

RNAi Suppression of *AGAMOUS*-like Genes Causes Field Sterility in *Populus*

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Forest Tree Workshop
PAG XXVI, San Diego, CA, 2018



Oregon State
University



The containment issue

- Coexistence and adventitious presence key GMO issues in agriculture and forestry – compromising public acceptance and regulatory approvals for field research, commercial use, and in trade
- Issue amplified with forest trees due to wild relatives, long distance pollen and seed movement, and ecological importance
- Invasive exotic trees also problematic for horticulture and forestry in many places
- Sexual sterility – a major approach to mitigate concerns over transgene dispersal from GE and exotic trees

Control

Barnase

Male sterility
highly effective
and stable in the
field



Eucalyptus



Pine



Poplar

Tree Genetics & Genomes (2014) 10:1583–1593
DOI 10.1007/s11295-014-0781-6

ORIGINAL PAPER

**A tapetal ablation transgene induces stable male sterility
and slows field growth in *Populus***

Estefania Elorriaga · Richard Meilan · Cathleen Ma · Jeffrey S. Skinner ·
Elizabeth Etherington · Amy Brunner · Steven H. Strauss

Negative impact on tree health was observed in poplar

Zhang et al. 2012; Elorriaga et al. 2014

Bisexual sterility desirable and should be feasible

- Seed dispersal and adventitious presence can be major problems
- Identification of many key floral genes
 - Bisexually active floral regulatory genes such as *LEAFY*, *APETALA1*, *AGAMOUS*, *SHORT VEGETATIVE PHASE*
- RNAi gene suppression powerful
- Gene knockout using nucleases
 - Research underway; not the focus of this talk

Female sterility previously demonstrated using RNA interference (RNAi) of meristem identity gene *LEAFY*



Control



RNAi-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 populations¹⁻⁴. Unlike other crops, trees are long-lived, weakly domesticated and their propagules can spread over several kilometers⁵. 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 growth⁶. Furthermore, barnase expression may not be fully stable⁸. Bisexual sterility would allay concerns over seed dispersal, could be used to control invasive exotic trees, and might increase wood production⁹. 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 Klocko¹, Amy M Brunner^{1,3}, Jian Huang², Richard Meilan^{1,3}, Haiwei Lu¹, Cathleen Ma¹, Alice Morel¹, Dazhong Zhao², Kori Ault¹, Michael Dow¹, Glenn Howe¹, Olga Shevchenko^{1,3} & Steven H Strauss¹

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The target gene *AGAMOUS* (*AG*) is a floral organ identity gene

- One of the first floral homeotic genes identified
- Regulates the differentiation of stamens and carpels
- Important to floral organ determinacy

NATURE · VOL 346 · 5 JULY 1990

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ARTICLES

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

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[†] 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 years⁹. The first published report of *Arabidopsis* flowers with an *ag* mutant phenotype was more than a century ago⁸, and another *Arabidopsis* mutant having similar flowers has been described by Conrad¹⁰. The extensively characterized³ 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 mutation¹². 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*.

The ABC model – combinatorial interactions control floral organ development

Development

Volume 112 (1)

May 1991

Development 112, 1–20 (1991)
Printed in Great Britain © The Company of Biologists Limited 1991

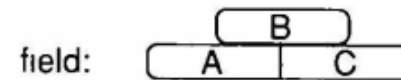
Genetic interactions among floral homeotic genes of *Arabidopsis*

JOHN L. BOWMAN, DAVID R. SMYTH* and ELLIOT M. MEYEROWITZ†

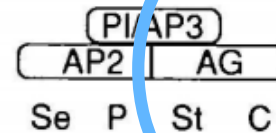
Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125, USA

*Permanent address: Department of Genetics and Developmental Biology, Monash University, Clayton, Victoria 3168, Australia
† Author for correspondence

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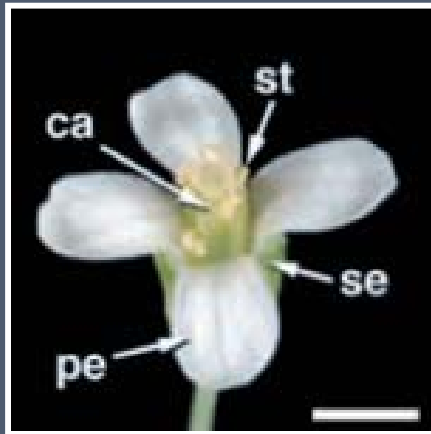
wild type



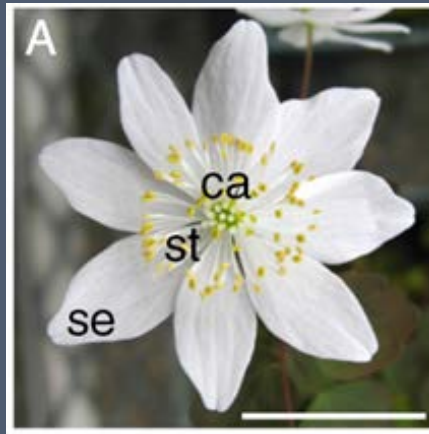
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Loss or reduced expression of AG orthologs results in sterility and reduced determinacy in several plant species

Arabidopsis



Ranunculid



Apple



Japanese gentian



Parcy *et al.* 2002

Galimba *et al.* 2012

Klocko *et al.* 2016

Nakatsuka *et al.* 2015

Two *AGAMOUS* (*AG*) orthologs identified in poplar

- Paralogs on different chromosomes (chromosomes IV and XI)
- 89% DNA sequence similarity in protein coding region of *P. alba* clone 6k10
- Simultaneous suppression with one RNAi construct
- Vegetative expression role?



Plant Molecular Biology 44: 619-634, 2000. © 2000 Kluwer Academic Publishers. Printed in the Netherlands.

619

Structure and expression of duplicate *AGAMOUS* orthologues in poplar

Amy M. Brunner, William H. Rottmann¹, Lorraine A. Sheppard², Konstantin Krutovskii, Stephen P. DiFazio, Stefano Leonardi³ and Steven H. Strauss*

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Received 9 November 1999; accepted in revised form 24 July 2000

