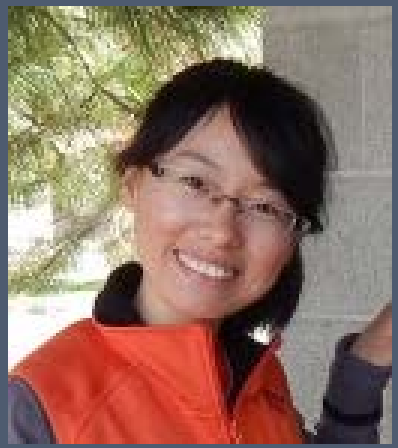
Cross-suppression of *AG* and *AG-LIKE 11* Genes Gives Sterility in Field Grown Poplar

Haiwei Lu and Steven H. Strauss
Oregon State University

Development and Application of Genome Engineering and Transgenic Technology to the Agriculture Workshop
PAG XXVI, San Diego, CA, 2018

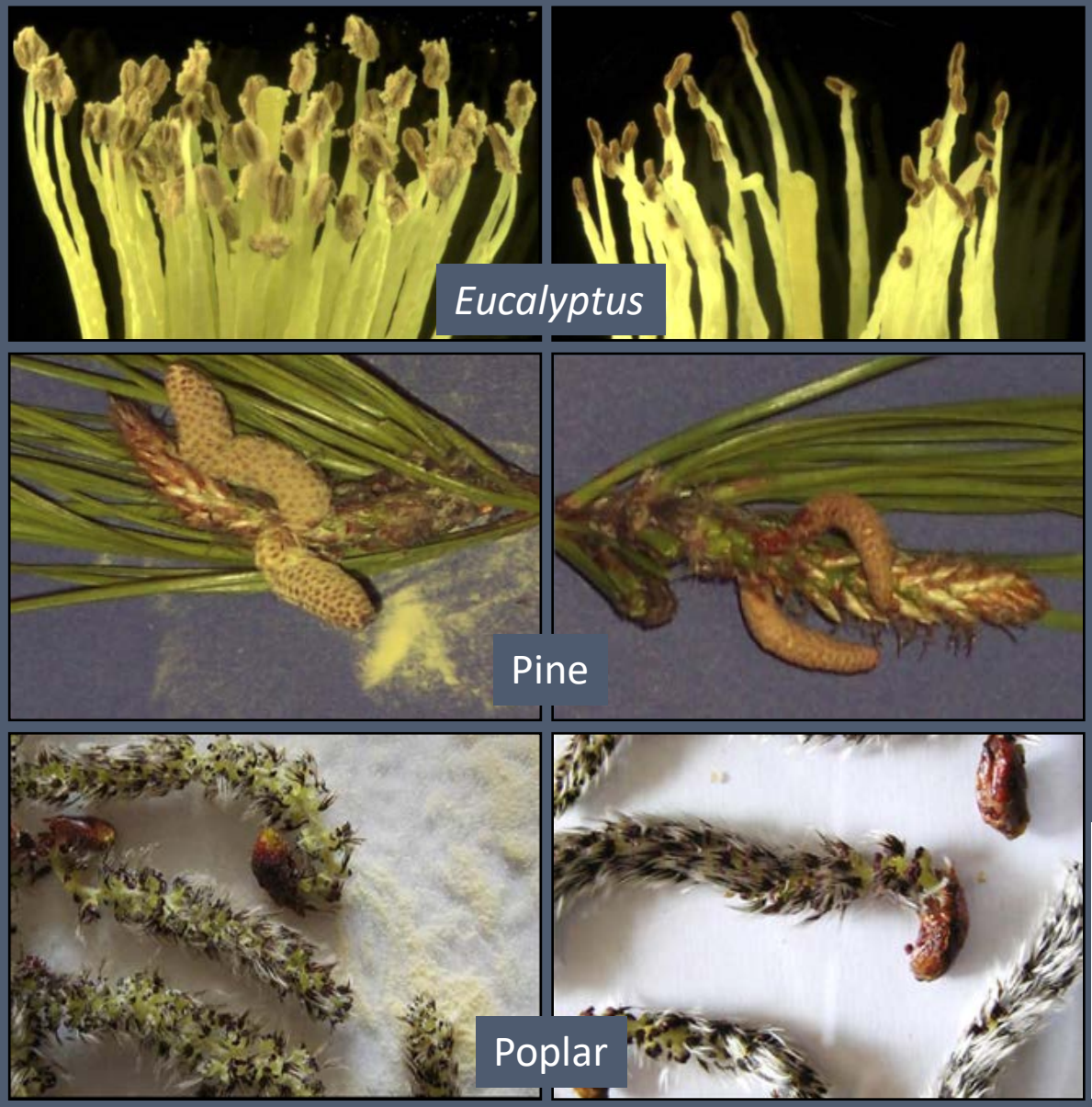
Oregon State University



The containment issue

- Coexistence and adventitous 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

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

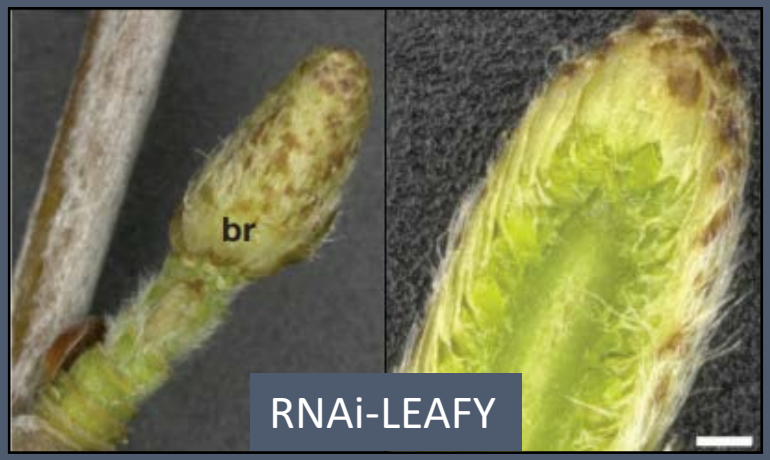
Negative impact on tree health was observed in poplar

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*



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

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

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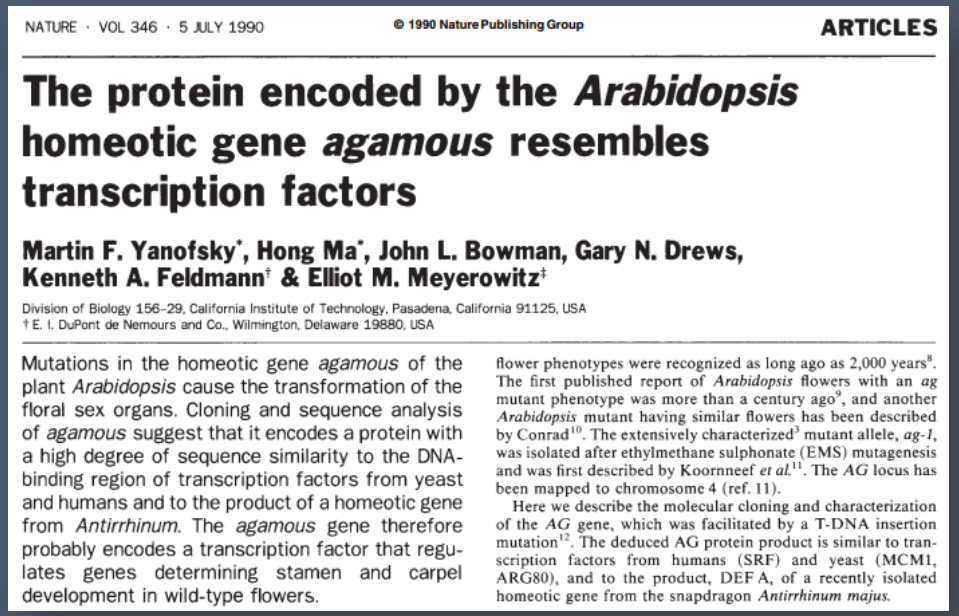
VOLUME 34 NUMBER 9 SEPTEMBER 2016 NATURE BIOTECHNOLOGY

