RNAi and gene editing as tools for containment of genetically engineered and exotic forest trees

SIVB Raleigh, NC / 2017

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Agenda

- Rationale and approaches
- RNAi
- Repressor overexpression
- CRISPR-Cas in brief
- Regulatory implications

Why genetic containment

- **Goal:** To develop robust male and female containment technologies for vegetatively propagated forest trees
- **Why:** Regulatory, market, and public acceptance with exotic and native trees can be costly or impossible – even for field research
  - Long distance gene flow, incomplete domestication, wild or feral relatives, perception of forests as wild
- **Advantage of RNAi:** No toxic genes like barnase used (which can be unstable and harm vegetative development), degree of suppression can be varied, and may be highly stable
- **Advantage of repressor overexpression**: No flowering at all, trees remain juvenile, most rapid vegetative growth?
- **Advantage of gene editing:** Expected to give strongest loss of function, and be most efficient, predictable, and stable

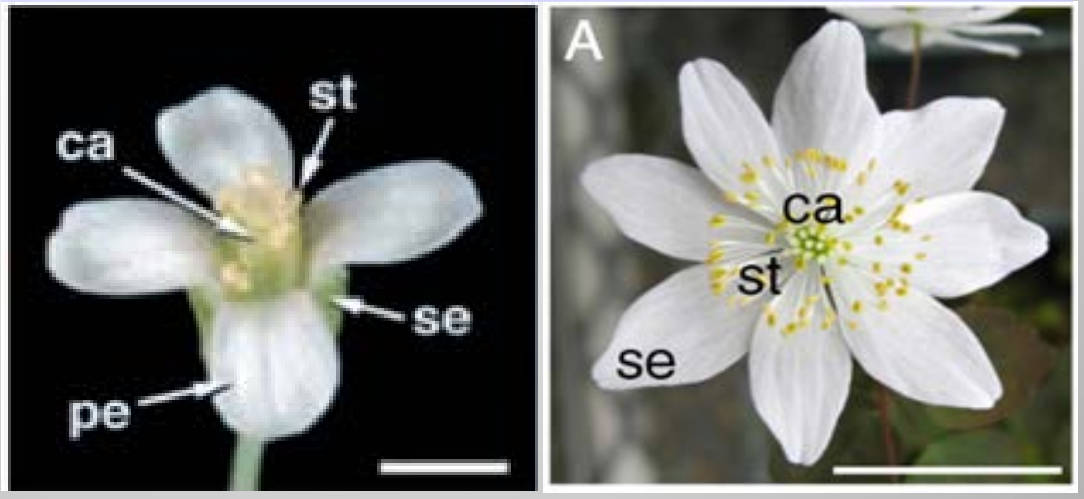
Approaches

- Bisexual sterility: Target is intensely managed, vegetatively propagated elite forest tree varieties (clones), thus targeting master regulators of sexual development
  - No further breeding, or create asexual restorer systems
- Suppress or mutate: Floral organ identity gene *AGAMOUS* and floral meristem identity gene *LEAFY*
- Repressor overexpression: Use of natural floral suppressor or dominant negative form of natural activator

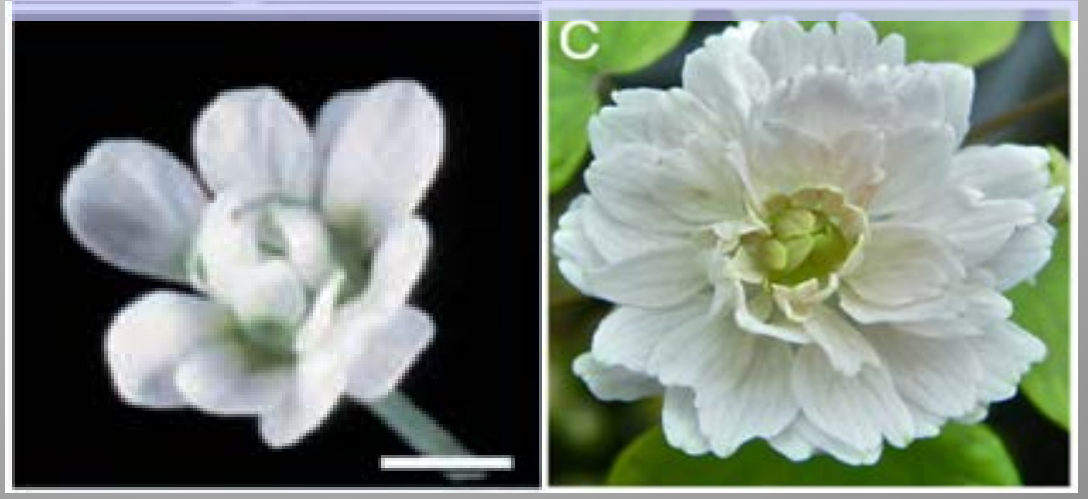
Flowers in strong *ag* mutants lack both stamens and carpels, and are indeter-minate

Arabidopsis Ranunculid

Wild type



ag mutants



Parcy et al. 2002; Galimba et al. 2012

Strong *lfy* mutants appear to have no flowers

Parcy et al. 2002; Moyroud et al. 2010

Experimental field trial (summer 2016)

Amy Brunner,
Virginia Tech

Experimental overview

1

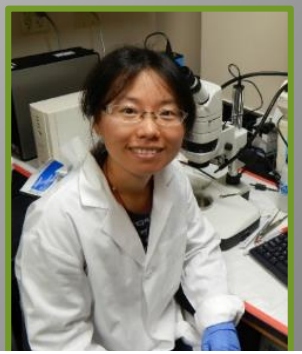
Create RNAi constructs based on the reference sequence from *Populus trichocarpa*

2

Produce transgenic poplars (*P. alba* genotype 6K10, Marizio Sabbati, Univ. Viterbo, Italy)

3

Evaluate phenotypic changes in field (*FT* accelerated flowering impeded RNAi effects)



Haiwei Lu, PhD student, OSU

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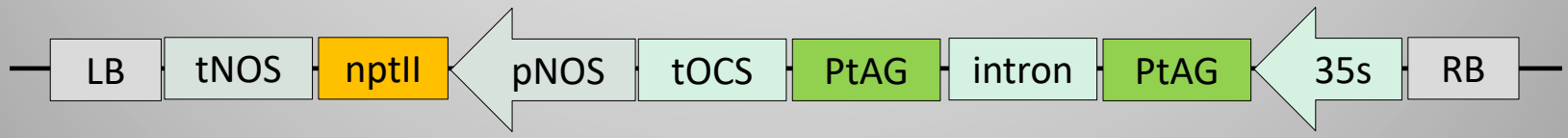
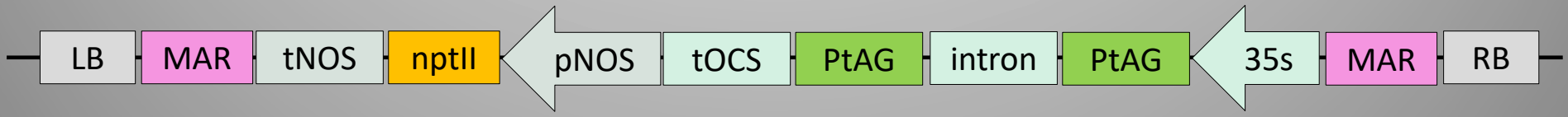
Evaluate gene expression

Two *PtAG*-RNAi constructs, with and without matrix attachment regions (MARs)

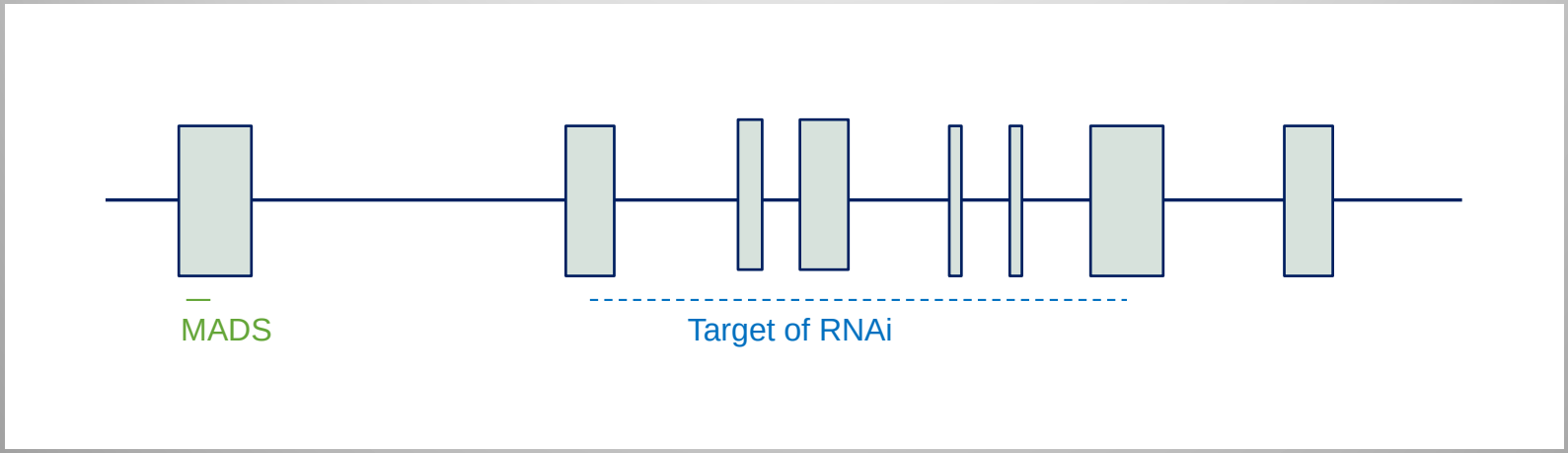
- PTG



- MPG



RNAi constructs contained an inverted repeat that targeted 393 bp of the non-MADS region



