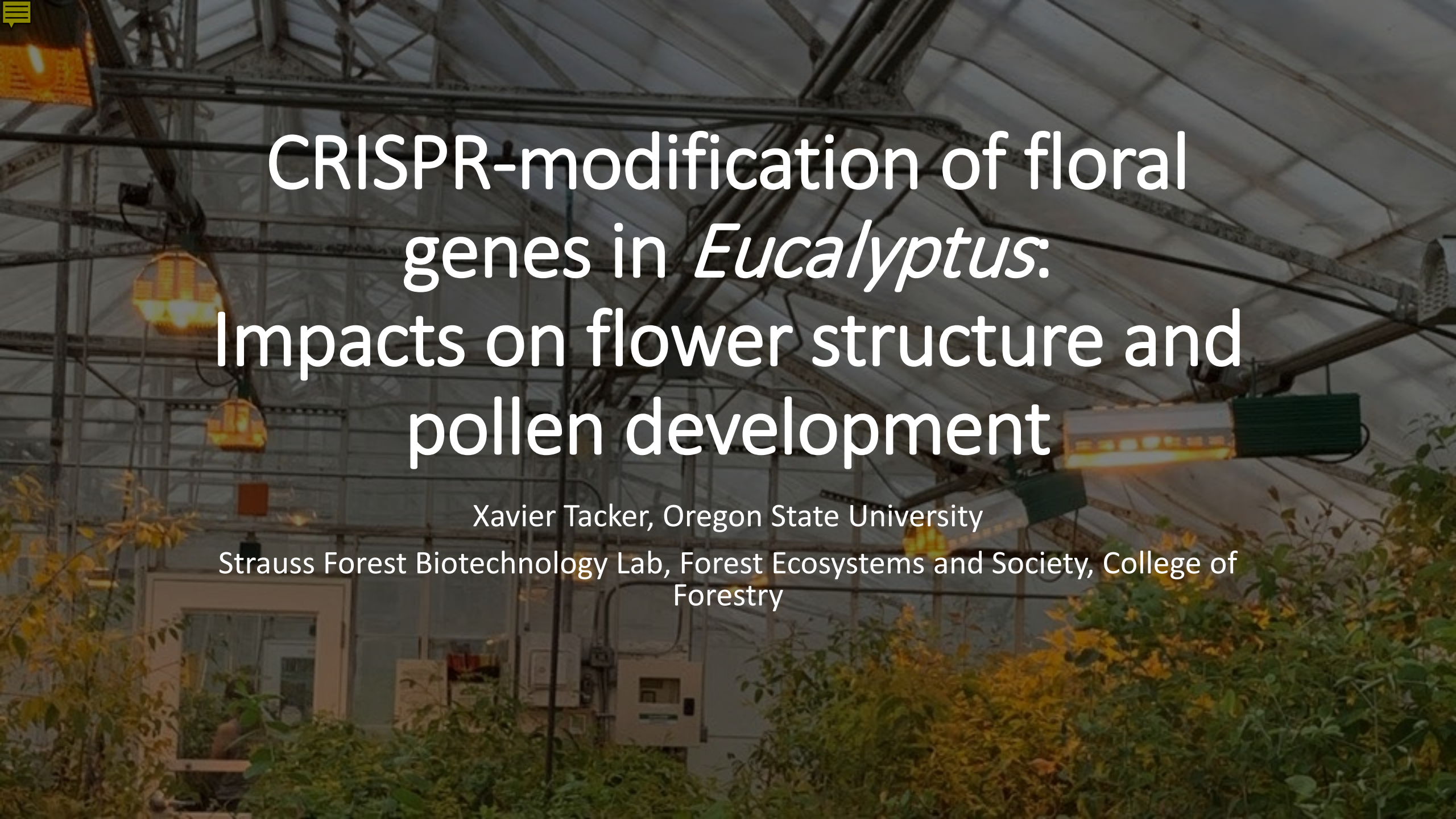


CRISPR-modification of floral genes in *Eucalyptus*: Impacts on flower structure and pollen development

Xavier Tacker, Oregon State University

Strauss Forest Biotechnology Lab, Forest Ecosystems and Society, College of Forestry

Agenda

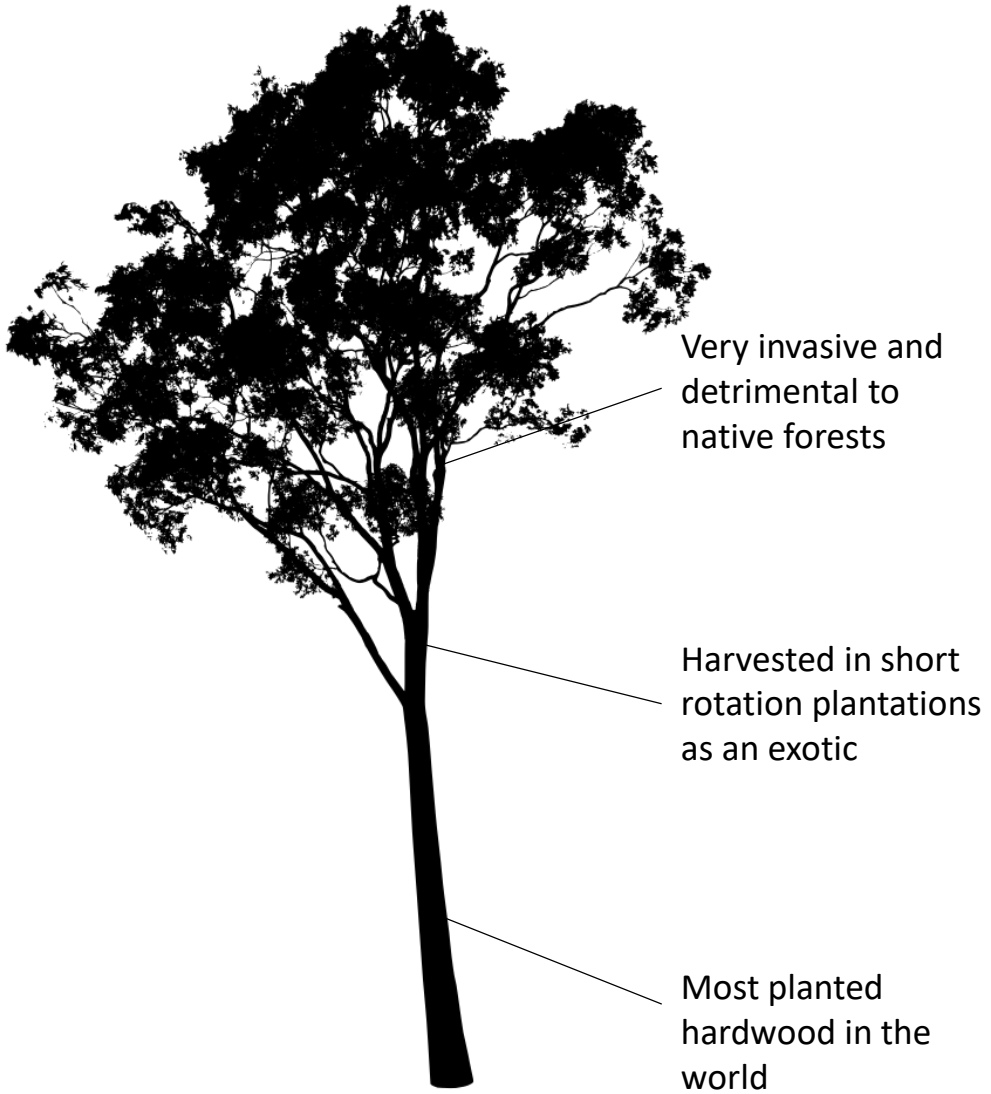
- Background
- Methods
- Results
- Conclusions