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

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‡

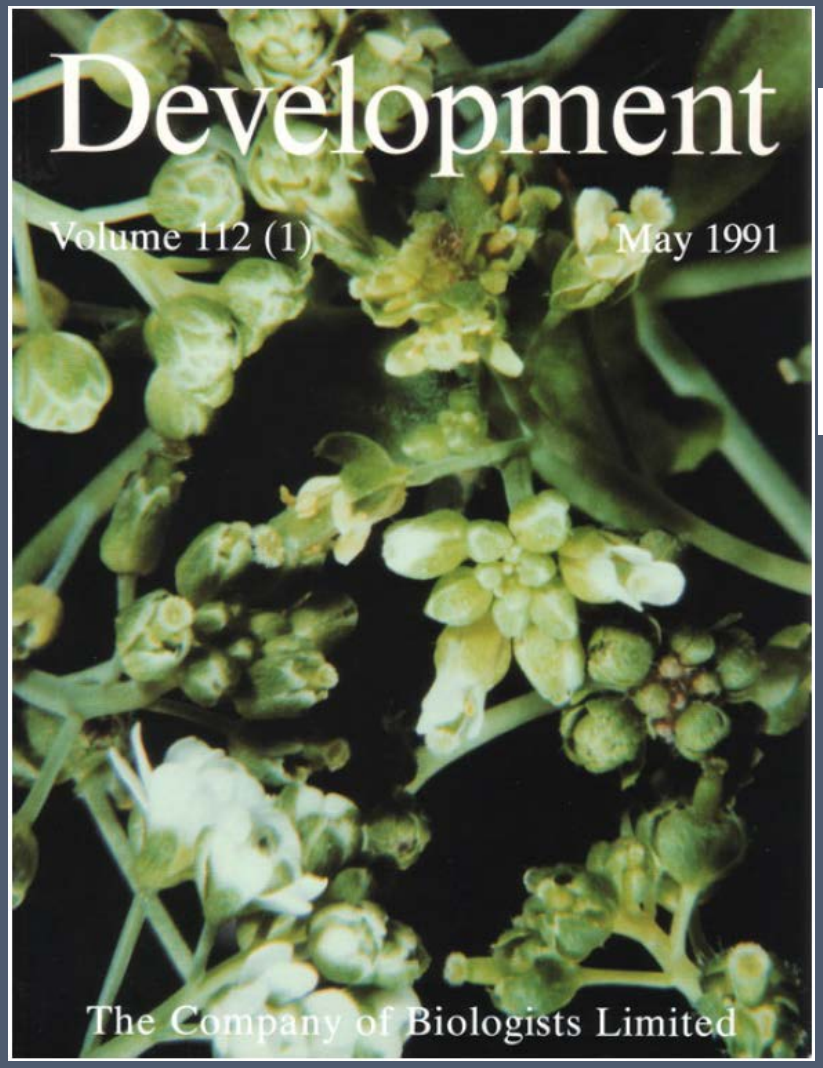
Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125, USA
† E. I. DuPont de Nemours and Co., Wilmington, Delaware 19880, USA

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, DEFA, of a recently isolated homeotic gene from the snapdragon *Antirrhinum majus*.

The ABC model – combinatorial interactions control floral organ development



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†

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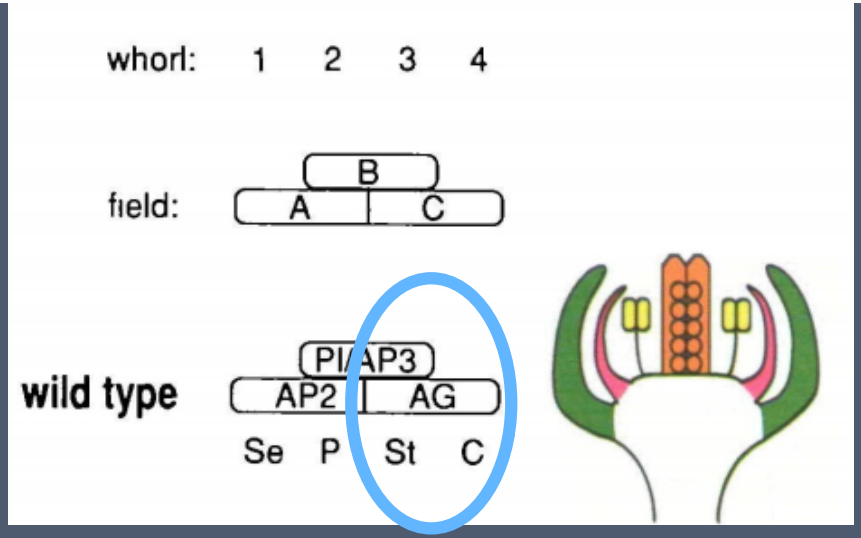
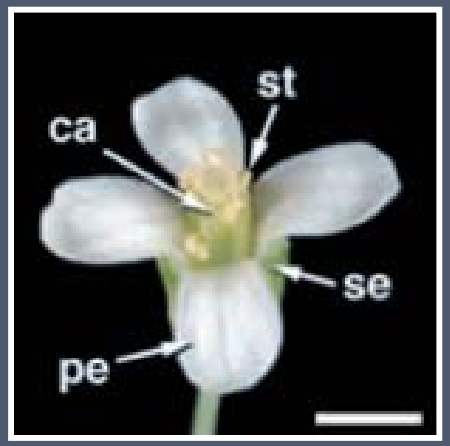


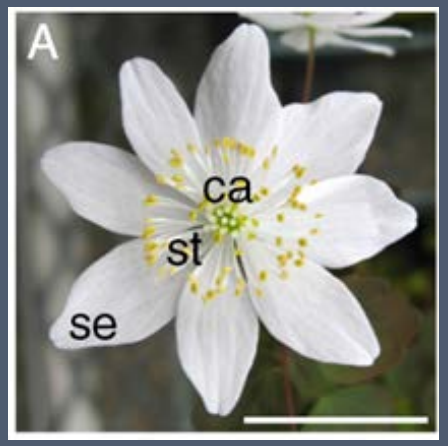
Loss or reduced expression of *AG* orthologs results in sterility and reduced determinacy in several plant species

Arabidopsis



Parcy *et al*. 2002

Ranunculid



Galimba *et al*. 2012

Apple

Klocko *et al*. 2016

Japanese gentian

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.

