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

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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
Male sterility is highly effective and stable in the field.

Negative impact on tree health was observed in poplar by Zhang et al. 2012; Elorriaga et al. 2014.
Bisexuality

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\textsuperscript{1-4}. Unlike other crops, trees are long-lived, weakly domesticated and their propagules can spread over several kilometers\textsuperscript{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\textsuperscript{6,7}, barnase can reduce rates of genetic transformation and vegetative growth\textsuperscript{8}. Furthermore, barnase expression may not be fully stable\textsuperscript{9}. Bisexual sterility would allay concerns over seed dispersal, could be used to control invasive exotic trees, and might increase wood production\textsuperscript{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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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
The ABC model – combinatorial interactions control floral organ development
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?
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
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:**

- LB
- tNOS
- nptII
- pNOS
- tOCS
- PtAG
- intron
- PtAG
- p35s
- RB

**MPG = MAR / RNAi-AG / MAR:**

- LB
- MAR
- tNOS
- nptII
- pNOS
- tOCS
- PtAG
- intron
- PtAG
- MAR
- p35s
- MAR
- RB
MARs can increase transgene expression level and possibly RNAi efficiency.

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

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We tested the value of a matrix attachment region (MAR) fragment derived from a neomycin resistance gene in the frequency of Agrobacterium-mediated transformation. A binary vector that carried an intron and an nptII gene was modified to contain flanking MAR elements. Vectors containing or lacking MARs were then used to transform tobacco, a readily transformable species, and a recalcitrant poplar clone (Populus trichocarpa × P. alba), and a recalcitrant poplar clone (Populus trichocarpa × P. alba). MARs also increased the frequency of kanamycin-resistant shoots recovered.

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

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Keywords: 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.
MARS induced a high rate of RNAi floral modification

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

MAR elements more than tripled RNAi suppression frequency
Floral buds on altered events flushed early

2015 data

2016 data
Altered events showed a “carpel-inside-carpel” phenotype

WT

AG-RNAi

stigma

ovule
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 $\geq 6$ bp identity with dsRNA – found 13 potential off-target genes
• Studied expression of homologs in *Arabidopsis* expression atlas ([ePlant](http://epic.plantbiology.msu.edu)) 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.

Diverse Roles for MADS Box Genes in Arabidopsis Development

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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 the to previously isolated MADS box genes, whereas the four genes

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

Nilo Mejía¹, 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.

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

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

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