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

Experimental overview

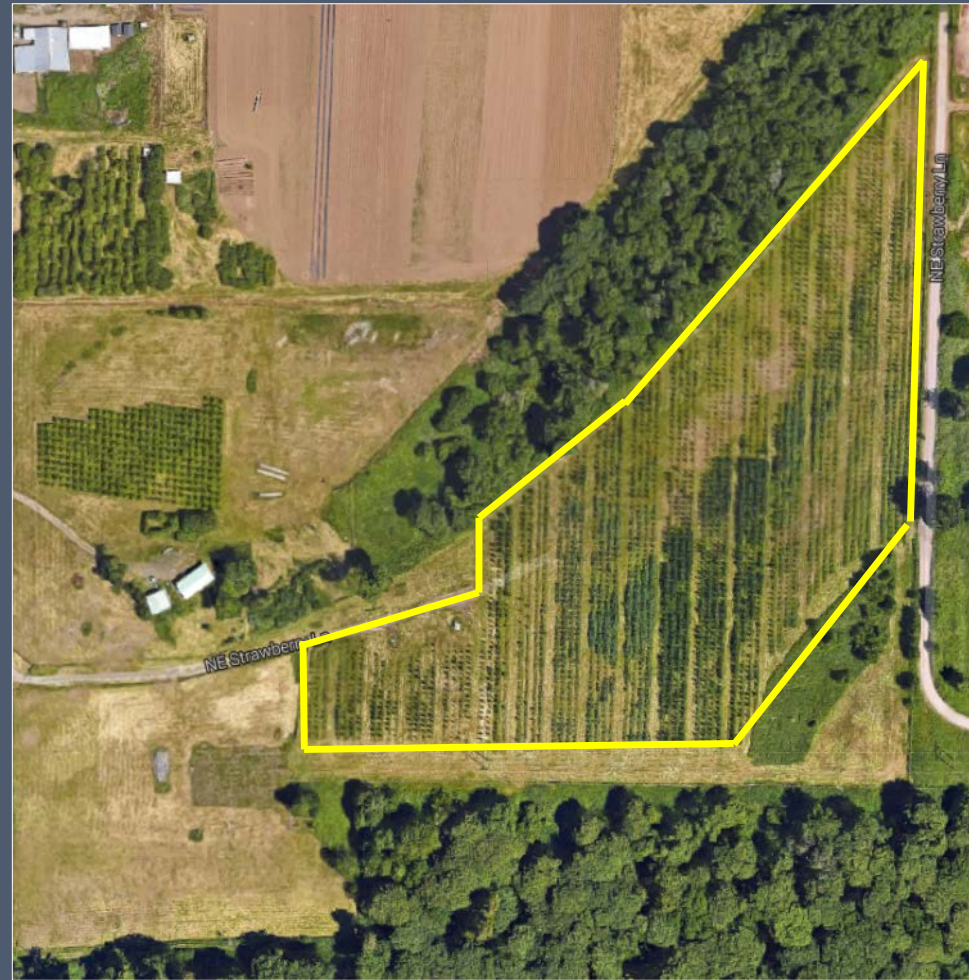
- Creation of RNAi constructs based on the v. 1.0 reference sequence from *Populus trichocarpa*
- Production of transgenic poplars
 - Female clone 6K10 (*P. alba*; early flowering) – focus of this study
 - Provided by Maurizio Sabatti, Tuscia University, Viterbo, Italy
 - Female clone 717 (*P. tremula* x *P. alba*)
 - Male clone 353 (*P. tremula* x *P. tremuloides*)
- Evaluation of phenotypic changes in field
- Evaluation of target gene suppression



Dr. Maurizio Sabatti

Test plantation in Oregon: 3.6 ha / 3,414 trees

- 23 constructs, 10-20 events each
- 2 x 2-tree row plots per event
- 96% survival since planting in 2011
- Mostly RNAi against a variety of floral genes



Most 6K10 trees initiated flowering in their third growing season

Jan 2014



Transformed 6K10 trees have now gone through seven growing seasons

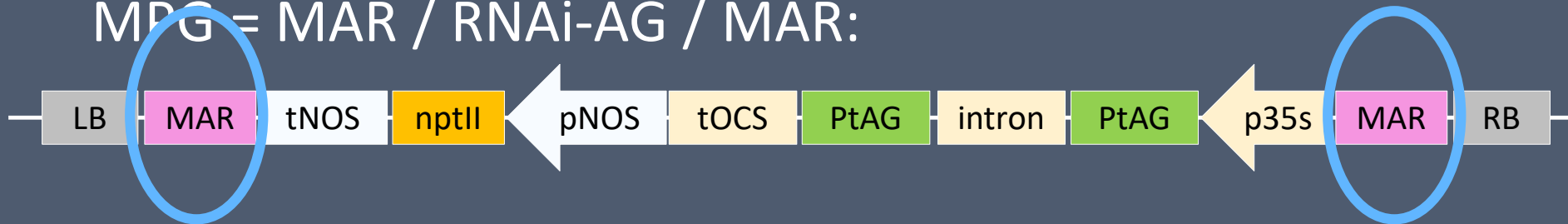


Two AG-RNAi constructs, with and without MARs

PTG = RNAi-AG:



MPG = MAR / RNAi-AG / MAR:



MARs can increase transgene expression level and possibly RNAi efficiency

Transgenic Research 6, 415–420 (1997)

Matrix attachment regions (MARs) enhance transformation frequency and transgene expression in poplar

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Department of Forest Science, Oregon State University, Corvallis, OR 97331, USA (Fax: +1 541 737 1393)

Received 20 May 1997; revised 25 June 1997; accepted 26 June 1997

We tested the value of a matrix attachment region (MAR) fragment derived from the frequency of *Agrobacterium*-mediated transformation. A binary vector that carries an intron and an *nptII* gene was modified to contain flanking MAR elements containing or lacking MARs were then used to transform tobacco, a readily transformable species (*Nicotiana glauca* × *N. glauca*), and a recalcitrant poplar clone (*Populus trichocarpa* × *P. trichocarpa*), and a recalcitrant poplar clone (*Populus trichocarpa* × *P. trichocarpa*). Transgene expression was approximately 10-fold in the two hybrid poplar clones and two tobacco clones with MARs compared to those without MARs. The frequency of kanamycin resistance gene expression was also increased in the two hybrid poplar clones and two tobacco clones with MARs compared to those without MARs. MARs also increased the frequency of kanamycin resistance gene recovered.

Transgenic Research (2005) 14:193–206
DOI 10.1007/s11248-004-5413-8

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Matrix attachment regions increase the efficiency and stability of RNA-mediated resistance to Tomato Spotted Wilt Virus in transgenic tobacco

Jennifer S. Levin^{1,*}, William F. Thompson², Alex S. Csinos³, Michael G. Stephenson⁴ & Arthur K. Weissinger¹

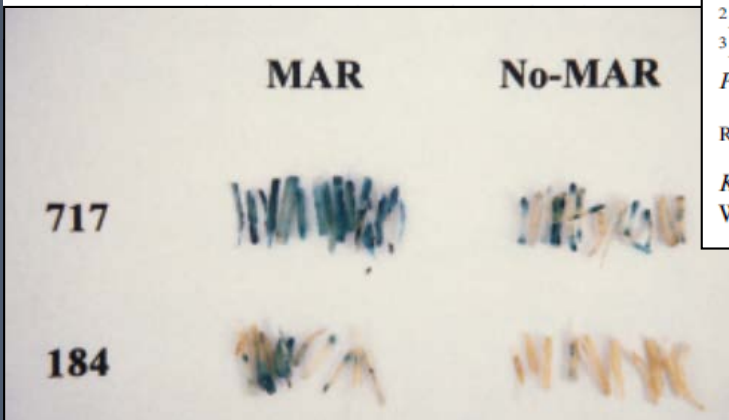
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Received 11 March 2004; revised 23 July 2004; accepted 18 October 2004

Key words: gene silencing, matrix attachment regions, RNA-mediated virus resistance, Tomato Spotted Wilt Virus



The AG-RNAi constructs contained an inverted repeat that targeted 386 bp of the non-MADS region