Structure and expression of duplicate AGAMOUS orthologues in poplar

Amy M. Brunner, William H. Rottmann[1], Lorraine A. Sheppard[2], Konstantin Krutovskii, Stephen P. DiFazio, Stefano Leonardi[3] and Steven H. Strauss*

*Department of Forest Science, Oregon State University, Corvallis, OR 97331, USA (*author for correspondence; e-mail: strauss@fsl.orst.edu); present addresses: 1 Westvaco Forest Science and Technology, PO. Box 1950, Summerville, SC 29484, USA; 21nstitute of Forest Genetics, USDA Forest Service c/o Department of Environmental Horticulture, One Shields Ave., University of California, Davis, CA, 95616, USA; 3Department of Environmental Science, University of Parma, Parco Area delle Scienze 33a, 43100 Parma, Italy*

Received 9 November *1999;* accepted in revised from *24 July 2000*

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

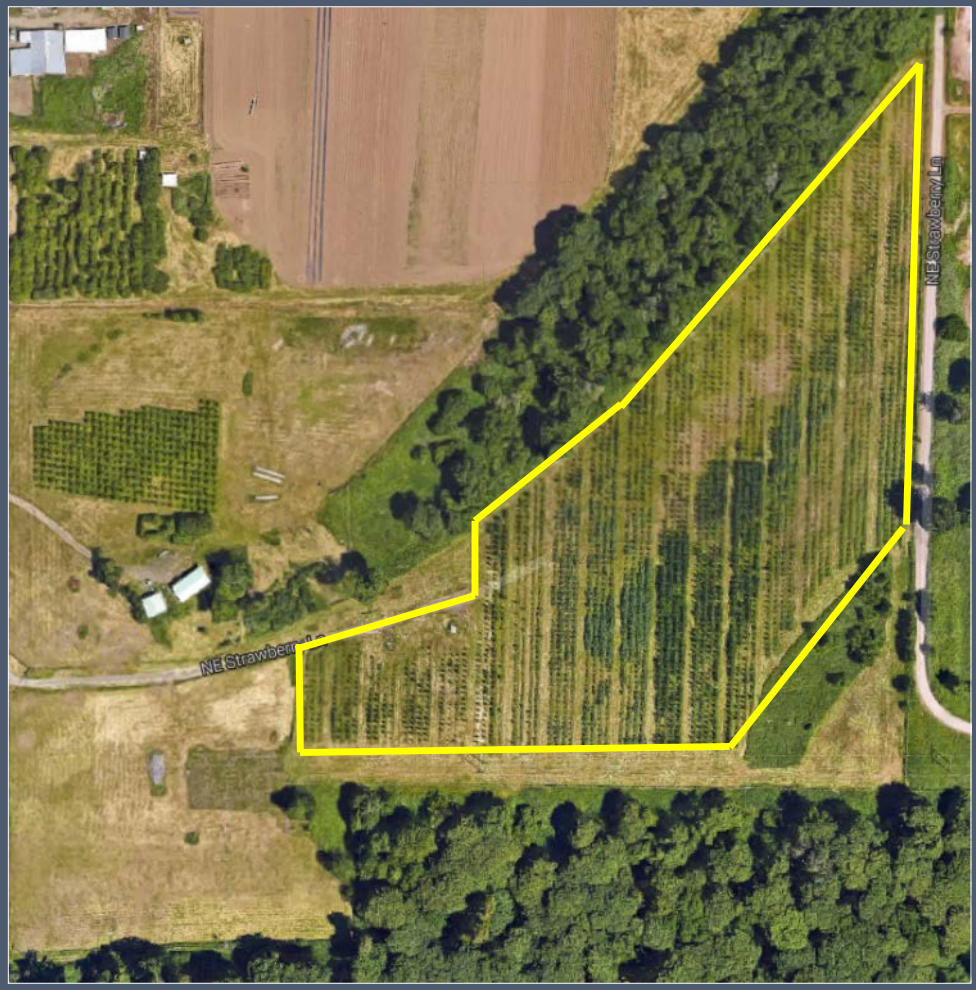
Experimental overview

- 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

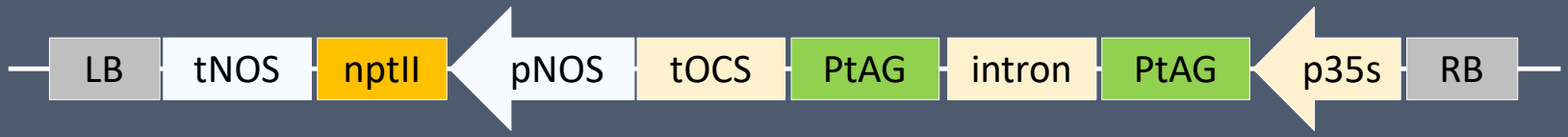


Most 6K10 trees initiated flowering in their third growing season

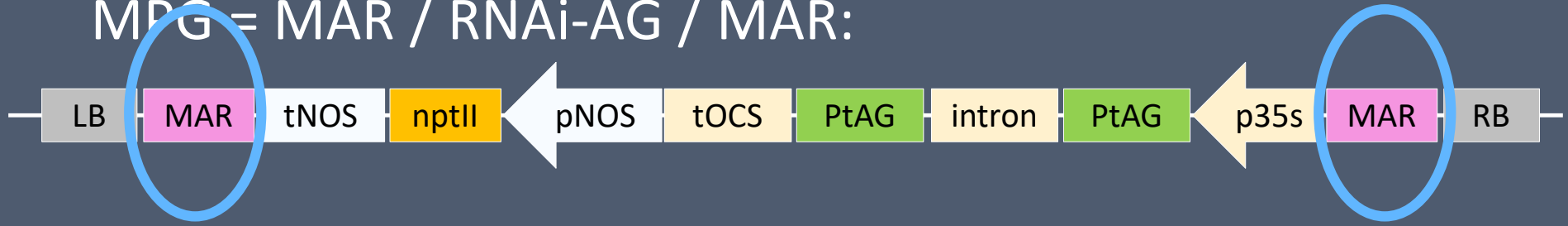
Transformed 6K10 trees have now gone through seven growing seasons

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)

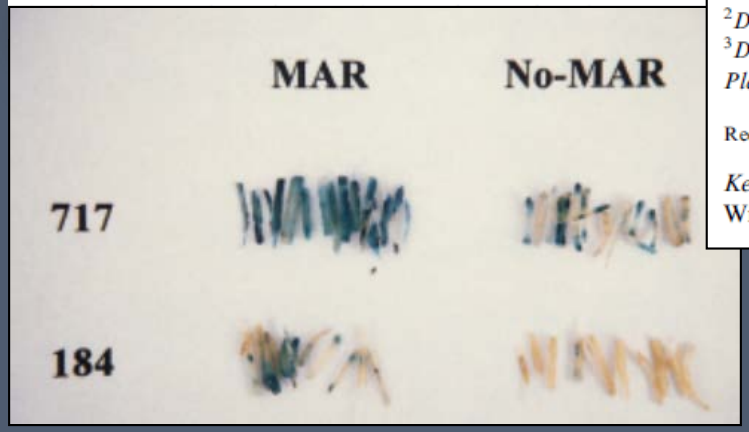
Matrix attachment regions (MARs) enhance transformation frequency and transgene expression in poplar

KYUNG-HWAN HAN‡, CAIPING MA and STEVEN H. STRAUSS*

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 fron
frequency of *Agrobacterium*-mediated transformation. A binary vector that carri
an intron and an *npt*II gene was modified to contain flanking MAR elements
containing or lacking MARs were then used to transform tobacco, a readily tra
tremula × *P. alba*), and a recalcitrant poplar clone (*Populus trichocarpa* × *P.*
gene expression approximately 10-fold in the two hybrid poplar clones and tw
cocultivation with *Agrobacterium*; MARs also increased the frequency of kana
recovered.