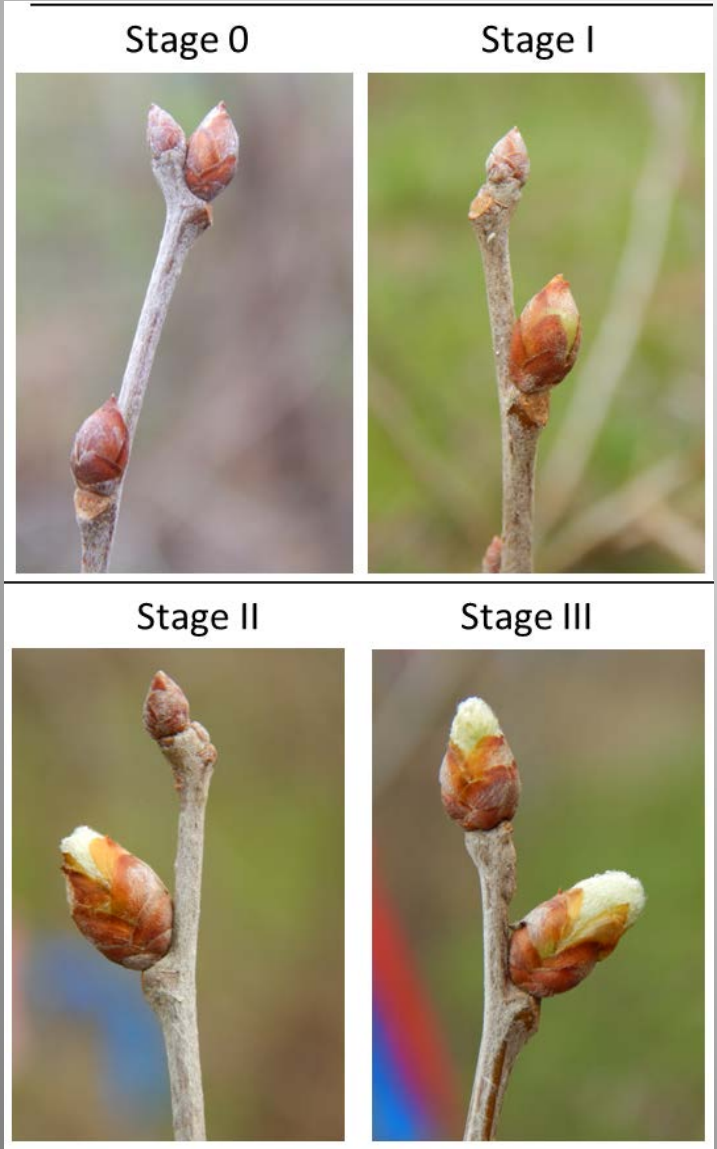
Targeting two paralogous (duplicated) highly similar *PtAG* genes in poplar

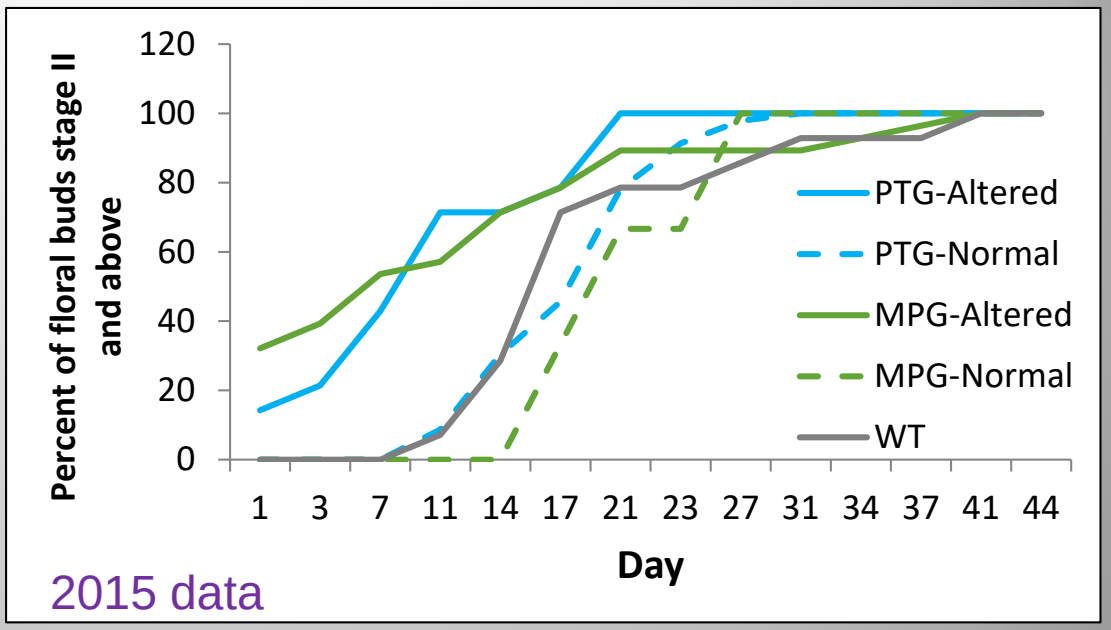
Summary of floral modifications

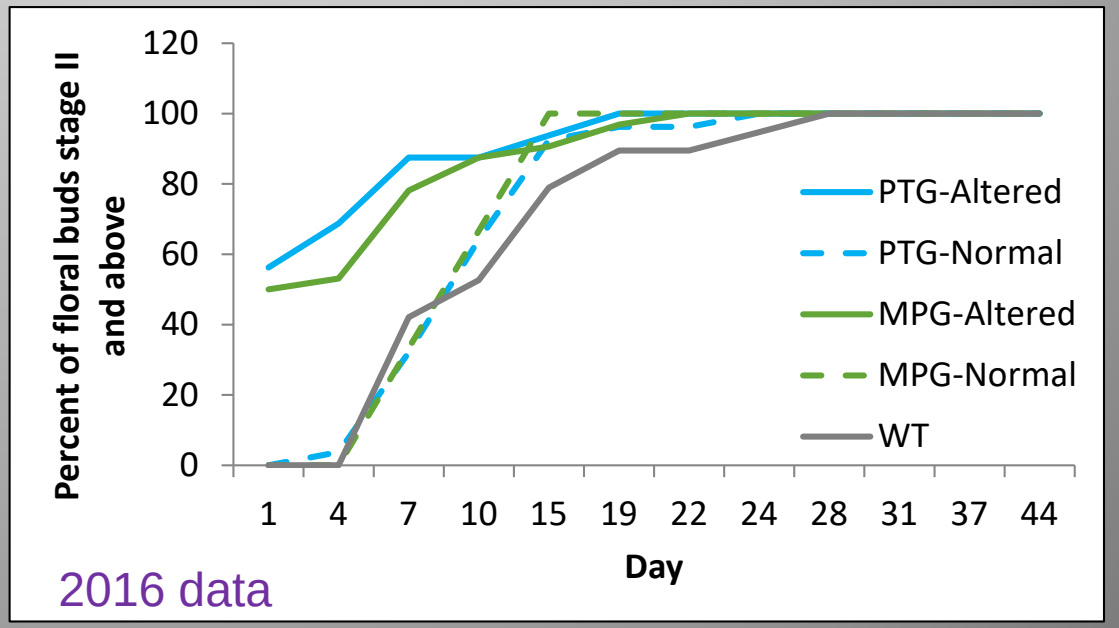
Construct ID	No. of insertion events	No. of events that flowered by 2017	No. of events with altered floral morphology
PTG	22	22	6 (27%)
MPG	13	12	11 (92%)
WT-CTR	24	19	0 (0%)

