Sterile Eucalyptus for Ornamental and Forestry Uses through CRISPR Disruption of *LEAFY* function

Steve Strauss / Oregon State University / USA

PhD thesis project, in part, by Estefania Elorriaga

Agenda

- Rationale for reproductive modification in Eucalyptus and other trees/ornamentals
- Experimental methods
- Results – floral and vegetative effects

Gene flow: A major reason for strict regulation and market barriers to GMOs

- Bigger for woody ornamentals and forest trees than most ag crops ? … for many reasons
  - **Record of many serious invasives from ornamental and forestry introductions**
  - Wild/feral populations
  - Keystone roles in ecosystems
  - Long distance pollen and/or seed movement
  - Limited domestication
  - Larger role in providing ecosystem services
  - Public view of forests/native ornamentals as natural or wild
  - Scientific uncertainty - Introgression experiments costly or impossible to do, models speculative
- **Gene flow prevention an essential tool, especially for more novel and high impact GMOs? For highly sensitive countries?**
- **Gene flow a major concern with eucalypts, GMO or not, in the USA – deregulation proposal for cold tolerant and male-sterile eucalypts help up for many years at USDA**

Many containment options

- Non-GE: Ploidy changes / irradiation / hybrids
- Cytotoxins / barnase driven by floral promoters
- Disruption of essential genes for flowering
  - Dominant interfering proteins
  - Suppressing expression
  - **Physical mutation**
- Various options for control: Male vs. female, induction & restorer
- Our focus has been on <u>bisexual</u> and <u>permanent</u> sterility for vegetatively propagated species
  - CRISPR mutation likely to be most reliable, predictable, and efficient

LEAFY gene target for bisexual sterility: Strong mutants appear to have no flowers

Snapdragon *Arabidopsis* Petunia

Wild type

***lfy* mutants**

Parcy et al. 2002; Moyroud et al. 2010

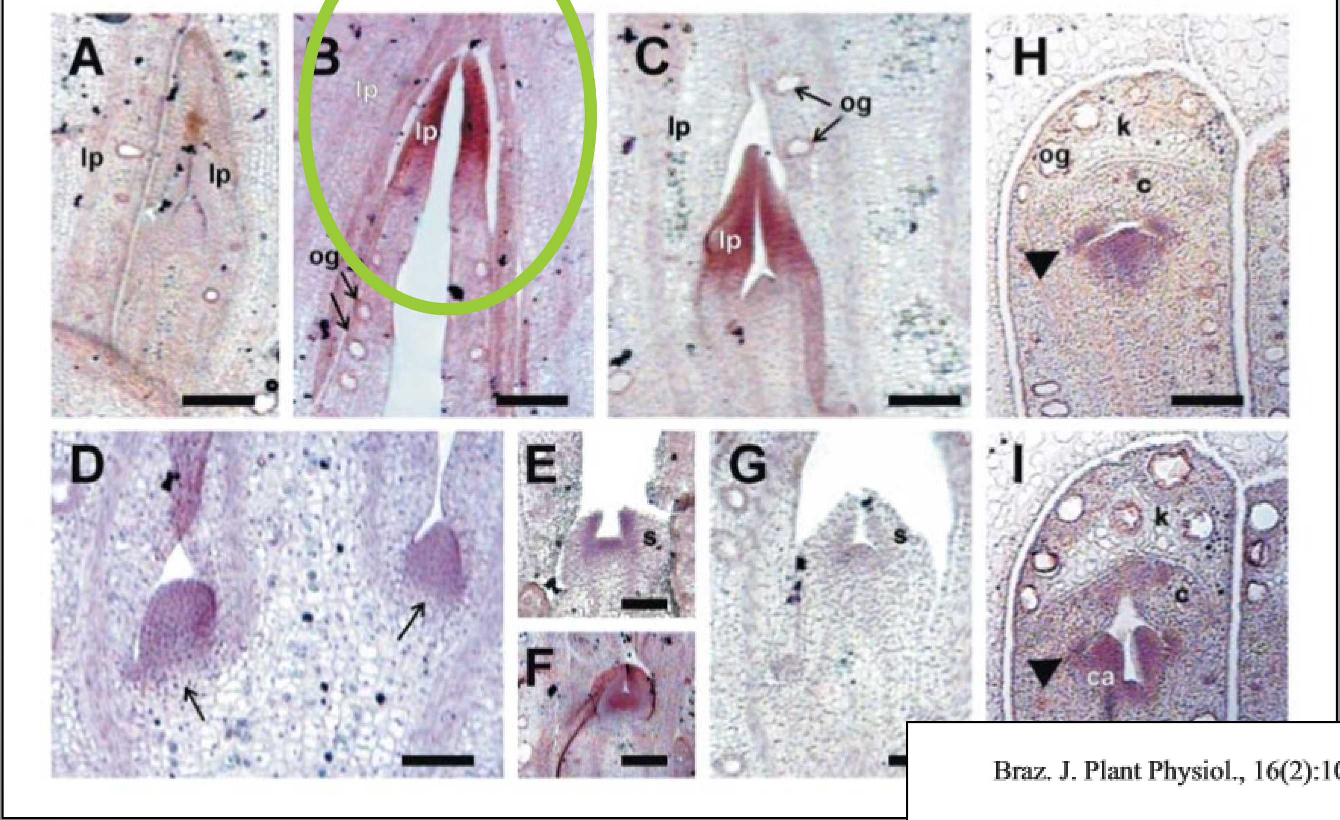
The full roles of *LFY* unclear

- Critical that *LFY* mutation does not depress tree productivity, though might increase it
  - Studies in model plants did not conduct significant analyses of vegetative/productivity effects
  - An absence of gene knock-out studies in the field
- No studies in the very divergent floral types of important forest tree taxa
  - *LFY* might have evolved different functions in species like poplar and eucalypts
- Found to have vegetative as well as floral expression in poplars and eucalypts, thus worrisome
  - Meristematic vegetative cell expression

Eucalyptus *LFY* vegetative expression

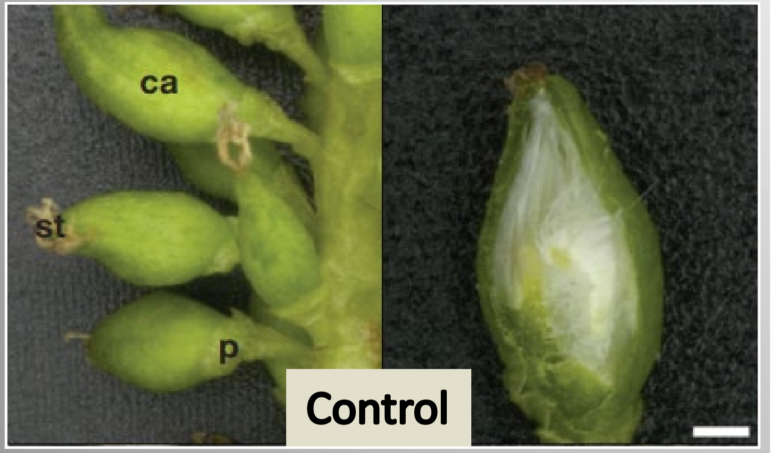
EgLFY, the _Eucalyptus grandis_ homolog of the _Arabidopsis_ gene _LEAFY_ is expressed in reproductive and vegetative tissues

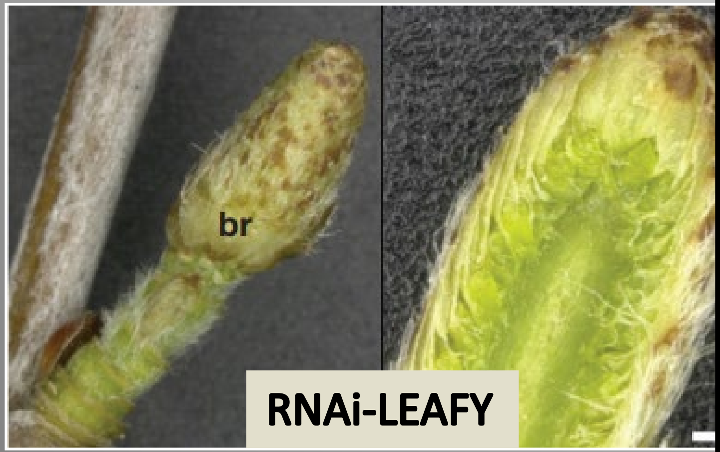
Marcelo Carnier Dornelas[1*], Weber A. Neves do Amaral[2] and Adriana Pinheiro Martinelli Rodriguez[1]

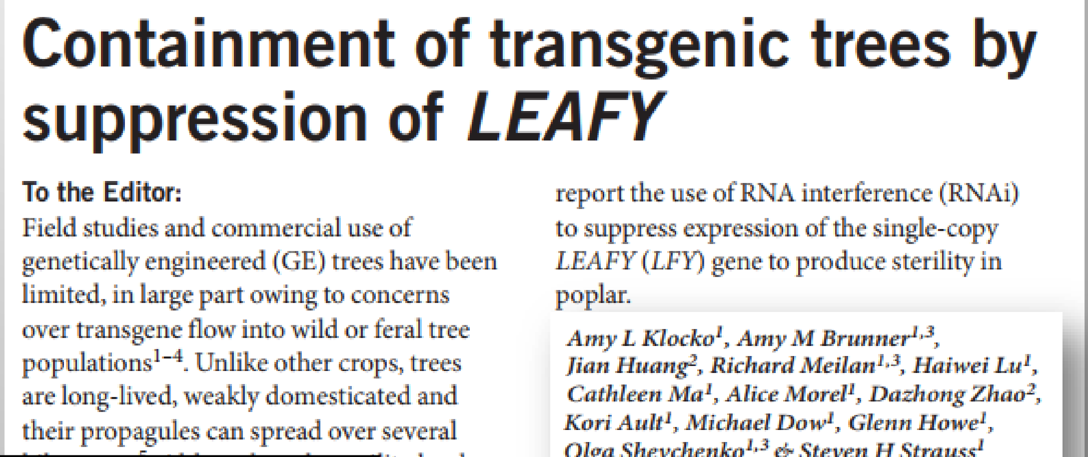


Braz. J. Plant Physiol., 16(2):105-114, 2004

Why not RNAi? Poplar sterility using RNAi against *LEAFY* works in the field, is stable, but only two of 15 events sterile in *P. alba* 6K10







Containment of transgenic trees by suppression of *LEAFY*

To the Editor:
Field studies and commercial use of genetically engineered (GE) trees have been limited, in large part owing to concerns over transgene flow into wild or feral tree populations[1–4]. Unlike other crops, trees are long-lived, weakly domesticated and their propagules can spread over several

ity has been
ucalyptus
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gene in
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nd vegetative
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sterility
ispersal,
exotic trees,
tion[9]. We

report the use of RNA interference (RNAi) to suppress expression of the single-copy *LEAFY* (*LFY*) gene to produce sterility in poplar.