Agenda

- **Background**
- Methods
- Results
- Conclusions

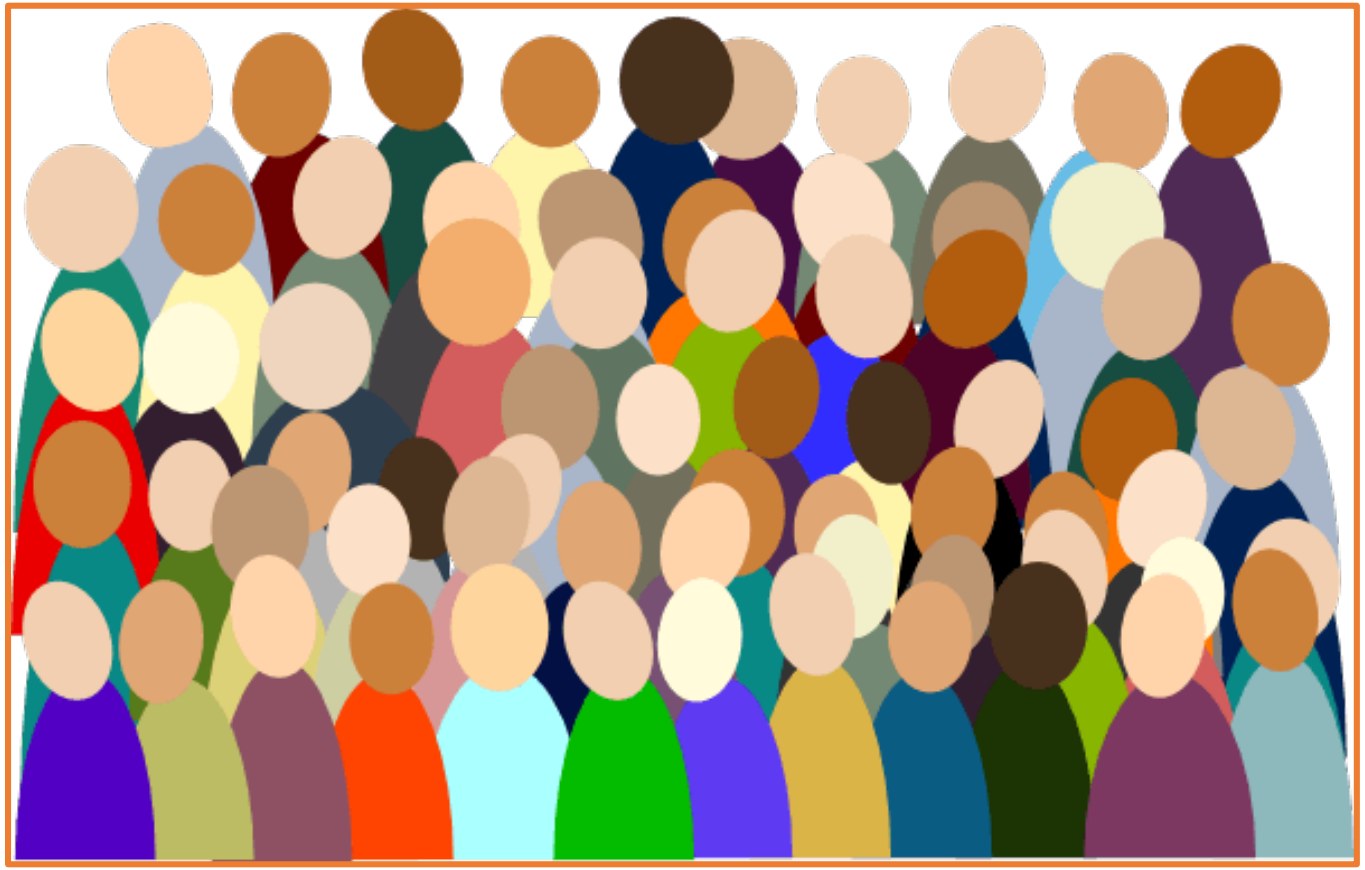
Project Goals

1. Develop tools for modifying flowers
2. Determine impacts of gene knockouts on vegetative and floral traits



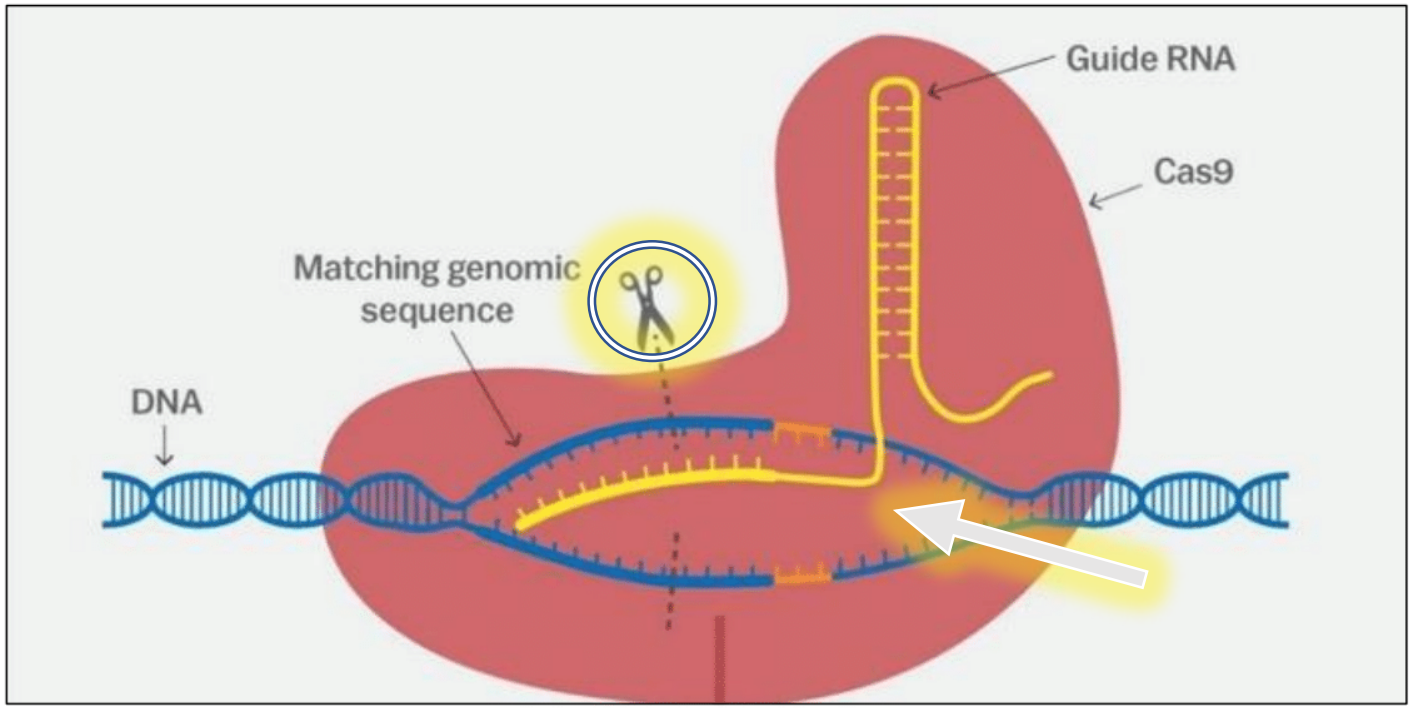
Project Rationale

- **Environmental:** The mitigation of gene flow
- **Social:** Publicly acceptable organisms
- **Economic:** Open the door to many other modifications for forestry purposes



CRISPR is the Key for Modifying Target Genes

- Cas9 nuclease (protein)
- Guide RNA with 20 base pairs that match target DNA



Flowering locus T (FT): Early flowering

- Induces flowering earlier
- Greater rates of flowering
- Minimal impacts to floral functionality

Early flowering Eucalyptus from FT over expression

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*

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Received 8 April 2015;
revised 29 May 2015;
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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,

Agenda

- Background
- **Methods**
- Results
- Conclusions

Methods

Gene Selection

Genetic Transformation

Floral Study

How we identified target genes in Eucalyptus

- Arabidopsis biology powered our search
- Key genes occur throughout the plant kingdom
- We identified target genes by searching the Eucalyptus genome for genes that matched with Arabidopsis