The MAR elements more than tripled RNAi suppression frequency

Floral buds on altered events flushed early - unexpected





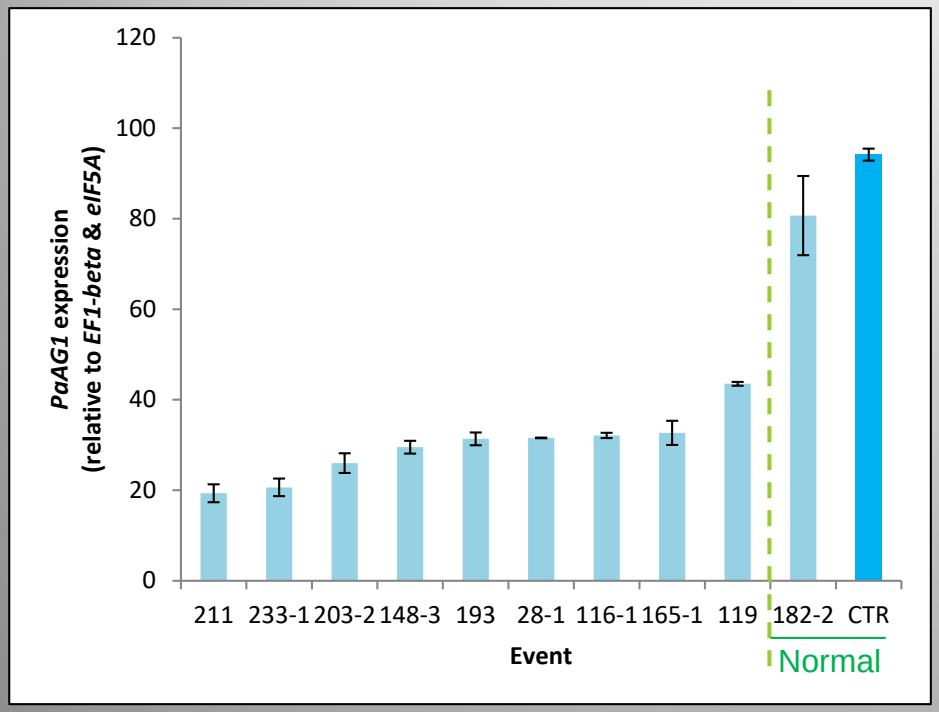


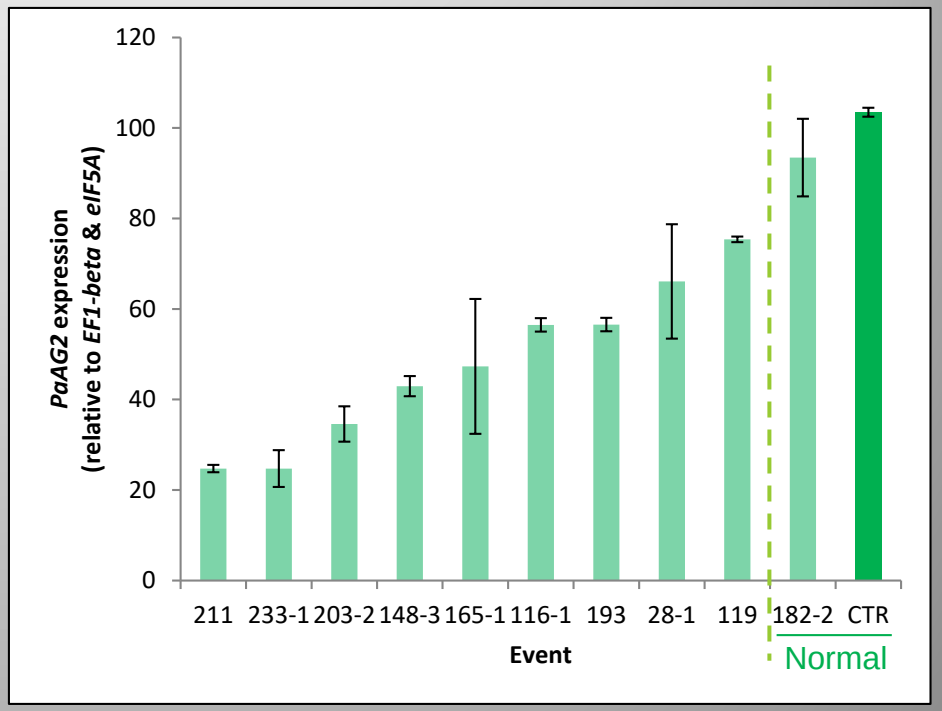
Altered events had highly modified, sterile flowers

Strongly altered events were stable within and among trees over 3 years

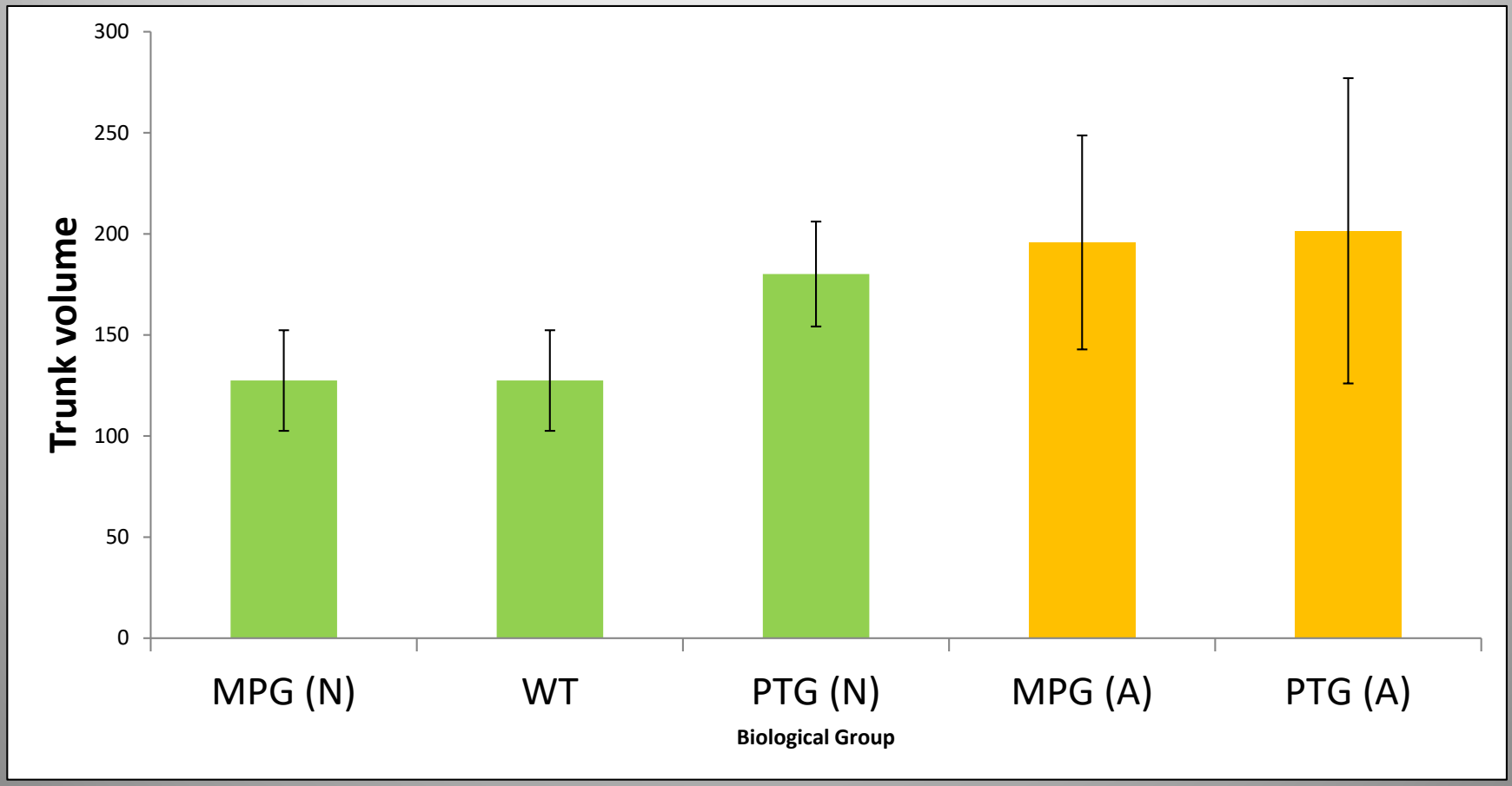
12 fully sterile events (2/3), 50 trees examined

Mild, correlated suppression of the two *PaAG* paralogs were associated with floral modification





