4], Alexander A. Myburg[5] and Steven H. Strauss[1,*]

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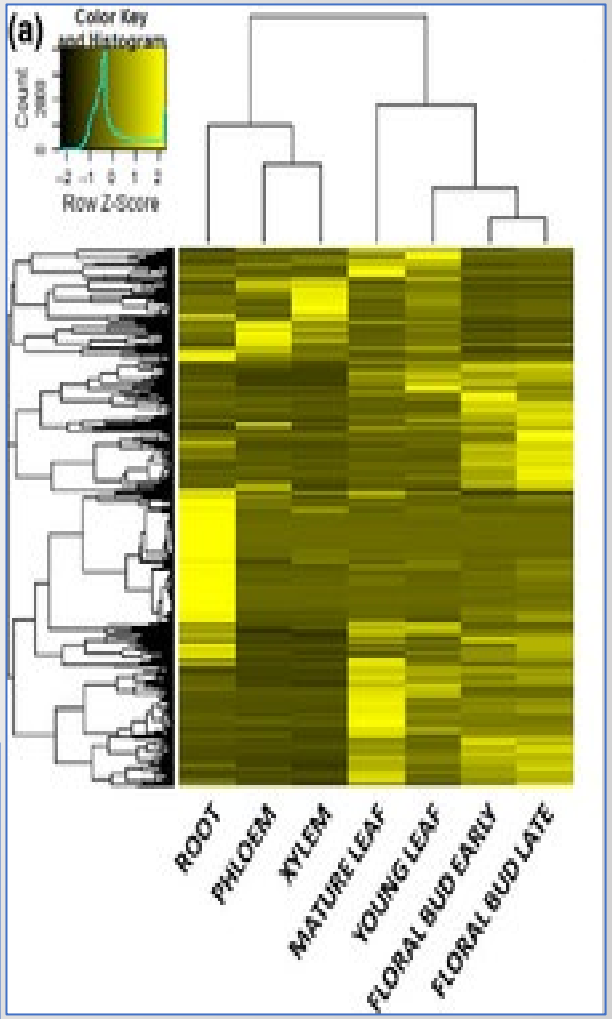
Strategy for selection of new target genes – male, female, and complete sterility goals

- Began with list of floral-specific genes from Vining et al. 2014 floral transcriptome
- Selected targets critical for reproduction, but not vegetative development
- Ran BLAST, Smith-Waterman alignment and examined function in Arabidopsis orthologs (literature review)
- Also examined Arabidopsis meiotic genes directly