Amy L Klocko[1], Amy M Brunner[1,3], Jian Huang[2], Richard Meilan[1,3], Haiwei Lu[1], Cathleen Ma[1], Alice Morel[1], Dazhong Zhao[2], Kori Ault[1], Michael Dow[1], Glenn Howe[1], Olga Shevchenko[1,3] & Steven H Strauss[1]

[1]Department of Forest Ecosystems and Society, Oregon State University, Corvallis, Oregon, USA. [2]Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, USA. [3]Present addresses: Department of Forest Resources and Environmental Conservation, Virginia Tech, Blacksburg, Virginia, USA (A.M.B.), Department of Forestry and Natural Resources, Purdue University, West Lafayette, Indiana, USA (R.M.), and Delaware Biotechnology Institute, Newark, Delaware, USA (O.S.).
e-mail: steve.strauss@oregonstate.edu

Two other LFY-RNAi poplar clones tested

No sterile trees obtained among ~30 insertion events in a 4 ha field trial in Oregon, USA

Clone 717 female

Clone 353 male

June 27, 2017

Eucalypt RNAi-LFY also tested in a field trial in Israel: No floral modified trees seen in ~30 events

FuturaGene
Yielding the Future

Field trail LFY:RNAi

A Suzano Pulp and Paper Company

28/2/17

Thus need a much more efficient, and complete, gene knock-out method – **CRISPR to the rescue**?

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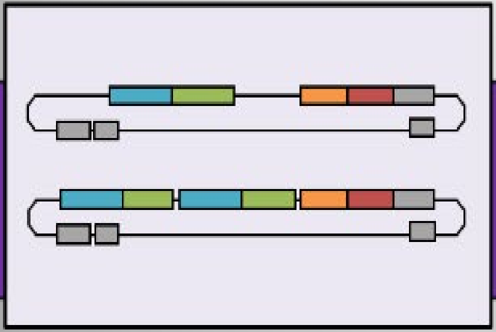
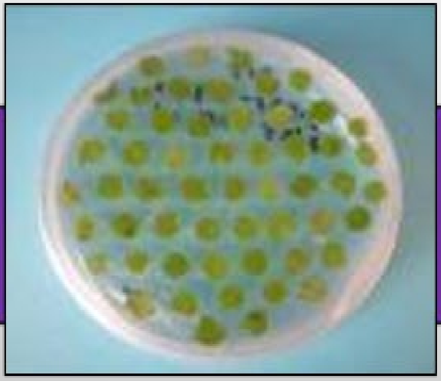
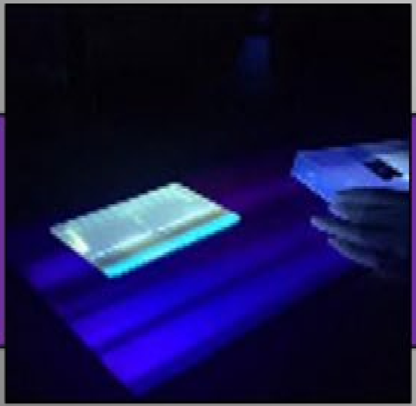
CRISPR pipeline



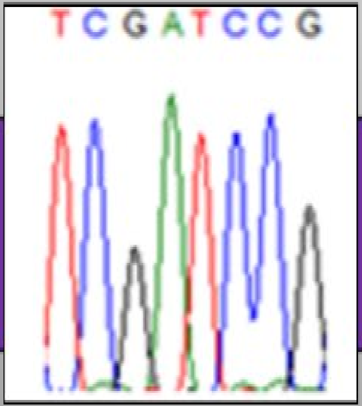
Construct

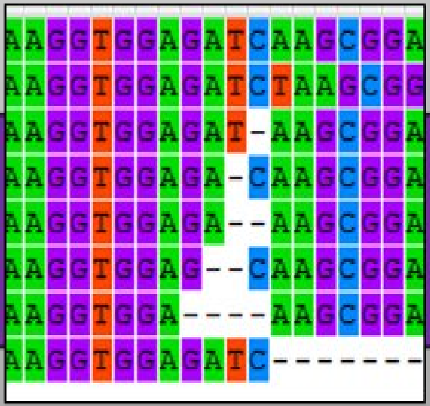


Transformation and regeneration



PCR and gel analysis
(allele specific)

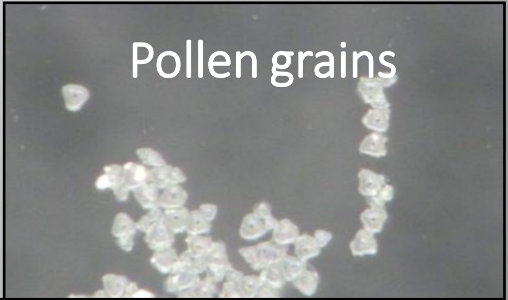


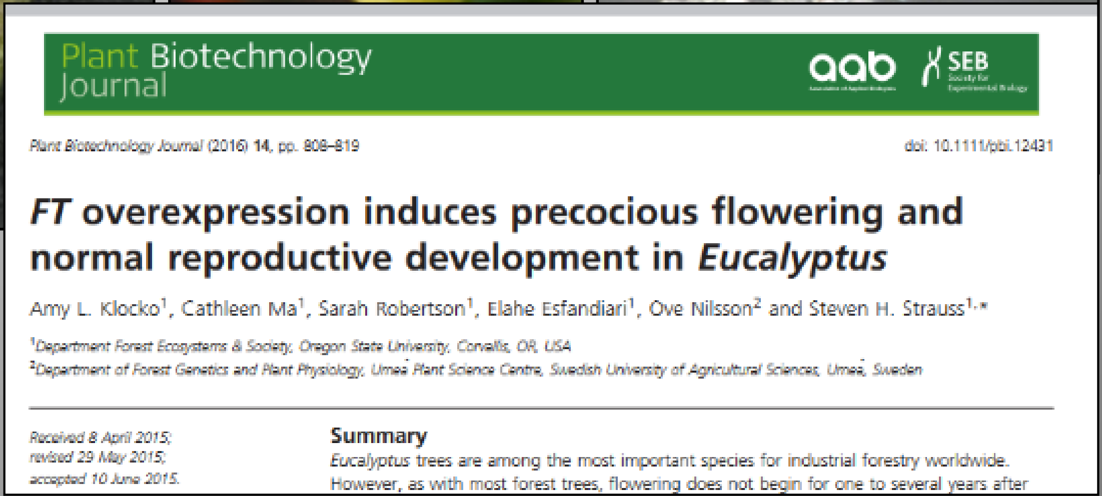


```
AAGGTGGAGATCAAGCGGA
AAGGTGGAGATCTAAGCGG
AAGGTGGAGAT-AAGCGGA
AAGGTGGAGA-CAAGCGGA
AAGGTGGAGA--AAGCGGA
AAGGTGGAG--CAAGCGGA
AAGGTGGA----AAGCGGA
AAGGTGGAGATC-------
```

Sequencing of targets, alignment, and phenotyping

Early flowering *FT*-eucalypts to speed floral 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,*]

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[2]Department of Forest Genetics and Plant Physiology, Umeå Plant Science Centre, Swedish University of Agricultural Sciences, Umeå, Sweden

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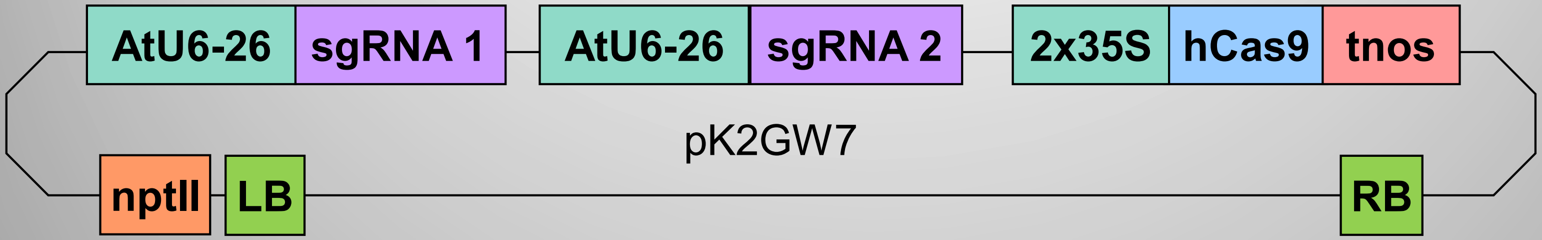
revised 29 May 2015;
accepted 10 June 2015.