Trees with altered flowers had normal vegetative growth and leaf morphology

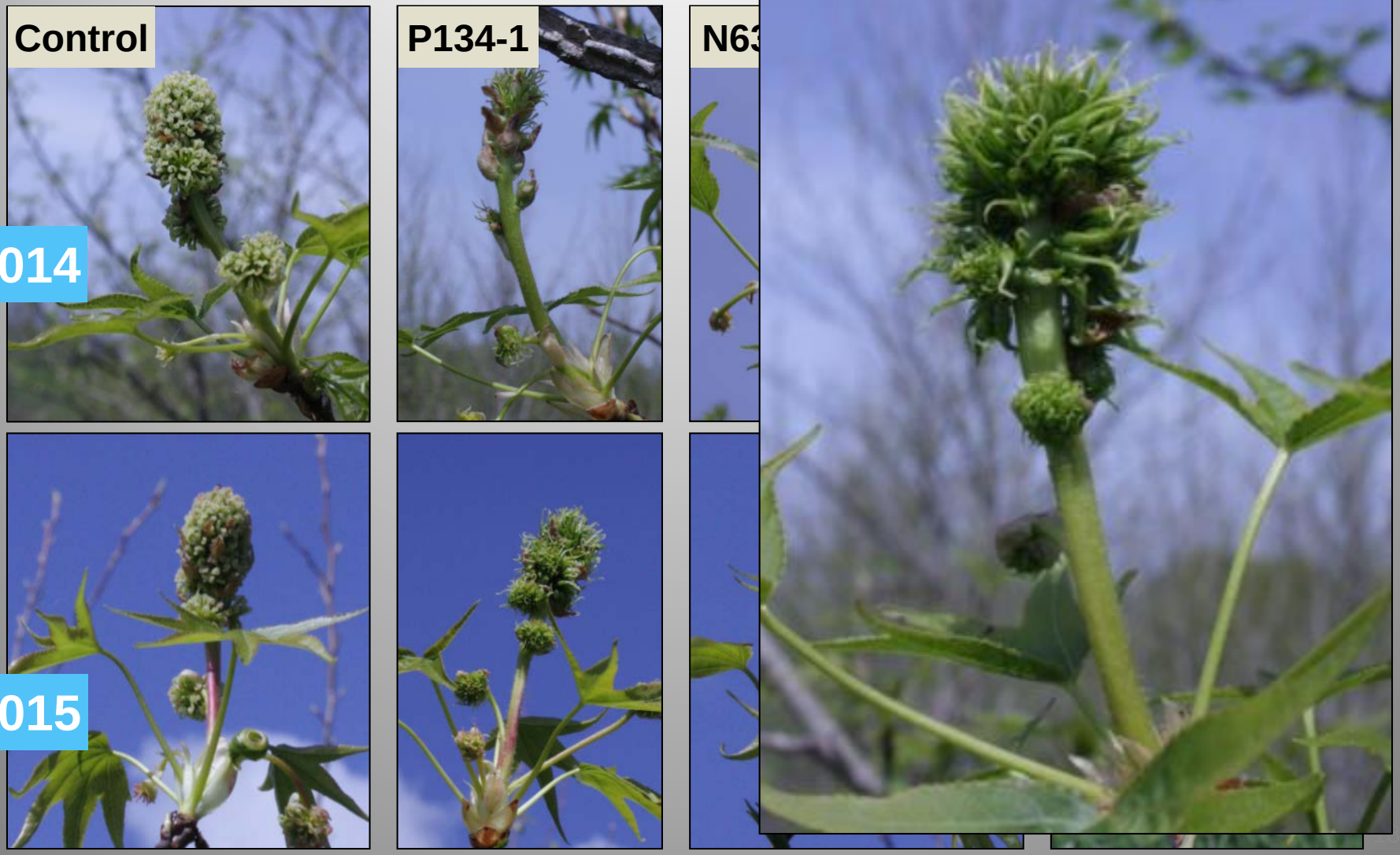


- A= Altered, N=Normal, Bars = SE of the mean

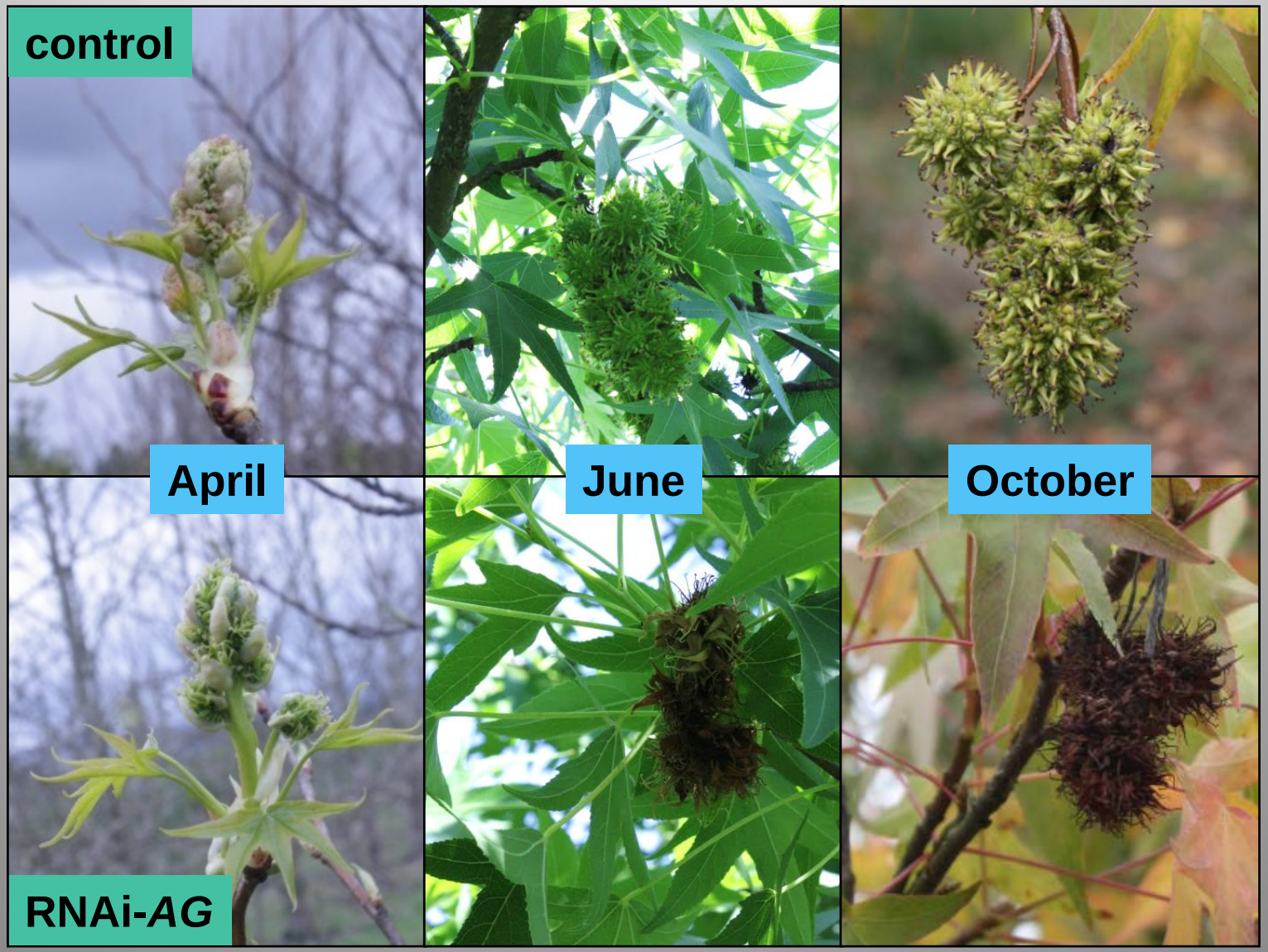
Other studies with similar effects

Sweetgum *LaAG*-RNAi – targeted two distinct *AG* genes

Altered phenotypes of RNAi-AG events were stable over 3 years



RNAi-*AG* flowers matured into sterile, brown papery fruits



Sterility, normal growth of *LEAFY*-RNAi poplars over four growing seasons

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 anther tapetal cells[6,7], barnase can reduce rates of genetic transformation and vegetative growth[6]. Furthermore, barnase expression may not be fully stable[8]. Bisexual sterility would allay concerns over seed dispersal, could be used to control invasive exotic trees, and might increase wood production[9]. We

poplar.

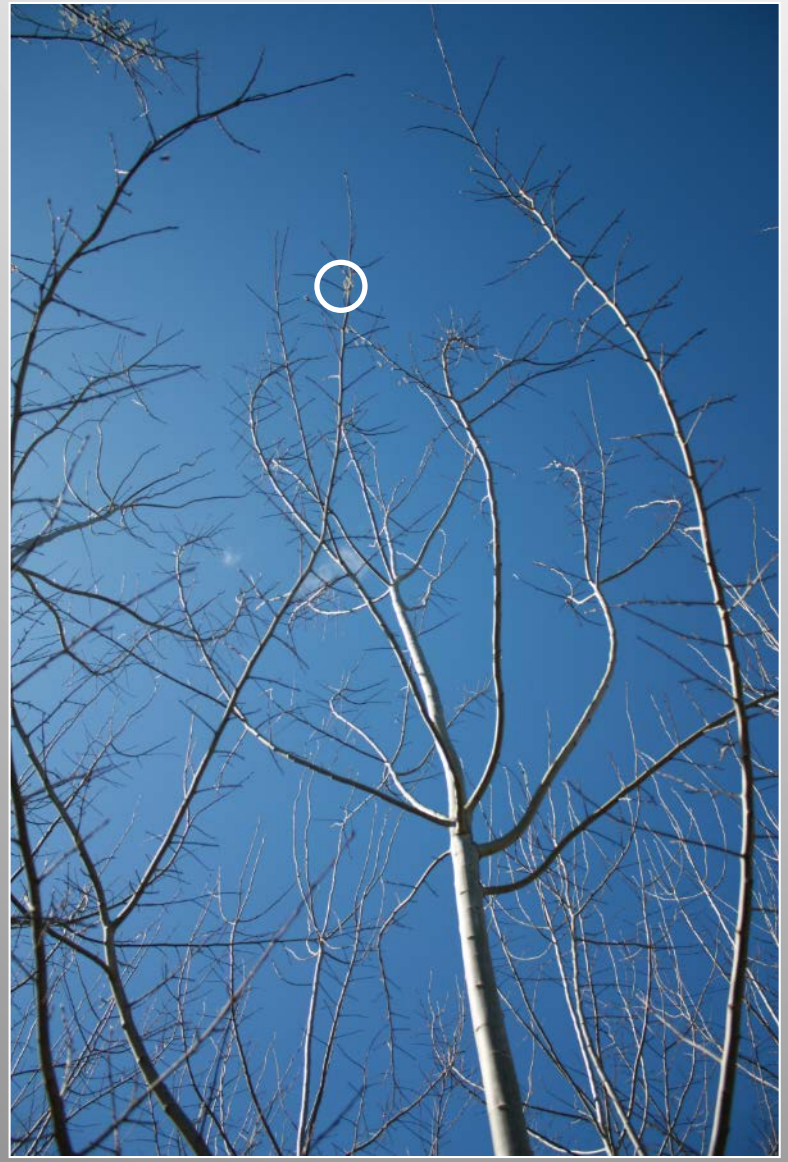
RNAi has been used to reduce gene expression in many plant species[10,11], and the reduction in gene expression that RNAi confers is highly stable in trees under field conditions[12]. *LFY* is required for the early stages of male and female floral organ formation in plants, and encodes a transcription factor that promotes floral meristem identity[13,14]. In *Arabidopsis thaliana*, loss of *LFY* function results in the formation of vegetative structures instead of floral meristems, whereas reduction of *LFY* expression decreases floral abundance and results in partial conversion of floral organs to leaf-like structures[13,14]. We selected *LFY*