ARTICLE

OPEN

doi:10.1038/nature13308

The genome of *Eucalyptus grandis*

Alexander A. Myburg[1,2], Dario Grattapaglia[3,4], Gerald A. Tuskan[5,6], Uffe Hellsten[5], Richard D. Hayes[5], Jane Grimwood[7], Jerry Jenkins[7], Erika Lindquist[5], Hope Tice[5], Diane Bauer[5], David M. Goodstein[5], Inna Dubchak[5], Alexandre Poliakov[5], Eshchar Mizrachi[1,2], Anand R. K. Kullan[1,2], Steven G. Hussey[1,2], Desre Pinard[1,2], Karen van der Merwe[1,2], Pooja Singh[1,2], Ida van Jaarsveld[8], Orzenil B. Silva-Junior[9], Roberto C. Togawa[9], Marilia R. Pappas[3], Danielle A. Faria[3], Carolina P. Sansaloni[3], Cesar D. Petroli[3], Xiaohan Yang[6], Priya Ranjan[6], Timothy J. Tschaplinski[6], Chu-Yu Ye[6], Ting Li[6], Lieven Sterck[10], Kevin Vanneste[10], Florent Murat[11], Marçal Soler[12], Hélène San Clemente[12], Naijib Saidi[12], Hua Cassan-Wang[12], Christophe Dunand[12], Charles A. Hefer[8,13], Erich Bornberg-Bauer[14], Anna R. Kersting[14,15], Kelly Vining[16], Vindhya Amarasinghe[16], Martin Ranik[16], Sushma Naithani[17,18], Justin Elser[17], Alexander E. Boyd[18], Aaron Liston[17,18], Joseph W. Spatafora[17,18], Palitha Dharmwardhana[17], Rajani Raja[17], Christopher Sullivan[18], Elisson Romanel[19,20,21], Marcio Alves-Ferreira[21], Carsten Külheim[22], William Foley[22], Victor Carocha[12,23,24], Jorge Paiva[23,24], David Kudrna[25], Sergio H. Brommonschenkel[26], Giancarlo Pasquali[27], Margaret Byrne[28], Philippe Rigault[29], Josquin Tibbits[30], Antanas Spokevicius[31], Rebecca C. Jones[32], Dorothy A. Steane[32,33], René E. Vaillancourt[32], Brad M. Potts[32], Fourie Joubert[2,8], Kerrie Barry[5], Georgios J. Pappas Jr[34], Steven H. Strauss[16], Pankaj Jaiswal[17,18], Jacqueline Grima-Pettenati[12], Jérôme Salse[11], Yves Van de Peer[2,10], Daniel S. Rokhsar[5] & Jeremy Schmutz[5,7]

Eucalypts are the world's most widely planted hardwood trees. Their outstanding diversity, adaptability and growth have made them a global renewable resource of fibre and energy. We sequenced and assembled >94% of the 640-megabase genome of *Eucalyptus grandis*. Of 36,376 predicted protein-coding genes, 34% occur in tandem duplications, the largest proportion thus far in plant genomes. *Eucalyptus* also shows the highest diversity of genes for specialized metabolites such as terpenes that act as chemical defence and provide unique pharmaceutical oils. Genome sequencing of the *E. grandis* sister species *E. globulus* and a set of inbred *E. grandis* tree genomes reveals dynamic genome evolution and hotspots of inbreeding depression. The *E. grandis* genome is the first reference for the eudicot order Myrtales and is placed here sister to the eurosids. This resource expands our understanding of the unique biology of large woody perennials and provides a powerful tool to accelerate comparative biology, breeding and biotechnology.

A major opportunity for a sustainable energy and biomaterials economy in many parts of the world lies in a better understanding of the molecular basis of superior growth and adaptation in woody plants. Part of this opportunity involves species of *Eucalyptus* L'Hér, a genus of woody perennials native to Australia[1]. The remarkable adaptability of eucalypts coupled with their fast growth and superior wood properties has driven their rapid adoption for plantation forestry in more than 100 countries across six continents (>20 million ha)[2], making eucalypts the most widely planted hardwood forest trees in the world. The subtropical *E. grandis* and the temperate *E. globulus* stand out as targets of breeding programmes worldwide. Planted eucalypts provide key renewable resources for the production of pulp, paper, biomaterials and bioenergy, while mitigating human pressures on native forests[3]. Eucalypts also have a large diversity and high concentration of essential oils (mixtures of mono- and sesquiterpenes), many of which have ecological functions as well as medicinal and industrial uses. Predominantly outcrossers[4] with hermaphroditic animal-pollinated flowers, eucalypts are highly heterozygous and display pre- and postzygotic barriers to selfing to reduce inbreeding depression for fitness and survival[4].

To mitigate the challenge of assembling a highly heterozygous genome, we sequenced the genome of 'BRASUZ1', a 17-year-old *E. grandis* genotype derived from one generation of selfing. The availability of annotated forest tree genomes from two separately evolving rosid lineages, *Eucalyptus* (order Myrtales) and *Populus* (order Malpighiales[5]), in combination with genomes from domesticated woody plants (for example, *Vitis*, *Prunus*, *Citrus*), provides a comparative foundation for addressing

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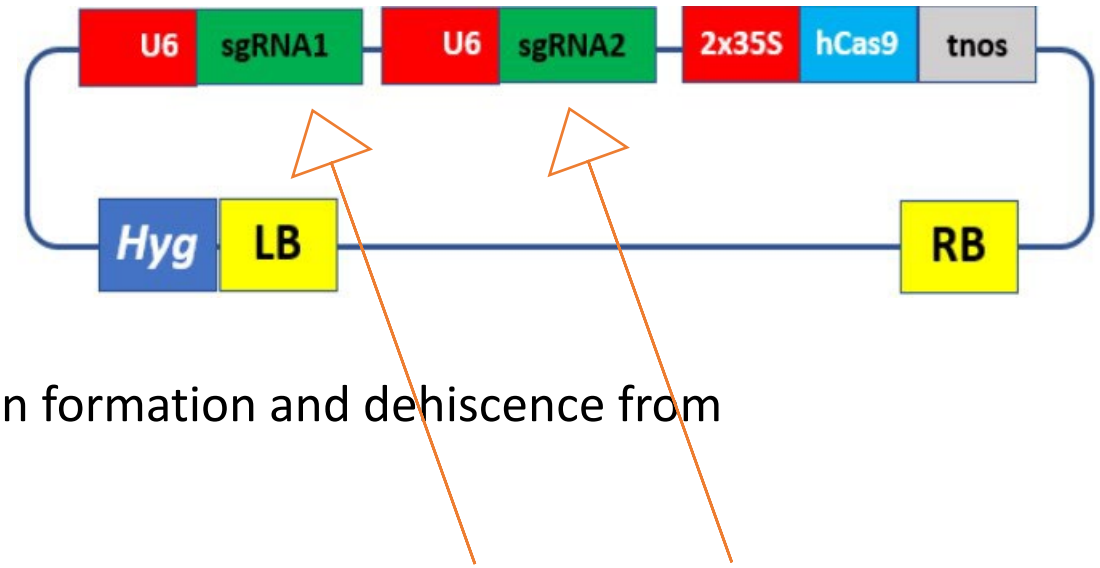
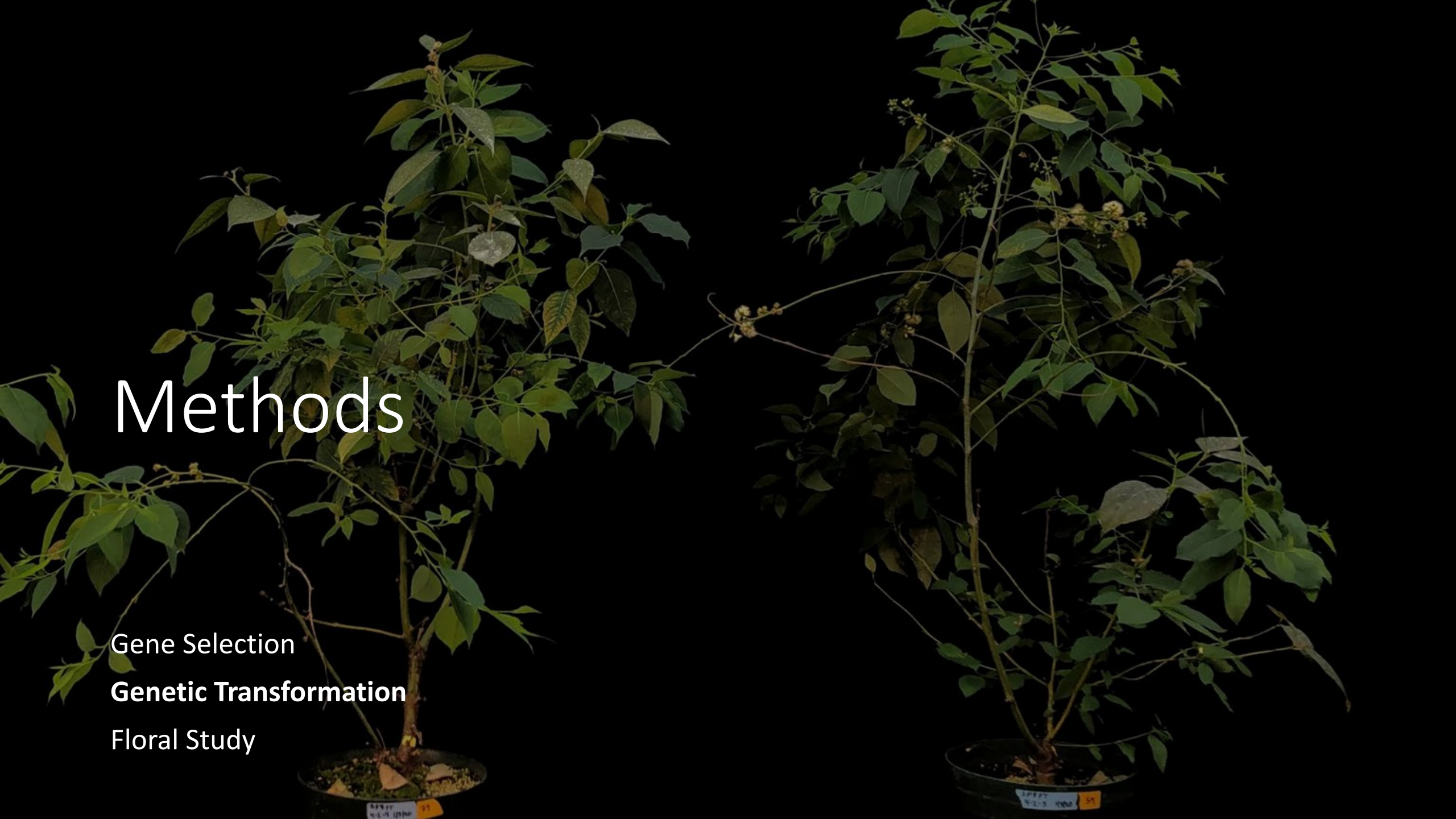
Target Flowering Genes for Mutation