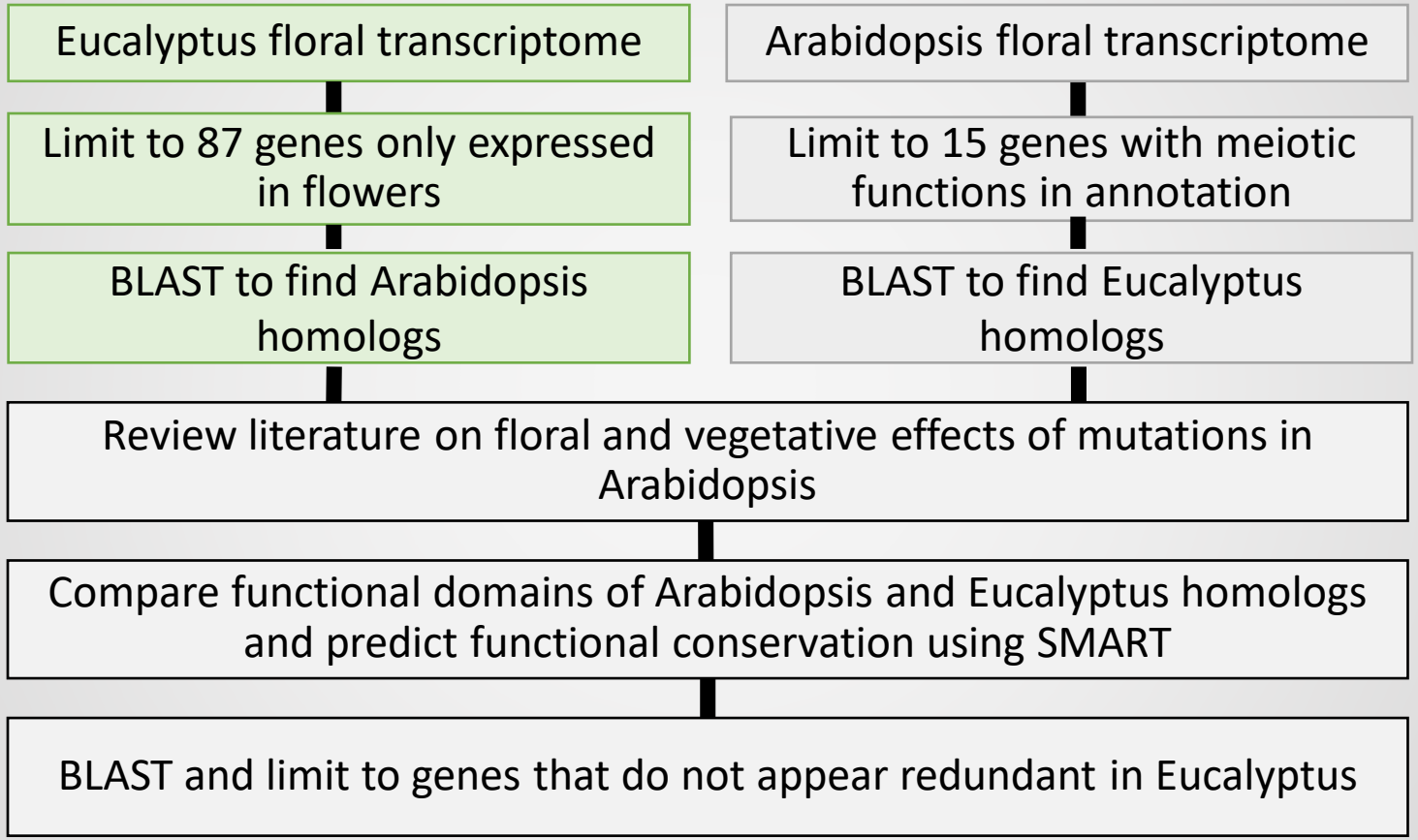


Research
New Phytologist

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 methods for gene target selection



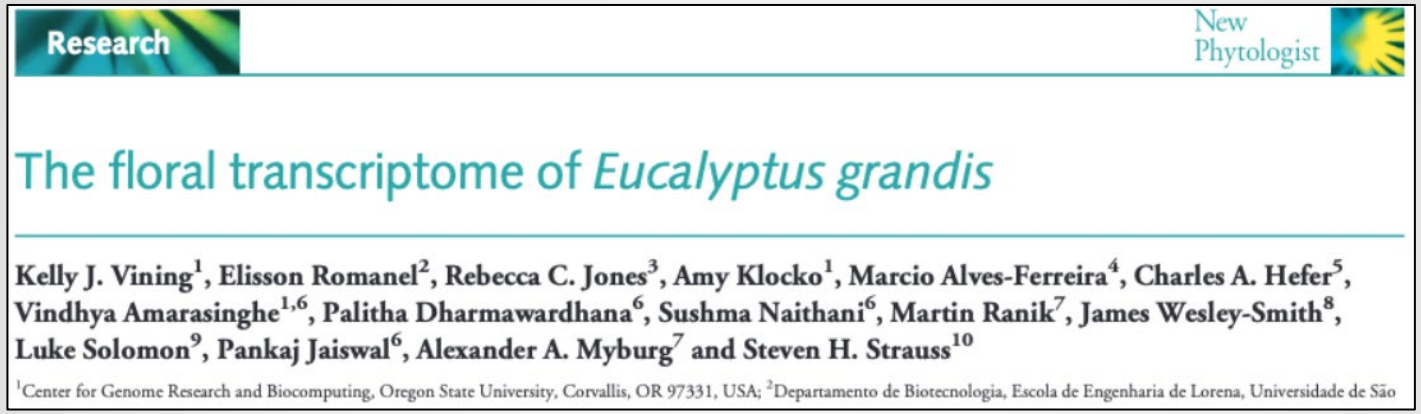
We selected three new eucalypt gene targets for transformation

Two expected to give male sterility, and one bisexual sterility but with ~normal flowers

Three gene targets selected for knockout

- *Male-sterility*
  - *Eucgr.I02017:* Ortholog of male flowering transcription factor *TAPETAL DEVELOPMENT AND FUNCTION 1*
  - *Eucgr.H04946:* Ortholog of flowering transcription factor *HECATE 3*
- *Complete sterility*
  - *Eucgr.G03083:* Ortholog of meiotic cohesin *REC8 (SYNAPTIC 1) so likely to produce ecologically functional flowers with pollen and nectar?*