Klocko et al. 2016, *Nature Biotechnology*

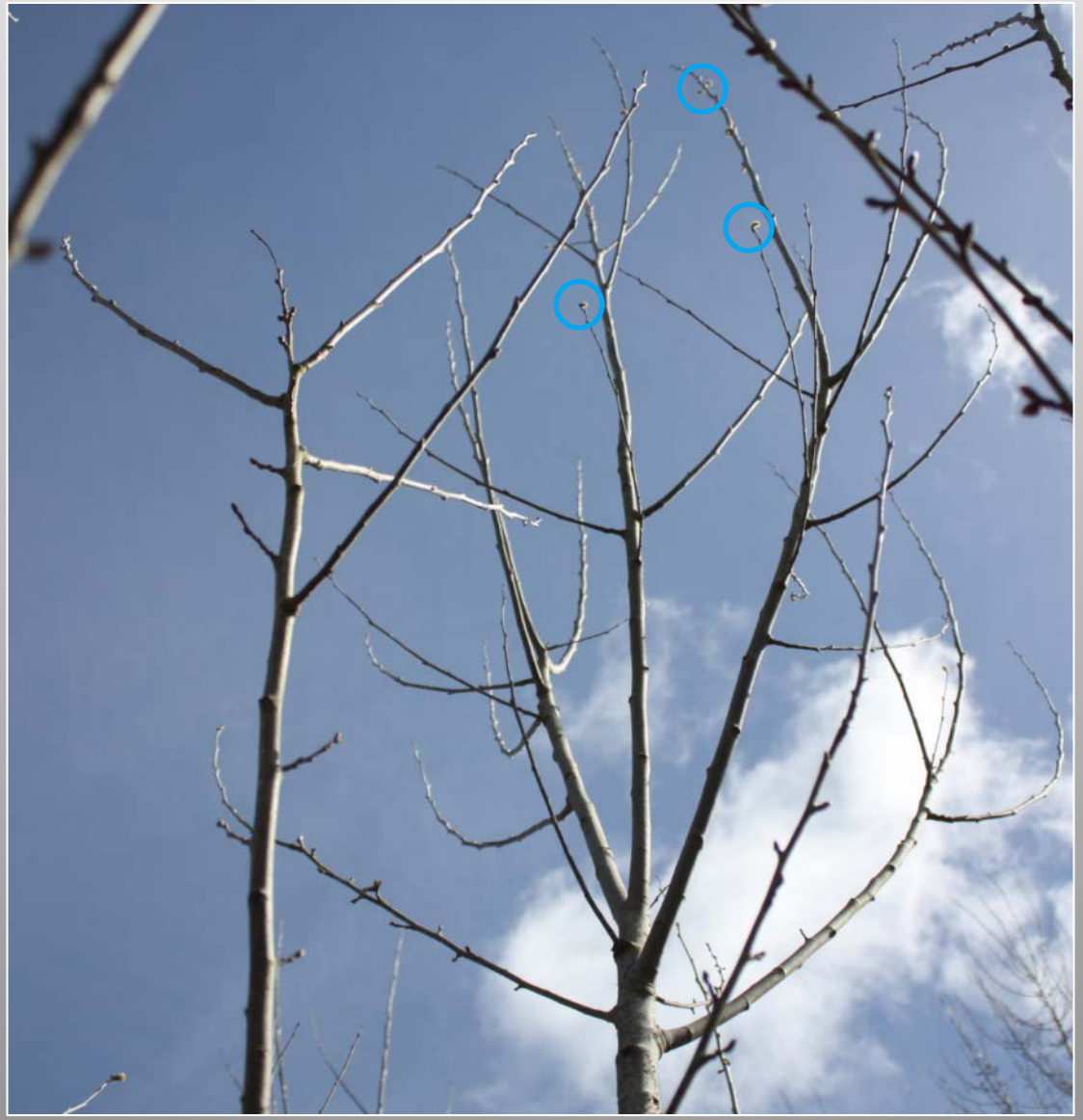
Floral suppressors: Scored extent of flowering in all trees