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

Constructs employed

CRISPR

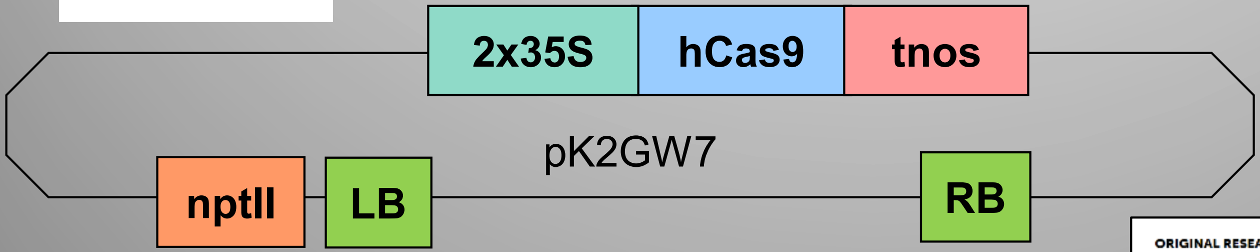


AtU6-26 | sgRNA 1 | AtU6-26 | sgRNA 2 | 2x35S | hCas9 | tnos

pK2GW7

nptII | LB | RB

Control



2x35S | hCas9 | tnos

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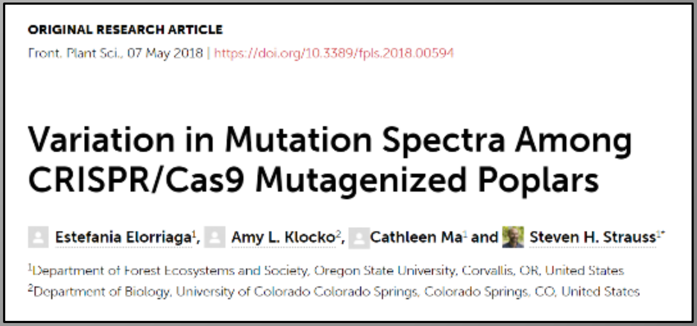

ORIGINAL RESEARCH ARTICLE

Front. Plant Sci., 07 May 2018 | https://doi.org/10.3389/fpls.2018.00594

Variation in Mutation Spectra Among CRISPR/Cas9 Mutagenized Poplars

Estefania Elorriaga[1], Amy L. Klocko[2], Cathleen Ma[1] and Steven H. Strauss[1*]

[1] Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR, United States
[2] Department of Biology, University of Colorado Colorado Springs, Colorado Springs, CO, United States

Agenda

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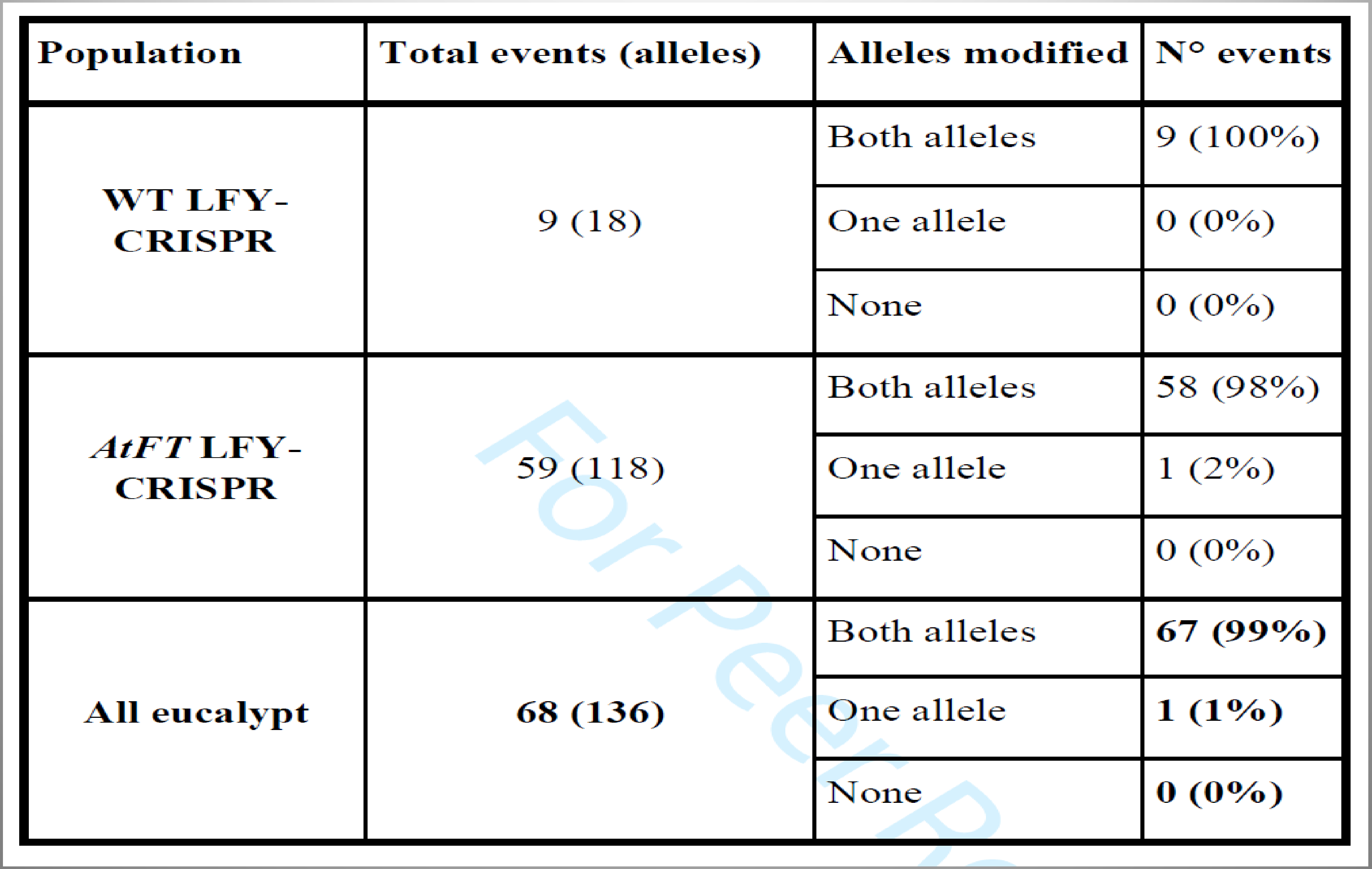
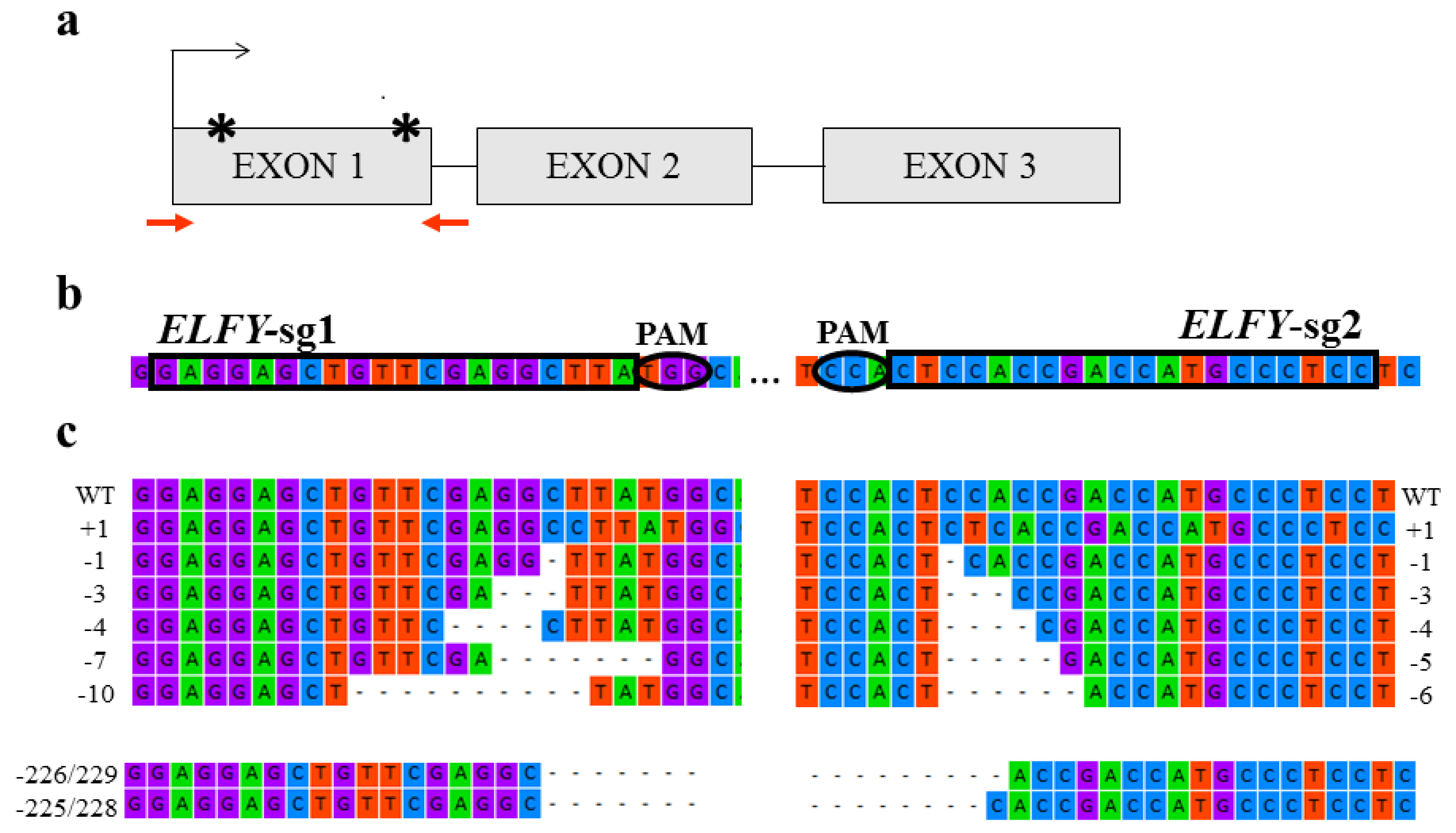
High knock-out mutation rate