Nearly exclusive floral-dominant expression



Research

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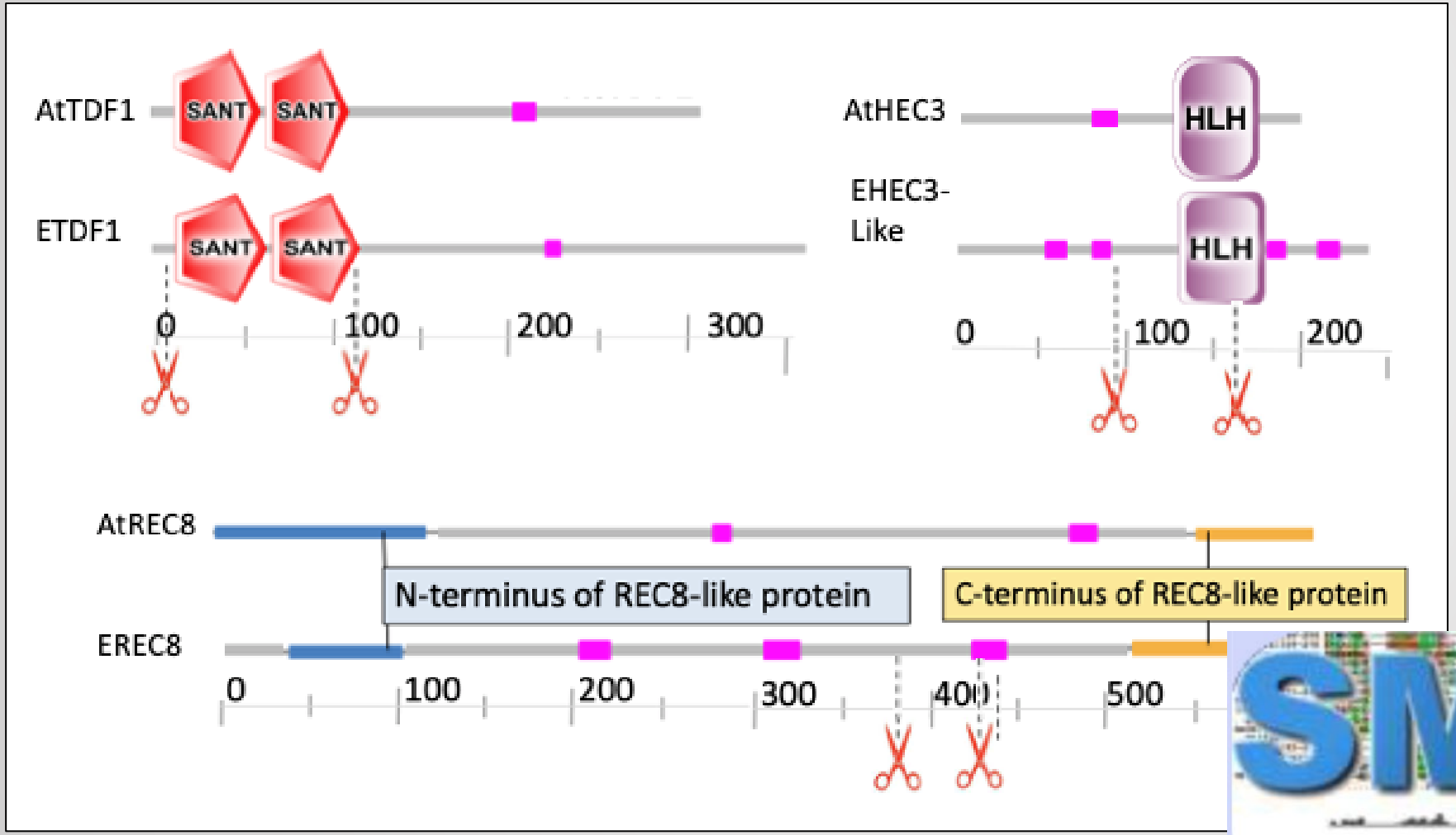
[1]Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR 97331, USA; [2]Departamento de Biotecnologia, Escola de Engenharia de Lorena, Universidade de São

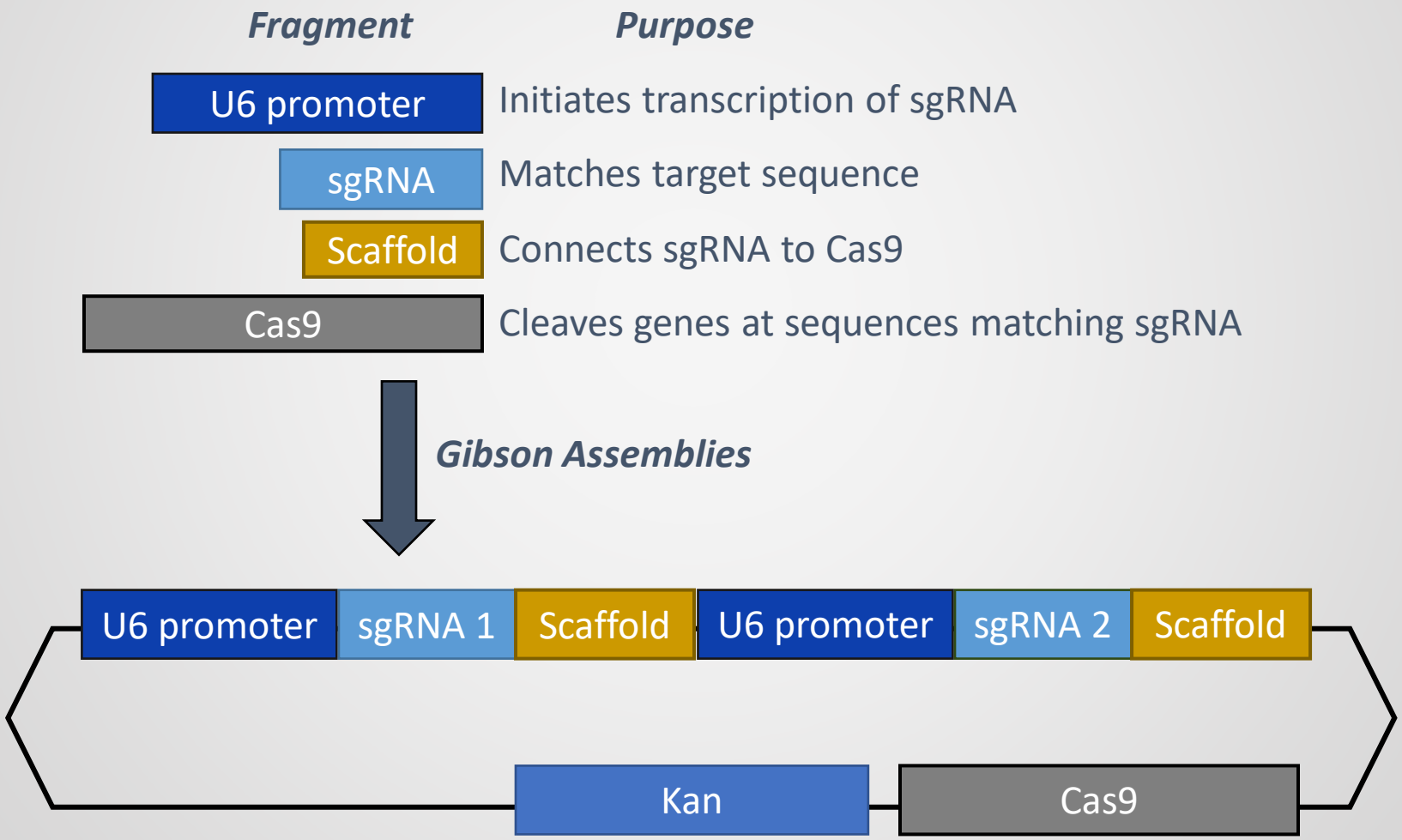
	***E. grandis* gene expression (FPKM)**							
Gene name	Roots	Phloem	Shoot tips	Immature xylem	Young leaf	Mature leaf	Flowers (early)	Flowers (late)
TDF1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.72
REC8	0.03	0.00	0.19	0.11	0.04	0.09	0.08	1.42
HEC3-Like	0.00	0.00	0.00	0.00	0.00	0.00	1.64	8.87