Score of 0

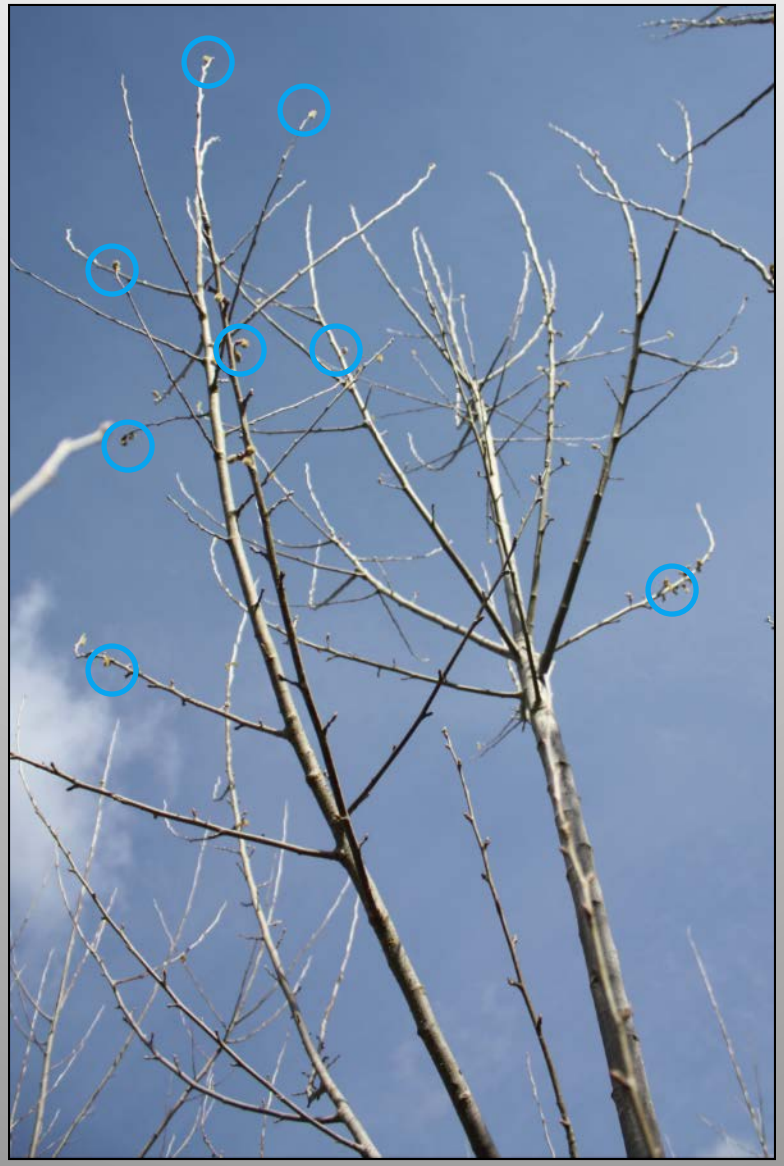
Score of 1



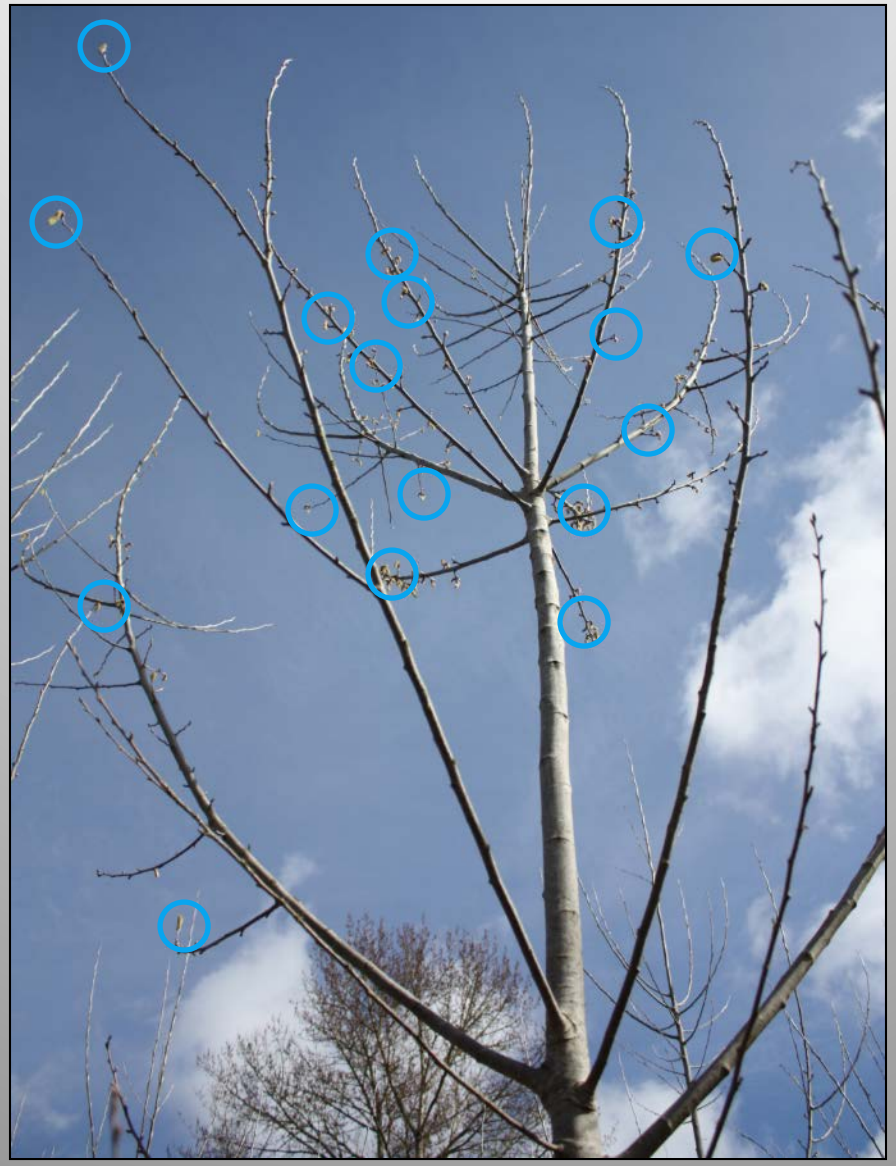
Score of 2



Score of 3

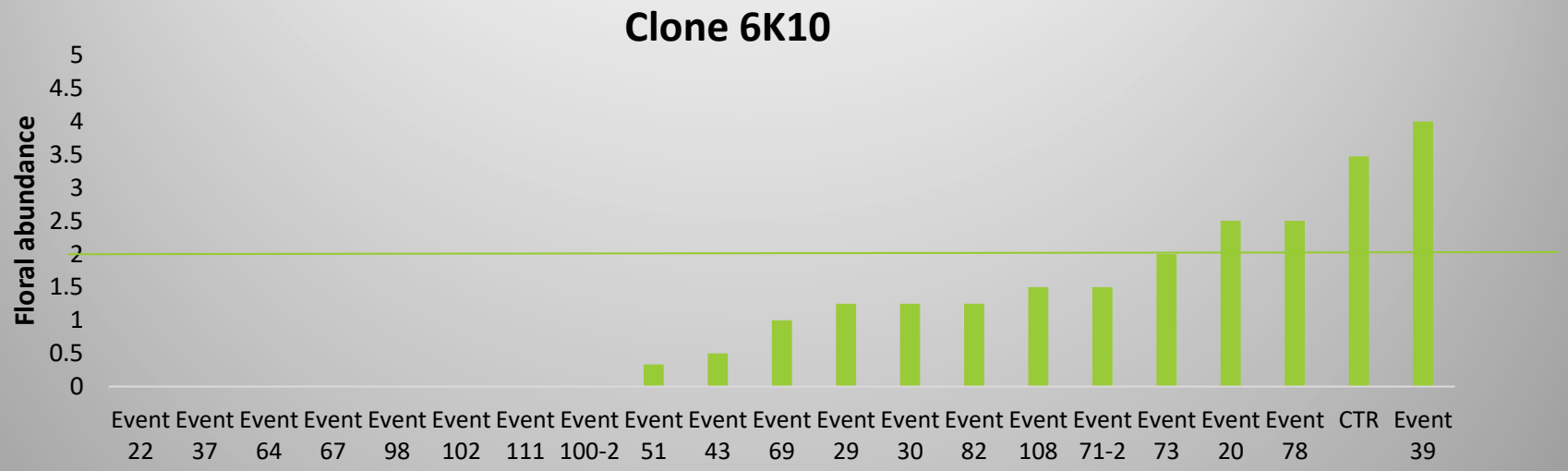


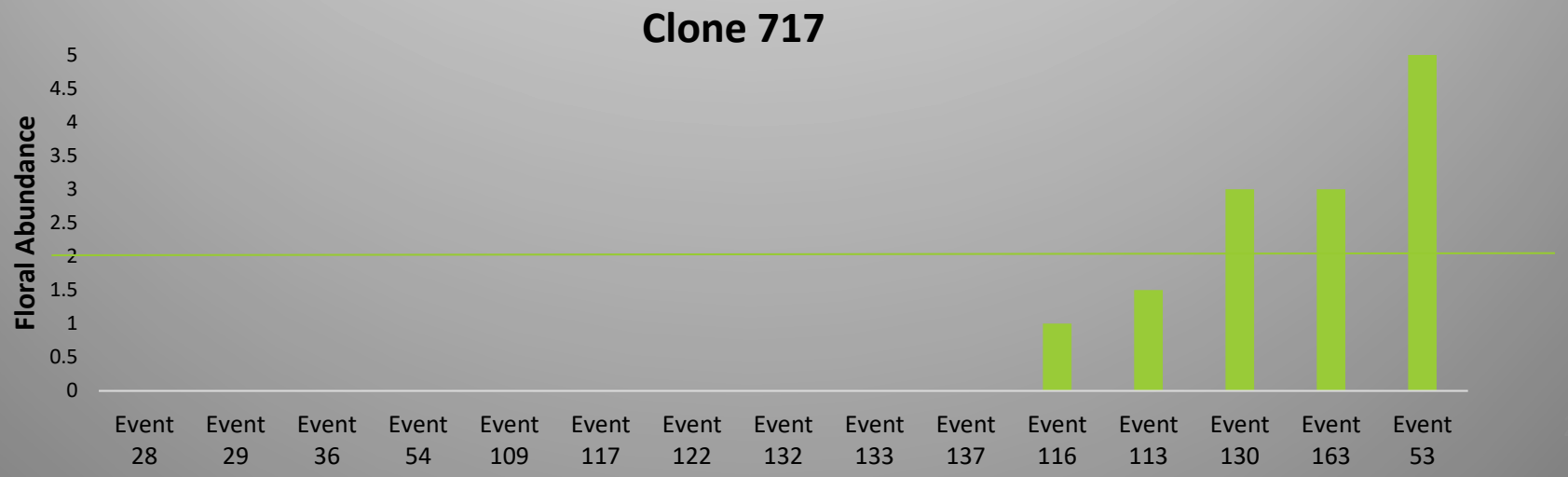
Score of 4



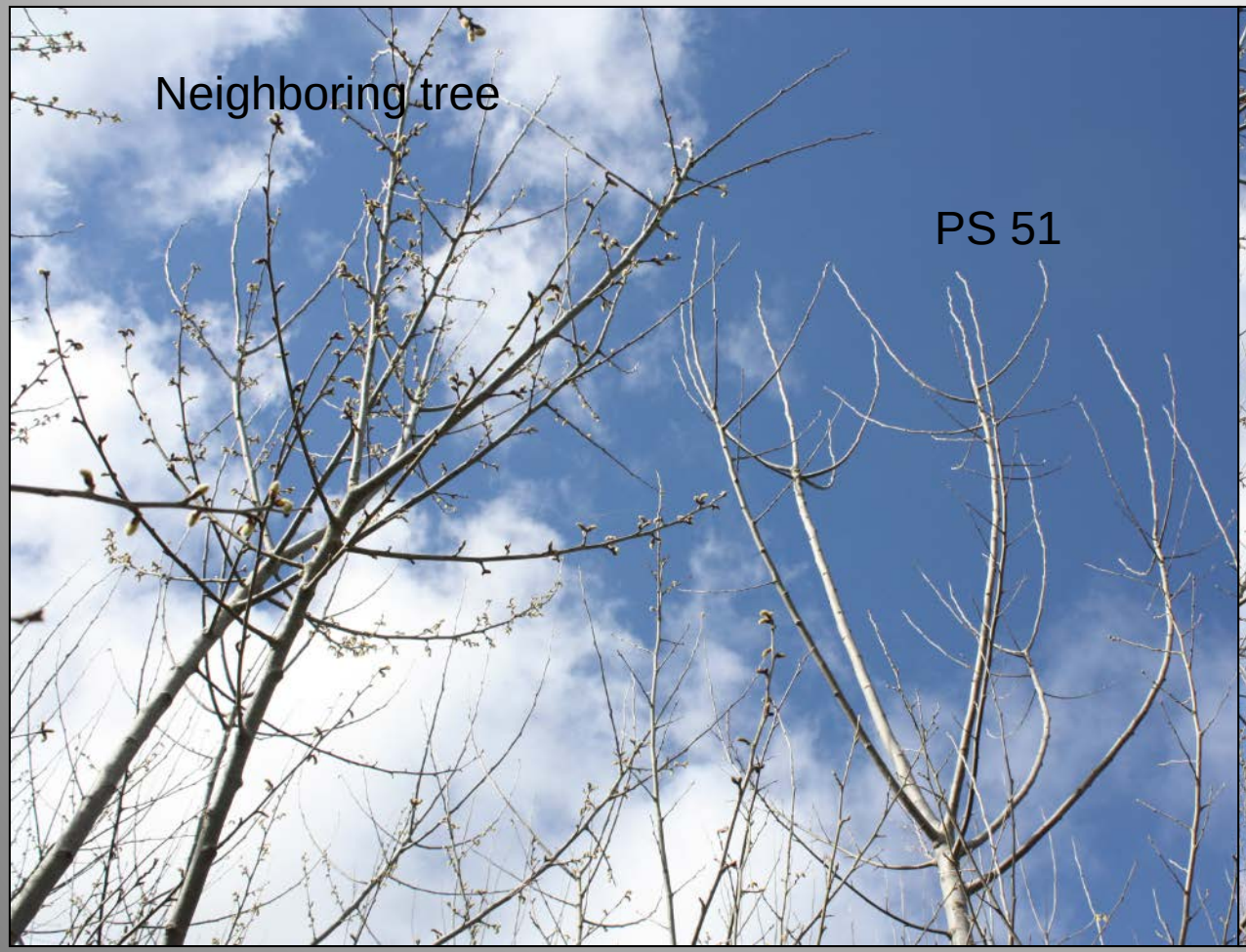