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

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. Thompson[2], Alex S. Csinos[3], Michael G. Stephenson[4] & Arthur K. Weissinger[1]

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[2]*Department of Botany, North Carolina State University, Raleigh, NC 27695–7612, USA*
[3]*Department of Plant Pathology and* [4]*Department of Crop and Soil Sciences, University of Georgia, Coastal Plain Experiment Station, Tifton, GA, 31793*

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

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RNAi      GGGTCAGTTTCTGAAGCCAATGCTCAGTTTTATCAGCAAGAAGCTGCCAAGCTGCGCTCG
PaAG1     GGGTCTGTTTCTGAAGCCAATGCTCAGTACTACCAGCAAGAAGCTGCCAAGCTGCGTTCC
PaAG2     GGGTCAGTTTCTGAAGCCAATGCTCAGTTCTATCAGCAAGAAGCTGCCAAGCTGCGCTCG
          ***** **********************  ** ************************ **

RNAi      CAAATTGGTAATTTGCAGAATTCAAACAGGAATATGCTGGGTGAATCACTTAGTGCATTG
PaAG1     CAAATTGGTAATTTGCAGAATTCAAACAGGCATATGCTGGGTGAAGCTCTTAGTTCATTG
PaAG2     CAAATTGGTAATTTGCAGAATTCAAACAGGAACATGCTGGGTGAATCACTTAGTGCATTG
          ****************************** * ************ * ****** *****

RNAi      AGTGTGAAGGAACTTAAGAGCTTGGAGATAAAACTTGAGAAAGGAATTGGTAGAATTCGT
PaAG1     AGTGTGAAGGAACTTAAGAGTTTGGAAATACGACTTGAGAAAGGAATAAGCAGAATTCGT
PaAG2     AGCGTGAAGGAACTTAAGAGCTTGGAGATAAAACTTGAGAAAGGAATTGGTAGAATTCGT
          ** ***************** ***** ***  ***************  * *********

RNAi      TCGAAAAAGAATGAGCTGTTGTTTGCTGAAATTGAGTATATGCAGAAGAGGGAGATTGAC
PaAG1     TCCAAAAAGAATGAGCTGTTGTTTGCAGAAATCGAGTATATGCAGAAGAGGGAGGTTGAC
PaAG2     TCGAAAAAGAATGAGCTGTTGTTTGCTGAAATAGAGTATATGCAGAAGAGGGAGATTGAC
          ** ********************** ***** ********************** *****

RNAi      TTGCACAACAATAACCAGCTTCTCCGAGCAAAGATTGCAGAGAATGAAAGAAAGCGACAG
PaAG1     TTGCACAACAACAACCAGCTTCTCCGAGCAAAGATTTCAGAGAATGAAAGAAAGCGACAG
PaAG2     TTGCACACCAATAACCAGCTTCTCCGAGCAAAGATTGCAGAGAACGAAAGAAAGCGACAG
          ******* *** ********************** ******* ***************

RNAi      CACATGAATTTGATGCCGGGAGGTGTCAACTTCGAGATCATGCAGTCTCAACCATTTGAC
PaAG1     AGCATGAATTTGATGCCAGGAGGAGCAGACTTTGAGATCGTGCAGTCTCAACCATACGAC
PaAG2     CACATGAATTTGATGCCAGGAGGTGTCAACTTCGAGATCATGCAGTCTCAACCATTTGAC
            *************** ***** *   **** ****** ***************  ***

RNAi      TCTCGGAACTATTCTCAAGTTAATGG
PaAG1     TCTCGCAACTATTCTCAAGTGAATGG
PaAG2     TCTCGGAACTATTCTCAAGTTAATGG
          ***** ************** *****
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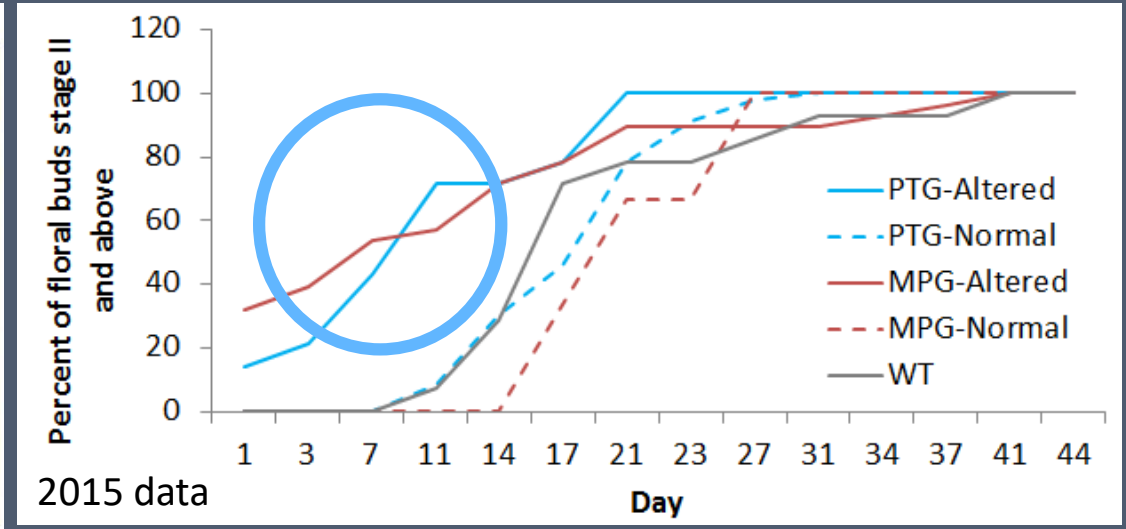
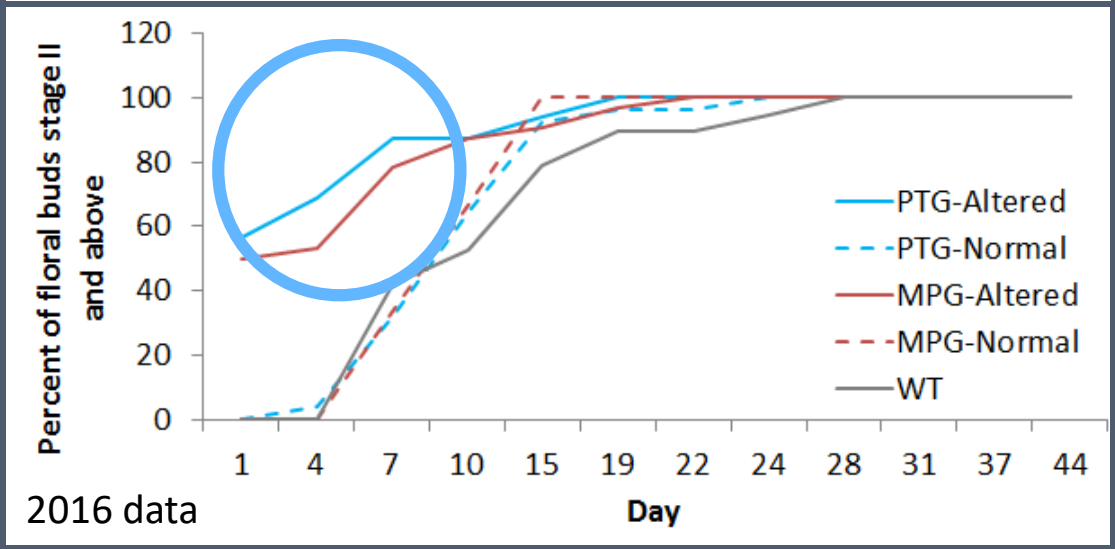
MARS induced a high rate of RNAi floral modification