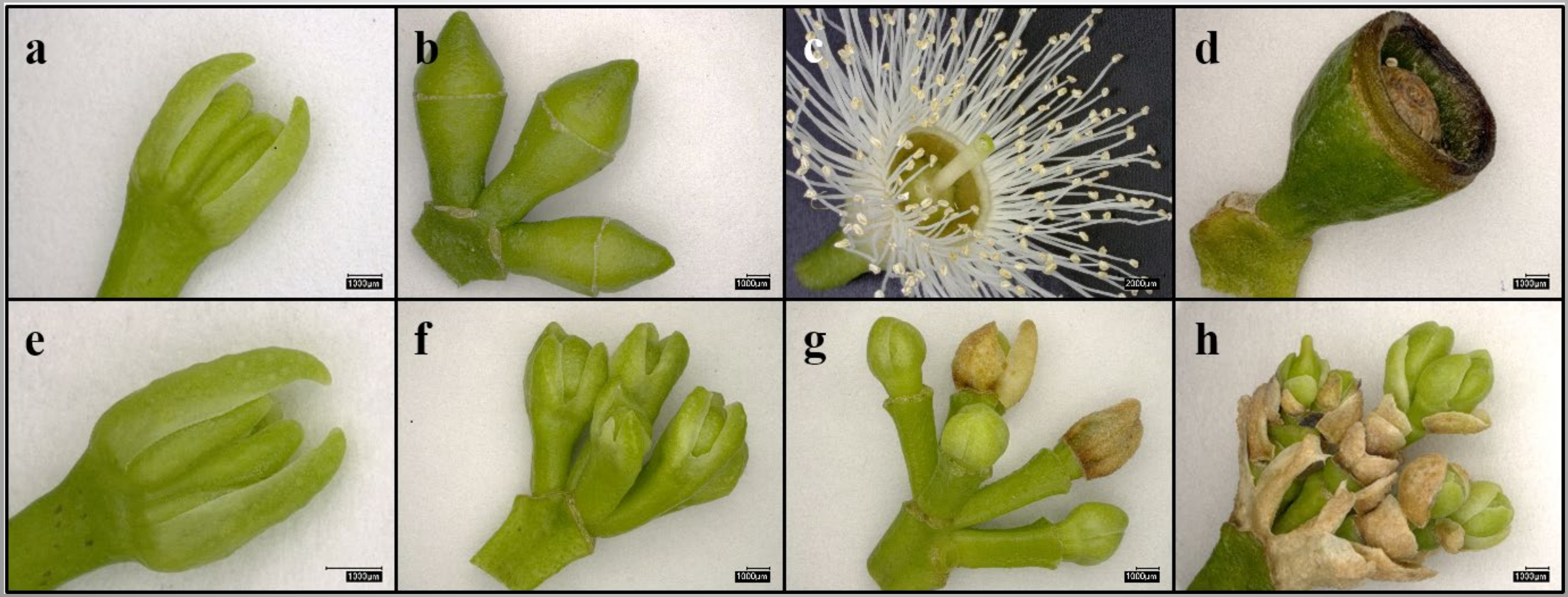
Population	**Total events (alleles)**	**Alleles modified**	**N° events**
WT LFY-CRISPR	9 (18)	Both alleles	9 (100%)
		One allele	0 (0%)
		None	0 (0%)
***AtFT* LFY-CRISPR**	59 (118)	Both alleles	58 (98%)
		One allele	1 (2%)
		None	0 (0%)
All eucalypt	**68 (136)**	Both alleles	**67 (99%)**
		One allele	**1 (1%)**
		None	**0 (0%)**

sgRNA locations, example mutations

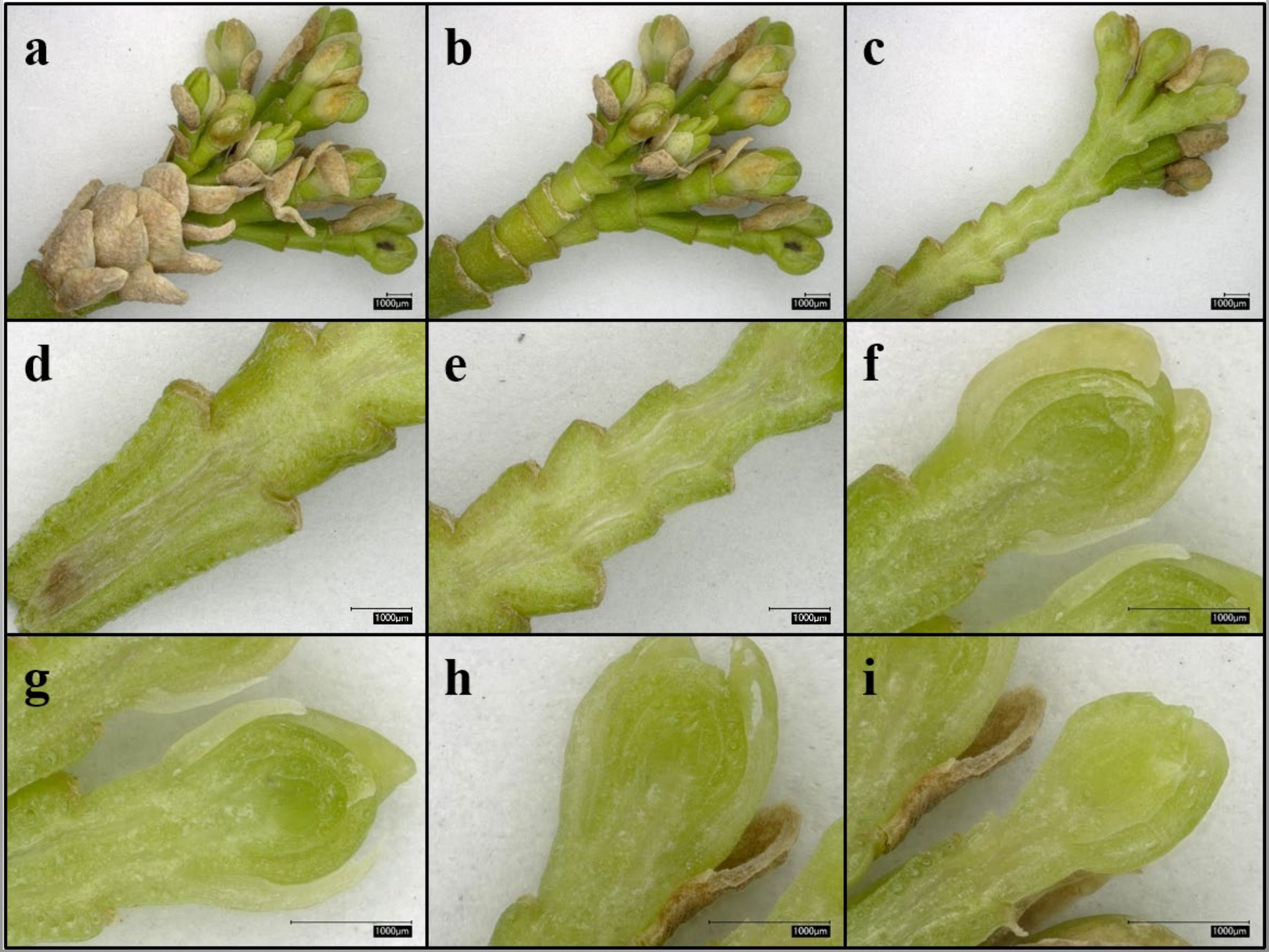


```
GGAGGAGCTGTTCGAGGCTTATGGC ... TCCACTCCACCGACCATGCCCTCCTC

WT    GGAGGAGCTGTTCGAGGCTTATGGC    TCCACTCCACCGACCATGCCCTCCT  WT
+1    GGAGGAGCTGTTCGAGGCCTTATGG    TCCACTCTCACCGACCATGCCCTCC  +1
-1    GGAGGAGCTGTTCGAGG-TTATGGC    TCCACT-CACCGACCATGCCCTCCT  -1
-3    GGAGGAGCTGTTCGA---TTATGGC    TCCACT---CCGACCATGCCCTCCT  -3
-4    GGAGGAGCTGTTC----CTTATGGC    TCCACT----CGACCATGCCCTCCT  -4
-7    GGAGGAGCTGTTCGA-------GGC    TCCACT-----GACCATGCCCTCCT  -5
-10   GGAGGAGCT----------TATGGC    TCCACT------ACCATGCCCTCCT  -6

-226/229 GGAGGAGCTGTTCGAGGC--------   ----------ACCGACCATGCCCTCCTC
-225/228 GGAGGAGCTGTTCGAGGC--------   ---------CACCGACCATGCCCTCCTC
```

