Modifying Reproductive Traits of Forest Trees

Experience from gene-targeting technologies in the lab and field

Steve Strauss / Oregon State University / USA

FTMB - Harbin, China - 2018

Agenda

- A look back on where we have come from and how it has affected our work: Breakthroughs and “breakdowns”
- Reproductive modification rationale and context
- Experience with three major approaches
  - RNAi against floral meristem development genes
  - Overexpression of floral onset repressors
  - CRISPR-Cas9 mutation effects

Breakthroughs – Basic molecular biology and genomic methods

- Revolution in methods: PCR
  - 1980s – Subtractive hybridization was standard
- Master floral gene identification
  - Late 1980s-1990s: *AGAMOUS*, *LEAFY* and more
  - PCR to isolate tree versions
- Age of genomics: cDNA sequencing, ESTs
  - Catalogs of genes from various tissues
  - Comparative genomics
- Whole genome (re)sequencing, short read method explosion, RNA-seq, computation

Cell, Vol. 69, 843–859, May 29, 1992, Copyright © 1992 by Cell Press

LEAFY Controls Floral Meristem Identity in Arabidopsis

Detlef Weigel,* John Alvarez,† David R. Smyth,†
Martin F. Yanofsky,*‡ and Elliot M. Meyerowitz*
*California Institute of Technology

Bowman et al., 1989, 1991
al., 1989; Sommer et al.,
Drews et al., 1991; Schwarz

Cell

Volume 63, Issue 6, 21 December 1990, Pages 1311-1322

Article

floricaula: A homeotic gene required for flower development in antirrhinum majus

Enrico S. Coen, JoséM. Romero *, Sandra Doyle, Robert Elliott, George Murphy, Rosemary Carpenter

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PNAS
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NEW RESEARCH IN Physical Sciences | Social Sciences

A *Populus* EST resource for plant functional genomics

Fredrik Sterky, Rupali R. Bhalerao, Per Unneberg, Bo Segerman, Peter Nilsson, Amy M. Brunner, Laurence Charbonnel-Campaa, Jenny Jonsson Lindvall, Karolina Tandre, Steven H. Strauss, Björn Sundberg, Petter Gustafsson, Mathias Uhlén, Rishikesh P. Bhalerao, Ove Nilsson, Göran Sandberg, Jan Karlsson, Joakim Lundeberg, and Stefan Jansson

PNAS September 21, 2004. 101 (38) 13951-13956; https://doi.org/10.1073/pnas.0401641101

NATURE · VOL 346 · 5 JULY 1990 ARTICLES

The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors

Martin F. Yanofsky*, Hong Ma*, John L. Bowman, Gary N. Drews, Kenneth A. Feldmann† & Elliot M. Meyerowitz‡

Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125, USA
† E. I. DuPont de Nemours and Co., Wilmington, Delaware 19880, USA

Mutations in the homeotic gene *agamous* of the plant *Arabidopsis* cause the transformation of the floral sex organs. Cloning and sequence analysis of *agamous* suggest that it encodes a protein with a high degree of sequence similarity to the DNA-binding region of transcription factors from yeast and humans and to the product of a homeotic gene from *Antirrhinum*. The *agamous* gene therefore probably encodes a transcription factor that regulates genes determining stamen and carpel development in wild-type flowers.

flower phenotypes were recognized as long ago as 2,000 years[8]. The first published report of *Arabidopsis* flowers with an *ag* mutant phenotype was more than a century ago[9], and another *Arabidopsis* mutant having similar flowers has been described by Conrad[10]. The extensively characterized[3] mutant allele, *ag-1*, was isolated after ethylmethane sulphonate (EMS) mutagenesis and was first described by Koornneef *et al.*[11]. The *AG* locus has been mapped to chromosome 4 (ref. 11).

Here we describe the molecular cloning and characterization of the *AG* gene, which was facilitated by a T-DNA insertion mutation[12]. The deduced AG protein product is similar to transcription factors from humans (SRF) and yeast (MCM1, ARG80), and to the product, DEF A, of a recently isolated homeotic gene from the snapdragon *Antirrhinum majus*.

Breakthroughs – genetic modification methods

- Transformation capacity and knock-out libraries
  - Leaf disc general plant transformation
  - Arabidopsis floral dip, T-DNA mutagenesis – large scale functional discovery and validation
  - Poplar transformation and regeneration
- Antisense and RNAi
  - Single genes within trees can be specifically modified for the first time !
- Gene editing revolution
  - Beyond ZFNs and TALENs – The CRISPR-Cas miracle age of today

A Simple and General Method for Transferring Genes into Plants

Abstract. *Transformed petunia, tobacco, and tomato plants have been produced by means of a novel leaf disk transformation-regeneration method. Surface-sterilized leaf disks were inoculated with an* Agrobacterium tumefaciens *strain containing a modified tumor-inducing plasmid (in which the phytohormone biosynthetic genes from transferred DNA had been deleted and replaced with a chimeric gene for kanamycin resistance) and cultured for 2 days. The leaf disks were then transferred to selective medium containing kanamycin. Shoot regeneration occurred within 2 to 4 weeks, and transformants were confirmed by their ability to form roots in medium containing kanamycin. This method for producing transformed plants combines gene transfer, plant regeneration, and effective selection for transformants into a single process and should be applicable to plant species that can be infected by* Agrobacterium *and regenerated from leaf explants.*

Efficient methods for introducing cloned genes into plants are important for understanding and controlling plant gene expression. The ability to manipulate genes could lead to rational, deliberate alterations of the genome of crop plants for improvement of their agronomic performance. Production of morphologically normal plants that contain and express foreign genes has been made possible by use of the natural gene-transfer capacity of *Agrobacterium tumefaciens*, a soil bacterium that causes crown gall disease in plants (*1*). Modified *A. tumefaciens* strains were used in which the tumor-inducing (Ti) genes had been deleted from the transferred DNA (T-DNA) and replaced with chimeric genes for bacterial antibiotic resistance that had been engineered to express in plant cells (*2*).

In previous studies the transformed plants were regenerated from calli derived from protoplasts (single cells without a cell wall) transformed by cocultivation with *A. tumefaciens* cells (*1*). However, the protoplast culture method has certain limitations: not all species of plants can be readily regenerated from protoplasts; the entire process can take up to 6 months from protoplast to plant; and plants derived from protoplasts can be subject to mutations or chromosomal abnormalities (*3*). Protoplast culture technology can also be difficult to reproduce in a new laboratory or to control from one experiment to the next. Transformation of stem or root explants in vitro is a simple substitute for cocultivation (*4*) but is laborious for large scale experiments and not easy to use with modified Ti plasmids that lack the tumor-inducing genes.

To overcome these limitations, we have developed an approach to transfer of an *A. tumefaciens* strain (GV3Ti11SE) containing a modified octopine Ti plasmid (pTiB6S3SE) in which all phytohormone biosynthetic genes and the T_L-DNA right border sequence have been deleted has been described (*2*). Formation of a cointegrate between pTiB6S3SE and the intermediate vectors pMON120 or pMON200 results in a functional, aviruleent T-DNA (*2*, *7*). Plasmid pMON200 is a derivative of pMON120, which contains a translationally-improved chimeric NOS/NPTII/NOS gene for kanamycin resistance and confers a high degree of resistance to aminoglycoside antibiotics on transformed plant cells (*8*). The vectors also contain the nopaline synthase gene, which provides a second marker in the transformed plant cells (*1*).

Disks were punched from surface-sterilized leaves with a paper punch (6 mm in diameter) and submerged in a culture of *A. tumefaciens* grown overnight in luria broth at 28°C. After gentle shaking to ensure that all edges were infected, the disks were blotted dry and incubated upside-down on nurse culture plates prepared as described (*7*) containing medium that induces regeneration of shoots of the species being transformed. The age and titer of the bacterial inoculum had little influence on the effectiveness of the transformation; however, it was important to avoid excessive soaking of the internal tissues of the leaf disk by the bacterial culture. After 2 to 3 days, the disks were transferred to petri plates containing the same medium but without feeder cells or filter papers and containing carbenicillin (500 μg/ml) and kanamycin (300 μg/ml).

After 2 to 4 weeks, shoots that developed were excised from calli and transplanted to appropriate root-inducing medium containing carbenicillin (500 μg/ml) and kanamycin (100 μg/ml). Rooted plantlets were transplanted to soil as soon as possible after roots appeared. *Nicotiana tabacum* varieties Samson and Havana 425 (*9*) and a first-generation cross-fertilized (F_1) hybrid of *Petunia hybrida* (*10*) were easily transformed by this system. L2 tomato plants (*11*) responded better when the feeder plate medium was modified by reducing the amount of inorganic salts to one-tenth the usual concentration.

Uninoculated petunia leaf disks and those inoculated with *A. tumefaciens* strains containing pTiB6S3SE::pMON-120 (which lacks the chimeric gene for kanamycin resistance) did not produce calli or shoots on medium containing 300 μg of kanamycin per milliliter (Fig. 1). In contrast, leaf disks inoculated with *A.*

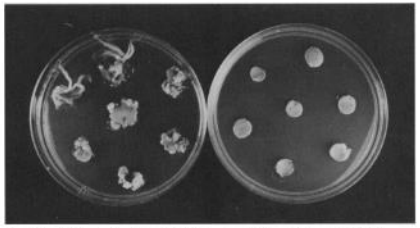
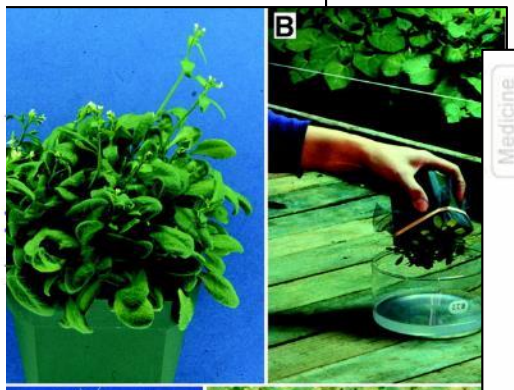
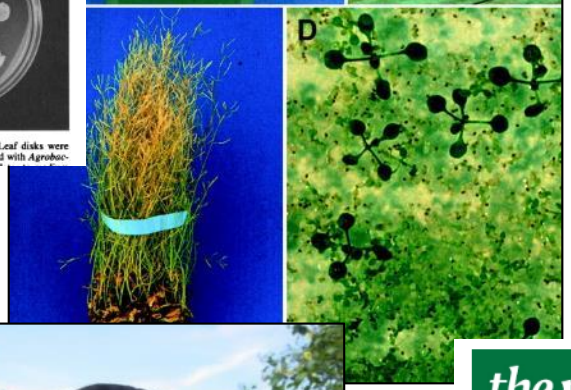
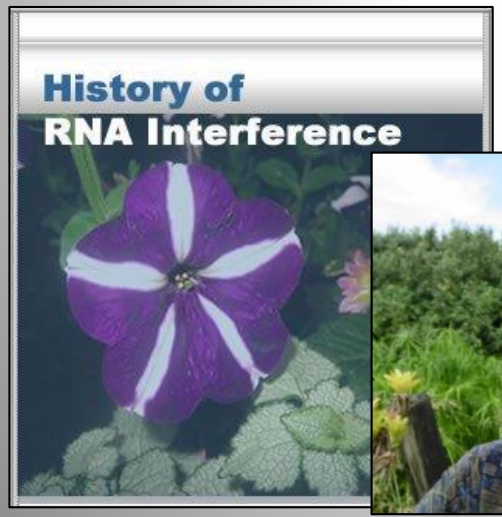
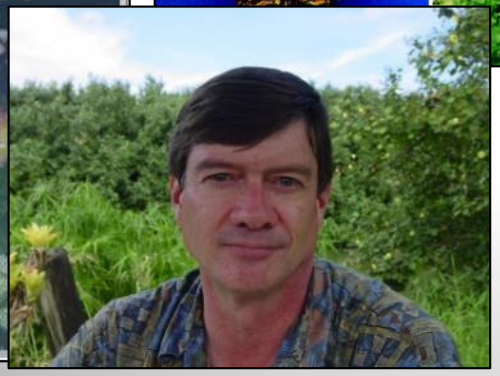


Fig. 1. Leaf disk transformation and selection of antibiotic-resistant cells. Leaf disks were punched from a surface-sterilized leaf of *Petunia hybrida* (Mitchell), inoculated with *Agrobac-*









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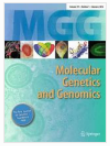

Molecular and General Genetics MGG

February 1987, Volume 206, Issue 2, pp 192–199 | Cite as

Agrobacterium mediated transformation and regeneration of *Populus*

Authors | Authors and affiliations

JoAnne J Fillatti, James Sellmer, Brent McCown, Bruce Haissig, Luca Comai

Medicine

The Nobel Prize in Physiology or Medicine 2006

"for their discovery of RNA interference - gene silencing by double-stranded RNA"

Photo: L. Cicero/Stanford

Andrew Z. Fire

1/2 of the prize

Photo: R. Carlin/UMMAS

Craig C. Mello

1/2 of the prize

the plant journal

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Free Access

Populus CEN/TFL1 regulates first onset of flowering, axillary meristem identity and dormancy release in *Populus*

Rozi Mohamed, Chieh-Ting Wang, Cathleen Ma, Olga Shevchenko, Sarah J. Dye, Joshua R. Puzey, Elizabeth Etherington, Xiaoyan Sheng, Richard Meilan, Steven H. Strauss, Amy M. Brunner

First published: 11 May 2010 | https://doi.org/10.1111/j.1365-313X.2010.04185.x | Cited by: 86