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





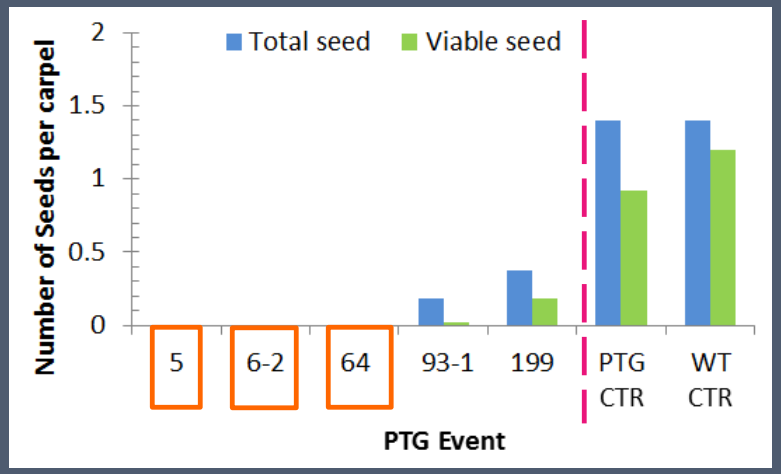
Altered events showed a “carpel-inside-carpel” phenotype

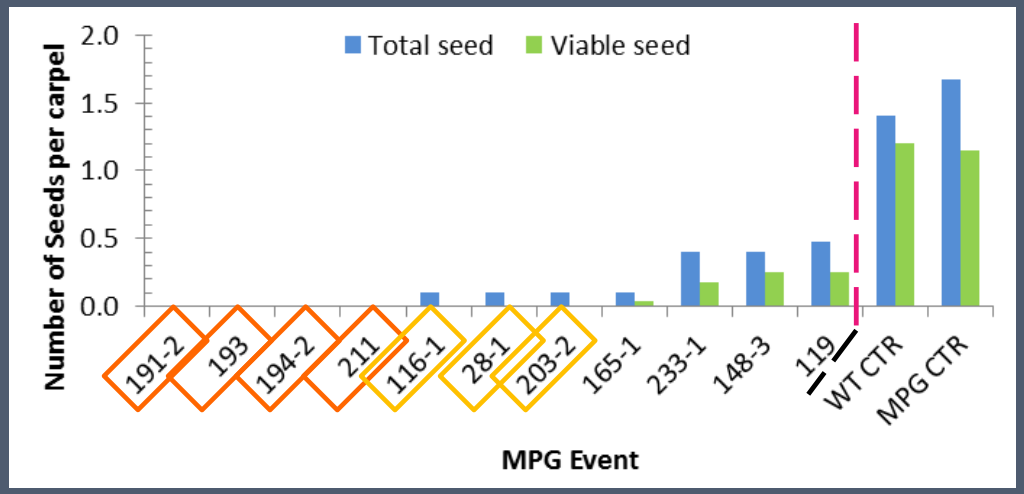
Morphological variation was commonly observed among and within events

- 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



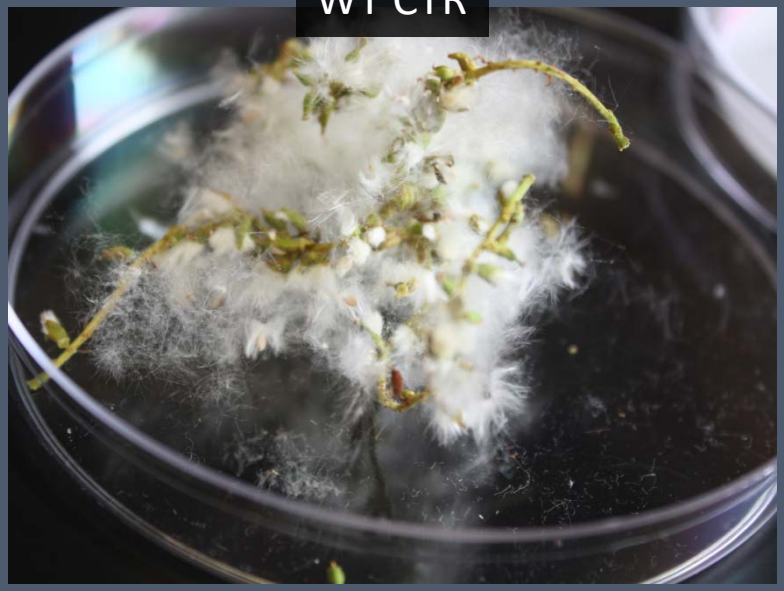


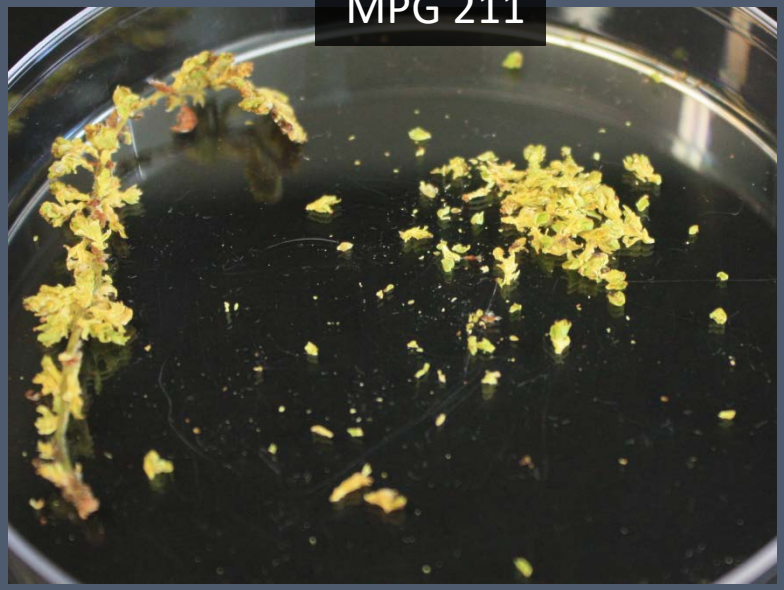
- Seedless
- Non-viable seeds
- Viable seeds at a low rate