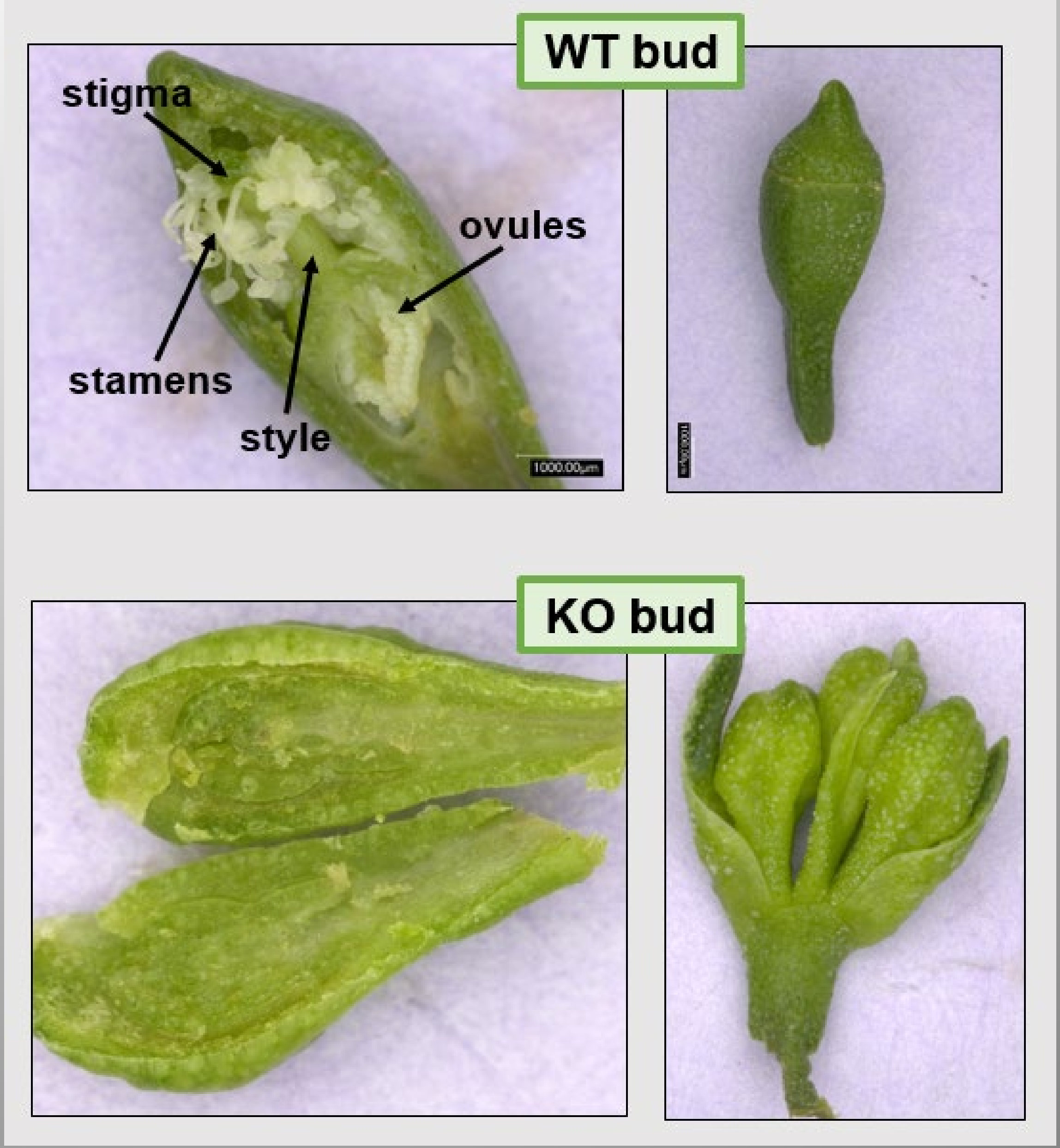
Wild-type vs. knockout stages



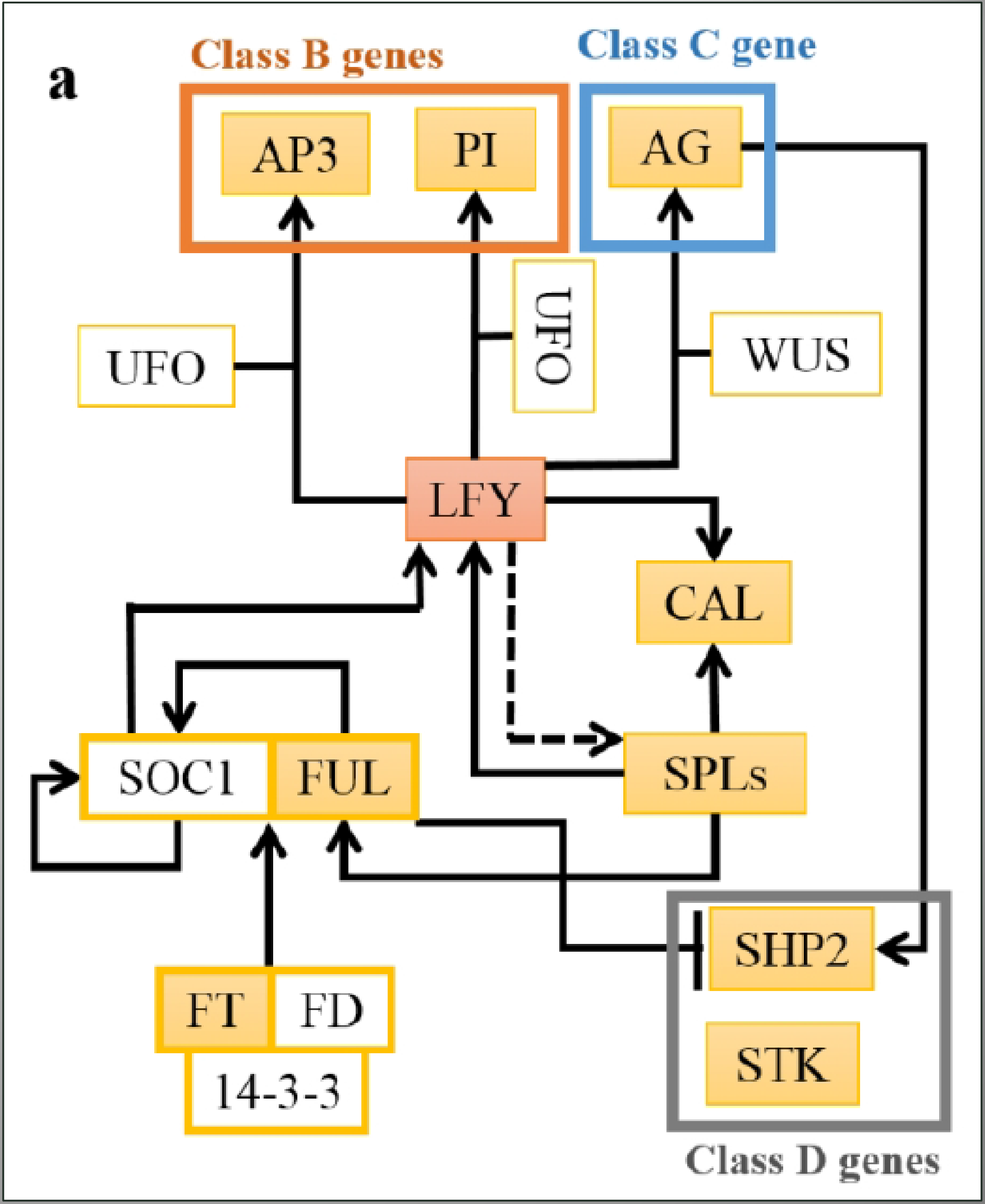
Prominent indeterminacy in knock-outs



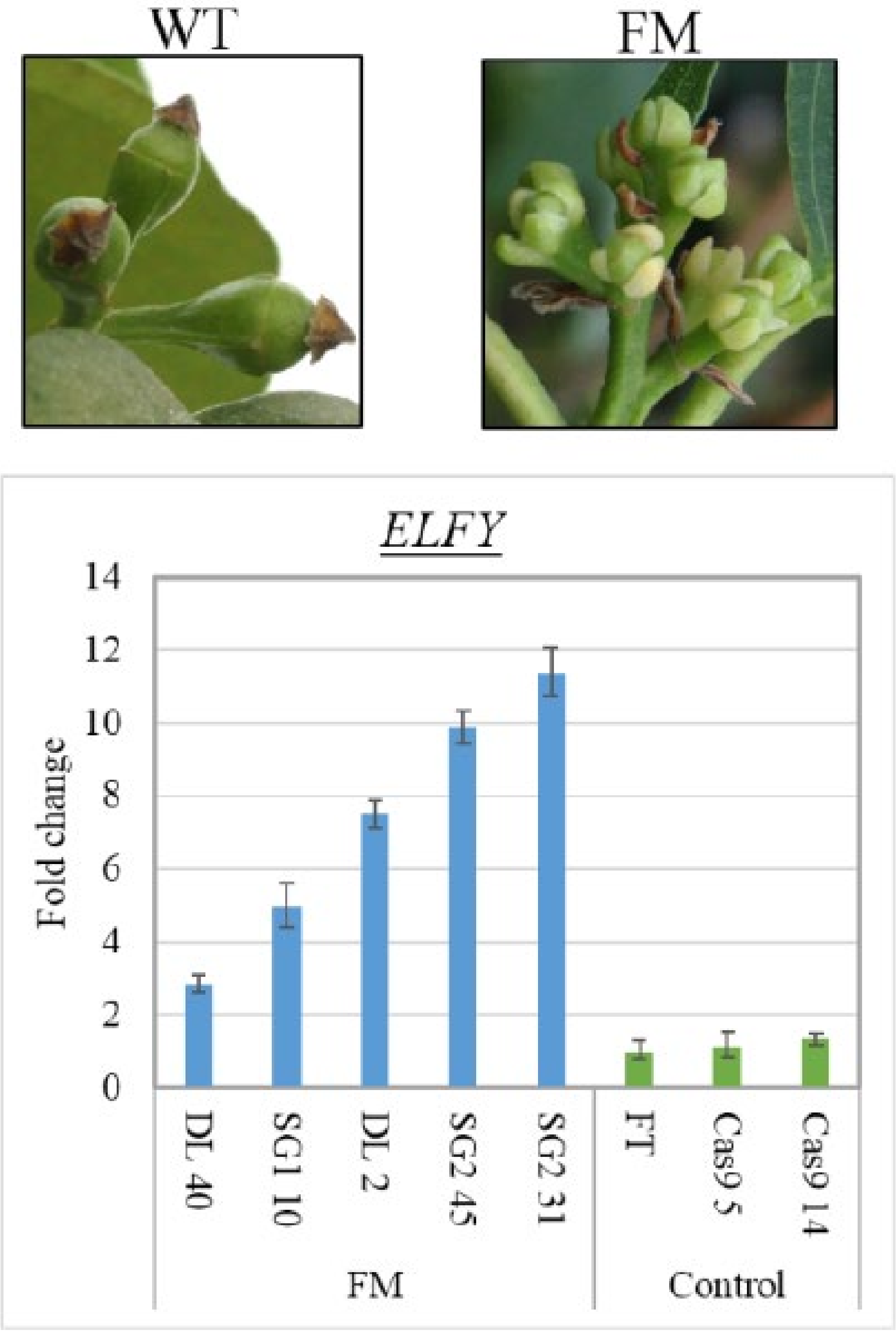
Knockout buds appear devoid of any floral organs



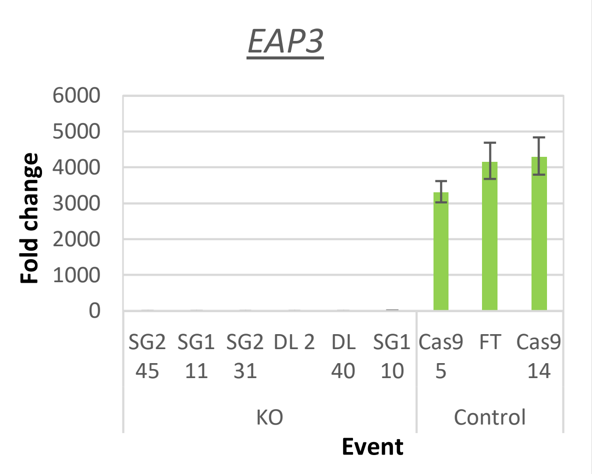
qPCR studies of genes up- and down-stream of *LEAFY*