GE trees: Reliable in the field

Lessons from Two Decades of Field Trials with Genetically Modified Trees in the USA: Biology and Regulatory Compliance

Steven H. Strauss, Cathleen Ma, Kori Ault and Amy L. Klocko

Abstract We summarize the many field trials that we have conduct
beginning in 1995 and continuing to this day. Under USDA APHIS
latory notifications and permits, we have planted nearly 20,000 trees
approximately 100 different constructs in more than two dozen fiel
The large majority of the trials were in *Populus* and included hybrid

Lecture by Amy Klocko at GMO Biosafety Meeting in Mexico, 2017

22 years and 22,979 trees later: Lessons from field-testing GM trees in the USA

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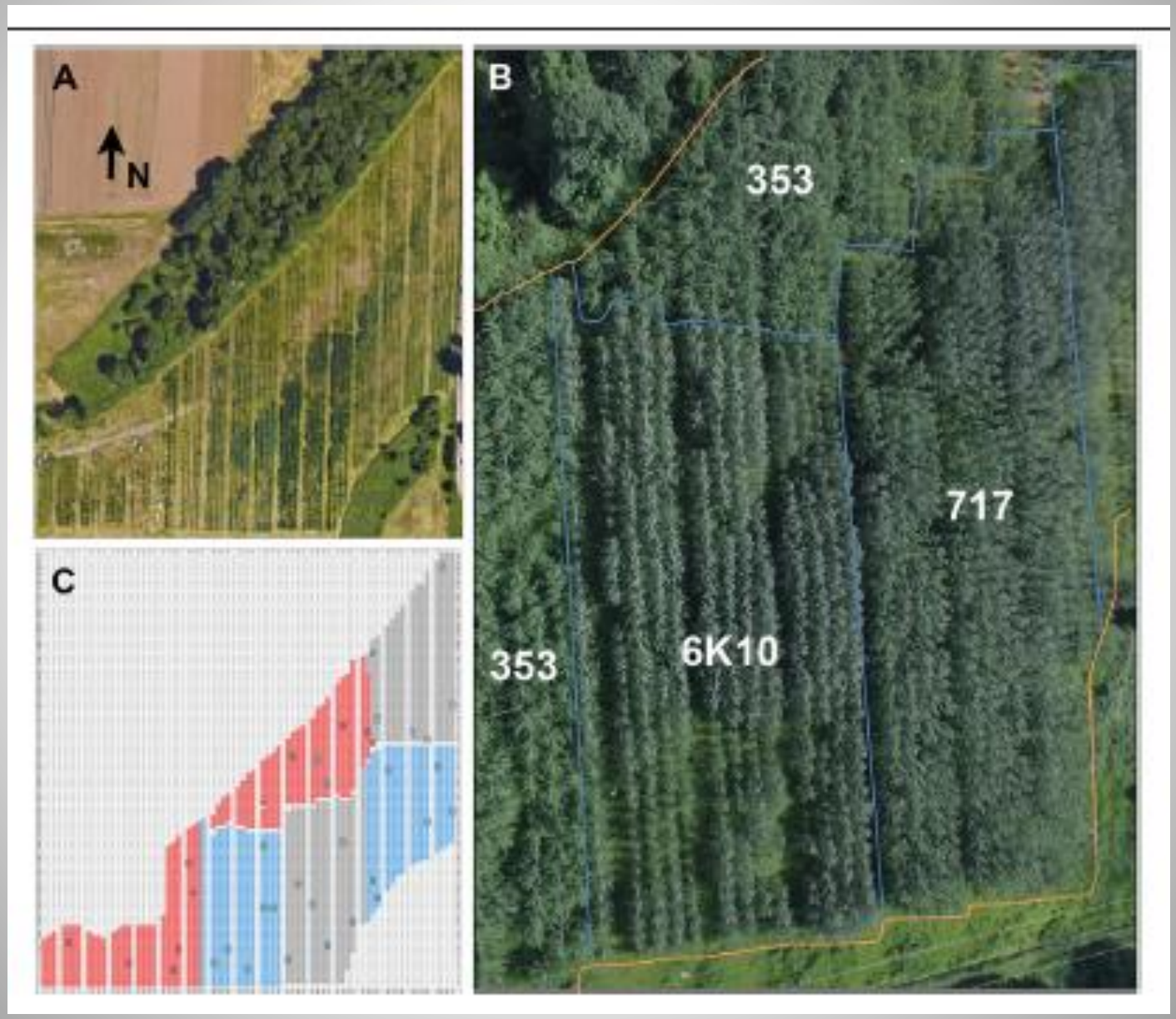
Current ~4 ha trial – flowering modification genes

Image taken in summer 2016

Overview of field site



GE trees in the field: Reliable floral modification

frontiers in Bioengineering and Biotechnology | Biosafety and Biosecurity

ORIGINAL RESEARCH ARTICLE Provisionally accepted The full-text will be published soon. Notify me

Front. Bioeng. Biotechnol. | doi: 10.3389/fbioe.2018.00100

Phenotypic expression and stability in a large-scale field study of genetically engineered poplars containing sexual containment transgenes

Amy L. Klocko[1], Haiwei Lu[2], Anna Magnuson[2], Amy Brunner[3], Cathleen Ma[2] and Steven H. Strauss[2*]

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[2]Forest Ecosystems and Society, Oregon State University, United States
[3]Department of Forest Resources and Environmental Conservation, Virginia Tech, United States

Genetic engineering (GE) can help meet demand for forest products and ecological services. However, high research and development costs, market restrictions, and regulatory obstacles to performing field tests have severely limited the extent and duration of field research. There is a notable paucity of field studies of flowering GE trees due to the time frame required

Breakdowns – genetic modification methods

- Transformation incapacity, inefficiency, cost
  - Hardly relevant beyond poplars and scientific studies
  - Hardly studied, developed for most forestry species
- Why?
  - Biological recalcitrance to regeneration
  - Huge genetic diversity in response
  - Little application of modern developmental science

DuPont Pioneer breakthrough advances

The Plant Cell, Vol. 28: 1998–2015, September 2016, www.plantcell.org

BREAKTHROUGH REPORT

Morphogenic Regulators *Baby boom* and *Wuschel* Improve Monocot Transformation[OPEN]

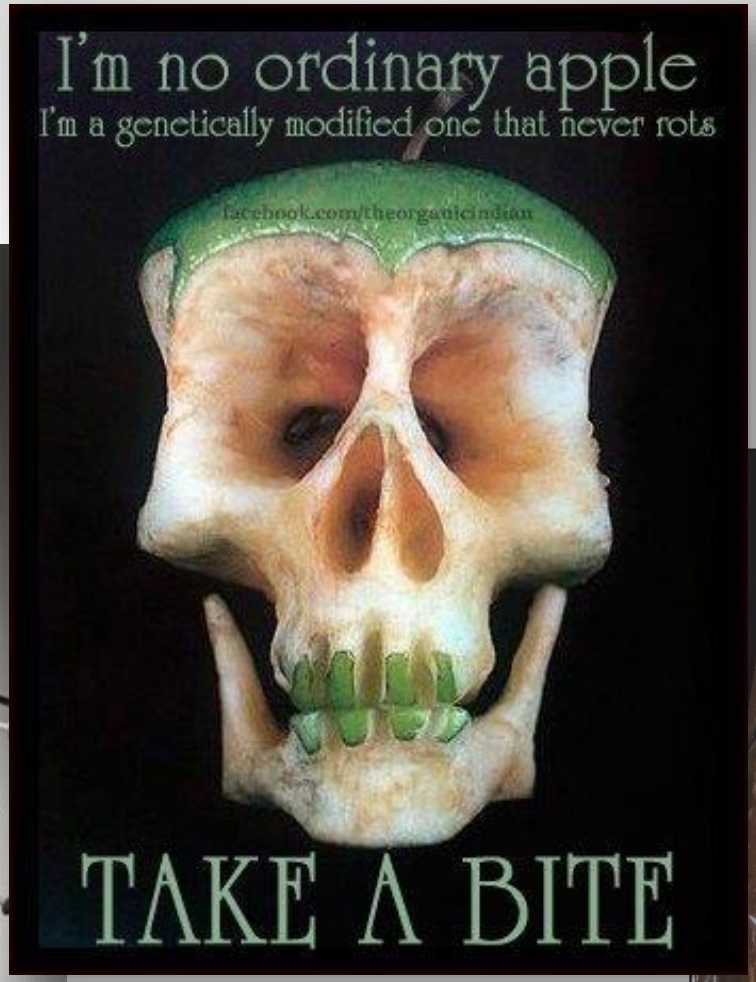
Keith Lowe,[a] Emily Wu,[a] Ning Wang,[a] George Hoerster,[a] Craig Hastings,[a] Myeong-Je Cho,[b] Chris Scelonge,[a] Brian Lenderts,[a] Mark Chamberlin,[a] Josh Cushatt,[a] Lijuan Wang,[a] Larisa Ryan,[a] Tanveer Khan,[c] Julia Chow-Yiu,[a] Wei Hua,[a] Maryanne Yu,[b] Jenny Banh,[b] Zhongmeng Bao,[a] Kent Brink,[d] Elizabeth Igo,[d] Bhojaraja Rudrappa,[e] PM Shamseer,[e] Wes Bruce,[f] Lisa Newman,[a] Bo Shen,[a] Peizhong Zheng,[g] Dennis Bidney,[a] Carl Falco,[a] Jim Register,[a] Zuo-Yu Zhao,[a] Deping Xu,[a] Todd Jones,[a] and William Gordon-Kamm[a,1]

[a] DuPont Pioneer, Johnston, Iowa 50131

Breakdowns – GMO stigma

- Little and decreasing public investment in transformation technology or transgenic products
- Limited and decreasing private investment in transformation technology or transgenic products
  - Very little experience, science, or transformation technology is shared, available for scientific advancement
  - Work is mostly short-term focused; little overall progress apart from genotype by genotype projects?
- Negative public views, anti-GMO activism, unsustainable industry management, have led to increasing regulatory and market barriers

Anti-GMO messaging everywhere in marketplace



Poor weed management has led to rapid development of herbicide-resistant weeds

And motivated development of new kinds of herbicide tolerant crops with their own problems

nature biotechnology

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NATURE BIOTECHNOLOGY | NEWS

Glyphosate resistance threatens Roundup hegemony

Emily Waltz

Nature Biotechnology **28**, 537–538 (2010) | doi:10.1038/nbt0610-537
Corrected online **13 October 2010**
Corrigendum (October, 2010)

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Weeds are becoming increasingly resistant to glyphosate, a report from the US National Academy of Sciences (NAS) released in April has found. The driving force, according to the report, is farmers' dependence on the weed killer accompanied by the widespread adoption of genetically modified (GM) herbicide-tolerant crops. Seed makers are hoping to forestall the problem by developing GM crops with 'stacked' traits that tolerate multiple herbicides. But weed scientists warn that if farmers manage these new crops in the same way as they managed their glyphosate-tolerant predecessors, weeds will simply become resistant to the new technologies.

"The number of weed species evolving resistance to glyphosate

BILL BARKSDALE / AGSTOCKUSA /

“Green” certification of forests create severe barriers to field research, markets

Plantation Certification & Genetic Engineering

FSC’s Ban on Research Is Counterproductive

Steven H. Strauss, Malcolm M. Campbell, Simon N. Pryor, Peter Coventry, and Jeff Burley

ABSTRACT

Genetic engineering, also called genetic modification (GM), is the isolation, recombinant modification, and asexual transfer of genes. It has been banned in forest plantations certified by the Forest Stewardship Council (FSC) regardless of the source of genes, traits imparted, or whether for research or commercial use. We review the methods and goals of tree genetic engineering research and argue that FSC’s ban on research is counterproductive because it makes it difficult for certified companies to participate in the field research needed to assess the value and biosafety of GM trees. Genetic modification could be important for translating new discoveries about tree genomes into improved growth, quality, sustainability, and pest resistance.

Keywords: biotechnology; entomology and pathology; ethics; genetics; silviculture

Genetic engineering, commonly called genetic modification (GM) in much of the world, is the use of recombinant DNA and asexual gene transfer methods to breed more productive or pest-resistant crops. It has been the subject of considerable controversy, with concerns raised from biological, socioeconomic, political, and ethical perspectives. Some of the issues are similar to those raised by the use of molecular biology and genetic engineering in medicine, which we see in the news headlines daily. However, genetic modification in agriculture and forestry raises environmental issues as well.

GM crops, mainly herbicide- and pest-resistant varieties of soybeans, maize, or cotton, have been vigorously adopted by farmers in North America because they are easy to manage and they improve yields, reduce costs, or reduce pesticide ecotoxicity (Carpenter and Gianessi 2001). However, the controversy, primarily embodied in regulatory barriers to trade of GM crops with Europe and Japan, has slowed their adoption considerably in recent years.

If GM trees are used in forestry in the near future, they are likely to occur primarily in intensively managed environments, such as urban forests or plantations. In urban forestry, genetic modification is expected to help trees adapt to the stresses and special demands of human-dominated systems. Examples would be trees that are more tolerant of heavy metals or other pollutants, resist urban pests or diseases, grow slower, or do not produce fruits when these create hazards in street environments (Brunner et al. 1998).

Plantations, although very different from natural forests in structure and function, are considered part of the spectrum of methods in sustainable forest management (Romm 1994). Plantations can relieve pressure on natural forests for exploitation and can be of great social value by supplying community and industrial wood needs and fueling economic development. The environmental role of plantations is recognized by the Forest Stewardship Council (FSC), an international body for certification of sustainably managed forests. FSC Principle 10 states that plantations should “complement the management of, reduce pressures on, and promote the restoration and conservation of natural forests” (FSC 2001).