Score of 5

80% of all SVP-OE events showed floral abundance scores of less than 2

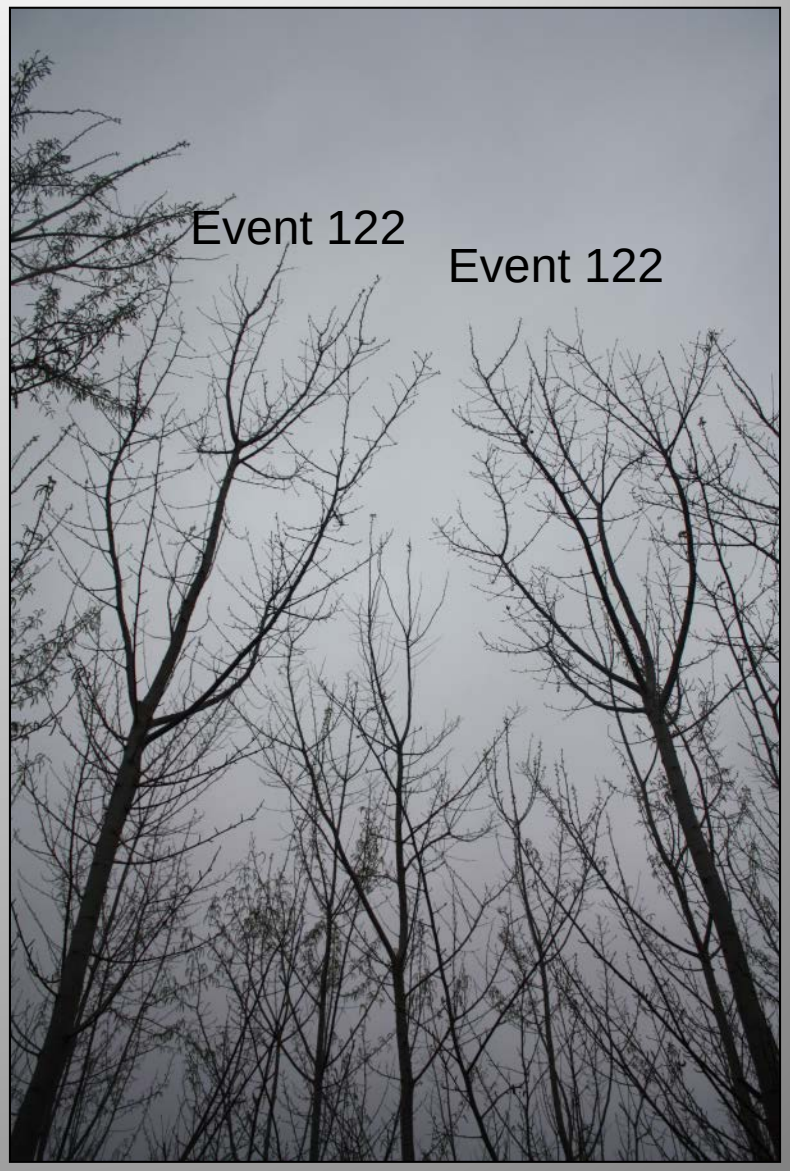




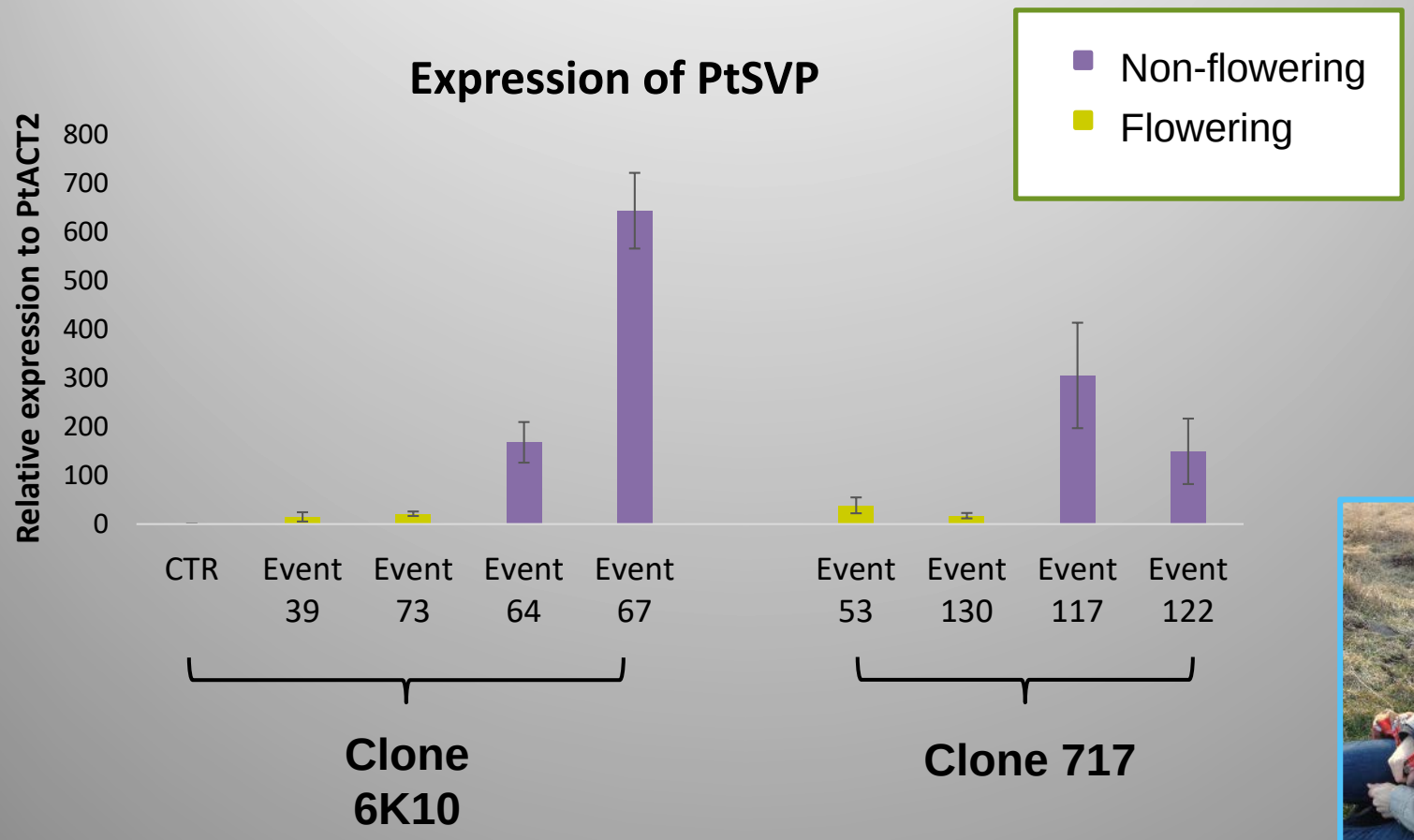
Striking differences among flowering vs. non-flowering adjacent events