- *Tapetal Development Function 1 (TDF1)*: Pollen formation and dehiscence from anthers
- *Embryo Development Arrest 33 (EDA33)*: Fruit development and seed detachment
- *Meiotic Recombinase 8 (REC8)*: Meiosis in flowers



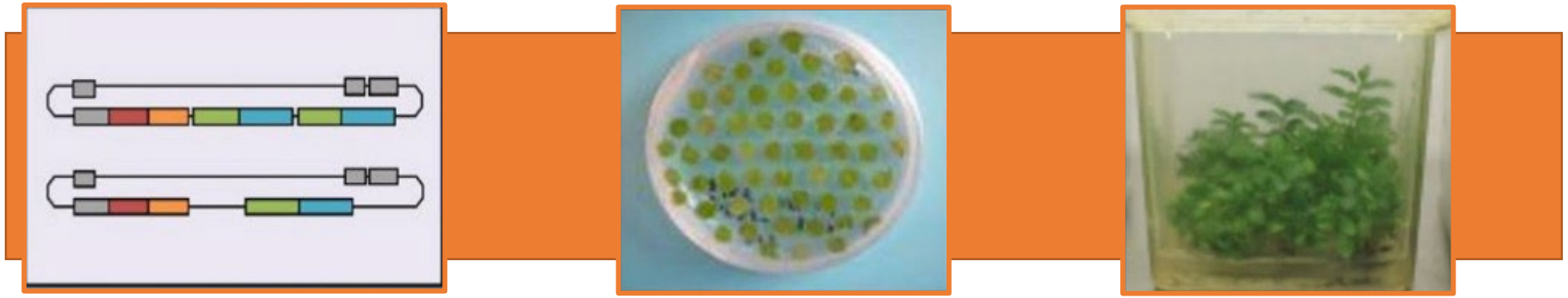
Methods

Gene Selection

Genetic Transformation

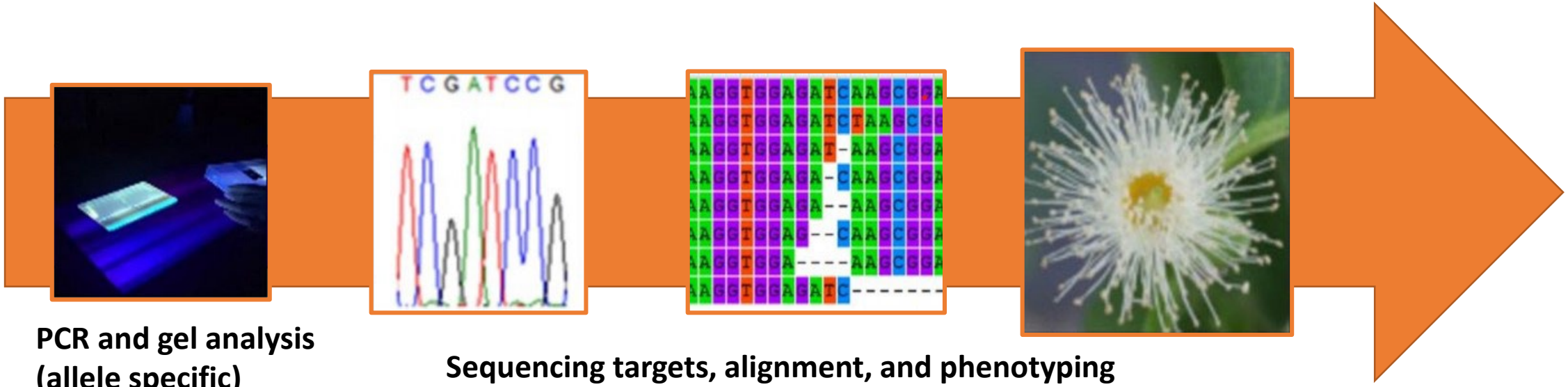
Floral Study

CRISPR For Gene Editing: How it works



Construct

Transformation and Regeneration



PCR and gel analysis (allele specific)

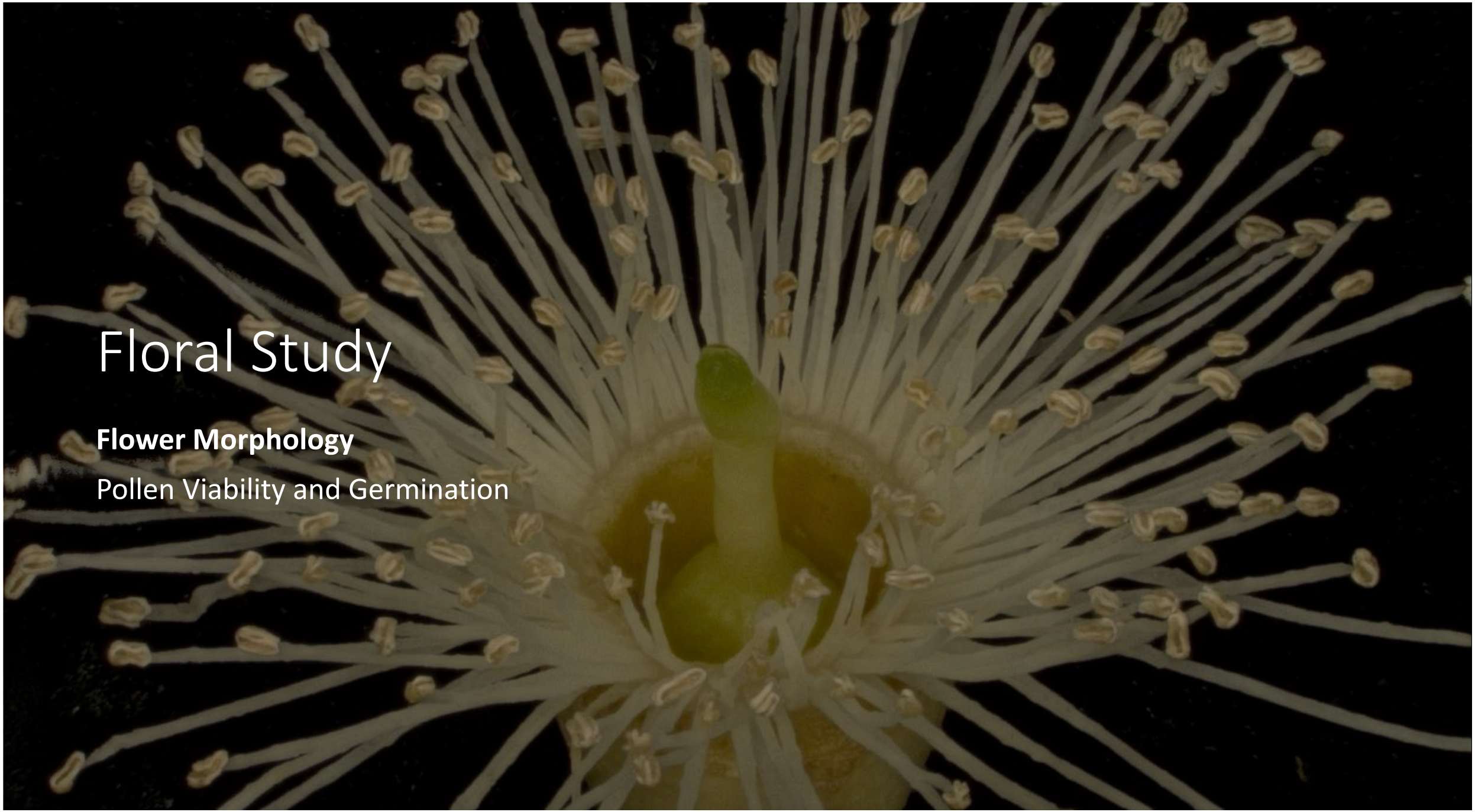
Sequencing targets, alignment, and phenotyping