FSC has certified some of the most intensively managed plantations in the world, including poplar plantations and the intensive pine and eucalypt plantations of the Southern Hemisphere. Although many environmental mitigations are built into these certified plantation systems, within the areas dedicated to wood production they function as tree farms. Such intensive plantation systems often use highly bred genotypes, possibly including exotic species, hybrids, and clones, as well as many other forms of intensive silvicultural management. It is in the context of these biointensive systems that the additional expense of GM trees is likely to be worthwhile.

However, FSC currently prohibits all uses of GM trees, and is the only certification system to have done so

Forest Stewardship Council

“…genetically modified trees are prohibited…”

Regulations and certification render GE ineffective as a tool for forest health

Traces of the emerald ash borer on the trunk of a dead ash tree in Michigan, USA. This non-native invasive insect from Asia threatens to kill most North American ash trees.

BIOTECHNOLOGY

Genetically engineered trees: Paralysis from good intentions

Forest crises demand regulation and certification reform

By **Steven H. Strauss[1]*, Adam Costanza[2], Armand Séguin[3]**

Intensive genetic modification is a long-standing practice in agriculture, and, for some species, in woody plant horticulture and forestry (*1*). Current regulatory systems for genetically engineered

recently initiated an update of the Coordinated Framework for the Regulation of Biotechnology (*2*), now is an opportune time to consider foundational changes.

Difficulties of conventional tree breeding make genetic engineering (GE) methods relatively more advantageous for forest trees than for annual crops (*3*). Obstacles

Although only a few forest tree species might be subject to GE in the foreseeable future, regulatory and market obstacles prevent most of these from even being subjects of translational laboratory research. There is also little commercial activity: Only two types of pest-resistant poplars are authorized for commercial use in small areas in China and two types of eucalypts, one approved in Brazil and another under lengthy review in the USA (*5*).

METHOD-FOCUSED AND MISGUIDED. Many high-level science reports state that the GE method is no more risky than conventional breeding, but regulations around the world essentially presume that GE is hazardous and requires strict containment

Agenda

- A look back on where we have come from: Breakthroughs and “breakdowns”
- Reproductive modification rationale and context
- Experience with three major approaches
  - RNAi against floral development genes
  - Floral onset repressor overexpression
  - CRISPR-Cas9 mutation

Roundup tolerant bentgrass escape in Oregon

GMO grass that 'escaped' defies eradication, divides grass seed industry

http://www.oregonlive.com/business/index.ssf/2017/01/grass_seed_industry_fearful_ab.html

Gene flow regulation and ethics a major reason for GMO stigma

- Bigger for forest trees than most ag crops – for many reasons
  - Wild/feral populations
  - Record of invasiveness of many exotic trees/shrubs
  - Keystone roles in ecosystems
  - Long distance pollen and/or seed movement
  - Limited domestication
  - Larger role in providing ecosystem services
  - Public view of forests 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?

Sterility genes are tools – to be used with discretion and management

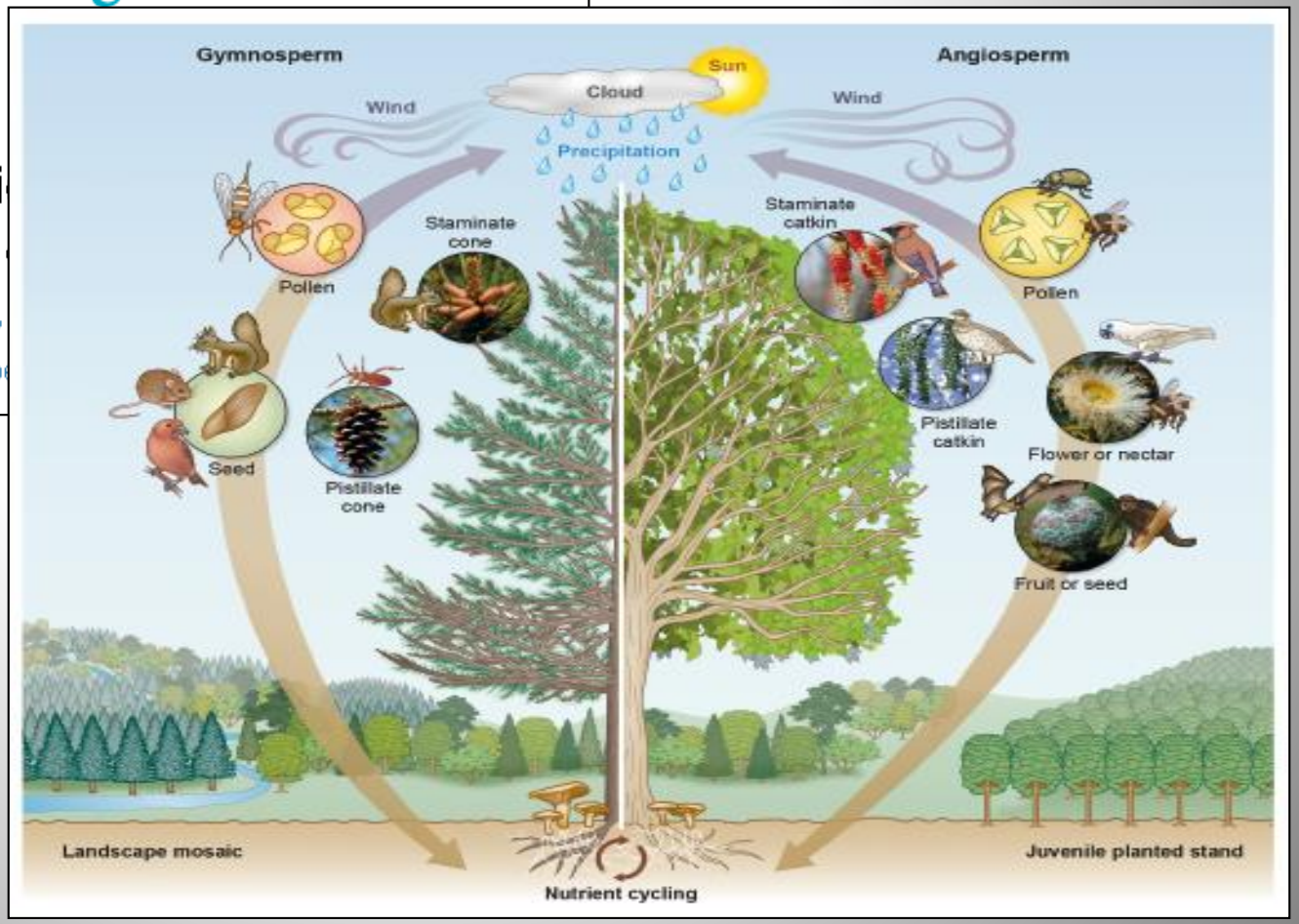
2017 Tansley Review

New Phytologist

Tansley review

Reproductive modificati
impacts on biodiversity

Steven H. Strauss ✉, Kristin N. Jones,
Matthew G. Betts, Berry J. Brosi, Robe



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, etc (not studied)
- Our focus has been on bisexual and permanent sterility for vegetatively propagated species

Strong *lfy* mutants appear to have no flowers

Snapdragon *Arabidopsis* Petunia

WT

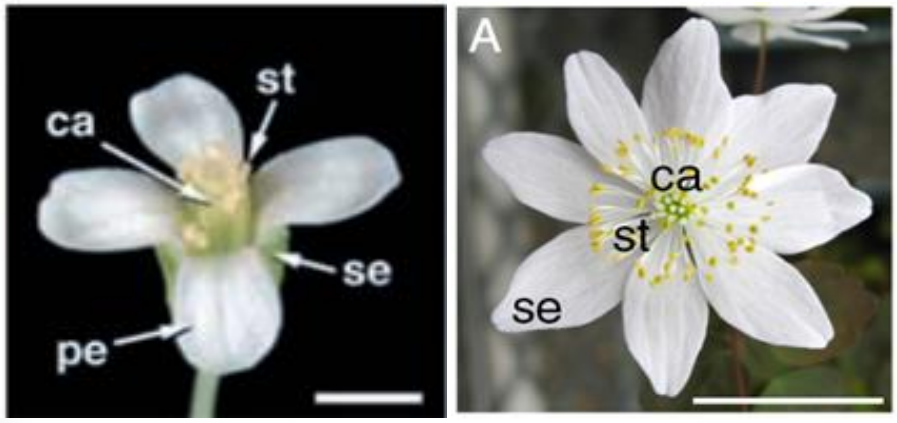
***lfy* mutants**

Parcy et al. 2002; Moyroud et al. 2010

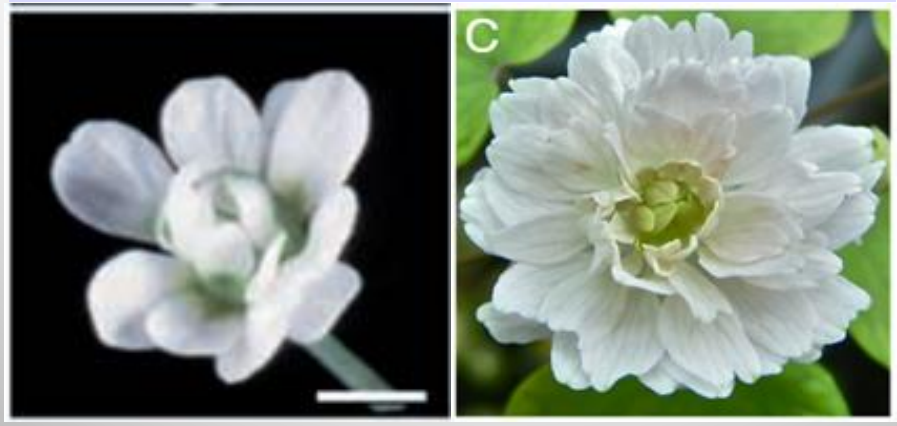
Flowers in strong *ag* mutants are missing both stamens and carpels

Arabidopsis Ranunculid

WT



***ag* mutants**



Parcy et al. 2002; Galimba et al. 2012

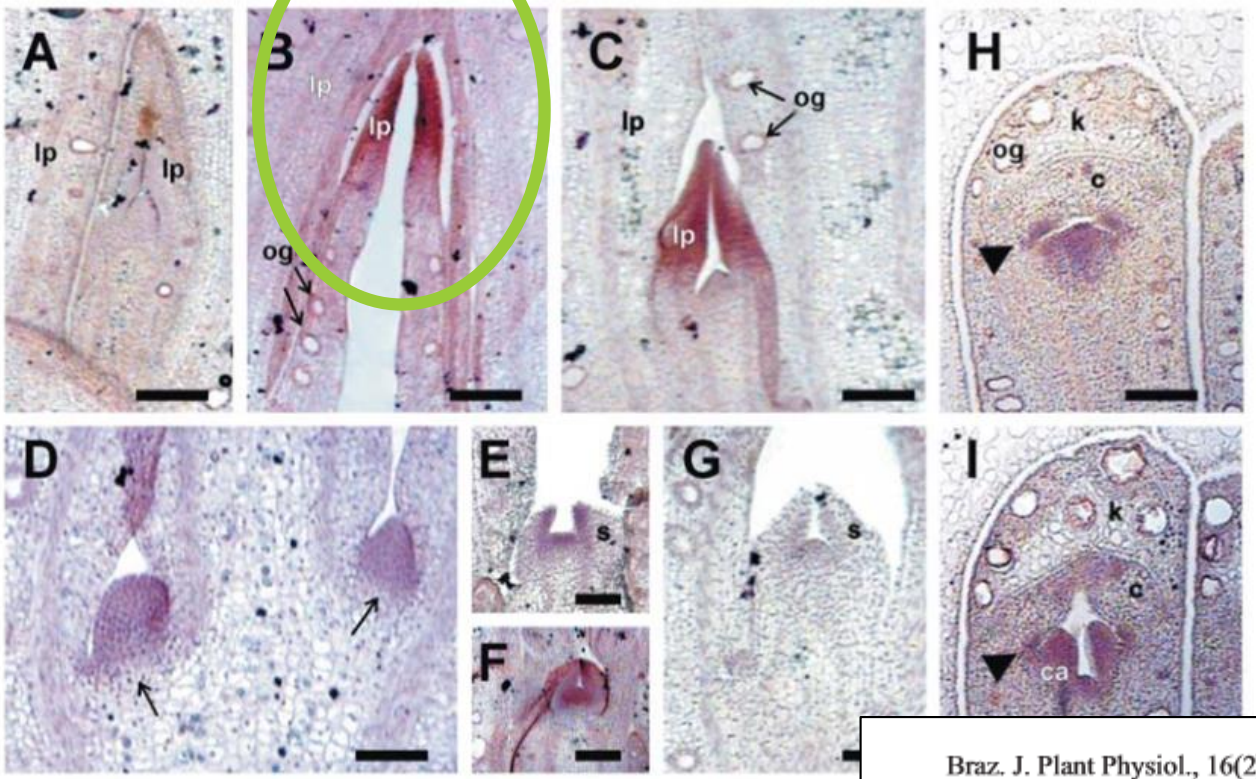
At the time our work started, the full roles of *LFY* and *AG* unknown

- Discovery studies did not have significant analysis of vegetative/productivity effects
  - An absence of studies of gene mutation/knock-out in the field
- No studies in the very divergent floral types of important forest tree taxa
  - Often parts of gene families
- Found to have vegetative as well as floral expression
  - 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

Agenda

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  - Overexpression of floral onset repressors
  - CRISPR-Cas9 mutation effects