Targeting two duplicated AG genes in poplar

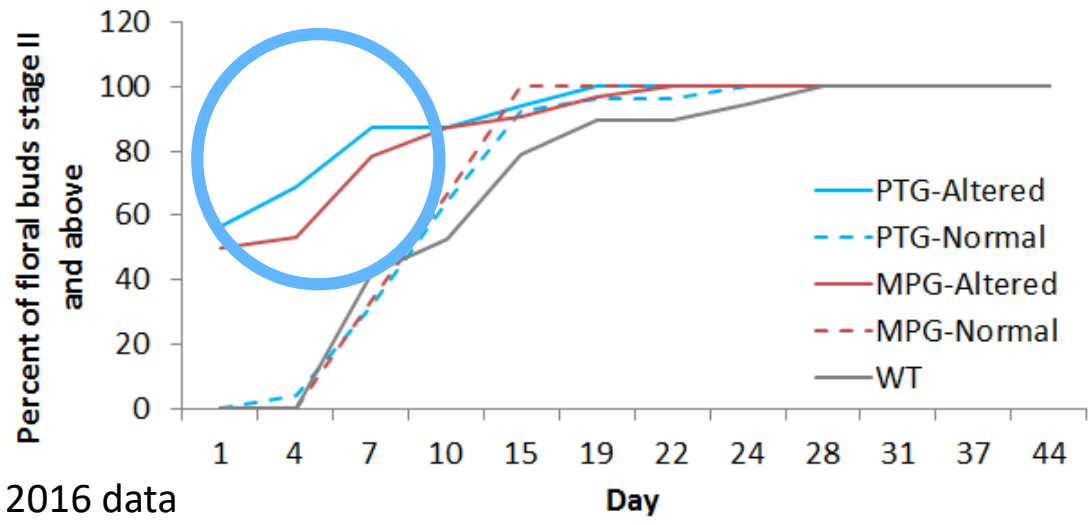
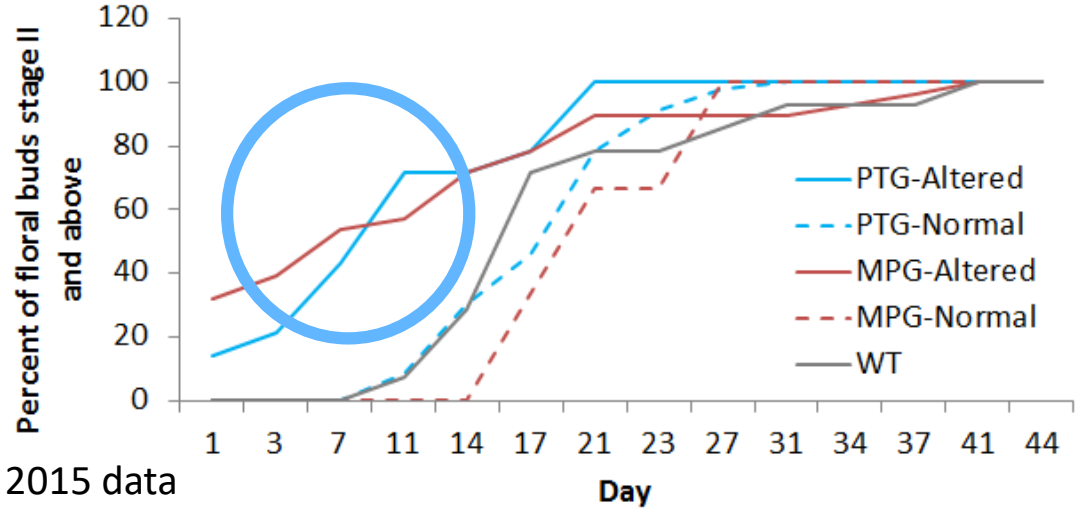
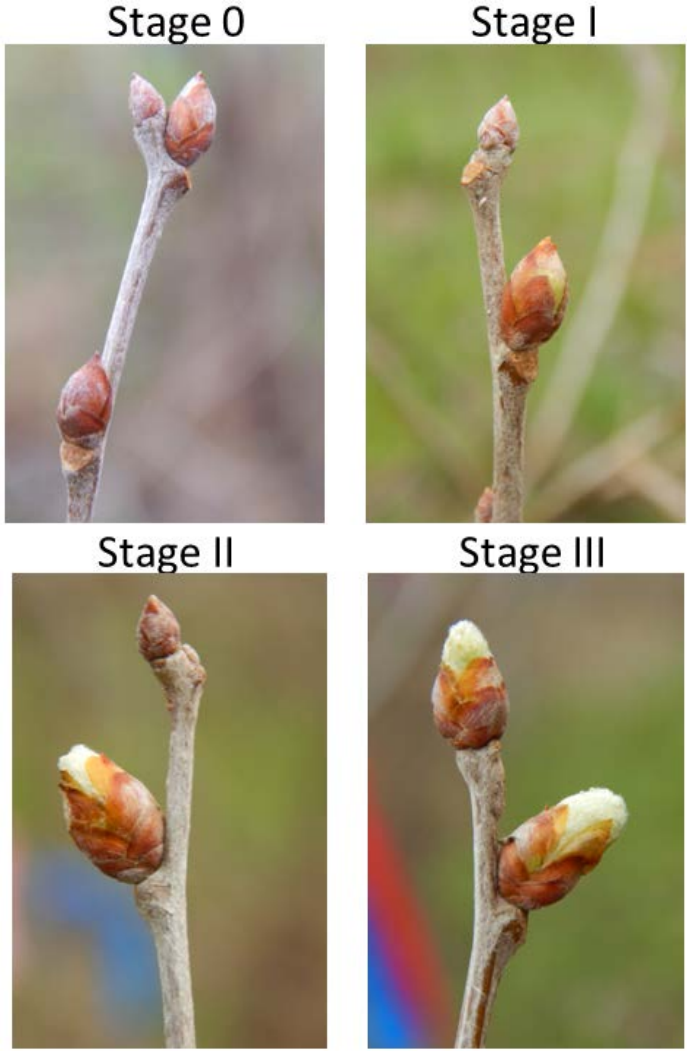
RNAi	GGGTCAGTTTCTGAAGCCAATGCTCAGTTTTATCAGCAAGAAGCTGCCAAGCTGCGCTCG
PaAG1	GGGTCGTTTTCTGAAGCCAATGCTCAGTACTACCAGCAAGAAGCTGCCAAGCTGCGTTCC
PaAG2	GGGTCAGTTTCTGAAGCCAATGCTCAGTTCTATCAGCAAGAAGCTGCCAAGCTGCGCTCG *****
RNAi	CAAATTGGTAATTTGCAGAATTCAAACAGGAATATGCTGGGTGAATCACTTAGTGCATTG
PaAG1	CAAATTGGTAATTTGCAGAATTCAAACAGGCATATGCTGGGTGAAGCTCTTAGTTCATTG
PaAG2	CAAATTGGTAATTTGCAGAATTCAAACAGGAACATGCTGGGTGAATCACTTAGTGCATTG *****
RNAi	AGTGTGAAGGAACCTTAAGAGCTTGGAGATAAACTTGAGAAAGGAATTGGTAGAATTCGT
PaAG1	AGTGTGAAGGAACCTTAAGAGTTTGGAAATACGACTTGAGAAAGGAATAAGCAGAATTCGT
PaAG2	AGCGTGAAGGAACCTTAAGAGCTTGGAGATAAACTTGAGAAAGGAATTGGTAGAATTCGT ** *****
RNAi	TGAAAAAGAATGAGCTGTTGTTTGTGCTGAAATTGAGTATATGCAGAAGAGGGAGATTGAC
PaAG1	TCCAAAAAGAATGAGCTGTTGTTTGCAGAAATCGAGTATATGCAGAAGAGGGAGTTGAC
PaAG2	TGAAAAAGAATGAGCTGTTGTTTGTGCTGAAATAGAGTATATGCAGAAGAGGGAGATTGAC ** *****
RNAi	TTGCACAACAATAACCAGCTTCTCCGAGCAAAGATTGCAGAGAATGAAAGAAAGCGACAG
PaAG1	TTGCACAACAACAACCAGCTTCTCCGAGCAAAGATTTAGAGAATGAAAGAAAGCGACAG
PaAG2	TTGCACACCAATAACCAGCTTCTCCGAGCAAAGATTGCAGAGAACGAAAGAAAGCGACAG ***** ** *****
RNAi	CACATGAATTTGATGCCCGGAGGTGTCAACTTCGAGATCATGCAGTCTCAACCATTTGAC
PaAG1	AGCATGAATTTGATGCCAGGAGGAGCAGACTTTGAGATCGTGCAGTCTCAACCATACGAC
PaAG2	CACATGAATTTGATGCCAGGAGGTGTCAACTTCGAGATCATGCAGTCTCAACCATTTGAC ***** ***** * ***** ***** *****
RNAi	TCTCGGAACTATTCTCAAGTTAATGG
PaAG1	TCTCGCAACTATTCTCAAGTGAATGG
PaAG2	TCTCGGAACTATTCTCAAGTTAATGG ***** *****