Methods

Gene Selection

Genetic Transformation

Floral Study



Floral Study

Flower Morphology

Pollen Viability and Germination

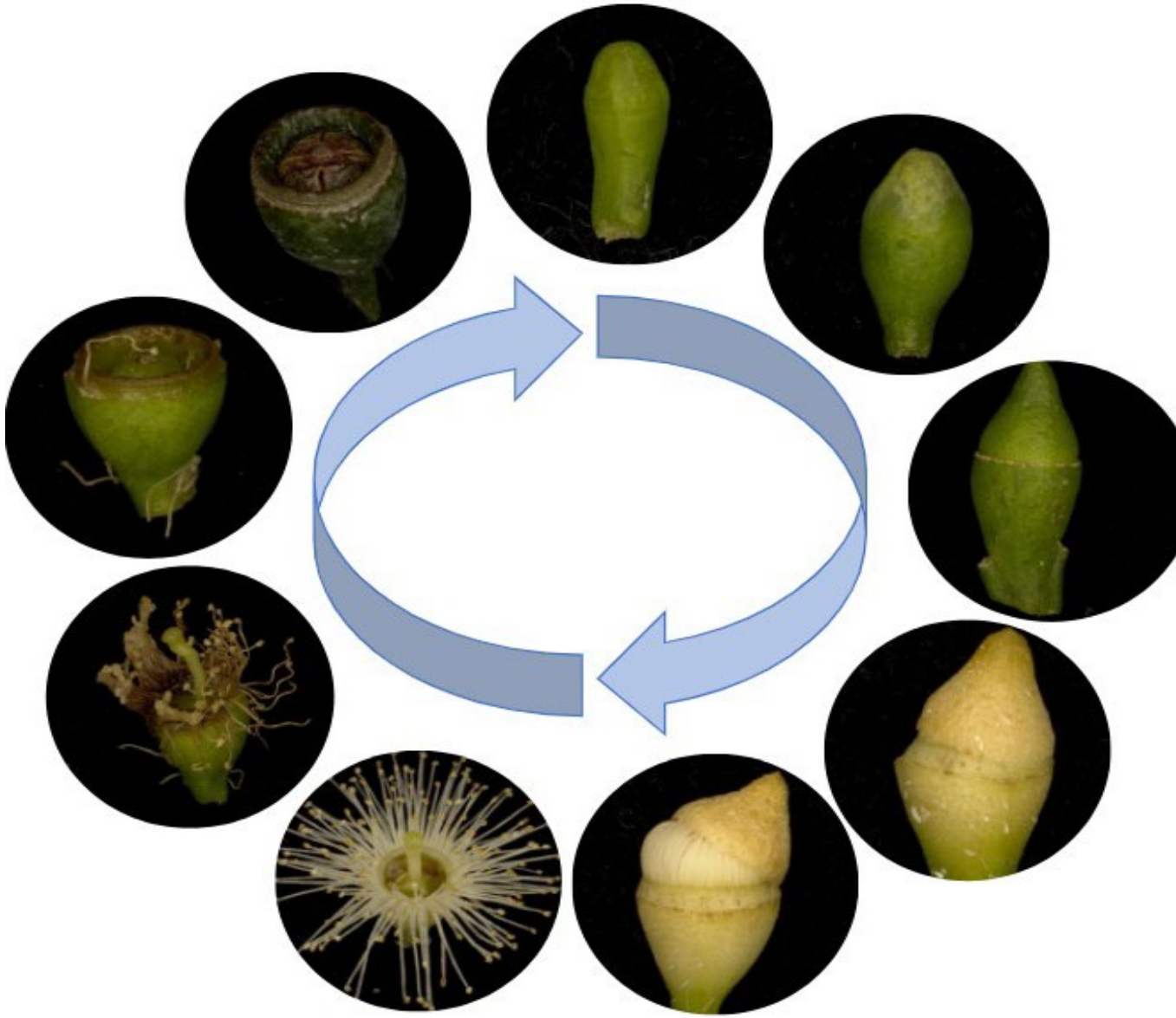
Flower morphology

Aim

- Document effects of KOs on morphology of subject trees

Methods

- Floral material collected at different stages of development
- Image and dissect flowers, compare to control flowers
- Document differences



Courtesy: Sonali Joshi

Floral Study

Flower Morphology

Pollen Viability and Germination

Differentiating Pollen: The Alexander Stain

- Differentiates between non-viable and viable pollen
- Stains protoplasm present viable pollen
- Viable magenta, non-viable translucent

International Journal of Plant Biology 2010; volume 1:e13

pagepress

A simplified method for differential staining of aborted and non-aborted pollen grains

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Abstract

The ability to use chemical staining to discriminate aborted from non-aborted pollen grains has well-known practical applications in agriculture. A commonly used technique for

cein diacetate that was initially developed to examine the viability of mammalian cells.[5,6] The uses of fluorescent dyes, however, require special equipment, such as fluorescence microscopes, which limit the application for field studies and most agricultural extension stations that do not have the available equipment.

A major improvement in differential pollen staining was reported by M.P. Alexander in 1969. Alexander's stain colors aborted pollen grains from most angiosperms and the spores of gymnosperms blue-green, and non-aborted pollen grains and spores stain magenta-red.[7] Unfortunately, three ingredients of Alexander's protocol exhibit possible health risks. Two of these reagents are chloral hydrate that is used in the stain solution and the mercuric chloride that is used in the fixative. Both are either highly regulated by worldwide government standards on chemical toxicities or threaten the health of those who use the stain solution.[8] The third harmful reagent is phenol. It can be absorbed through the skin and has shown to disrupt

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Key words: pollen staining, Alexander's stain, chloral hydrate, phenol.

Contributions: RP did most of experiments and contributed to the writing; JPS did further test and contributed to edit the manuscript; CC initiated this study, supervised RP for his bench-work and data collection and wrote this manuscript.

Acknowledgements: we deeply appreciated the comments from all three reviewers, which greatly improved the final protocol with the removal of phenol. The authors are grateful to Dr. Luca Comai for providing *Arabidopsis* autotetraploid seeds and Dr. Dong-Hoon Jeong for providing rice Nipponbare seeds. Our sincere thanks also go to Tao Li and Asmita Batajoo for plant care, Dr. Junhua Li and the former colleagues, Drs. Cary H Hord, Yoshitaka Azumi and Wuxing Li at the Pennsylvania State University for sharing their