Seedless events produced very little/no cotton

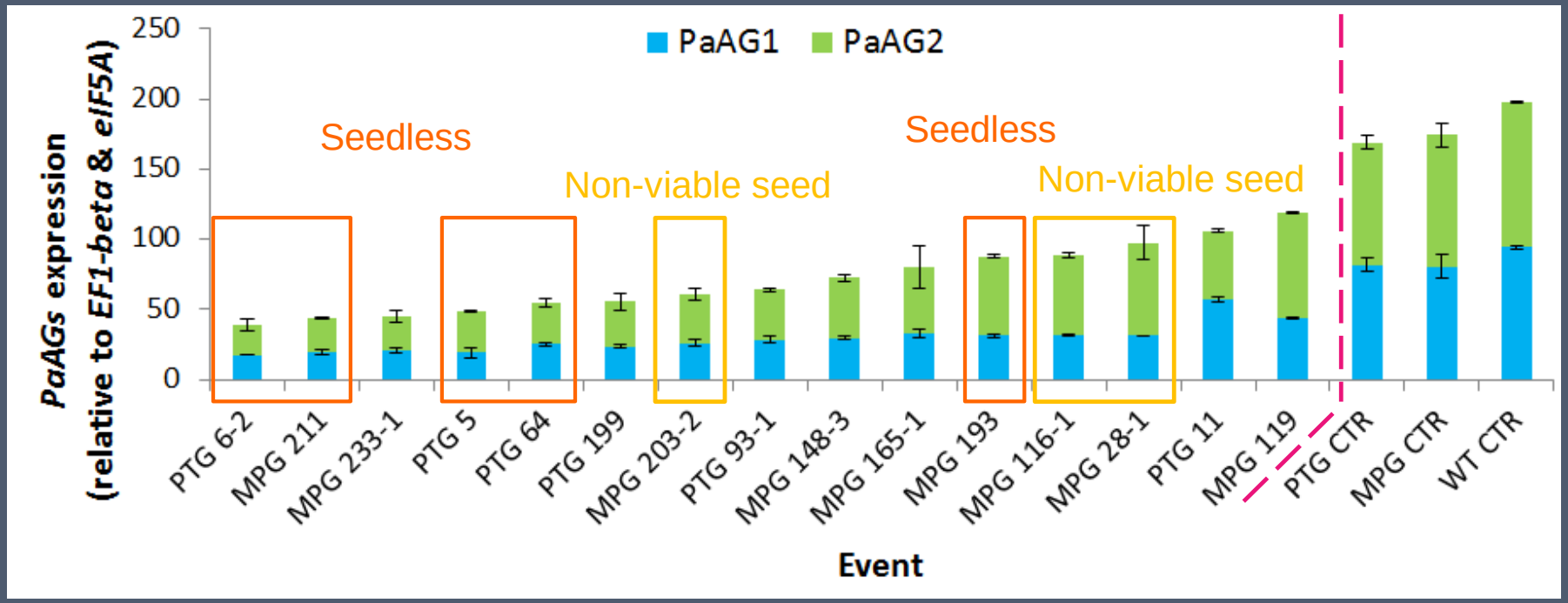
March

April



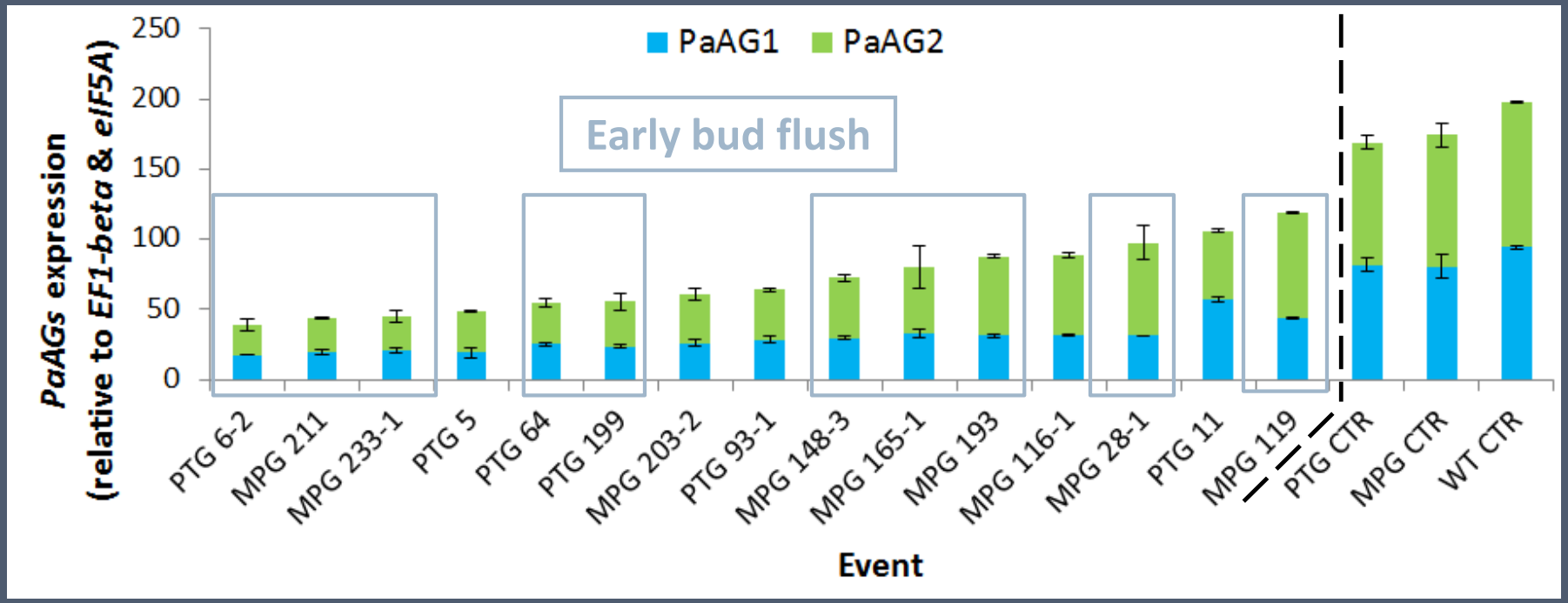


Suppression of the two *PaAG* paralogs were imperfectly associated with the sterility phenotype



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

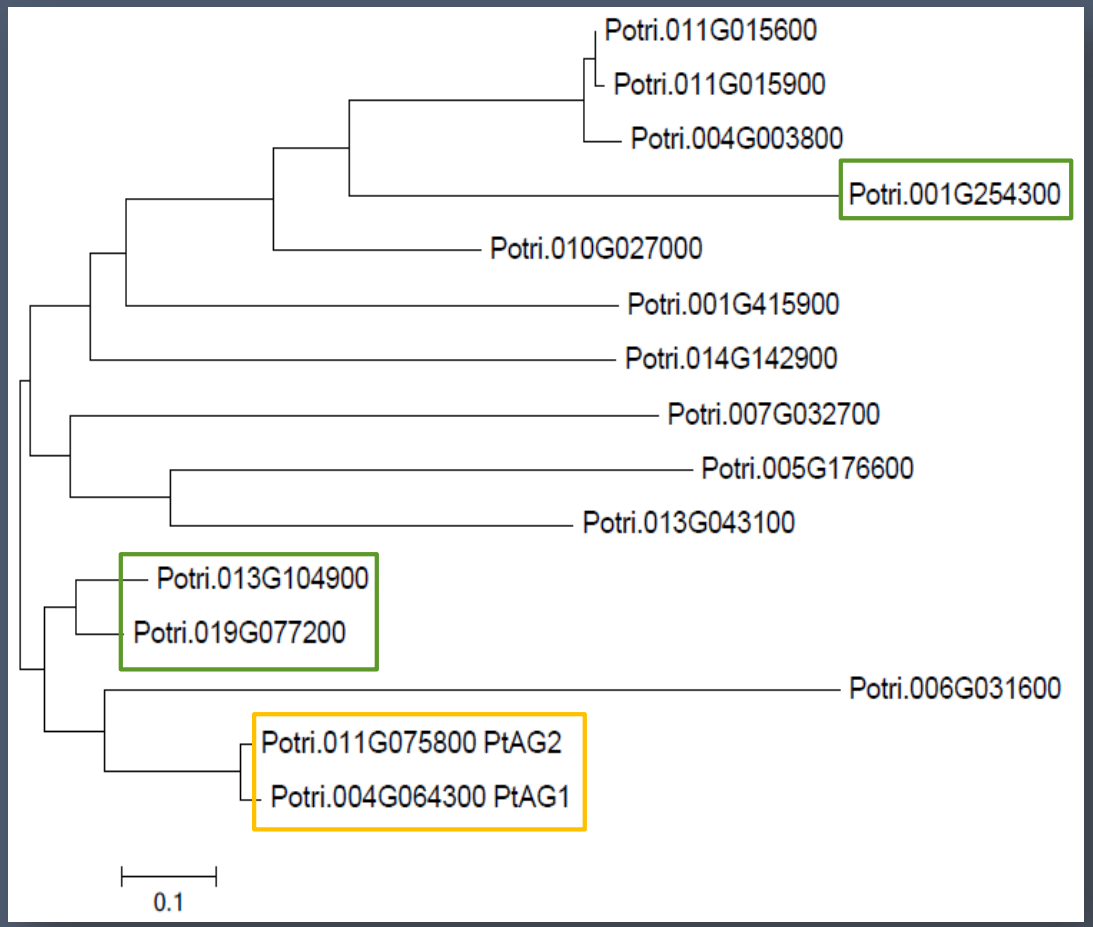
The timing of bud flush was also imperfectly associated with *PaAG* expression