MARS induced a high rate of RNAi floral modification

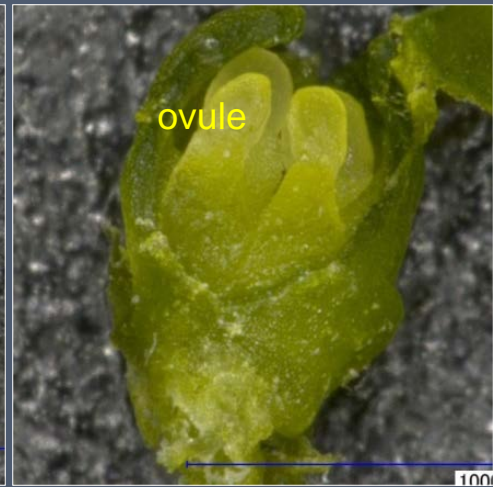
Construct ID	No. of Events Planted/Survived	No. of Events Flowered by 2017	No. (%) of Events with Altered Floral Morphology
AG-RNAi (PTG)	22/22	22 (100%)	6 (27%)
MAR-AG-RNAi (MPG)	13/13	12 (92%)	11 (92%)
Non-transgenic (WT)	24/24	19 (79%)	0 (0%)

MAR elements more than tripled RNAi suppression frequency

Floral buds on altered events flushed early



Altered events showed a “carpel-inside-carpel” phenotype

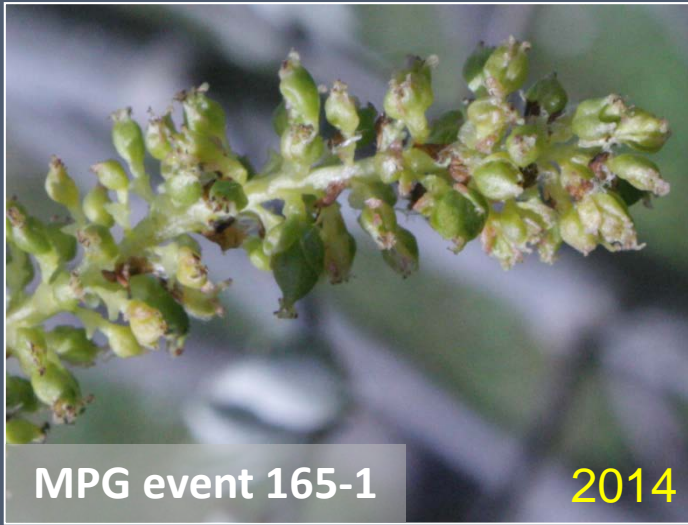


Morphological variation was commonly observed among and within events

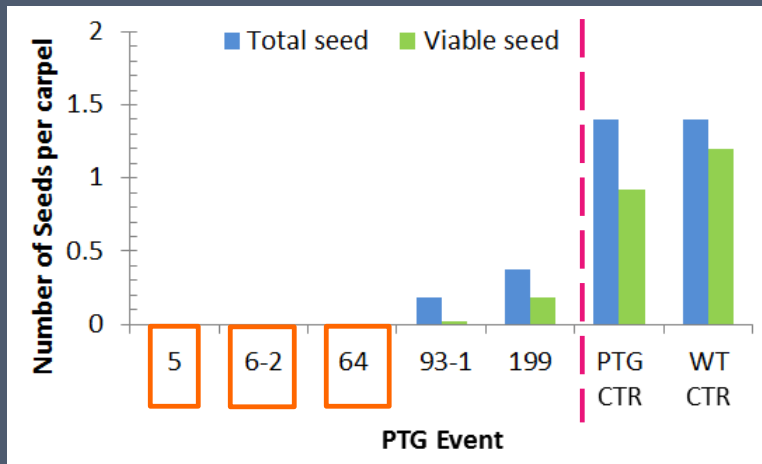
- Flowers differed in the number of layers of carpels
- Some had anther-looking structures



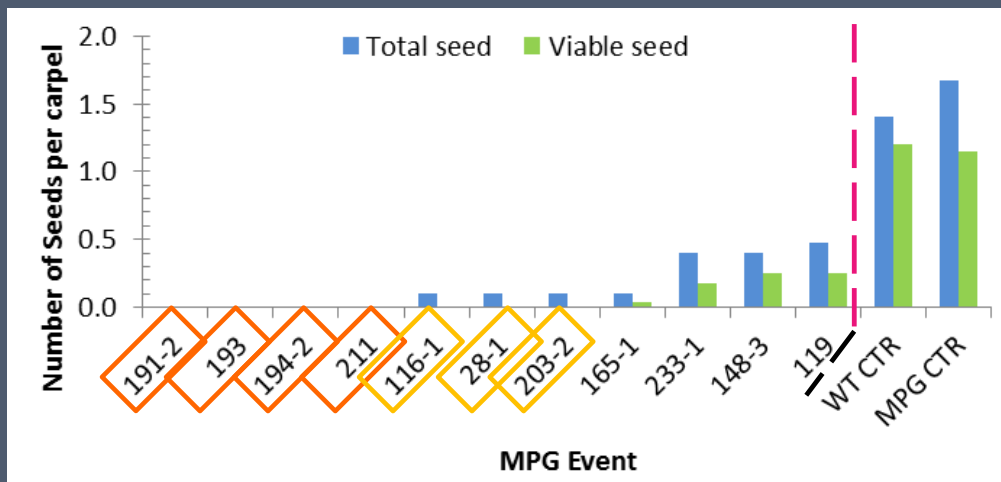
Altered events were stable in degree of modification within and among trees over four years



Up to 100% reduction in seed production and viability (= sterility) were observed in both constructs