PLOS ONE

RESEARCH ARTICLE

Transgenic Suppression of *AGAMOUS* Genes in Apple Reduces Fertility and Increases Floral Attractiveness

Amy L. Klocko[1], Ewa Borejsza-Wysocka[2], Amy M. Brunner[3], Olga Shevchenko[1], Herb Aldwinckle[2], Steven H. Strauss[1]*

1 Department of Forest Ecosystems and Society, Oregon State University, Corvallis, Oregon, United States of America, **2** Section of Plant Pathology and Plant-Microbe Biology, School of Integrative Plant Science, Cornell University, Geneva, New York, United States of America, **3** Department of Forest Resources and Environmental Conservation, Virginia Tech, Blacksburg, Virginia, United States of America

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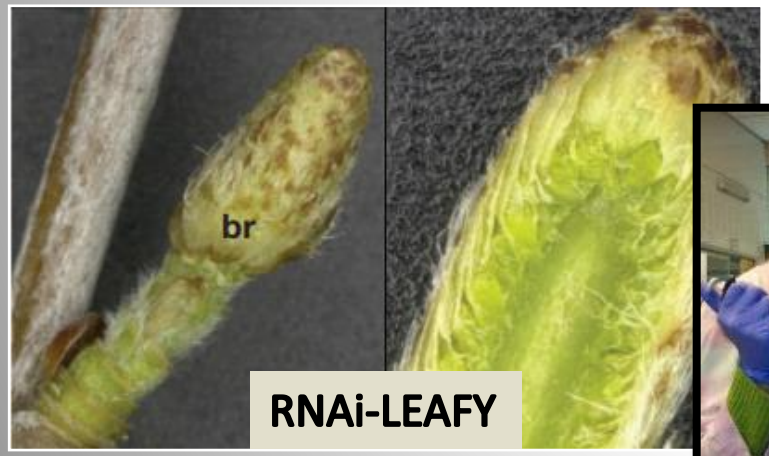
Abstract

We investigated the ability of RNA interference (RNAi) directed against two co-orthologs of *AGAMOUS* (*AG*) from *Malus domestica* (domestic apple, *MdAG*) to reduce the risks of

Poplar sterility using RNAi against *LEAFY*



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 kilometers[5]. Although male sterility has been engineered in pine, poplar, and eucalyptus trees grown under field conditions by expression of the barnase RNase gene in

can reduce

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expression

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ve exotic trees,

uction[9]. We

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

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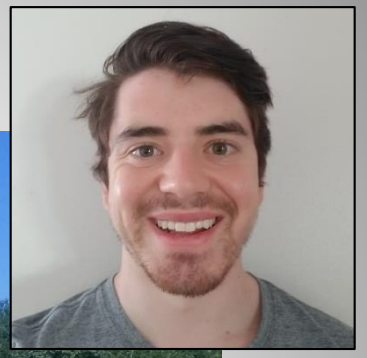
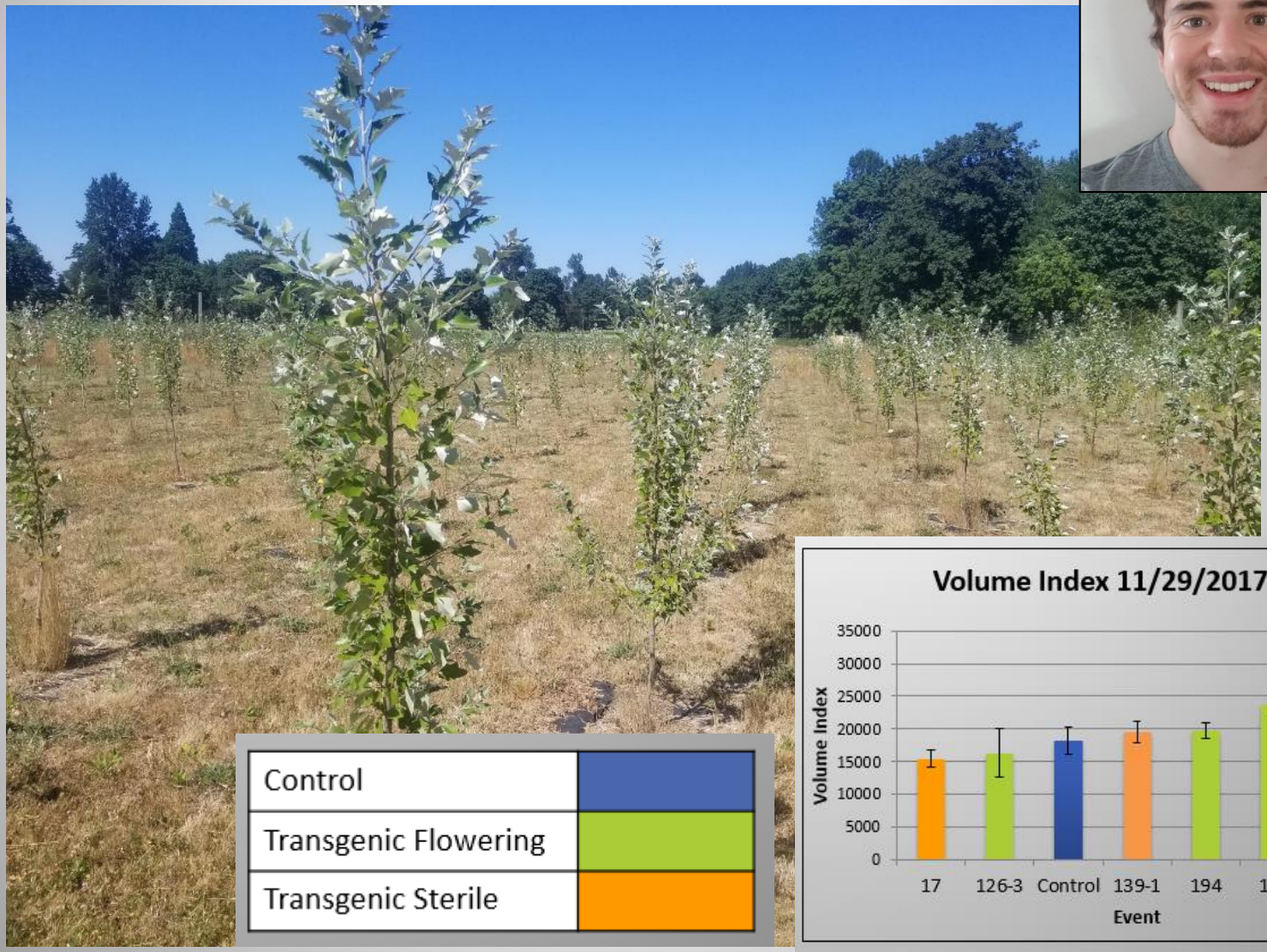
Expanded study of vegetative effects of *LFY* suppression/sterility





RNAi against *AGAMOUS (AG)* for sterility in poplar

- *AG* in poplar studied earlier: Amy Brunner
- Paralogs on different chromosomes - 89% DNA sequence similarity in protein coding region
- Simultaneous suppression with one RNAi construct
- Vegetative expression role

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Structure and expression of duplicate AGAMOUS orthologues in poplar

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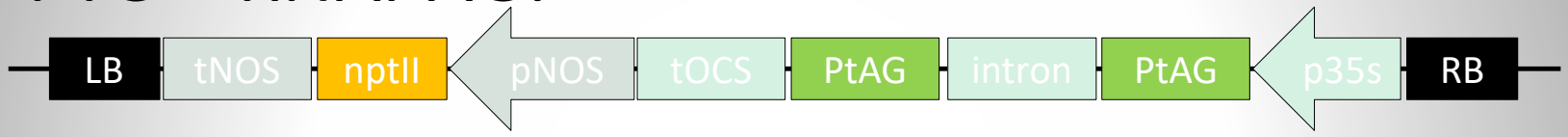
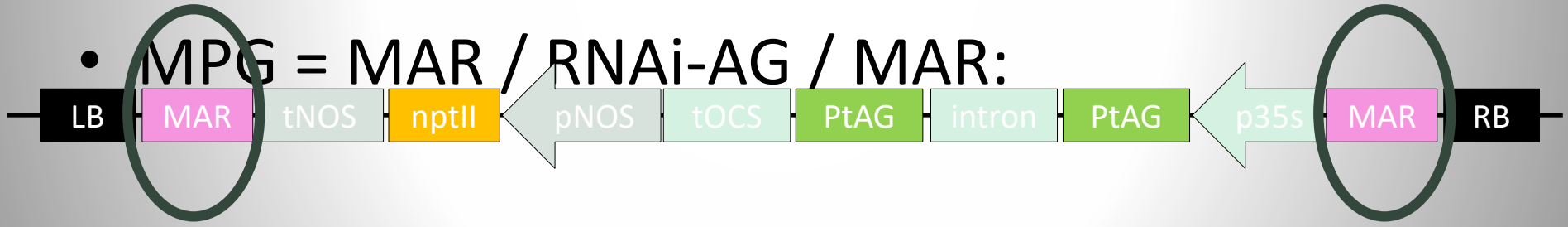
Key words: AGAMOUS, cottonwoods, dioecy, floral development, MADS-box, Populus

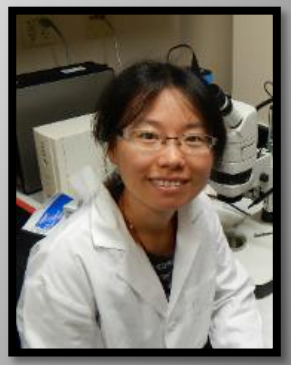
Two AG-RNAi constructs, with and without MARs

- PTG = RNAi-AG:



- MPG = MAR / RNAi-AG / MAR:





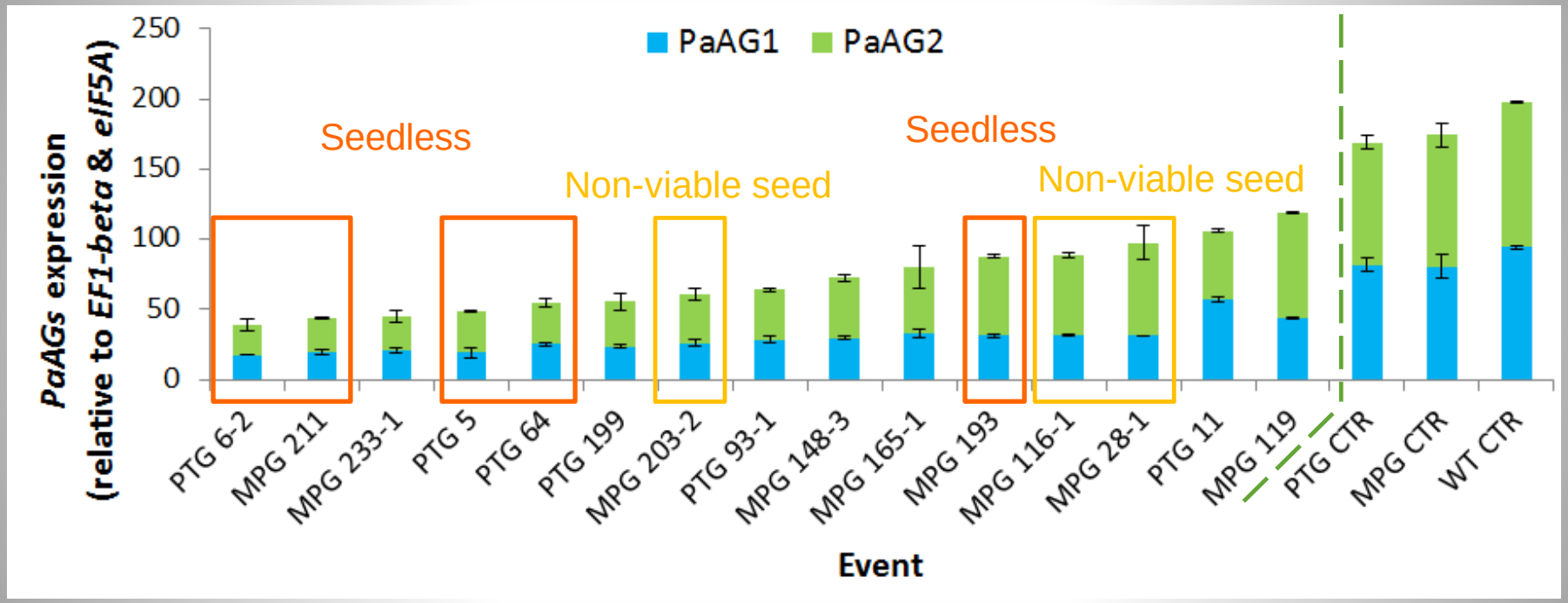
MARS induced a high rate of RNAi floral modification

Construct ID	No. of Events Planted/Survived	No. of Events Flowered by 2017	No. (%) of Events with Altered Floral Morphology
AG-RNAi (PTG)	22/22	22 (100%)	6 (27%)
MAR-*AG*-RNAi (MPG)	13/13	12 (92%)	11 (92%)
Non-transgenic (WT)	24/24	19 (79%)	0 (0%)