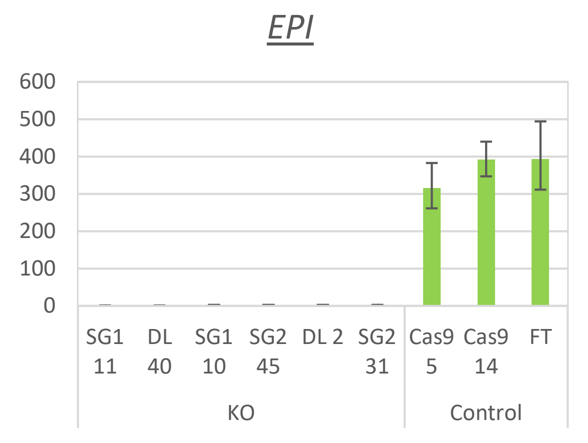


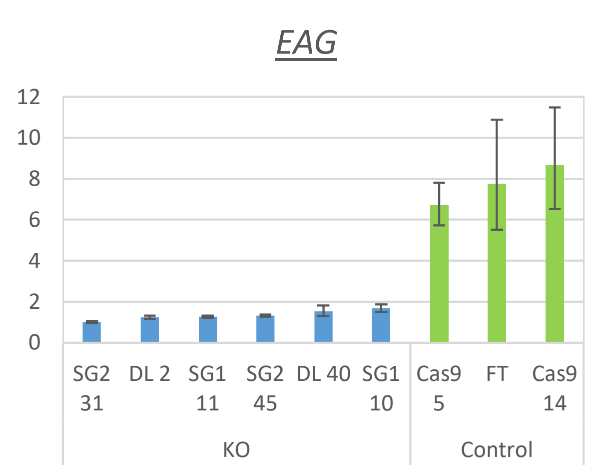
LEAFY itself is upregulated in knockout “floral” buds

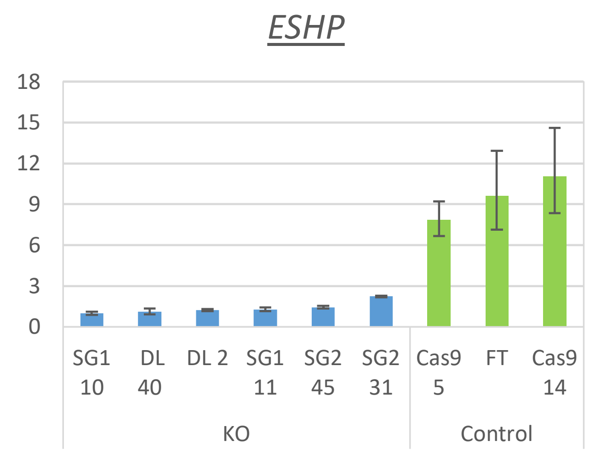


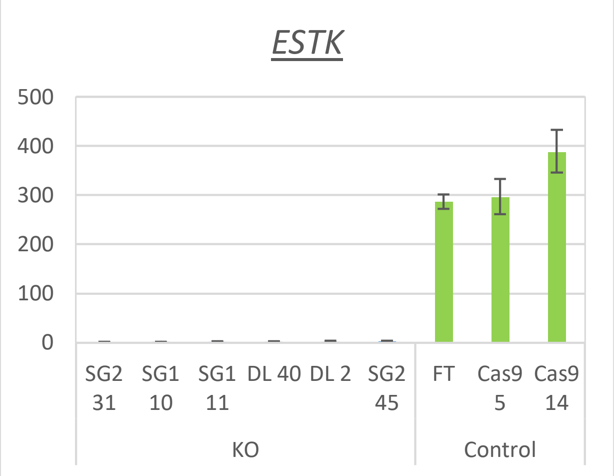
Knock-out buds nearly devoid of floral meristem gene expression based on qPCR