717 SVP event 122 no flowers



Non-flowering events had high expression of *PtSVP* in leaves

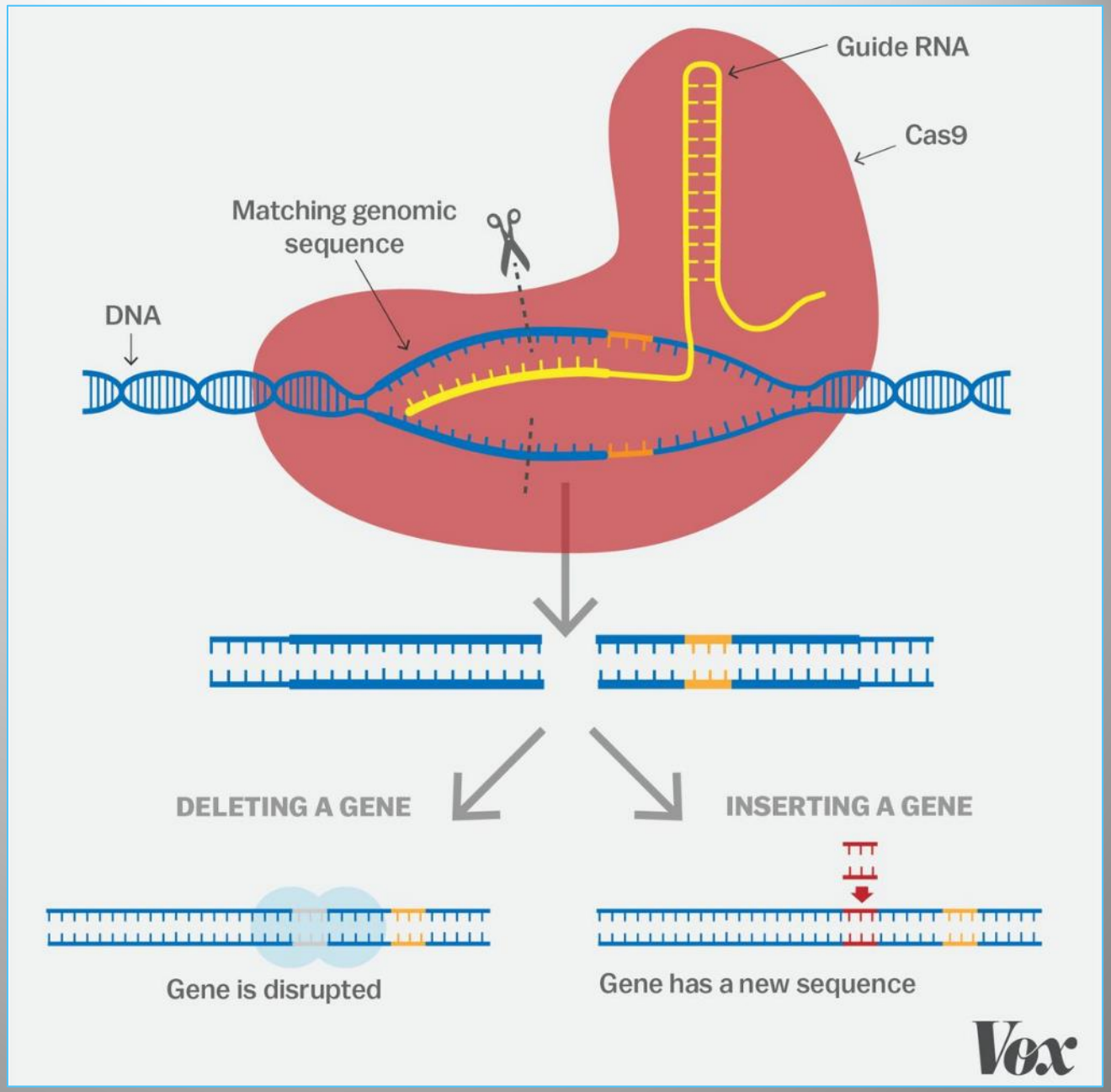


Emily Helliwell, Former postdoc

Future of floral suppressor studies

- Study of two additional successful suppressors based on mutated *AP1* gene
- Studies of growth effects underway – some appear likely
- Superior method likely to be CRISPR promoter engineering vs. simple 35S overexpression

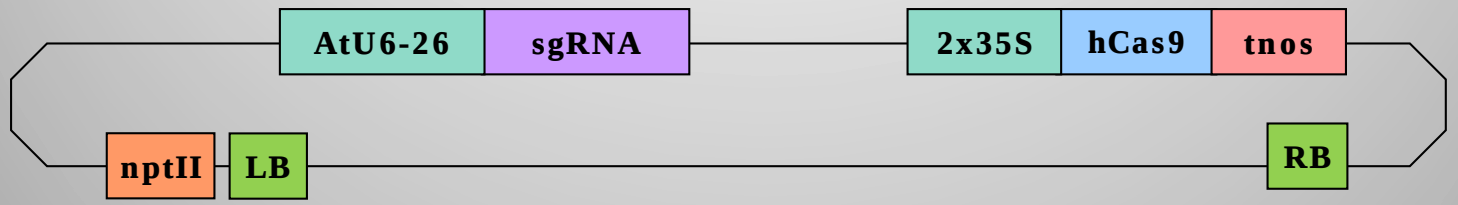
CRISPR Cas-gene editing

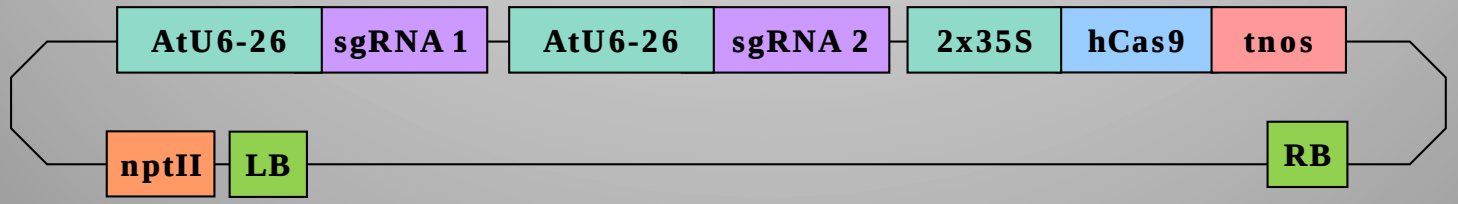


Gene editing knock-ou underway

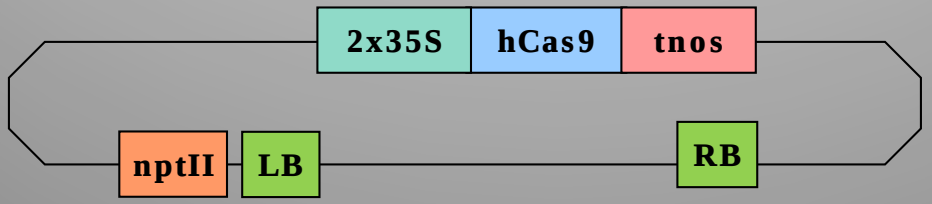
Experimental constructs – single and double targets per gene, no CRISPR removal

Nuclease constructs





Control construct



High CRISPR mutation rates observed

- Cas9-only control events
  - No mutations (62 events, poplar and eucalypts)
- CRISPR-Cas events
  - Poplar: 73% of events were knock-outs (488 events tested, *AG* and *LFY*)
  - Eucalypts: 97% knock-outs (70 events, *LFY*)
- Off-target studies underway

Estefania Elorriaga, PhD student

LFY knock-out in rapid flo background

Who did the work? Flowering research team 2016-17

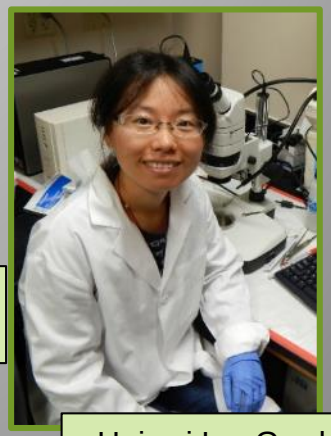
Cathleen Ma, Transformation & Greenhouse Experiments

Anna Magnuson, Program & Field Manager

Emily Helliwell, Post-Doc, Genomics and Bioinformatics

Amy Klocko, Post-doc, Cloning, Gene Expression, Flowering

Michael Nagle, Grad Student, Gene Targets



Haiwei Lu, Grad Student, ZFNs

Sarah Higgins, Technician, Floral Analysis

Estefania Elorriaga, Grad Student, CRISPRs

Jeremy Jacobson, Undergraduate Research

Thanks for support

Futuragene, SAPPI, SweTree, U. Pretoria, Arborgen