- Seedless
- Non-viable seeds
- Viable seeds at a low rate



Seedless events produced very little/no cotton

March

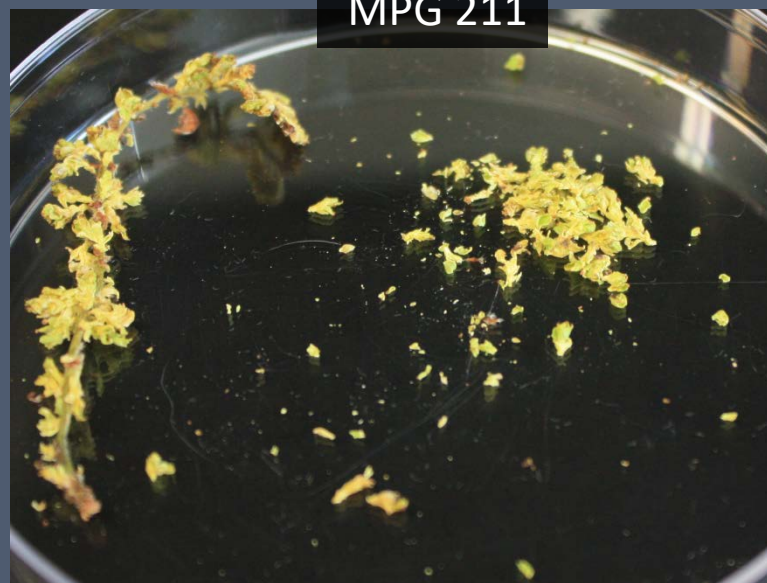
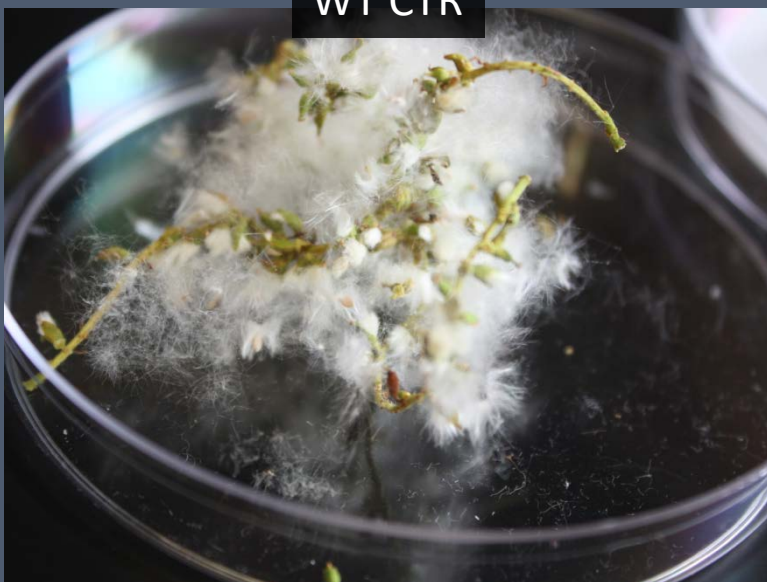