EAP3

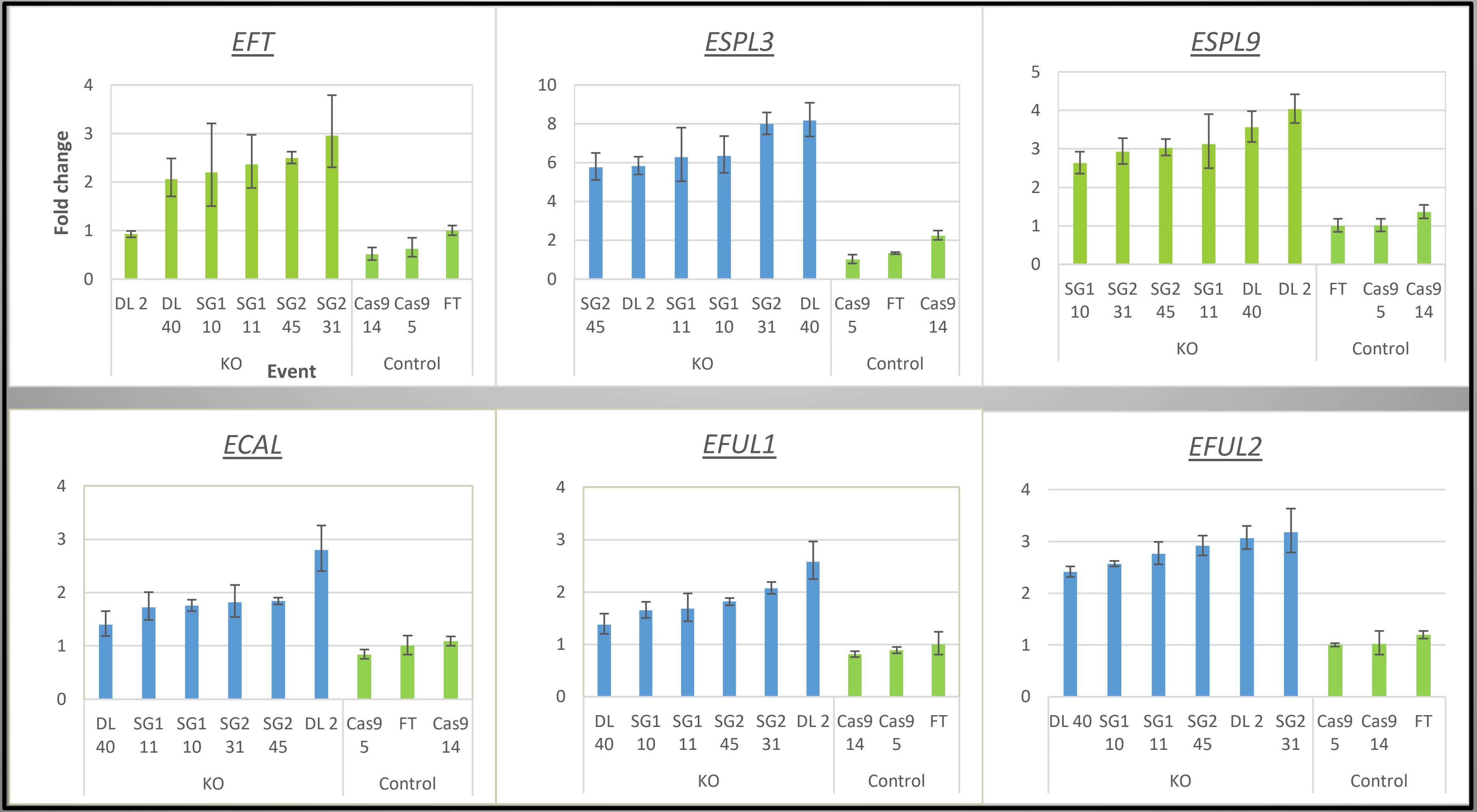

EPI


EAG


ESHP


ESTK

In contrast, knock-outs bud with enhanced pre-floral, inductive gene expression



Summary view of floral shoot development in knockouts vs. wild-type

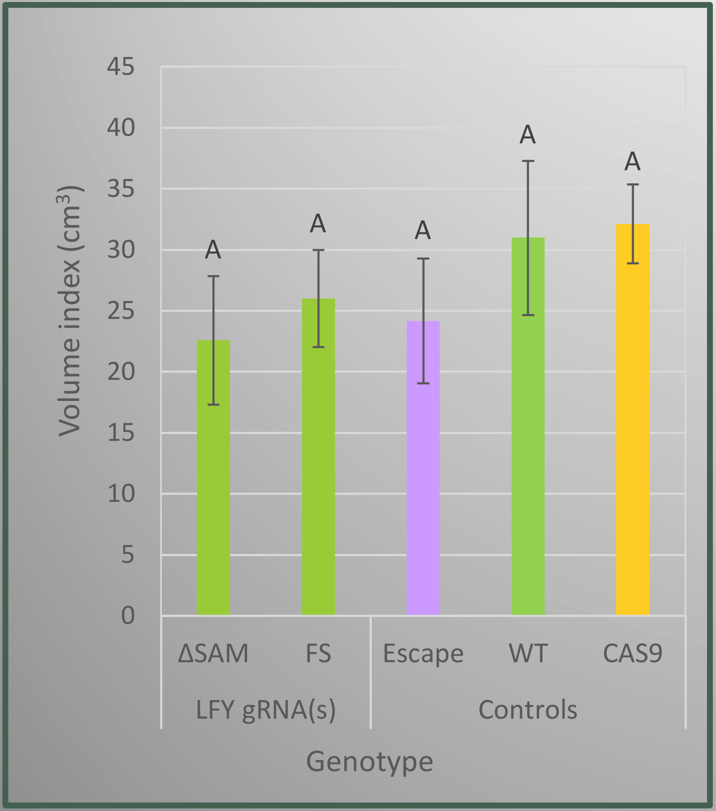
Control

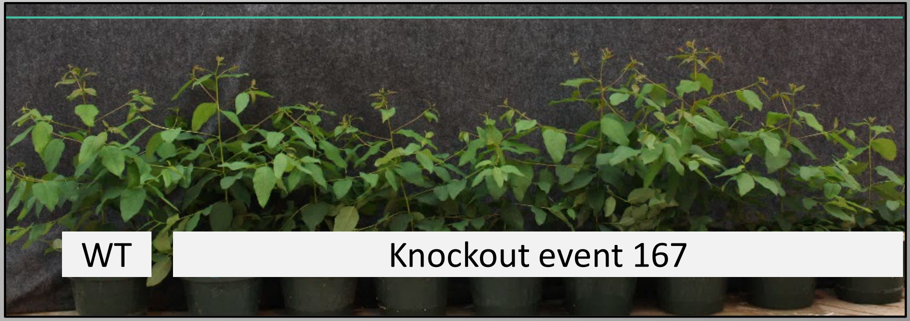
CRISPR

Control

CRISPR

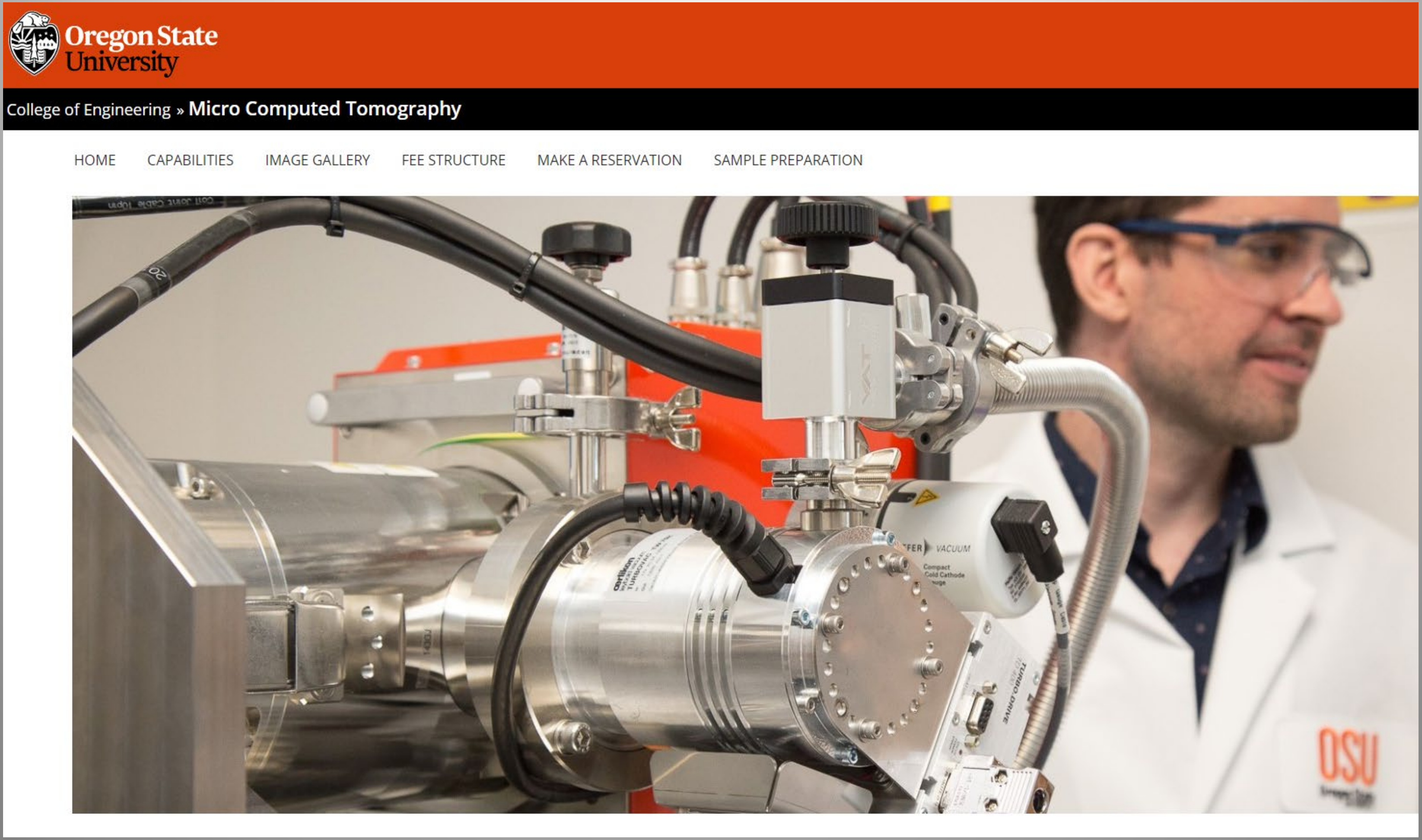
Vegetative growth and morphology in non-FT trees in greenhouse unaffected by knock-out





Other traits studied include chlorophyll density, leaf area, and leaf specific weight

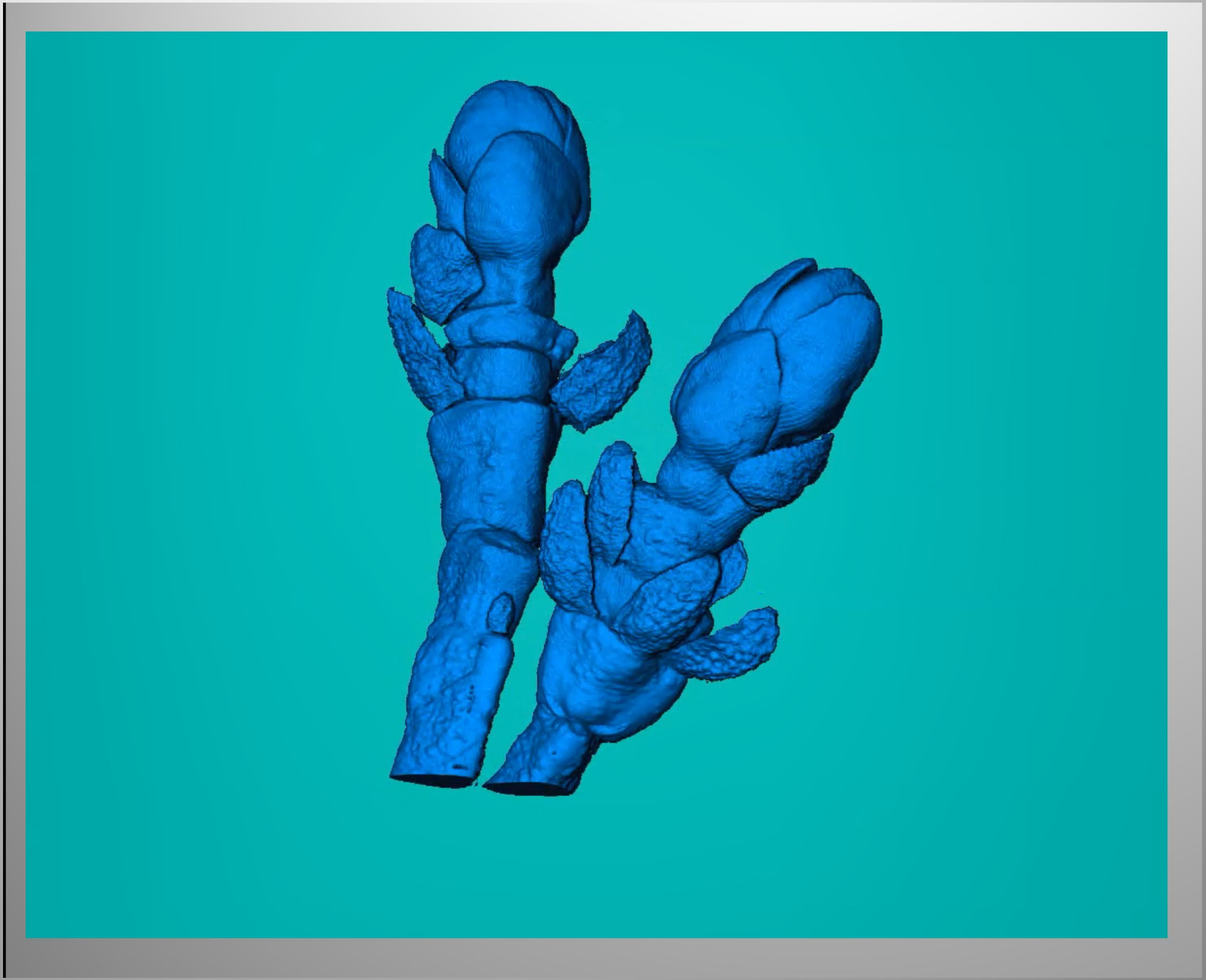
Floral scans – OSU MCT facility



Oregon State University

College of Engineering » Micro Computed Tomography

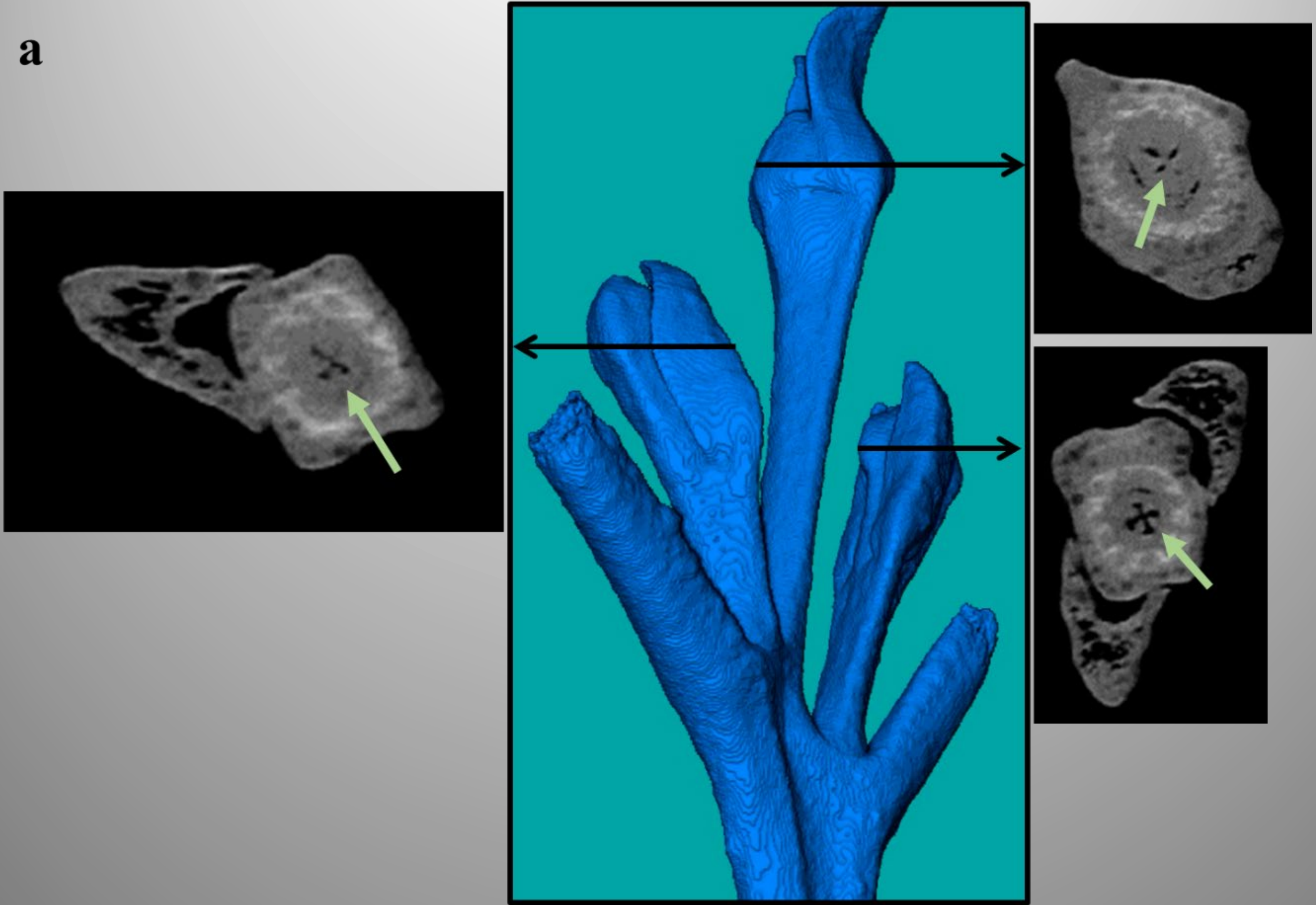
MCT scanning of shoots from top to bottom