MAR elements more than tripled RNAi suppression frequency

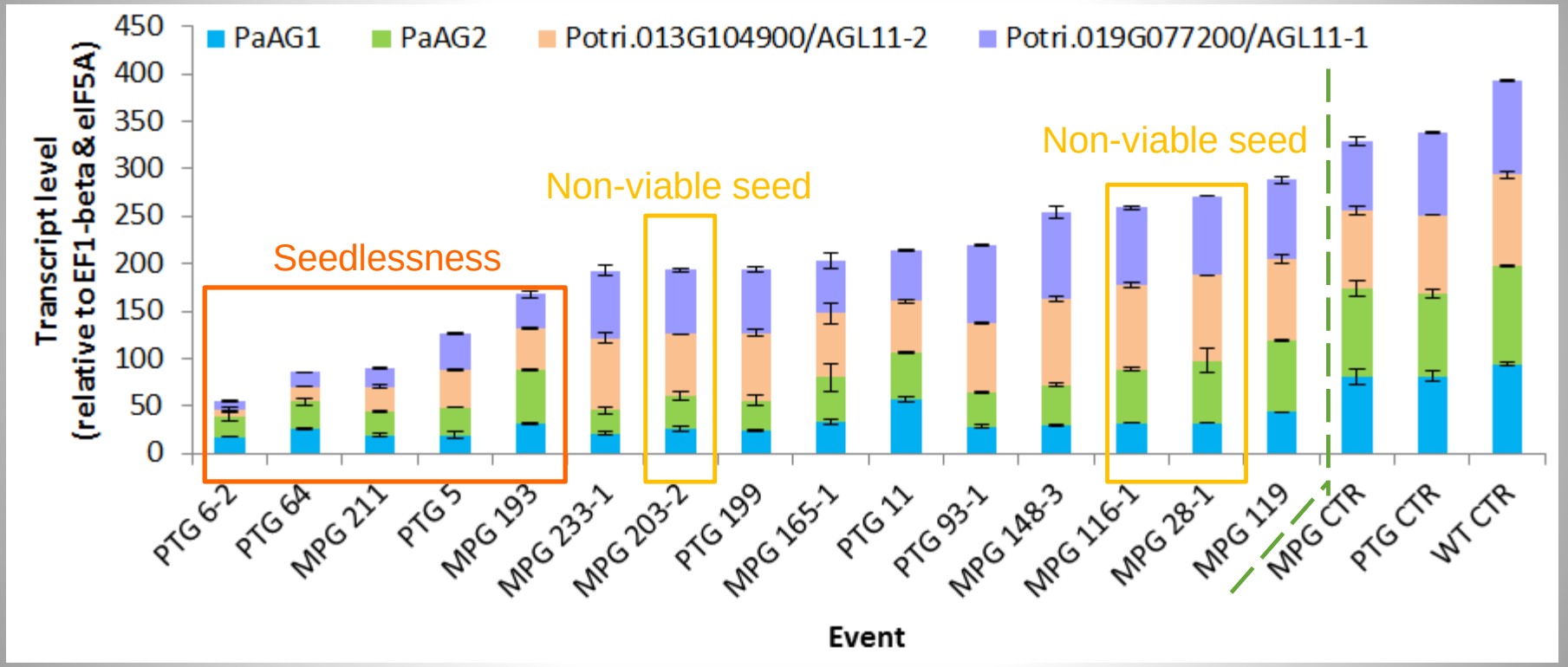
Strong poplar AG-RNAi events in the field with mutant flowers stable among/within trees over 4 years

Suppression of the two *PaAG* paralogs were imperfectly associated with the sterility phenotype



- *PaAG1* and *PaAG2* expression was highly correlated: r = 0.91

Full sterility phenotype strongly correlated with total expression of *AG* and *AGL11*

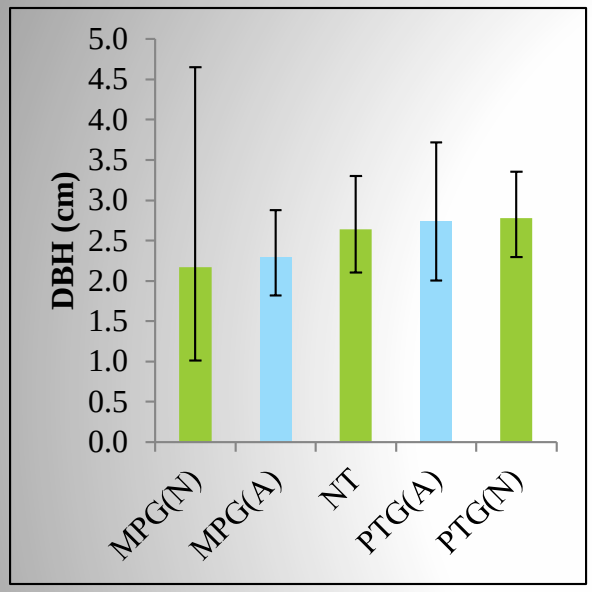


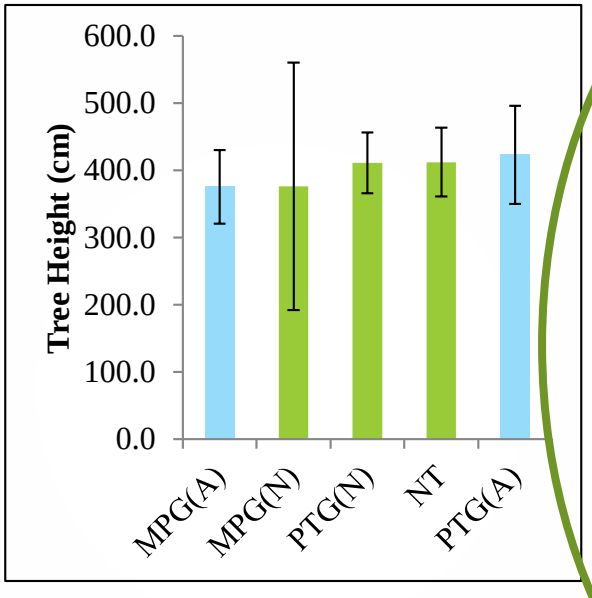
Correlation among AG and AGL paralogous pairs weak: r = 0 .50

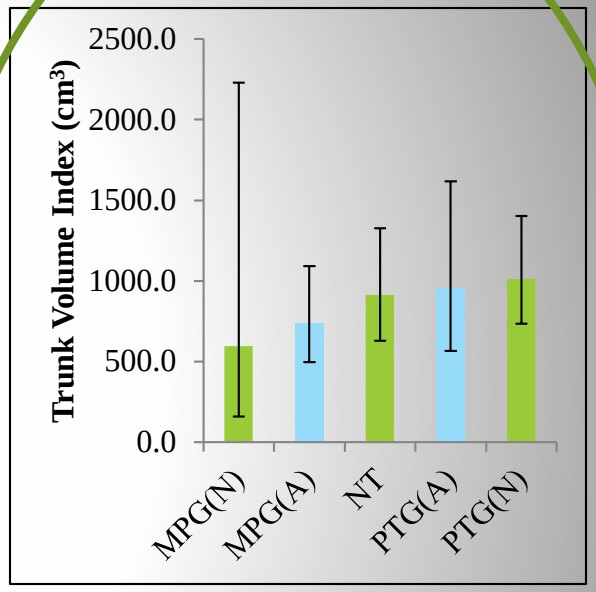
AG-RNAi events had normal tree and leaf form

- 3 leaves per tree scanned and analyzed for chlorophyll content, leaf area and weight, petiole length

No association between floral modification and tree growth







Mean or median (for back transformed estimates: DBH and trunk volume index) and 95% CI are shown in the figures

Key results – AG-RNAi

- MARs elevated RNAi efficiency greatly
- Stacked flowers within catkins
- Four-gene suppression gave ovule-free, seedless, cottonless, capsules
- Stable in the field over 4 years
- No detected vegetative effects
- (In male 353 clone, inadequate RNAi suppression to see strong sterility phenotype?)

Several constructs were designed to delay or prevent floral onset

Construct name	Field ID	Construct type	Predicted outcome
AG-M2	AM2	DNM	Delayed flowering
AG-M3	AM3	DNM	Delayed flowering
AP1-M2	AP2	DNM	Delayed flowering
AP1-M3	AP3	DNM	Delayed flowering
FT	FT	RNAi	Delayed flowering
SVP-OE	PS	OvExp	Delayed flowering
AGL20	A20	RNAi	Delayed flowering
AGL24	A24	RNAi	Delayed flowering
FT:AGL20:FPF1	FAP	RNAi	Delayed flowering

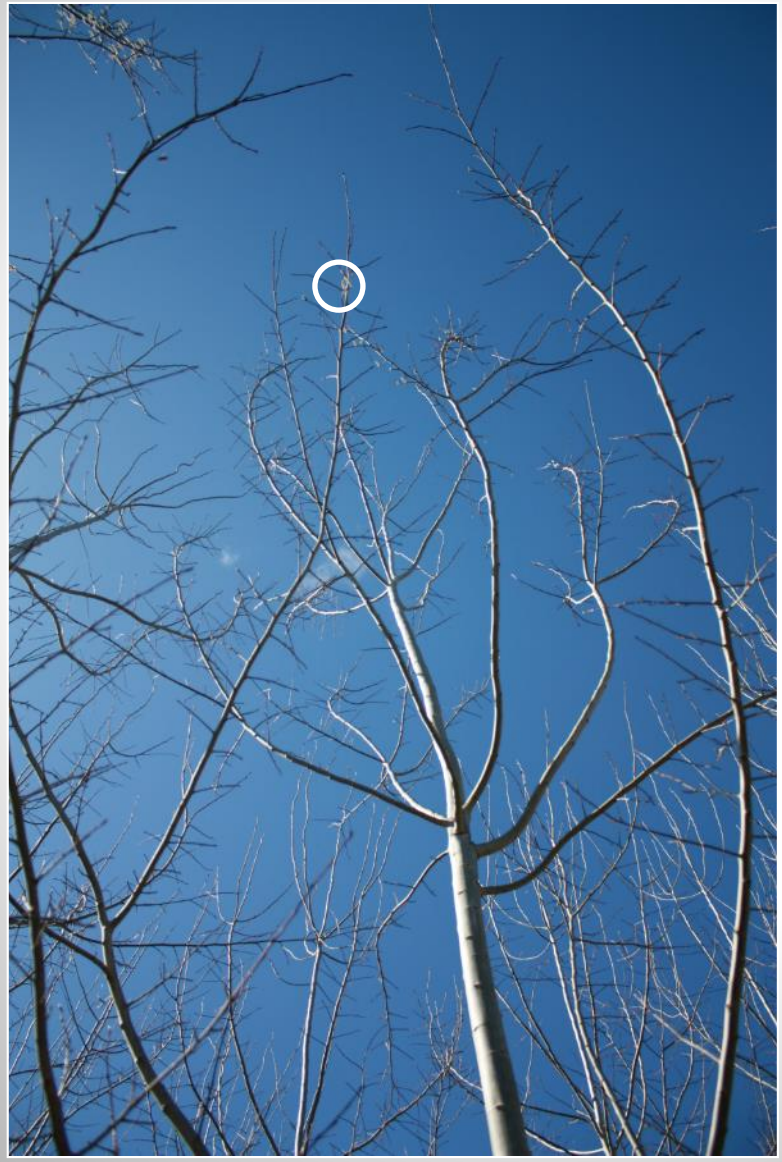
SVP background

- *SHORT VEGETATIVE PHASE* (*SVP*) is a MADS-domain transcription factor
- *SVP* suppresses flowering under non-inductive conditions (short days)
  - Suppresses gibberellin signaling at the shoot apex (in *Arabidopsis*)
- Interacts with *FLC (FLOWERING LOCUS C)* and suppresses *Flowering Locus T (FT)* in the leaves, and *SOC1* in the shoot apical meristem
- **Transformed three poplar clones with *35S:PtSVP***

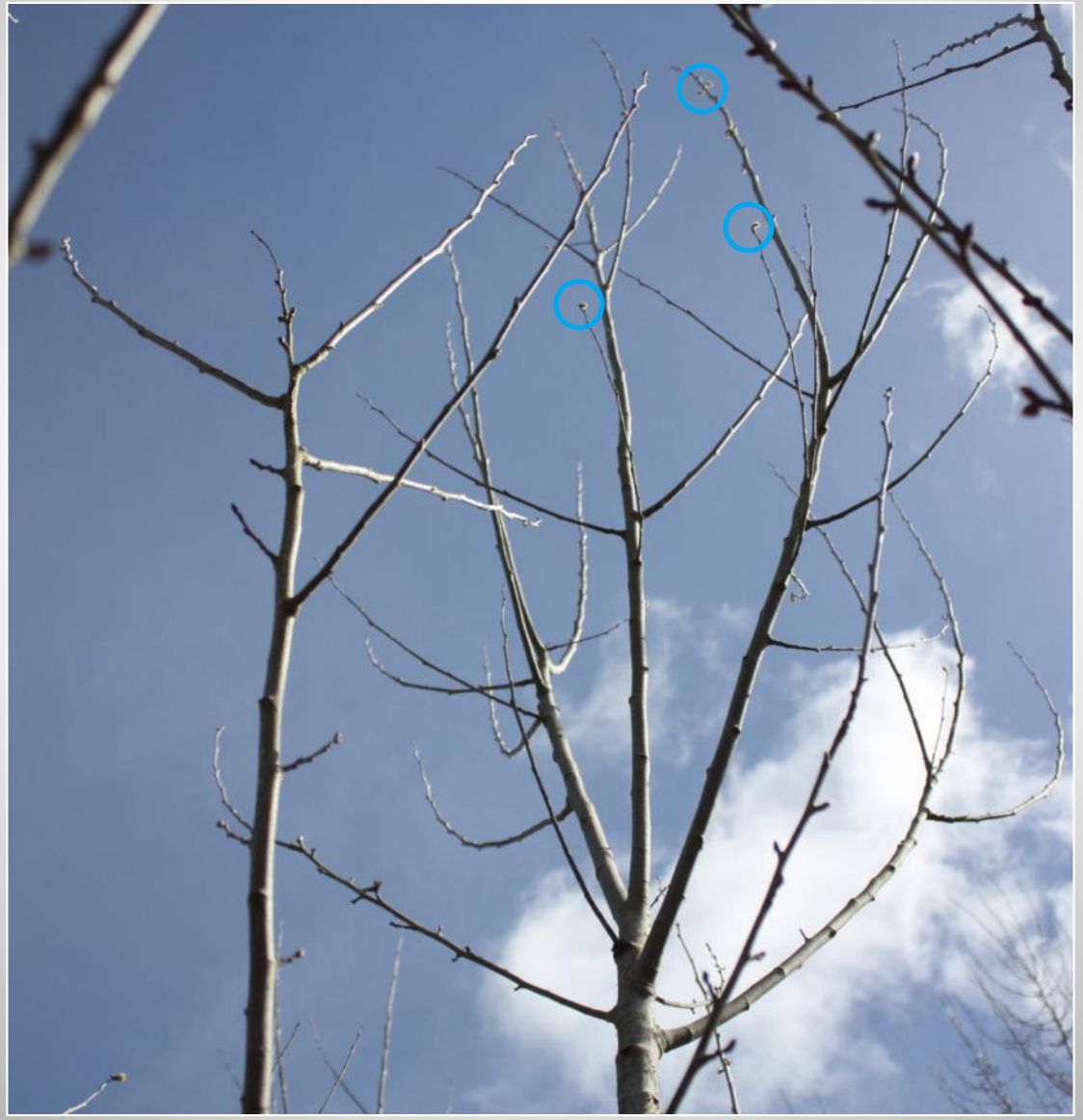
Scored flowering in all trees in ~4 ha trial