WT CTR

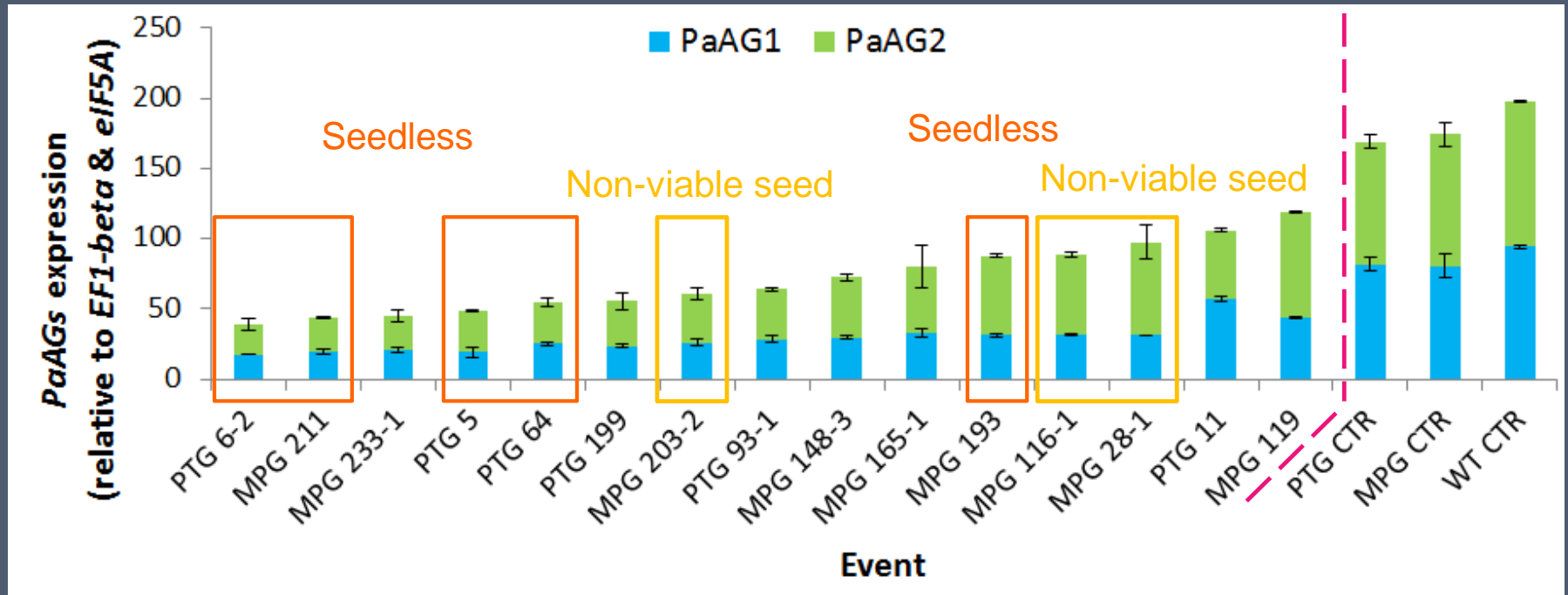


MPG 211

April

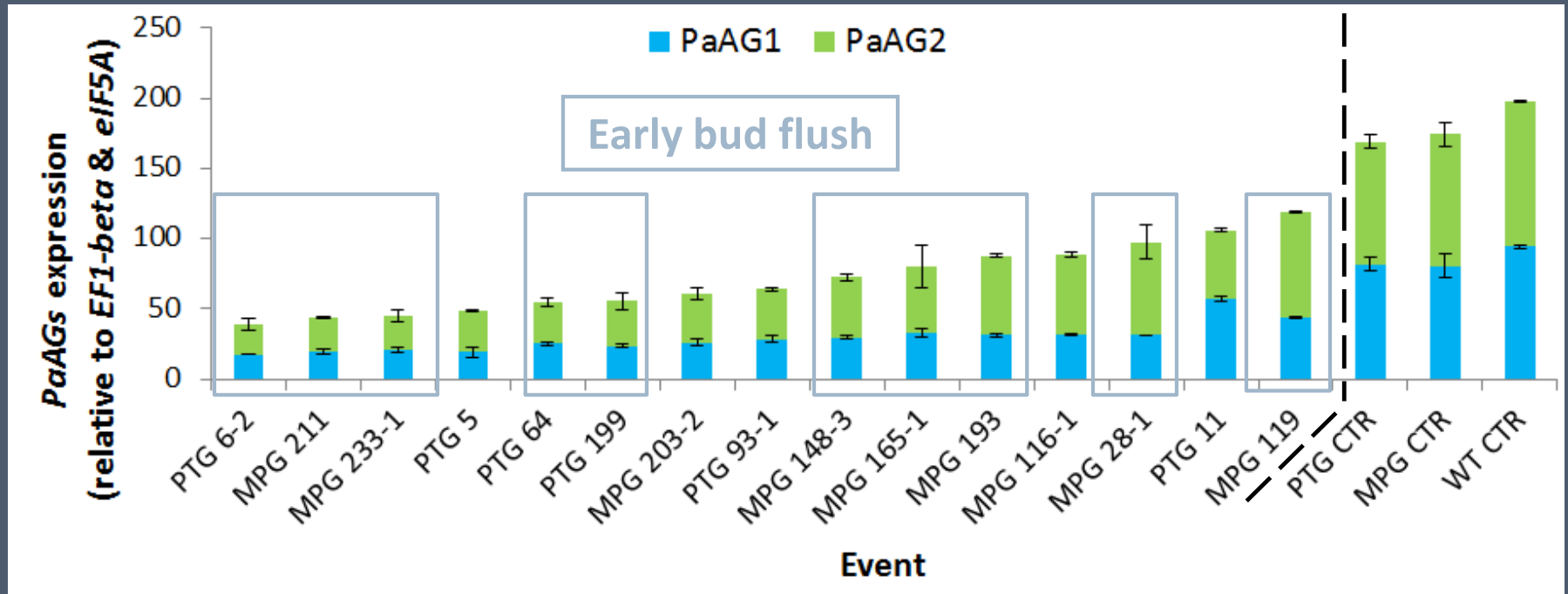


Suppression of the two *PaAG* paralogs were imperfectly associated with the sterility phenotype



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

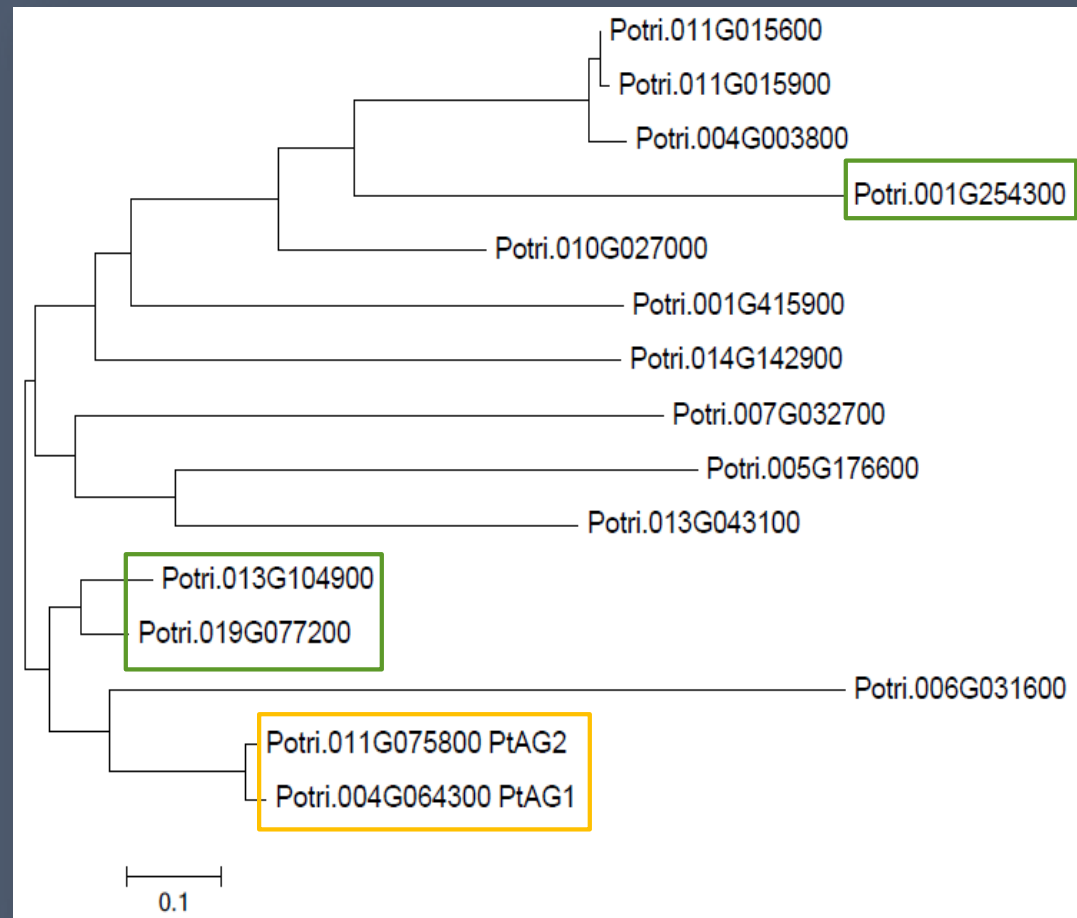
The timing of bud flush was also imperfectly associated with *PaAG* expression



Could off-target RNAi suppression be playing a role?

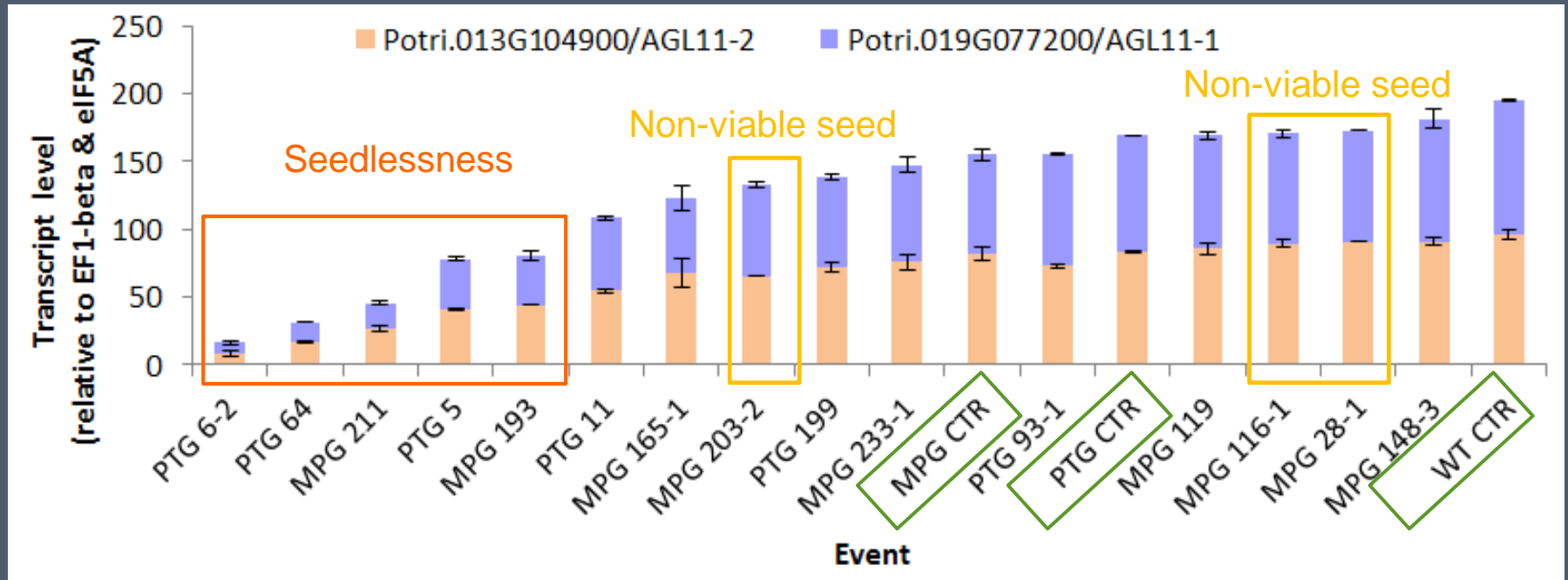
- Blasted poplar genome with dsRNA from RNAi constructs or parts thereof
- Aligned DNA sequences to identify regions with ≥ 6 bp identity with dsRNA – found 13 potential off-target genes
- Studied expression of homologs in *Arabidopsis* expression atlas ([ePlant](#)) and poplar ignored those without significant floral expression