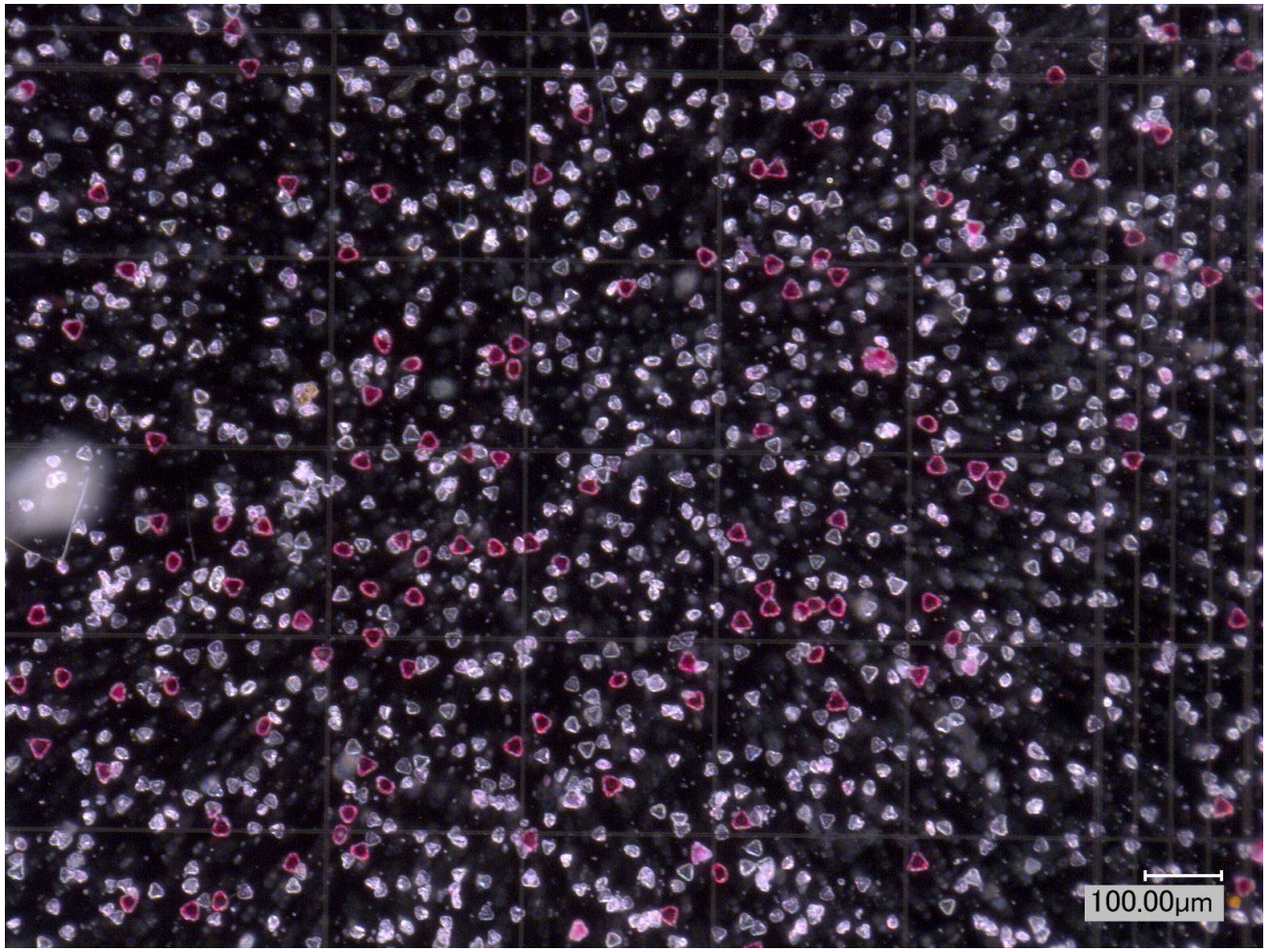
Pollen Production and Viability

Aim

- Image for pollen count and viability

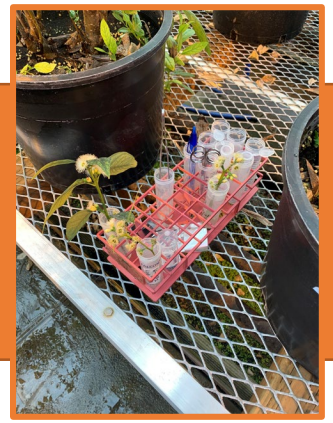
Methods

- Collect, process, and stain greenhouse pollen
- Image for viability percentage



Pollen viability stain on FT Control Tree

Pollen Viability Methods

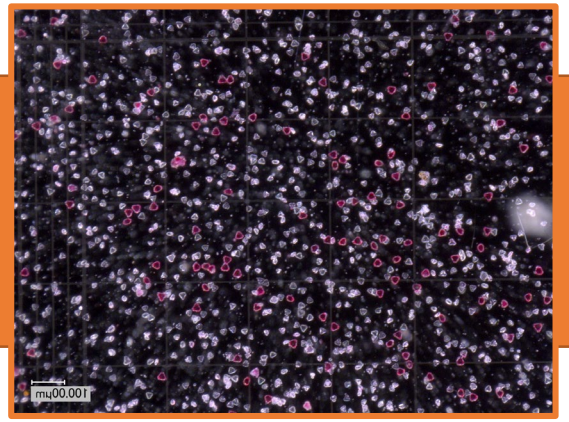


Floral Samples Collected

Viability Staining and Processing

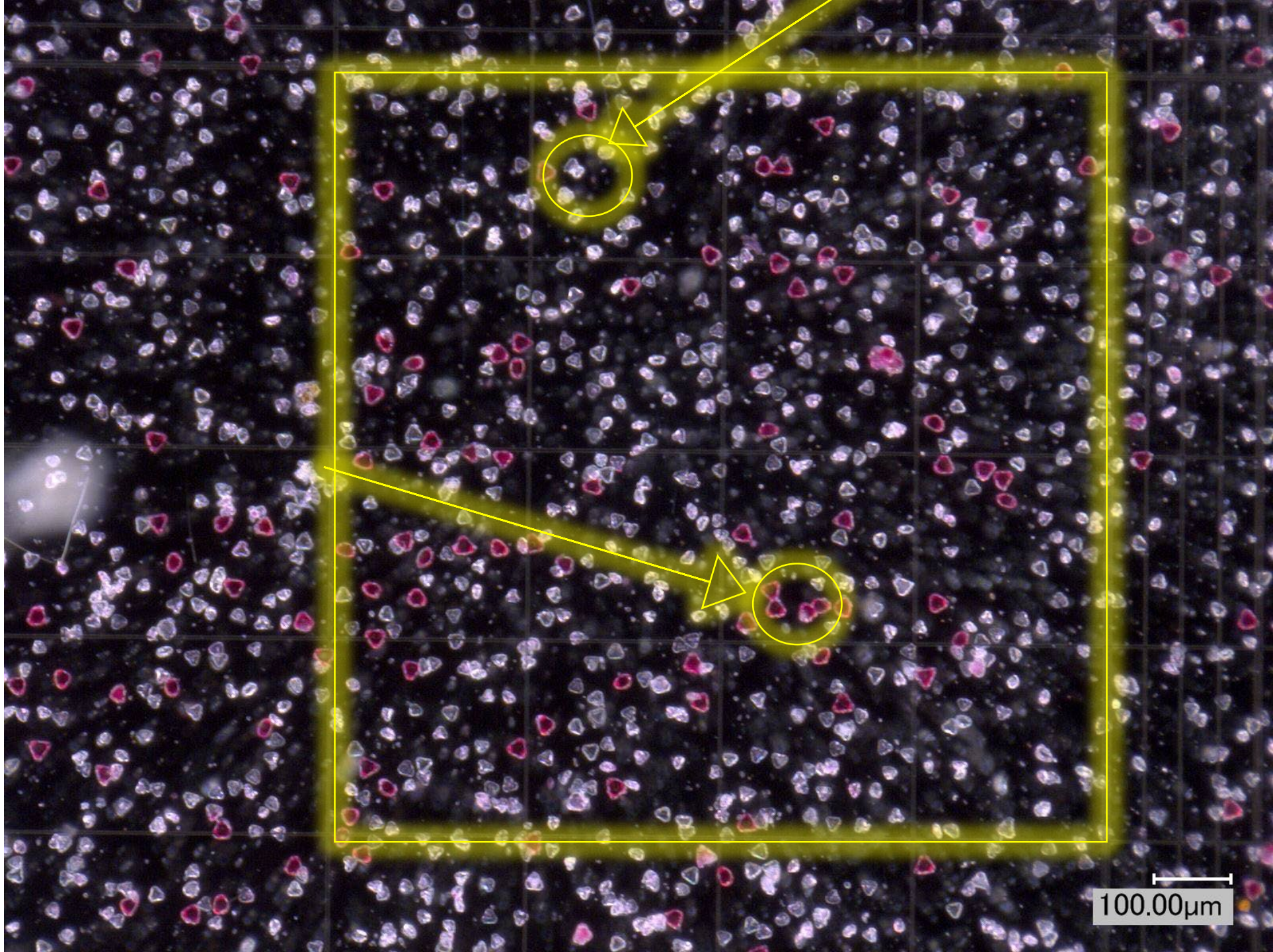