Score of 0

Score of 1

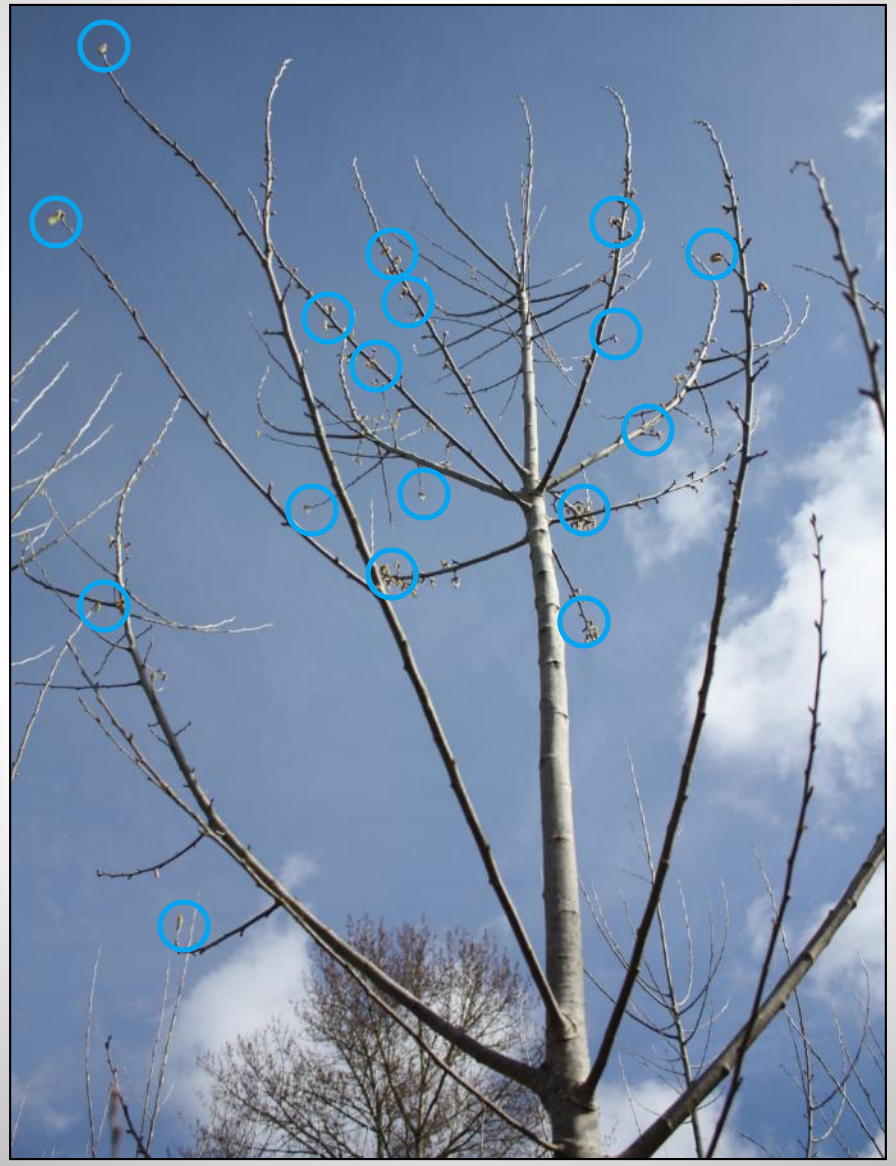


Score of 2



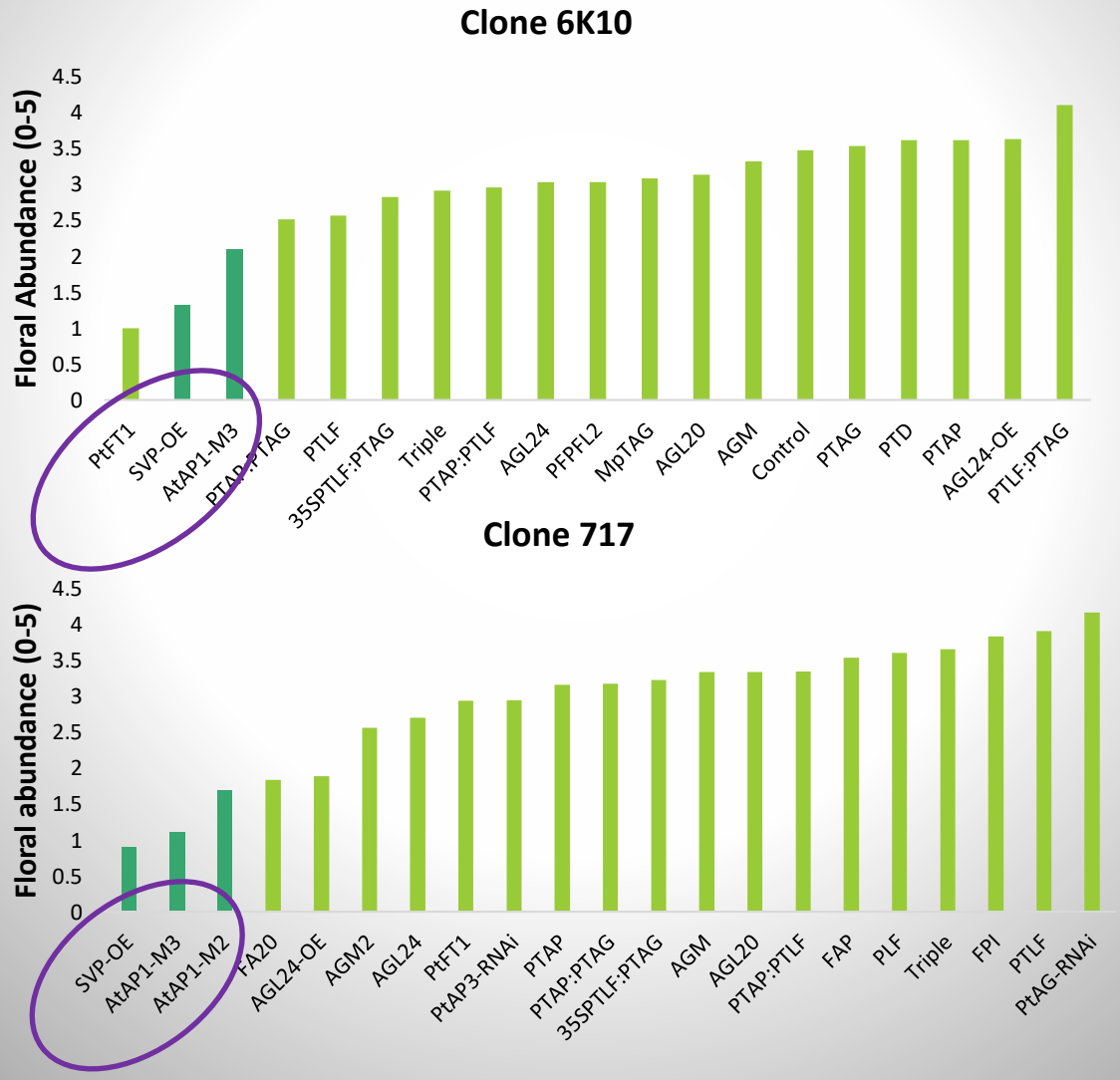
Score of 3

Score of 4



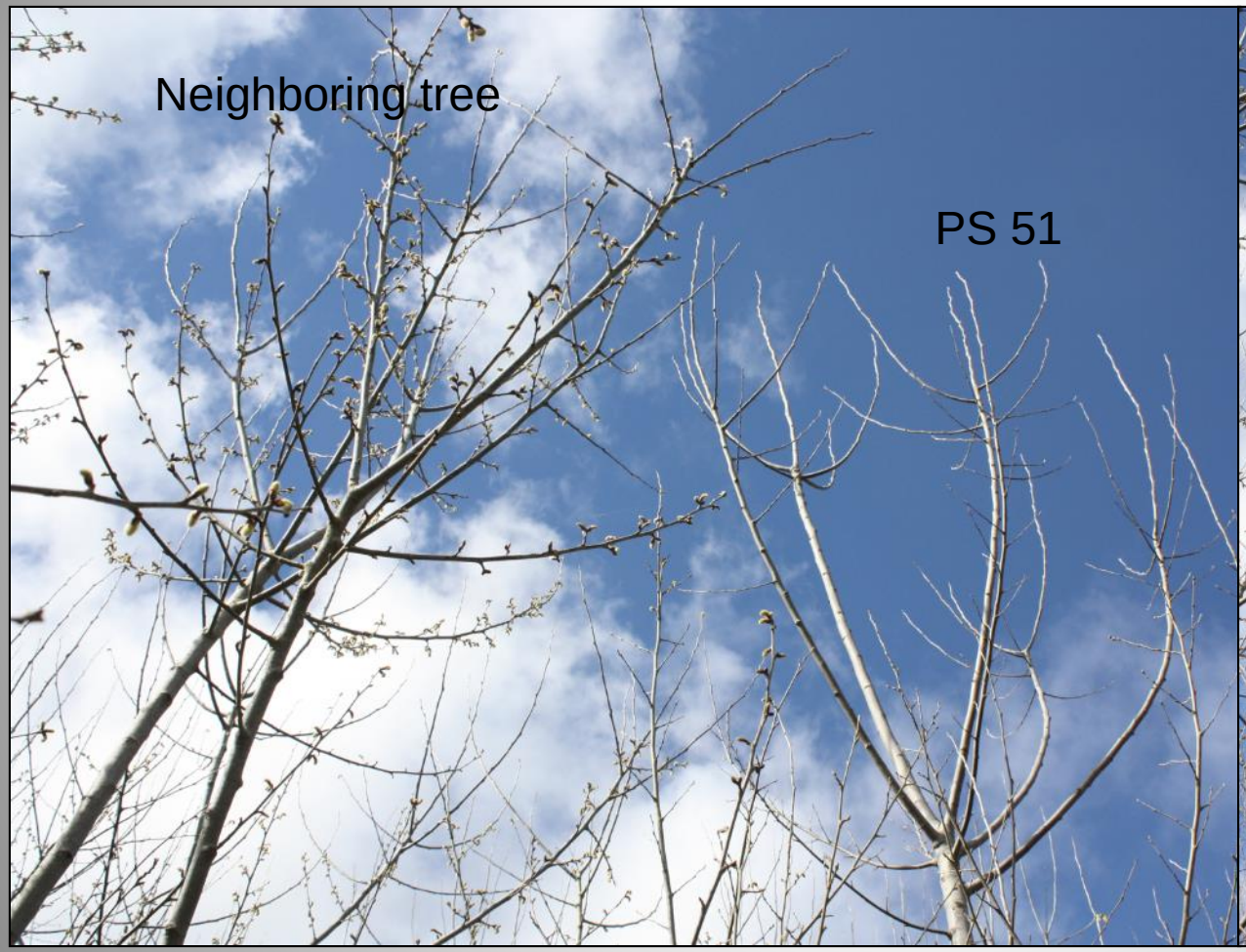
Score of 5

Three constructs resulted in very low floral abundance scores in all three clones