Selection of off-target genes for expression analysis



- Most genes not expressed in floral organs (no highlight)
- Examined genes **in green**
 - No significant changes in Potri.001G254300
 - Strong suppression of *PaAGL11* paralogs were observed

Suppression of *AGL11* paralogs strongly correlated with seedlessness



PaAGL11-1 and *PaAGL11-2* expression was highly correlated:
 $r = 0.98$

AGL11 and its orthologs play a major role in ovule development

The Plant Cell, Vol. 7, 1259–1269, August 1995 © 1995 American Society of Plant Physiologists

Diverse Roles for MADS Box Genes in Arabidopsis Development

Steven D. Rounsley, Gary S. Ditta, and Martin F. Yanofsky¹

Department of Biology and Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92093-0116

of them are floral specific. RNA expression analyses of the six genes reported here indicate that two genes, **AGL11** and **AGL13** (AGL for **AGAMOUS**-like), are preferentially expressed in ovules, but each has a distinct expression pattern. **AGL15**

of them are floral specific. RNA expression analyses of the six genes reported here indicate that two genes, **AGL11** and **AGL13** (AGL for **AGAMOUS**-like), are preferentially expressed in ovules, but each has a distinct expression pattern. **AGL15** is preferentially expressed in embryos, with its onset at or before the octant stage early in embryo development. **AGL12**, **AGL14**, and **AGL17** are all preferentially expressed in root tissue box genes expressed in roots. Phylogenetic analyses showed th to previously isolated MADS box genes, whereas the four genes Data from this and previous studies indicate that in addition to t are likely to play roles in many other aspects of plant develop

Mejía et al. *BMC Plant Biology* 2011, 11:57
<http://www.biomedcentral.com/1471-2229/11/57>



RESEARCH ARTICLE

Open Access

Molecular, genetic and transcriptional evidence for a role of *VvAGL11* in stenospermocarpic seedlessness in grapevine

Nilo Mejía^{1*}, Braulio Soto¹, Marcos Guerrero¹, Ximena Casanueva¹, Cléa Houel², María de los Ángeles Miccono¹, Rodrigo Ramos¹, Loïc Le Cunff³, Jean-Michel Boursiquot³, Patricio Hinrichsen¹ and Anne-Françoise Adam-Blondon²

The absence of cotton may be an indicator of disrupted ovule development

Ye et al. *BMC Genomics* 2014, **15**:475
<http://www.biomedcentral.com/1471-2164/15/475>



RESEARCH ARTICLE

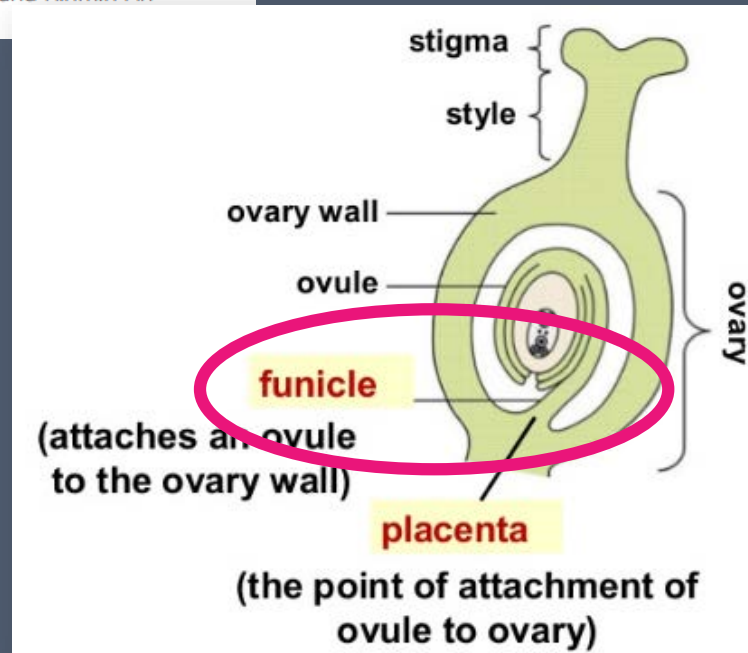
Open Access

Study of seed hair growth in *Populus tomentosa*, an important character of female floral bud development

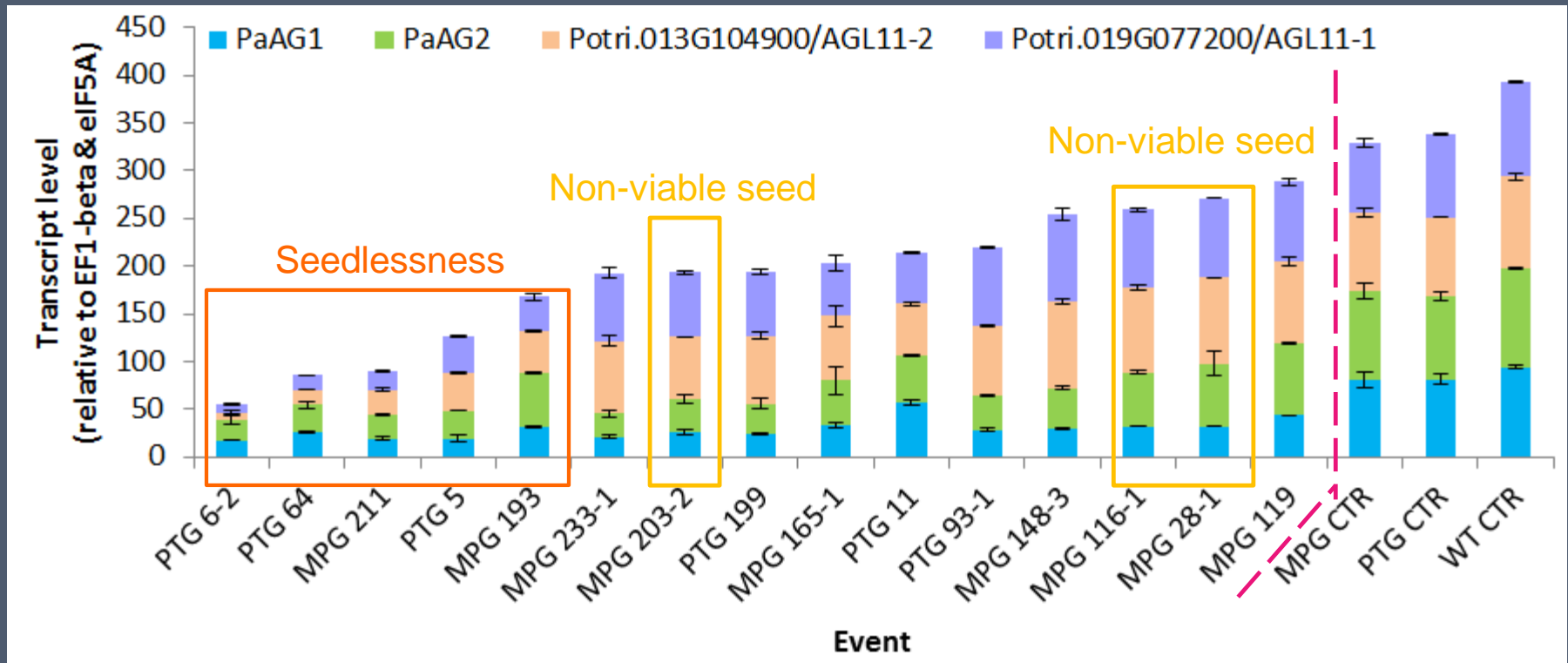
Meixia Ye¹, Zhong Chen^{1†}, Xiaoxing Su², Lexiang Ji¹, Jia Wang¹, Weihua Liao¹, Huandi Ma¹ and Xinmin An^{1*}