Peptide domains predicted with SMART informed selection of sgRNA targets



Letunic et al. (2017) *Nucleic Acids Res* doi: 10.1093/nar/gkx922
Letunic et al. (2020) *Nucleic Acids Res* doi: 10.1093/nar/gkaa937

Fragments assembled via Gibson Assembly



Transgenic CRISPR plants generated

- Use of Agrobacterium as gene transfer vector, producing stably transgenic CRISPR plants
- Early-flowering and wild-type backgrounds of model genotype-SP7 (provided by Futuragene) employed
- Allele-specific PCR and amplicon sequencing used to verify biallelic knockouts (frameshifts)

Plant Biotechnology Journal

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_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*

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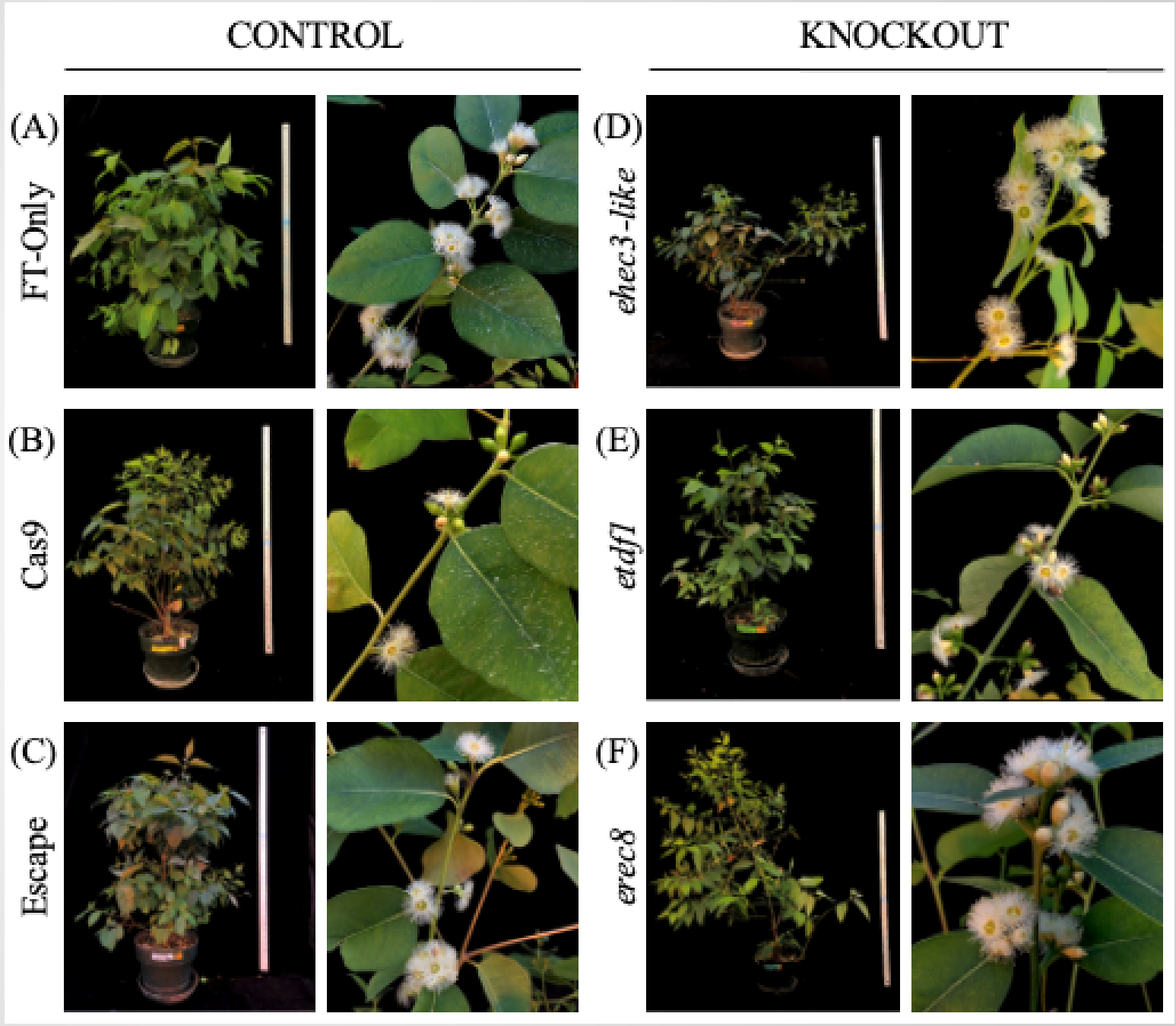
High rates of mutations across gene targets