Could off-target RNAi suppression be playing a role?

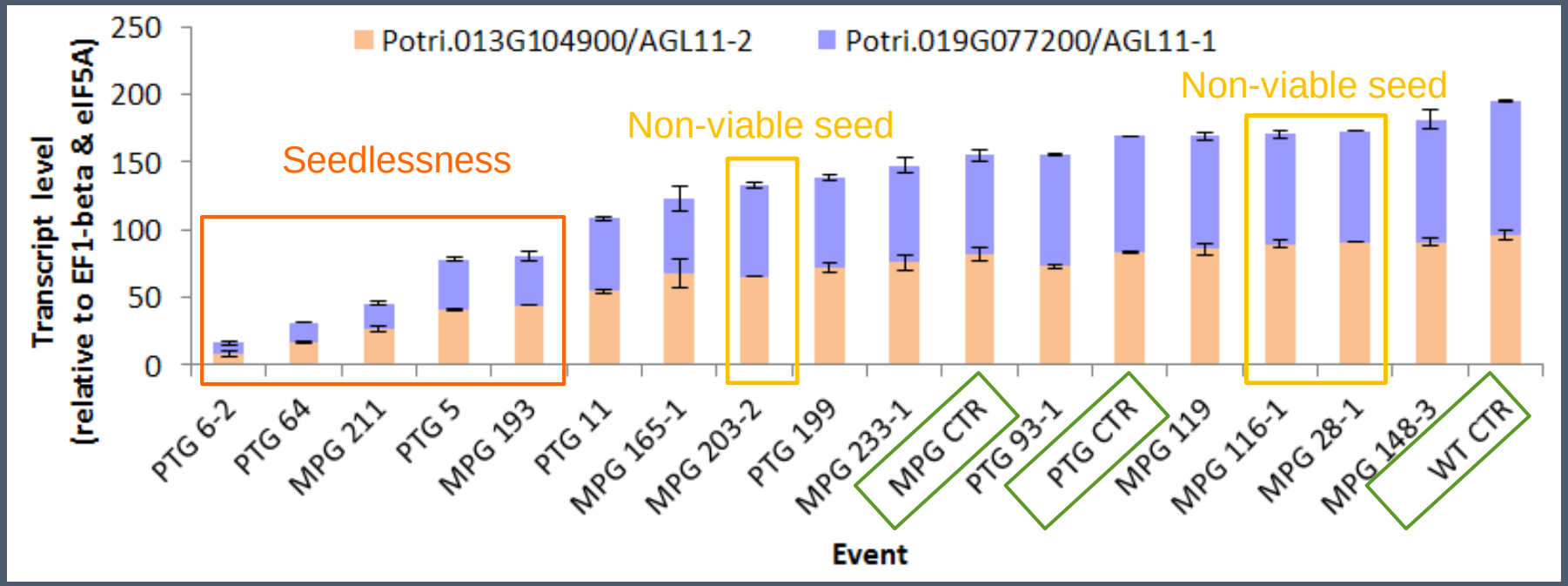
- Blasted poplar genome with dsRNA from RNAi constructs or parts thereof
- Aligned DNA sequences to identify regions with ≥ 6 bp identity with dsRNA – found 13 potential off-target genes
- Studied expression of homologs in *Arabidopsis* expression atlas (ePlant) and poplar ignored those without significant 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. Yanofsky[1]

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

BMC Plant Biology

RESEARCH ARTICLE Open Access

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

Nilo Mejía[1*], Braulio Soto[1], Marcos Guerrero[1], Ximena Casanueva[1], Cléa Houel[2], María de los Ángeles Miccono[1], Rodrigo Ramos[1], Loïc Le Cunff[3], Jean-Michel Boursiquot[3], Patricio Hinrichsen[1] and Anne-Françoise Adam-Blondon[2]