Seed hairs originate from the epidermal cells of the funicle

Suppressed ovule development, no funicle, no seed hairs?

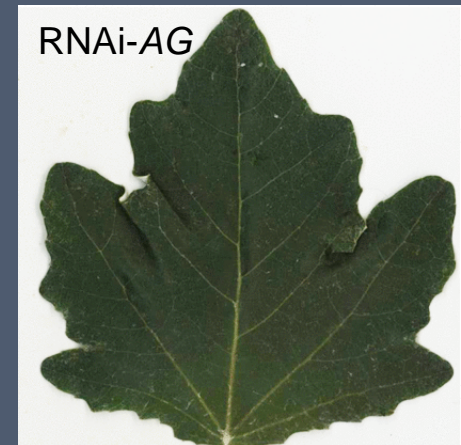


Seedlessness phenotype also strongly correlated with total expression of *AG* and *AGL11*



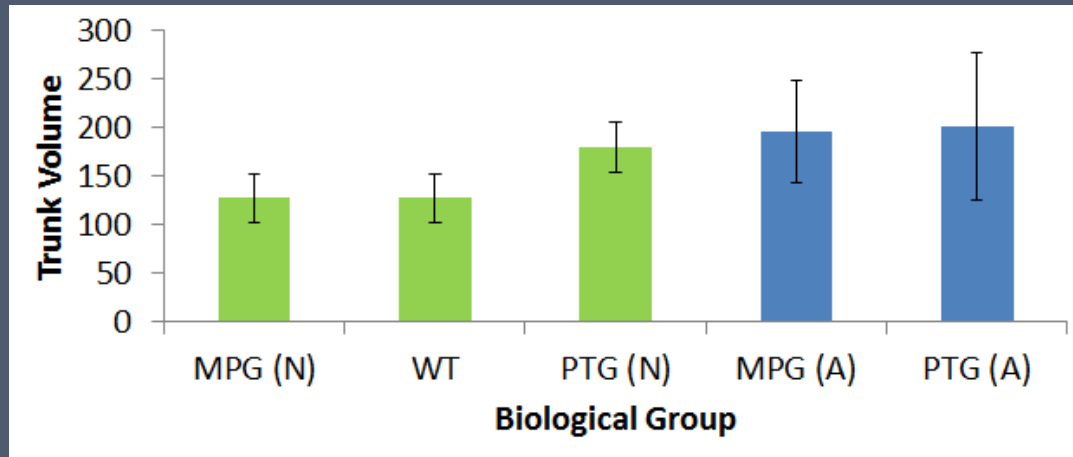
Correlation among *AG* and *AGL* paralogous pairs weak:
 $r = 0.50$

AG-RNAi events had normal tree and leaf form

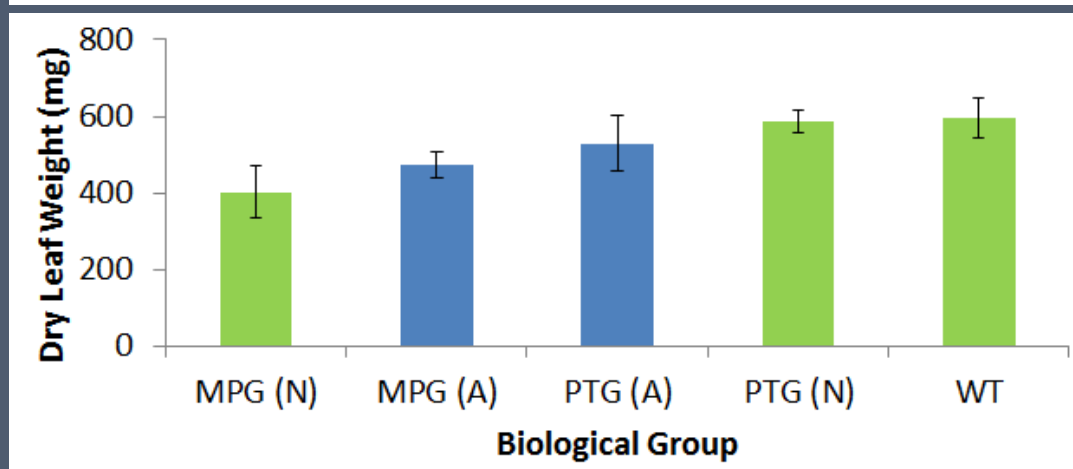


3 leaves per tree scanned and analyzed for chlorophyll content, leaf area and weight, petiole length

AG-RNAi events had normal vegetative growth



(A) = altered floral morphology
(N) = normal floral morphology



No significant differences in trunk volume, dry leaf weight, chlorophyll content, petiole length and petiole width, were detected

Summary

- Suppression of *AG* and *AGL11* expression leads to strong *ag*-like alteration of floral morphology
 - Complete female sterility
 - Early floral budburst
 - Indeterminacy of floral organs
 - *AGL11* suppression led to seedless/hairless phenotype
- No evidence for effects on biomass growth or leaf morphology
- RNAi-induced changes were stable over several years
- *AG* and *AGL11* appear to be good targets for genetic containment

Limitations and moving forward

- Sterility phenotype in male clones unclear
 - Investigation in male clone 353 underway
- Need to screen many insertion events to find those with sufficient knock-down with RNAi
- Need to wait for flowering to understand extent and pattern of knock-down
- Complete, easily predicted knockout using nucleases superior (e.g. CRISPR/Cas9), if not too strong?

Key collaborators and funding sources



Haiwei Lu



Amy Klocko



Amy Brunner,
now at Virginia Tech,
created the constructs



Cathleen Ma



Anna C. Magnuson