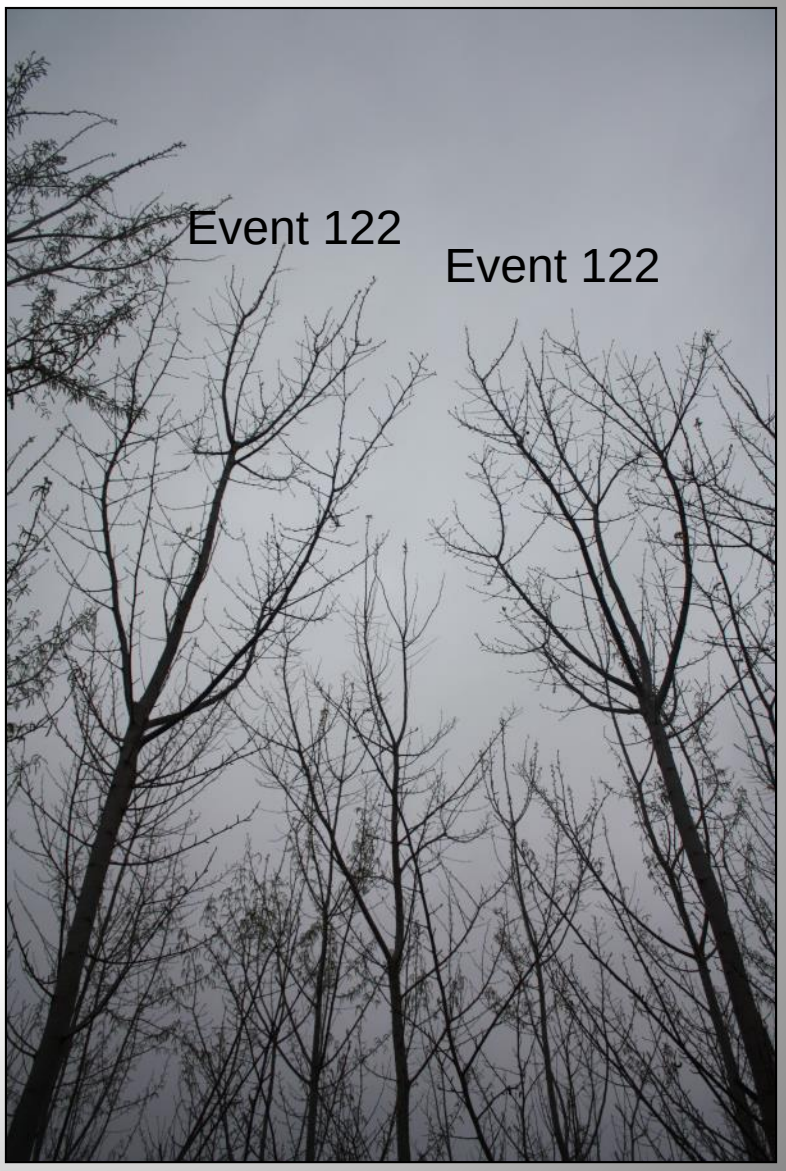


Data from 2016

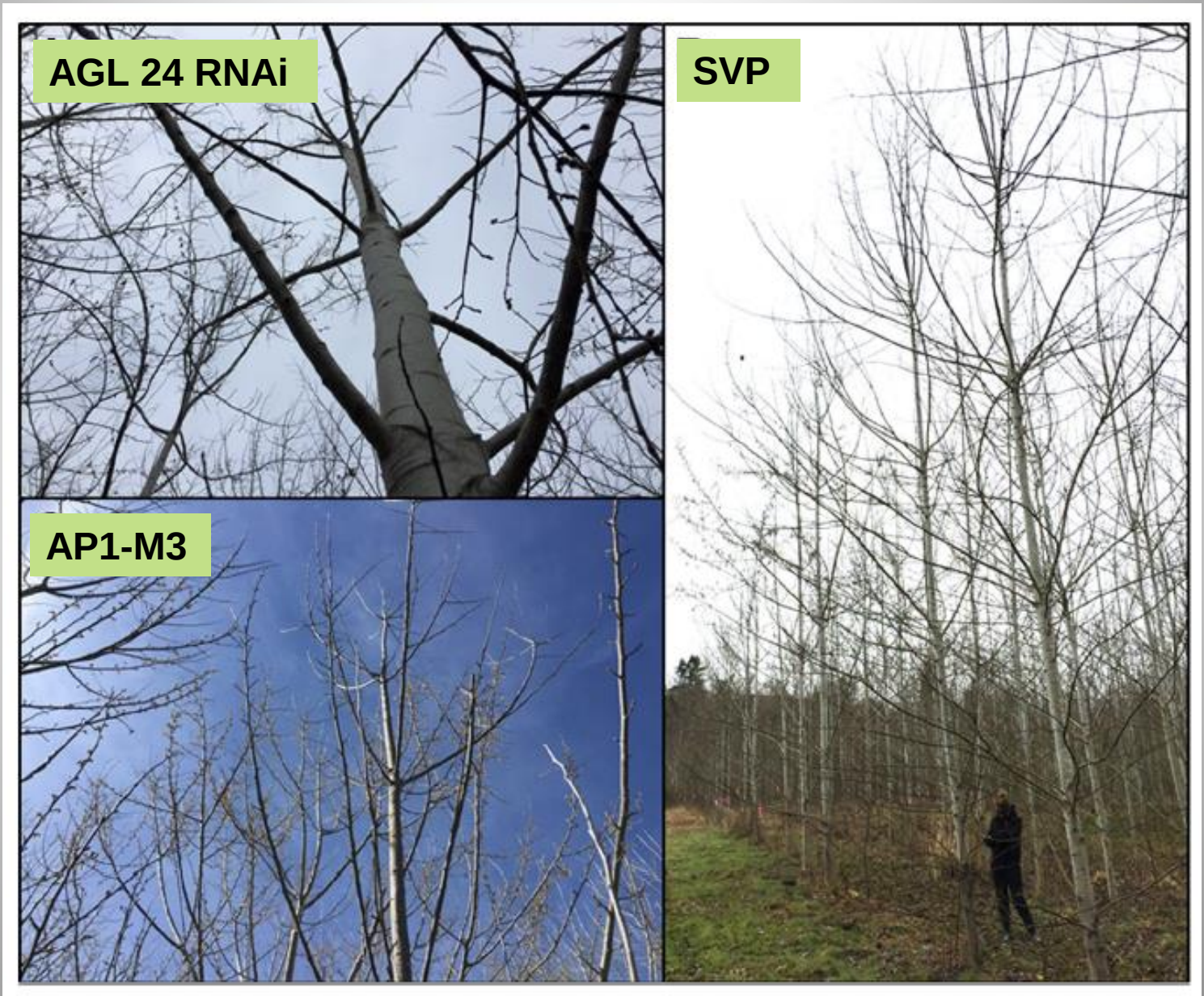
Striking differences among flowering vs. non-flowering adjacent events



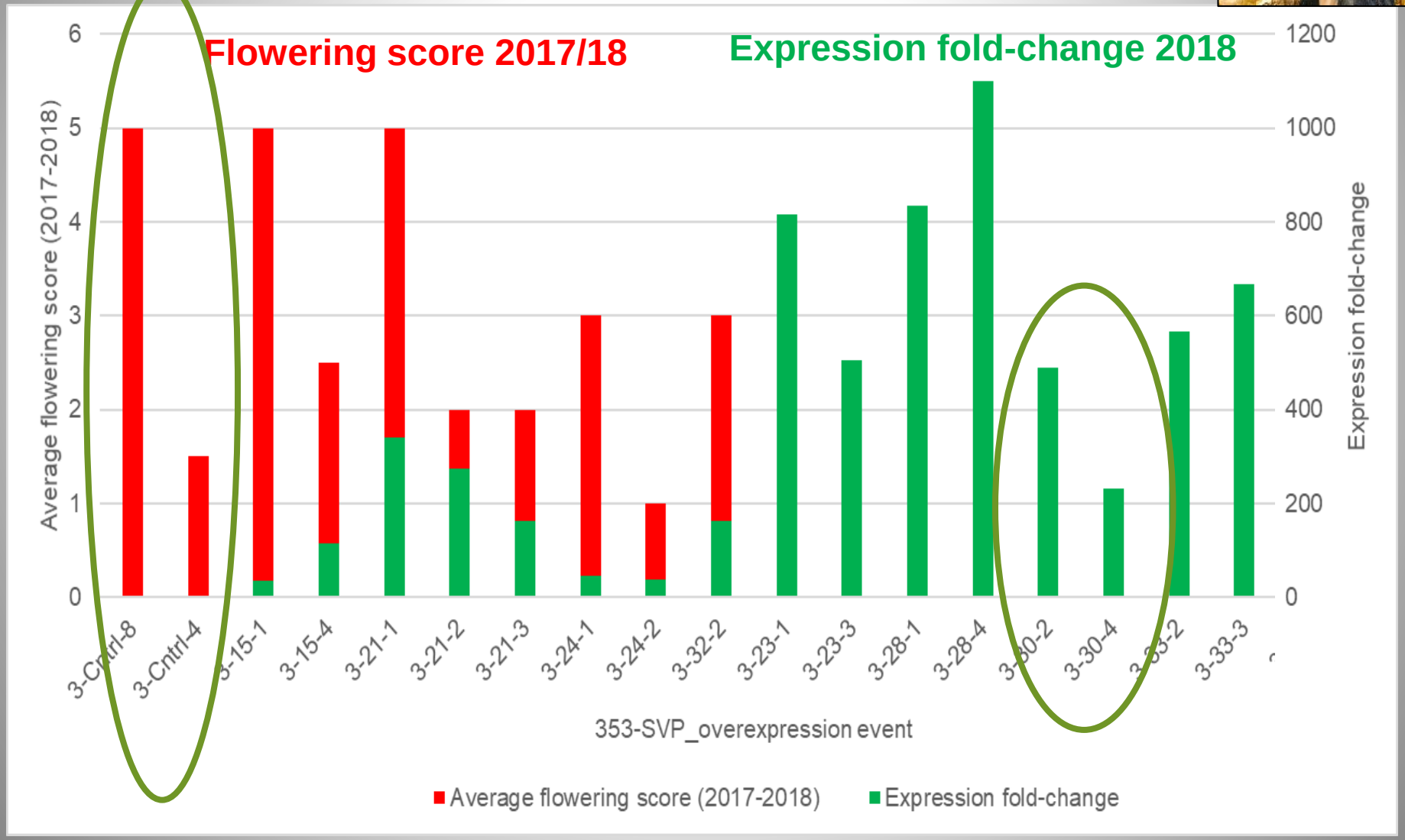
717 SVP event 122 no flowers



Other floral repressed trees



SVP overexpression in leaves and flowering are anti-correlated



Key results – *SVP*

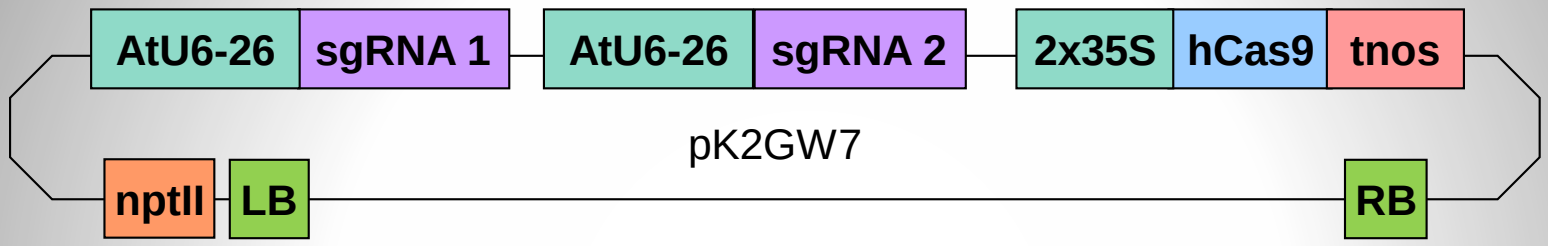
- Strong overexpression gave non-flowering or much reduced flowering
- Trees generally normal, but vegetative effects visible, awaits quantitative analysis
- More specific promoter desired – promoter editing/directed modification desirable ?
- Prevention of floral onset best for vegetative enhancement?

Eucalypt *LFY* CRISPR knock-outs

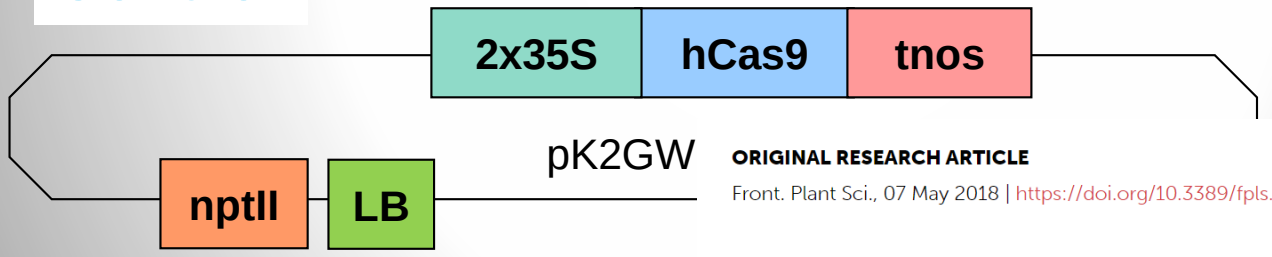
- Gene mutation/deletion the strongest and most stable form of genetic containment
- Created single and two-sgRNA constructs
- (Re)transformed into wild type and FT-early flowering *E. urophylla x grandis* hybrid (Futuragene/Suzano)
- Conducted allele-specific target PCR followed by gel isolation and sequencing
  - High knock out and deletion rate: **97% of transgenic events**
- Examined in greenhouse for growth rate and flowering/sterility

Constructs employed

CRISPR



Control



ORIGINAL RESEARCH ARTICLE
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Variation in Mutation Spectra Among CRISPR/Cas9 Mutagenized Poplars