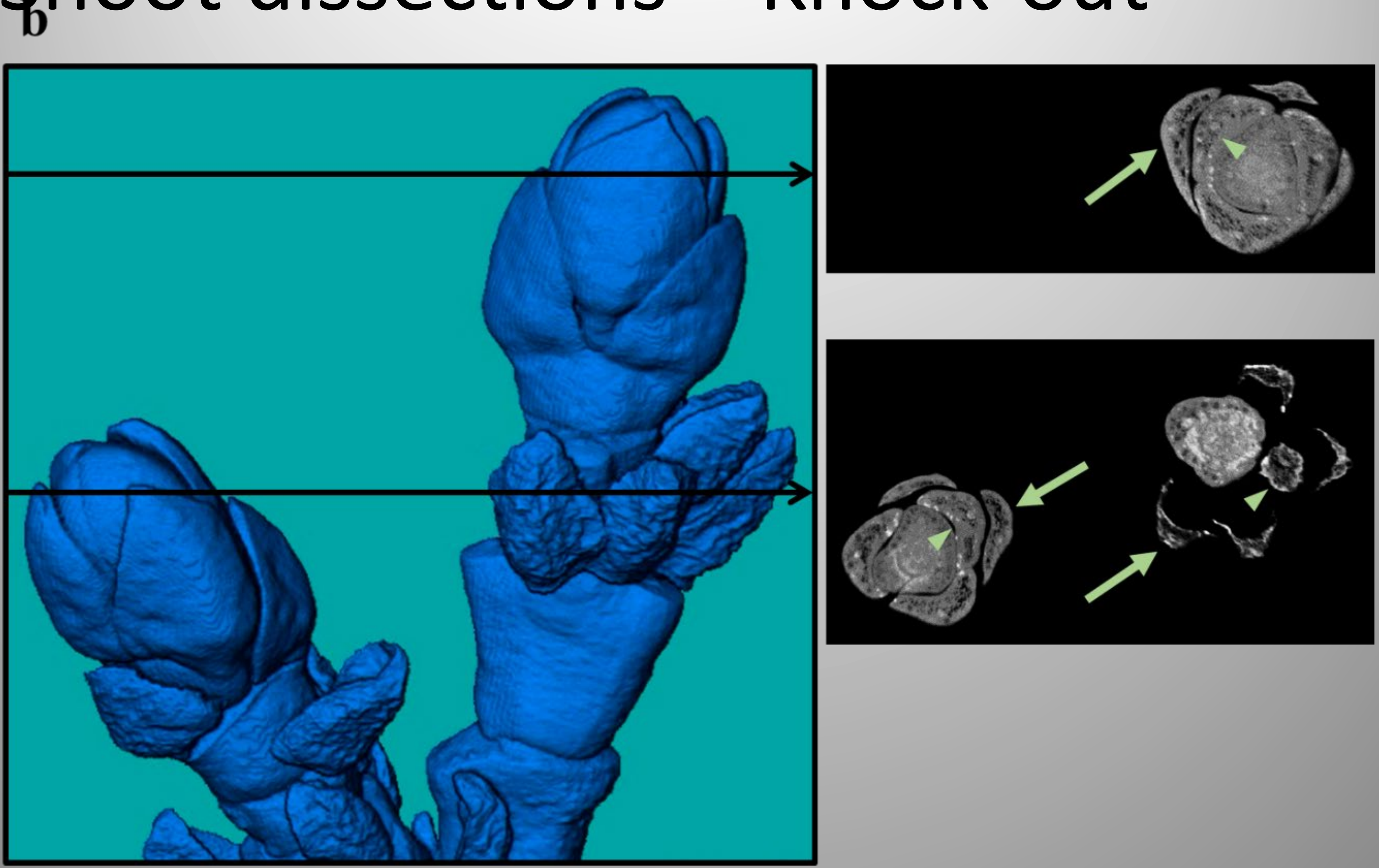
Shoot movies: Wild type

Shoot movies: knock-out

Shoot dissections – Wild type



Shoot dissections – Knock-out



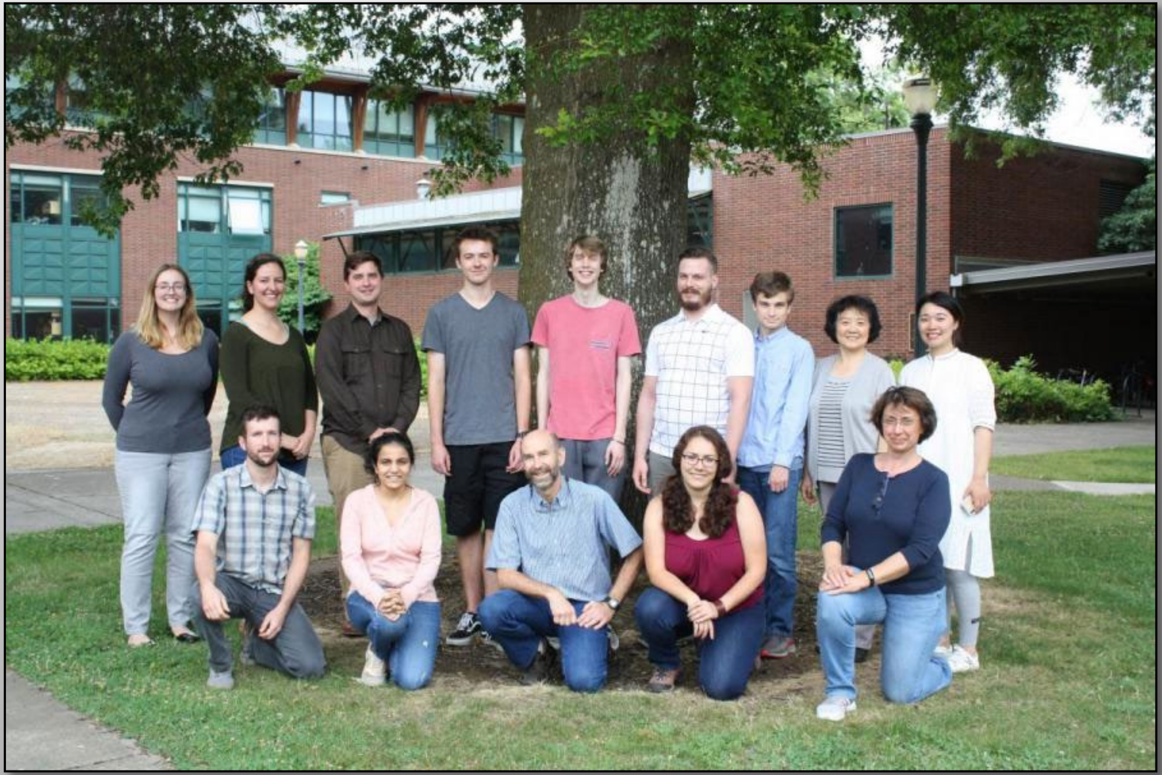
Summary – *LFY* CRISPR in Eucalyptus

- Nearly 100% biallelic knockout rate
- Flower buds devoid of reproductive structures and lack nearly all floral organ transcription factor expression
  - *LEAFY* and floral inductive genes hyperexpressed
- Partially indeterminate inflorescences
- No detectable vegetative effects on leaf morphology or biomass productivity in the greenhouse
- All CRISPR trees were transgenic: Work underway to develop CRISPR excision and other methods for “clean” knock-outs

Thanks to these key people, and many more over the years

Amy Klocko, now Univ of Colorado - Colorado Springs

Cathleen Ma, Lead transformation scientist



Lab group, summer 2019

Sources of support

TBGRC

USDA

United States Department of Agriculture

National Institute of Food and Agriculture

NSF

The National Science Foundation

TBGRC Coop: Futuragene, SAPPI, SweTree, U. Pretoria, Arborgen

Postdoc wanted – Quantitative genetics

Oregon State University, College of Forestry

Develop genomic resources for Doug-fir and Western White Pine

Design strategies for increasing efficiency of genomic selection

Develop a SNP genetic map of Doug-fir

Join the Pacific Northwest Tree Improvement Research Cooperative

☐ Experience in quantitative genetics, bioinformatics, and genomic analysis

☐ Experience programming in languages such as R, Perl, Python, C#, Java, Fortran

☐ Experience conducting statistical analyses in R, SAS, or ASReml

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- Contact Glenn.Howe@oregonstate.edu for more information

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