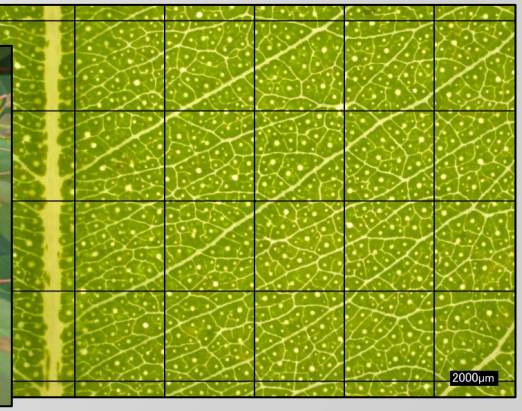
		TDF1	***REC8***	***HEC3***
Specific mutation types	Deletions	41.67%	51.14%	58.33%
	Insertions	9.72%	20.45%	13.89%
	Inversions	5.56%	0.00%	22.22%
	Compound mutations	2.78%	1.14%	1.39%
Summary statistics	Mutations / sgRNA sites	56.94%	72.73%	84.72%
	Frameshifts / alleles	55.56%	77.27%	88.89%

Visual inspection revealed no differences in vegetative, floral development in early flowering trees (normal bushy phenotypes)

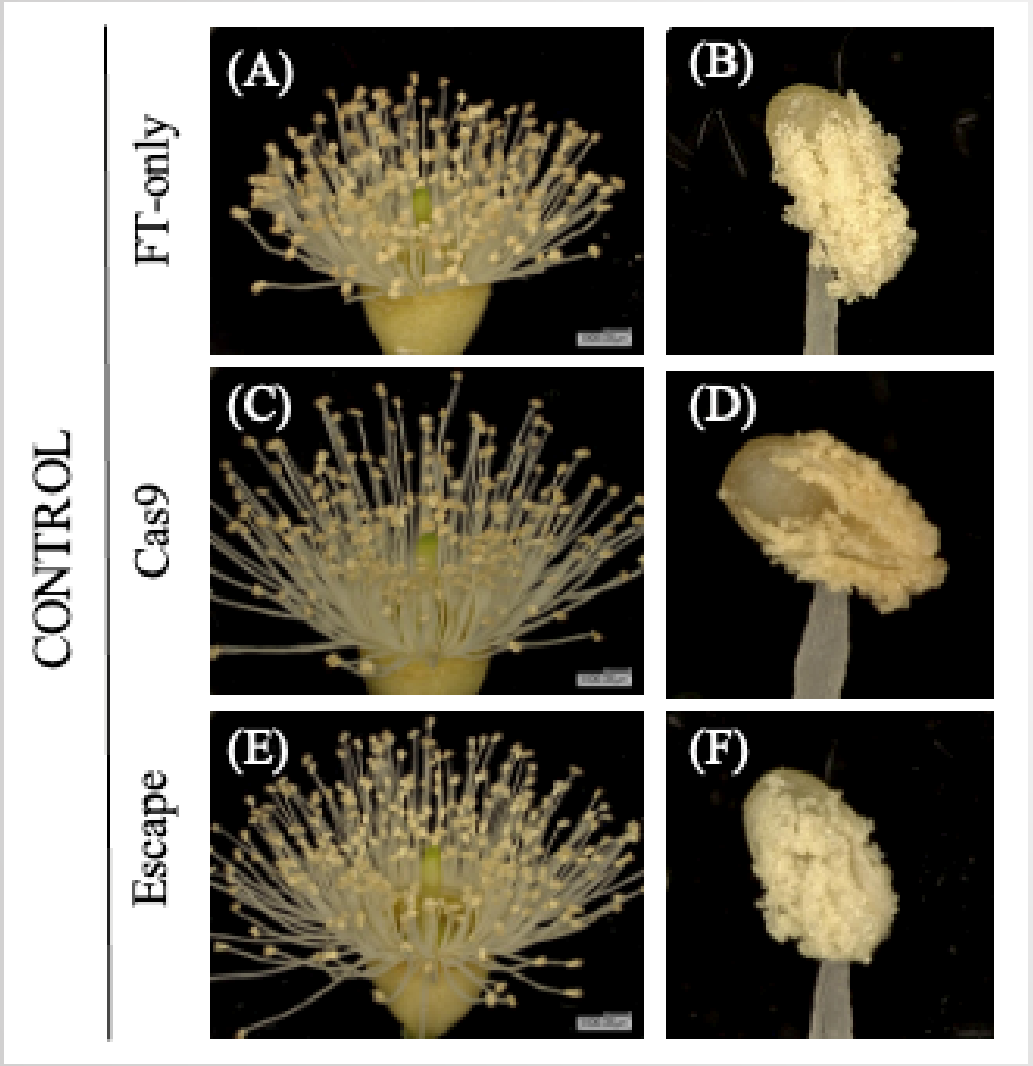


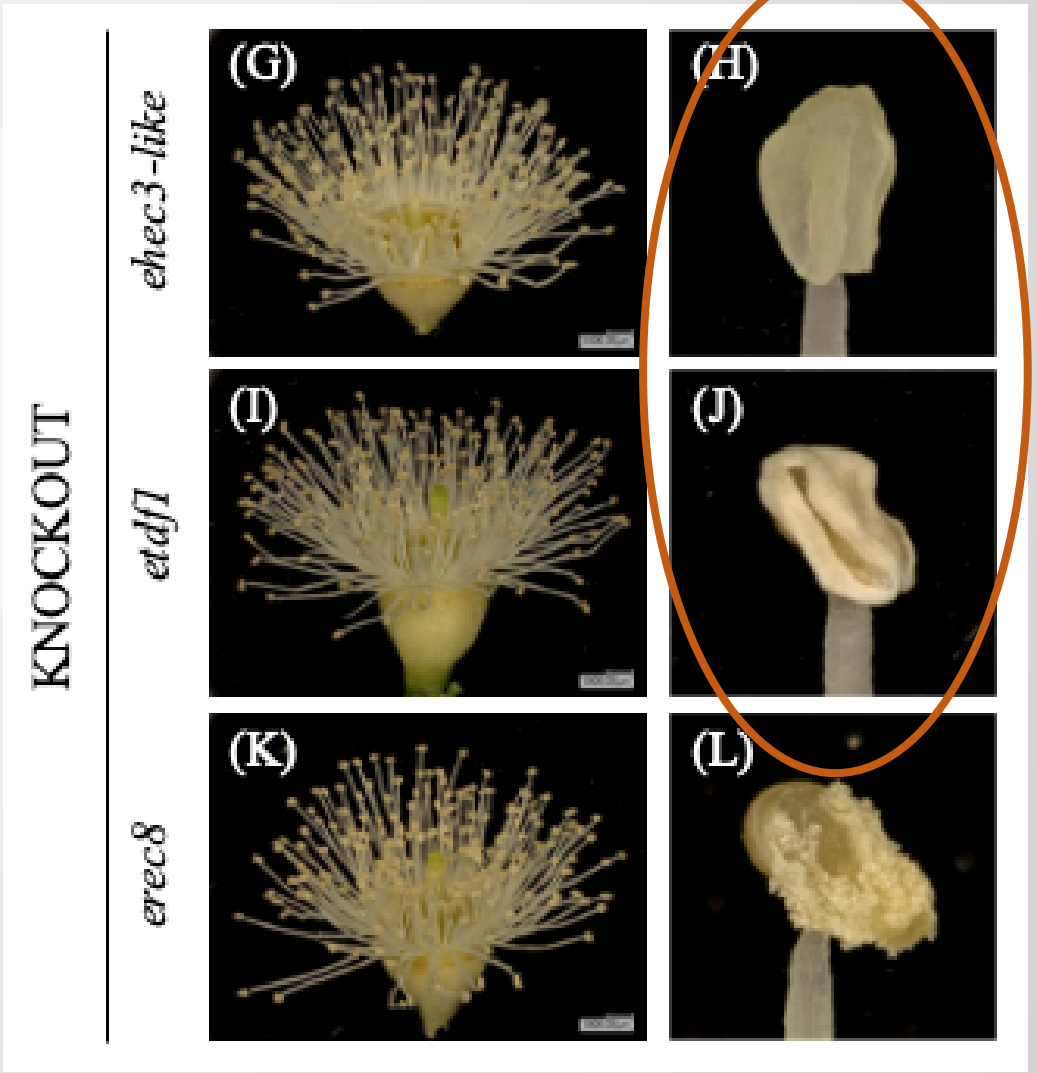
Plants without *FT* overexpression were closely evaluated for effects of knockout on vegetative traits

- Randomized greenhouse trials: KOs and Cas9 only and WT controls
- Measured markers of vegetative development: Leaf area, mass, SPAD (chlorophyll), oilduct count, plant diameter, tree height and stem diameter
- No statistically significant differences were found in any traits measured