Filtered for debris

Fixed for storage



Imaged and counted for viability

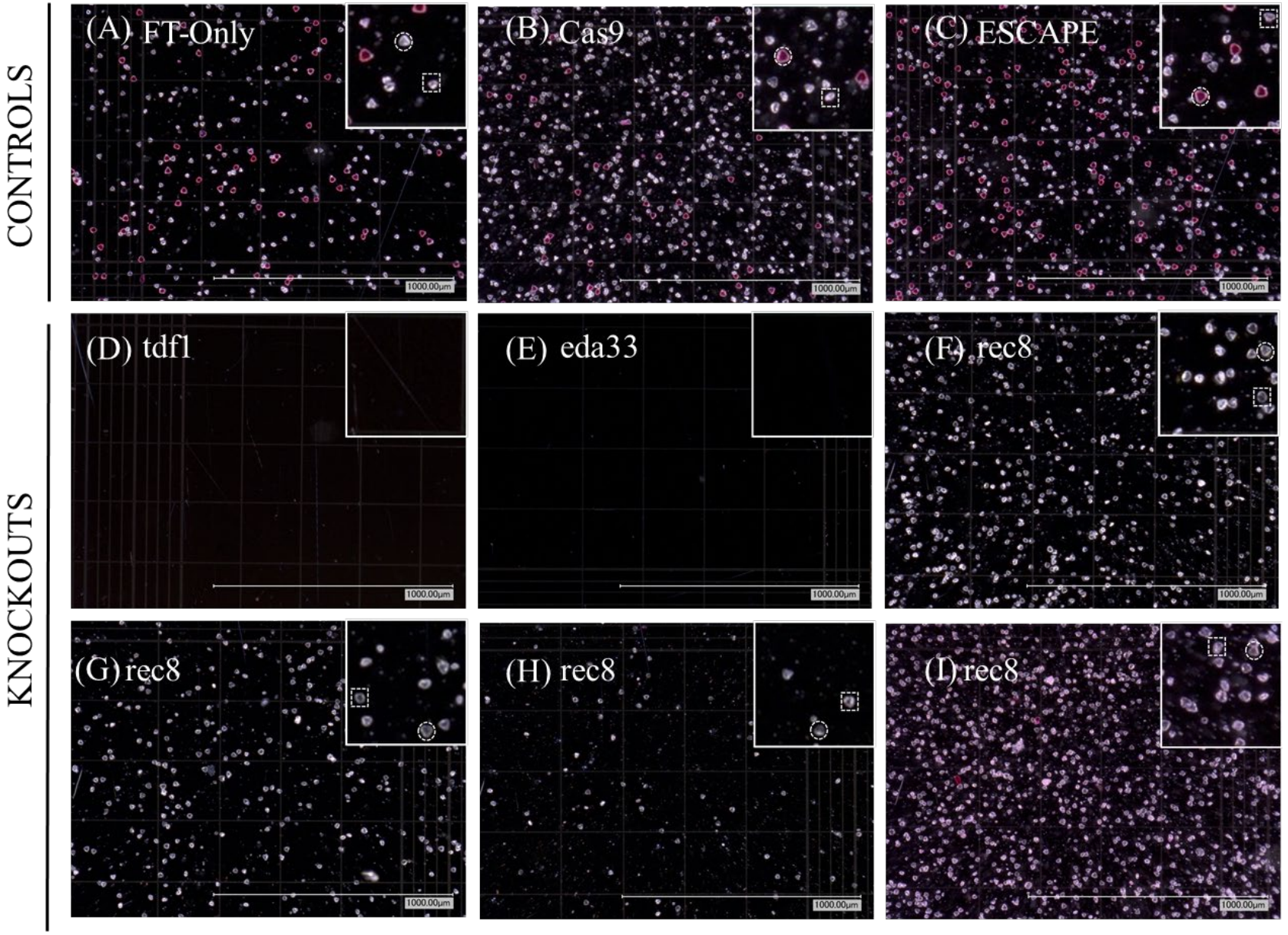
Imaging for Production and Viability



Agenda

- Background
- Methods
- **Results**
- Conclusions

Summary of Mutational Impacts



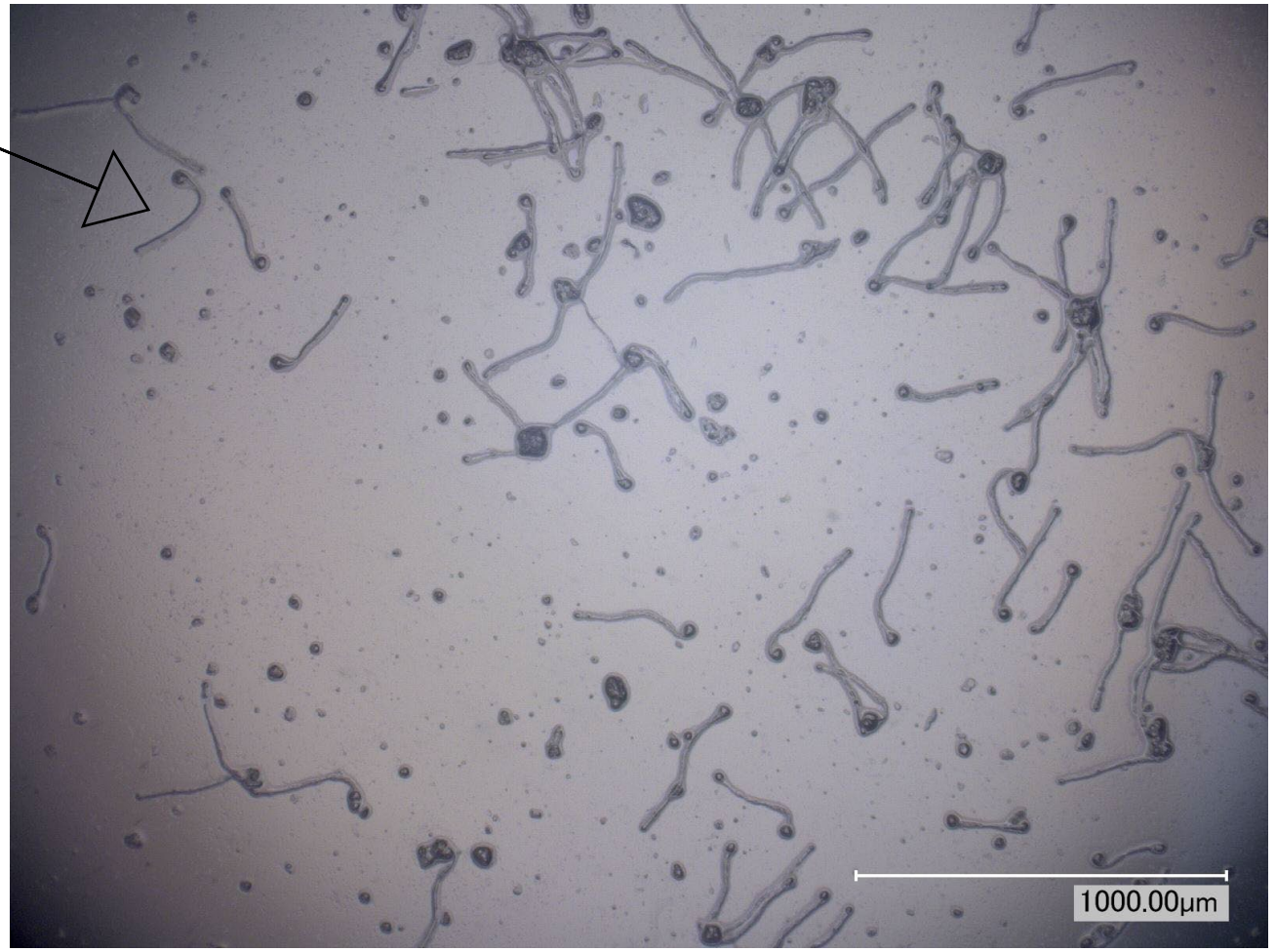
Pollen Germination

Aim

- Examine the potential viability of *rec8* pollen

Methods

- Collect, process, and germinate greenhouse pollen
- Score subjects based on percent germination of sample