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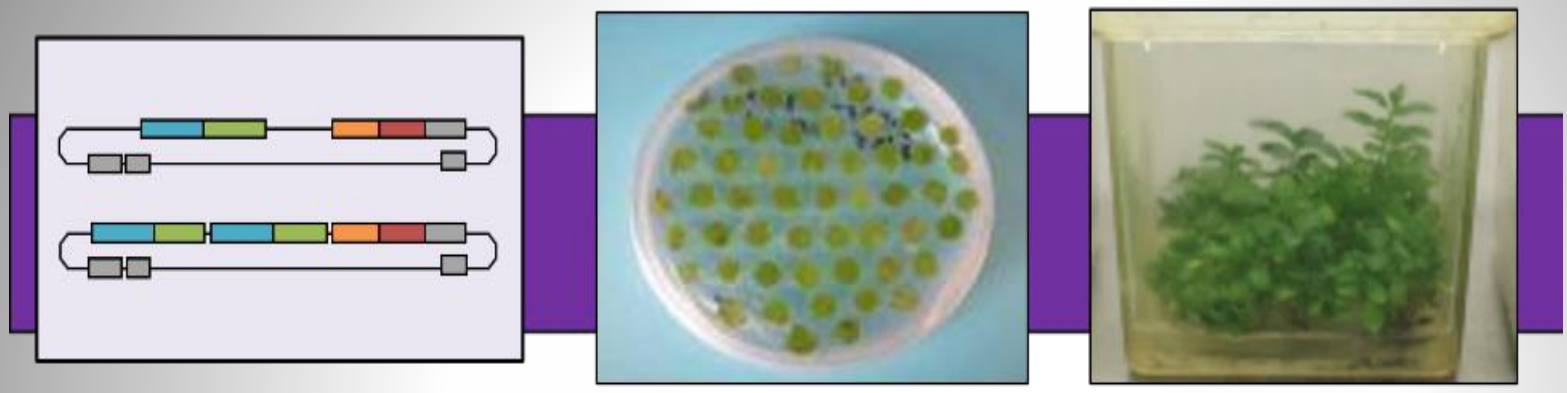
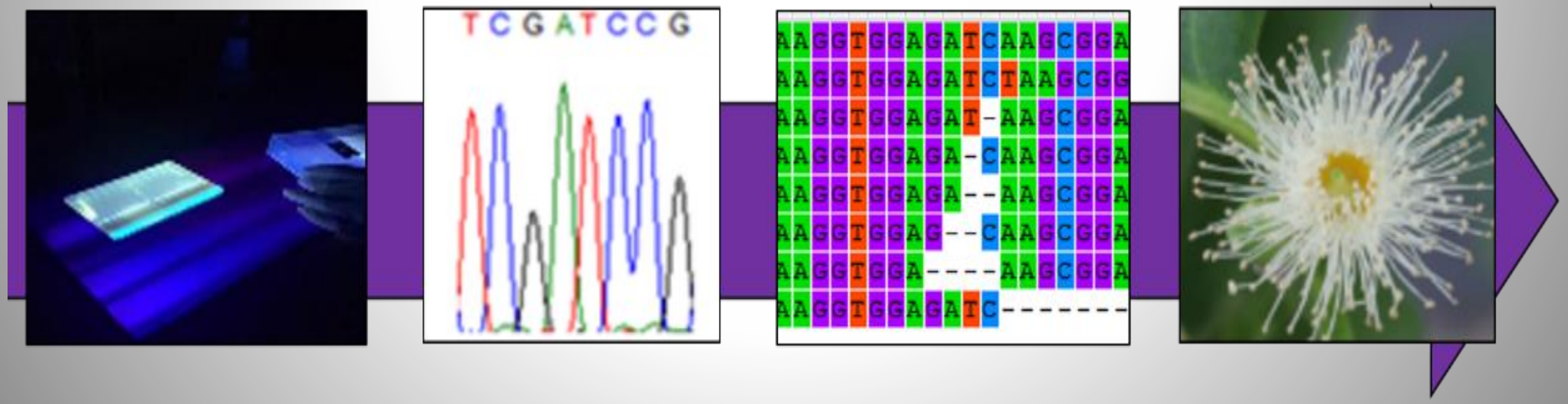
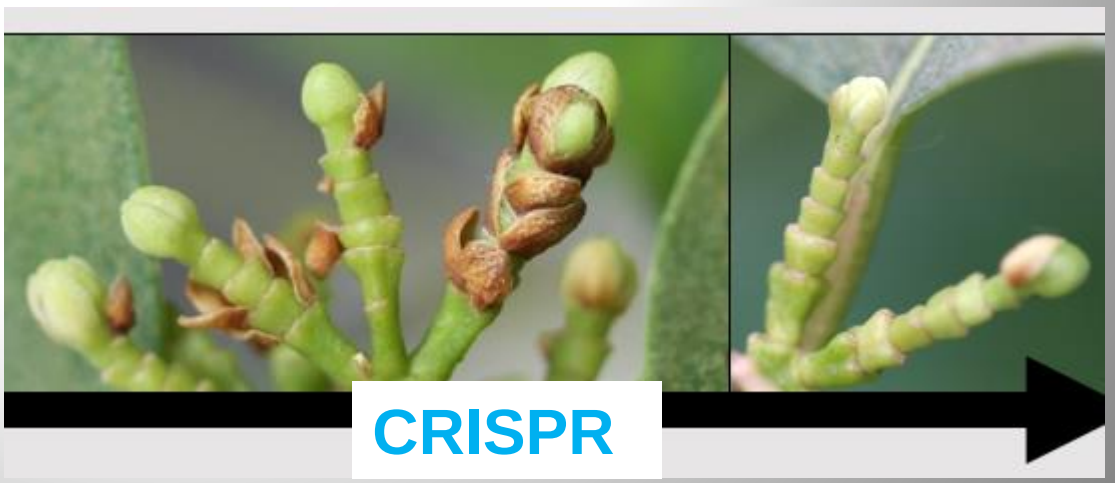
CRISPR pipeline

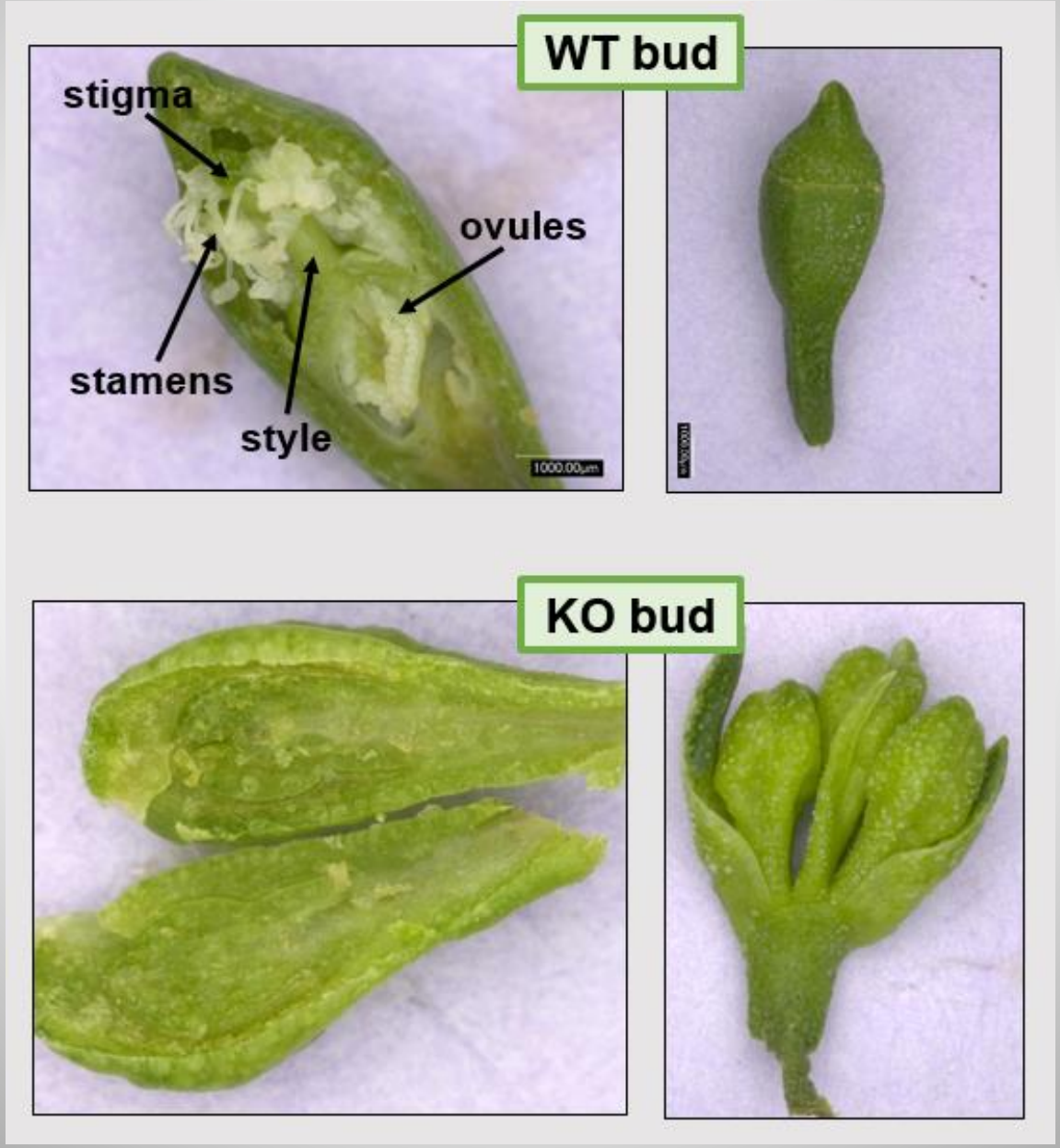




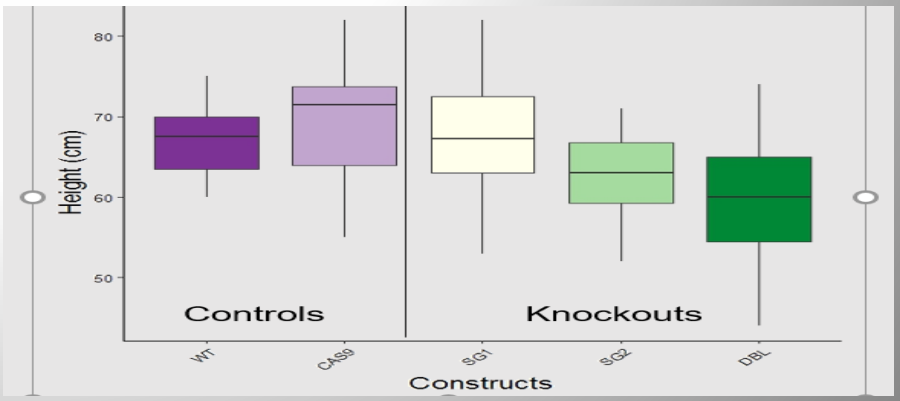
Knockouts had no stamens or carpels, shoots partially indeterminate



Knockout buds devoid of floral organs



No detectable effects of *LFY* knockout on vegetative growth in greenhouse



Key results – *LFY* CRISPR in Euc.

- Nearly 100% knockout rate
- Flower buds devoid of reproductive structures
- Partially indeterminate inflorescences
- No detectable vegetative effects
- Field trial planned – seeking location
- (Poplar *LFY* and *AG* CRISPR field trial underway in Oregon)

Summary

- Amazing breakthroughs & breakdowns ~ 30 years
- Gene flow control an important tool, feasible through several approaches, and stable in field
- RNAi of *LFY* and *AG* in poplar – highly effective
- *SVP* repressor overexpression effective, but with vegetative penalty, ripe for promoter editing?
- CRISPR of *LFY* in *Eucalyptus* – highly effective
- Containment genes valuable for field research? Wood properties and growth rate ?

Broader lessons

- **Social barriers = science barriers**: Need to field test thousands of "ideas" (= genes x traits x combinations x events x host genotypes x environments) and integrate with breeding to utilize. *Essentially impossible with regulatory and market obstacles*
- **It matters**: Ecological and environmental "opportunity costs" of lost productivity and biodiversity are likely to be large and become much larger as climate/pests change markedly
- **We must all work to resolve**: Within our own cultures and social systems, for rational and workable solutions; *it will be long and contentious*

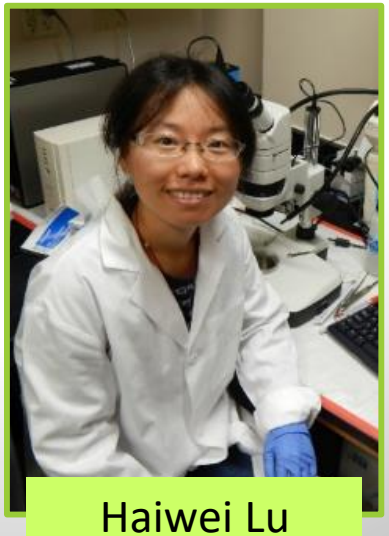
Thanks to these key people and many more over the years

Amy Klocko

Amy Brunner

Cathleen Ma

Estefania Elorriaga



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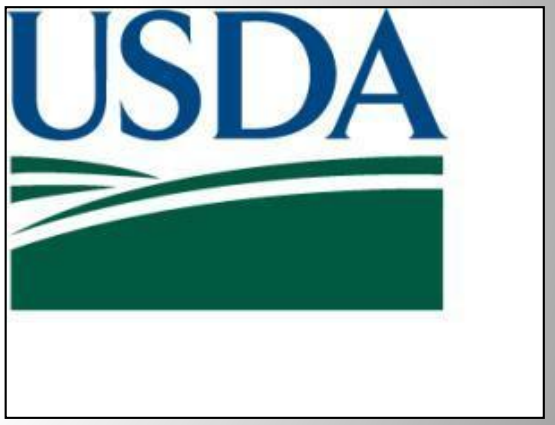

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