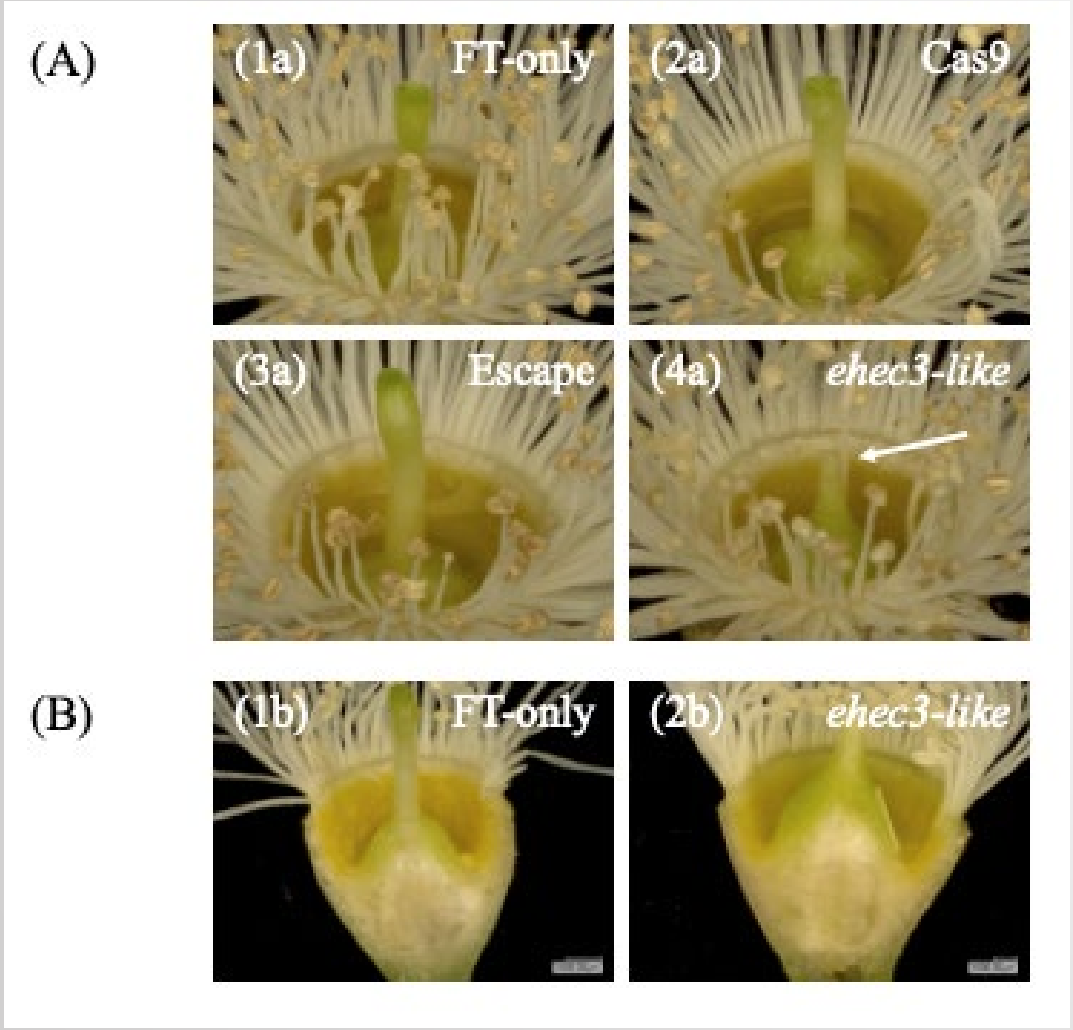


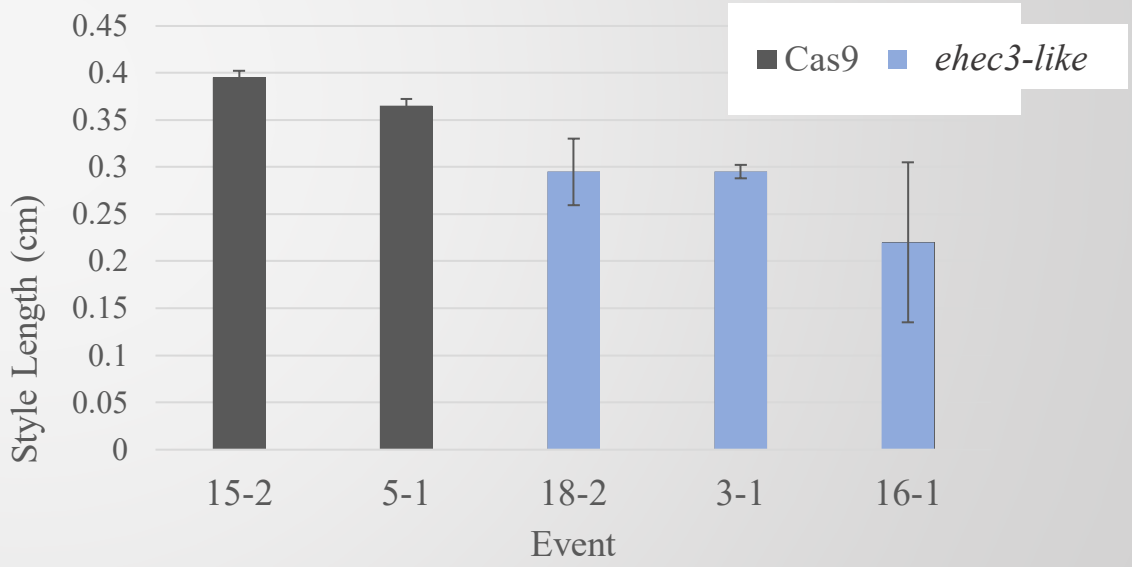
ehec3-like and *tdf1* knockouts feature anthers lacking any pollen production