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

BMC Genomics

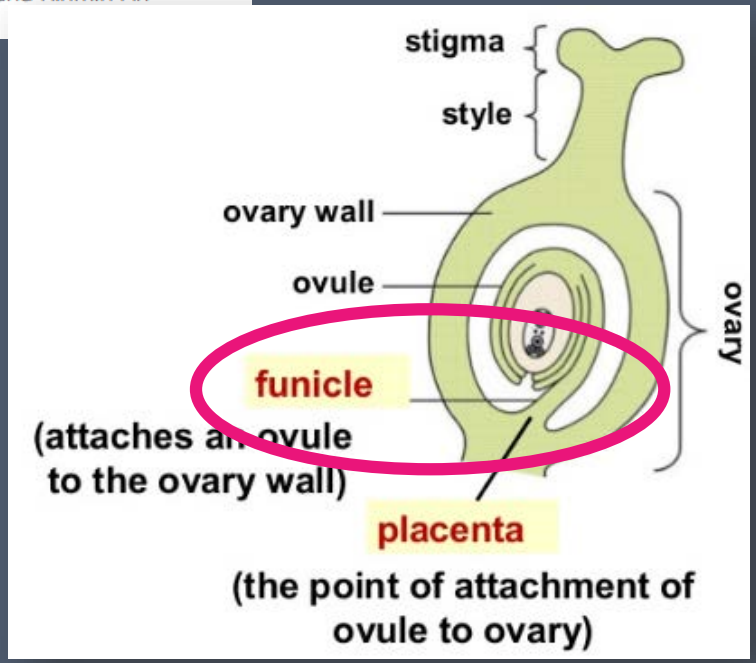
RESEARCH ARTICLE Open Access

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

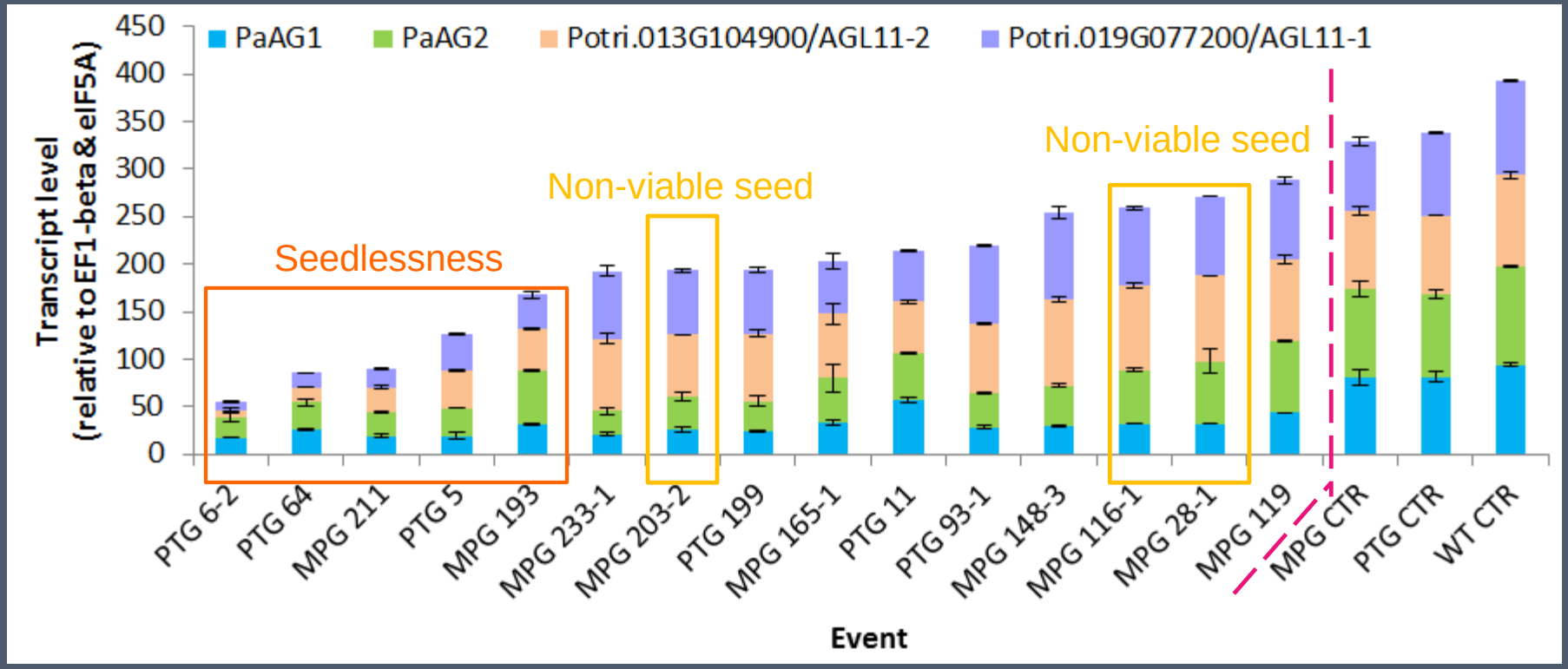
Meixia Ye[1], Zhong Chen[1†], Xiaoxing Su[2], Lexiang Ji[1], Jia Wang[1], Weihua Liao[1], Huandi Ma[1] and Xinmin An[1*]

Seed hairs originate from the epidermal cells of the funicle

Suppressed ovule development, no funicle, no seed hairs?

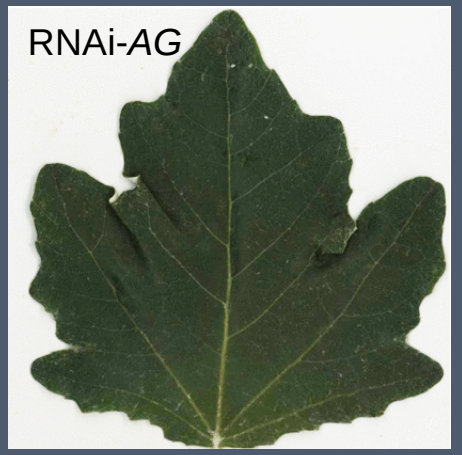


Seedlessness phenotype also strongly correlated with total expression of *AG* and *AGL11*



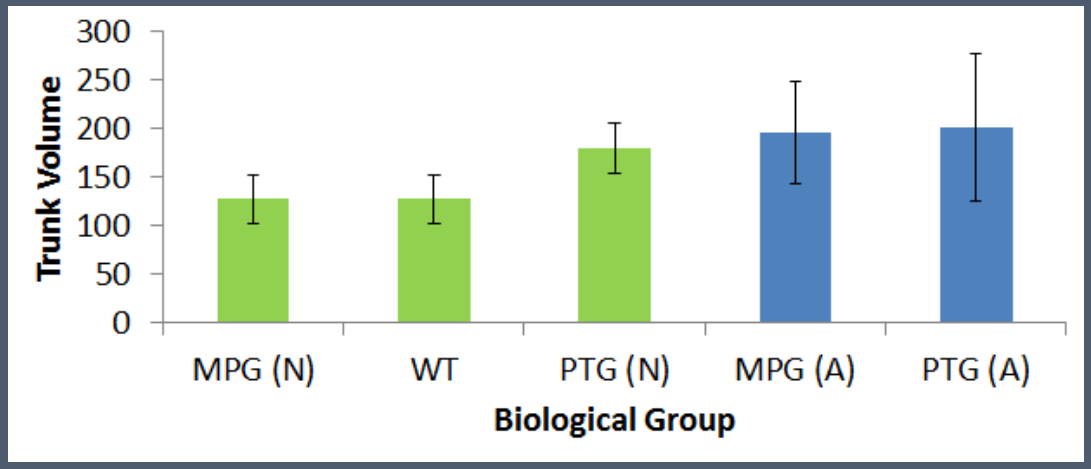
Correlation among AG and AGL paralogous pairs weak: r = 0 .50

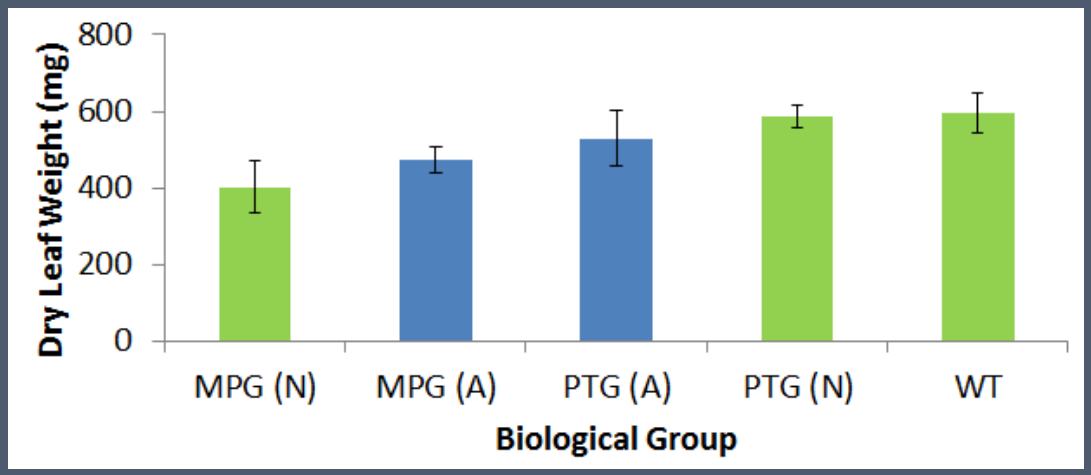
AG-RNAi events had normal tree and leaf form



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