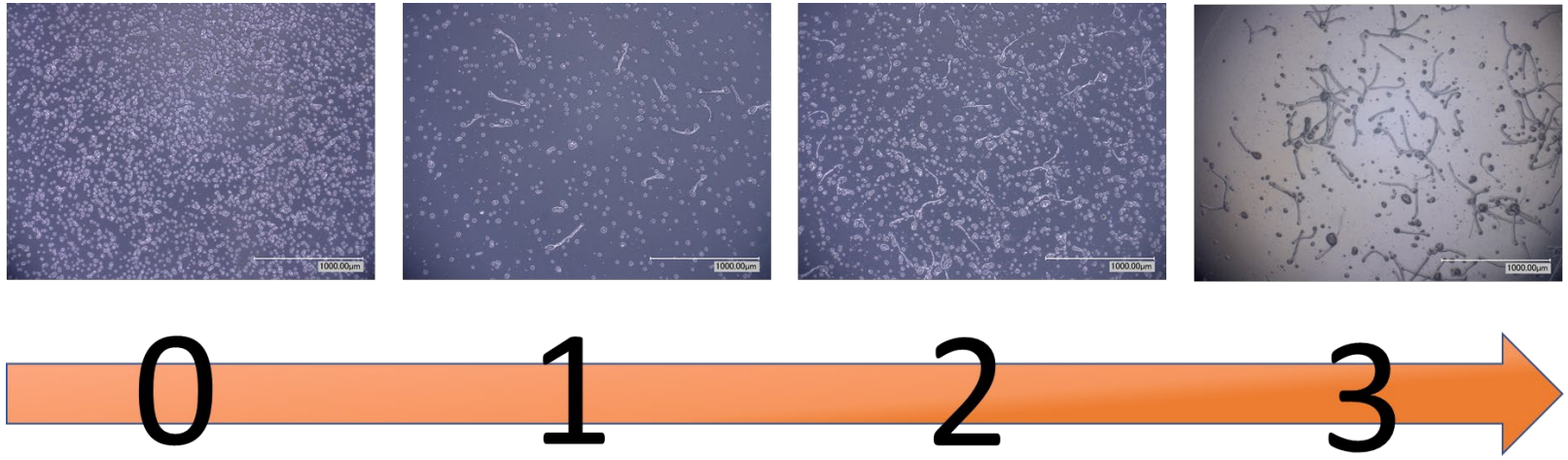
Wildtype *E. grandis* Pollen Germination

Pollen Germination Scoring & Results

As expected *rec8* was unable to germinate

- Wild type samples scored an average of 3
- *FT* controls scored low germination due to hybridization

Genotype	Germination Score
FT	1
rec8	0



Agenda

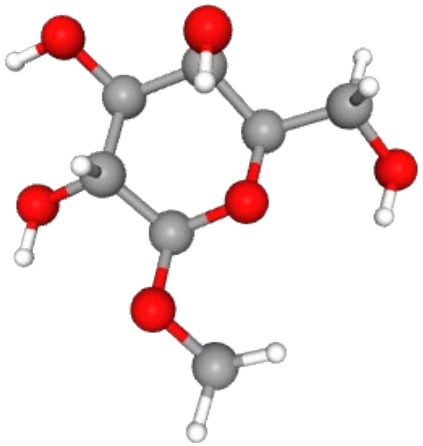
- Background
- Methods
- Results
- **Conclusions**

Conclusions

- CRISPR is an effective tool in mutating Eucalyptus genes
- Constructs behaved as hypothesized mostly
  - TDF1 male sterility
  - REC8 male sterility
  - EDA33 at least male sterility

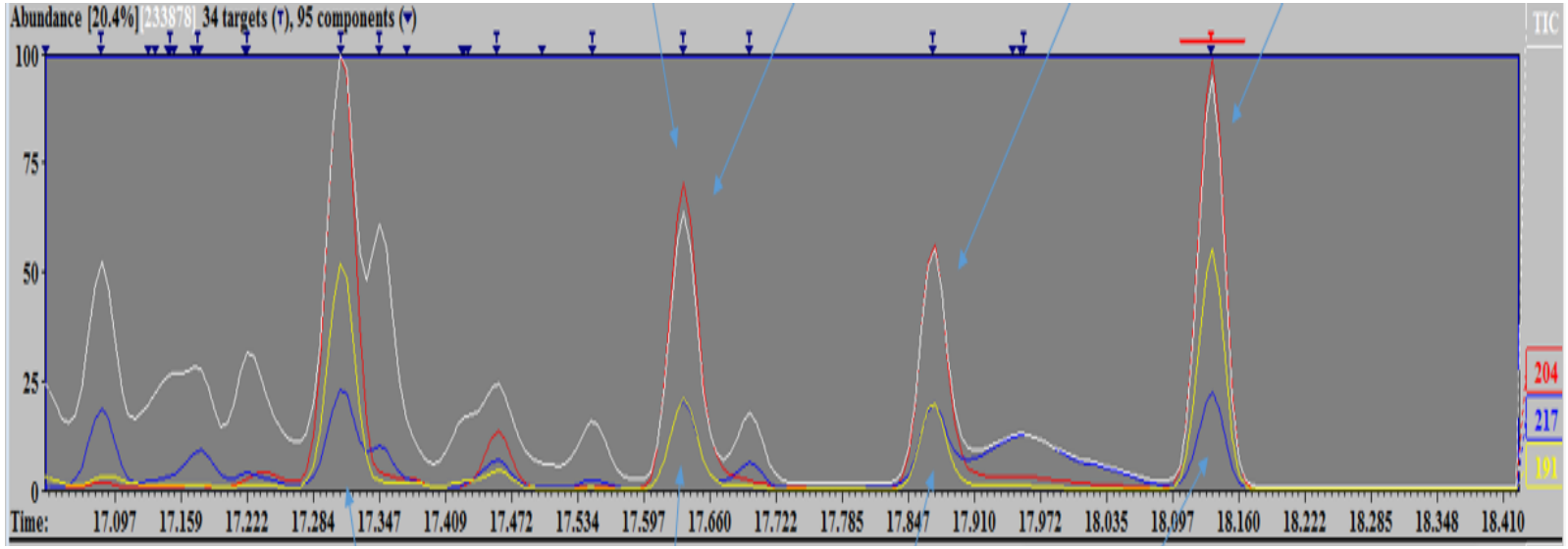
Next Steps

- Investigation in nectar chemistry of *rec8* flowers
- Investigation into female fertility
- Manuscript submission for review



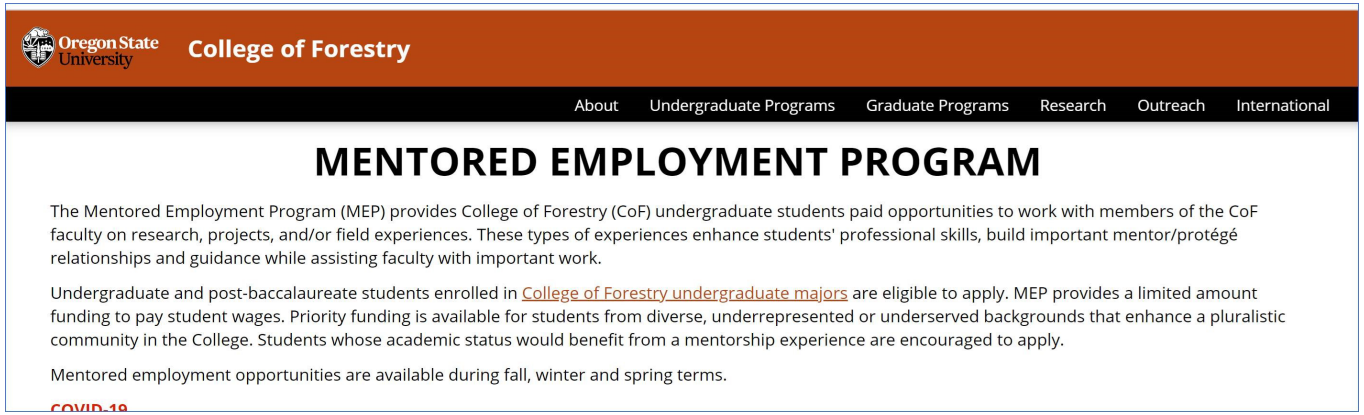
Methyl-beta-D-galactopyranoside

Gas chromatography-mass spectrometry of *rec8* nectar showing high concentrations of Methyl-beta-D-galactopyranoside



Thank you!

MANRRS



USDA NIFA