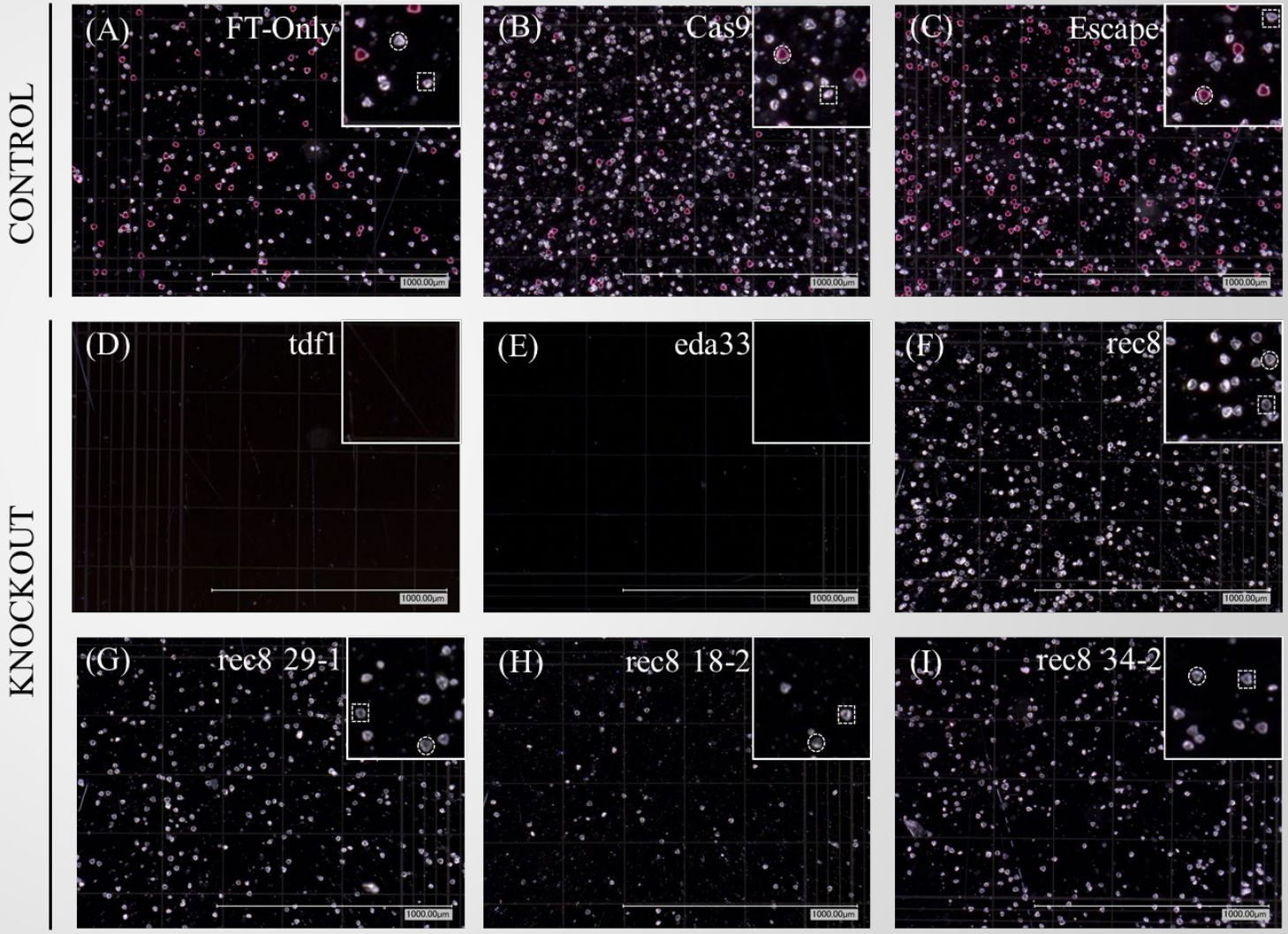
Absence of stigma in *ehec3-like* knockout flowers, along with statistically significant reduction in style length





Male infertility via lack of (viable) pollen for all knockouts

- Collection, staining and microscopy of pollen allows visualization of pollen presence and viability across samples
- Putatively viable pollen appears red



Future work: Female fertility evaluation, ecological function in *rec8* KOs, field studies

- Controlled pollination assays to determine if female flowers are sterile
- Evaluation of nectar composition and pollen nutrition to determine whether ecological value for pollinators is affected in *rec8* knockouts
- Study of flowering in plants without FT overexpression
- Field trials to study pleiotropic effects on vegetative development

Summary

- CRISPR/Cas9 with high KO rate
- KO phenotypes in flowers homologous to predictions from Arabidopsis/rice
- Male-sterility resulting from all three knockouts
- Putative functional but sterile flowers from *REC8* KOs
- Normal vegetative morphology and appearance in all KOs (no FT)
- Mutation of the Eucalyptus orthologs of *TDF1, REC8* and *HEC3-Like* show promise for imparting male-sterility or complete sterility without vegetative pleiotropy

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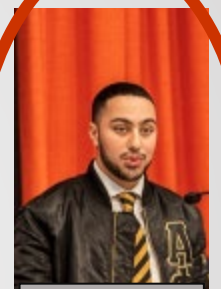
Transformation & Greenhouse Experiments

Amanda Goddard
Program & Field Manager

Greg Goralogia
Postdoc, Flowering & Gene Editing

Kate Peremyslova
GWAS, Transformation Experiments



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