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

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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.

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. Unlike other crops, trees are long-lived, weakly domesticated and their propagules can spread over several kilometers. 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, barnase can reduce rates of genetic transformation and vegetative growth. Furthermore, barnase expression may not be fully stable. Bisexual sterility would allay concerns over seed dispersal, could be used to control invasive exotic trees, and might increase wood production. 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

Genetic interactions among floral homeotic genes of *Arabidopsis*

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<tr>
<td>field</td>
<td>A</td>
<td>B</td>
<td>C</td>
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Wild type

- PI
- AP3
- AP2
- AG
- Se
- P
- St
- C

Diagram showing floral organ development with sectors labeled P (Pistil), St (Stamen), and C (Carpel).
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
- RB
MARs can increase transgene expression level and possibly RNAi efficiency
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>
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<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>
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<tbody>
<tr>
<td>AG-RNAi (PTG)</td>
<td>22/22</td>
<td>22 (100%)</td>
<td>6 (27%)</td>
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<td>MAR-AG-RNAi (MPG)</td>
<td>13/13</td>
<td>12 (92%)</td>
<td>11 (92%)</td>
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<tr>
<td>Non-transgenic (WT)</td>
<td>24/24</td>
<td>19 (79%)</td>
<td>0 (0%)</td>
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MAR elements more than tripled RNAi suppression frequency
Floral buds on altered events flushed early

![Stage 0](image1)
![Stage I](image2)
![Stage II](image3)
![Stage III](image4)

2015 data

![Line graph](image5)

2016 data

![Line graph](image6)
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.

MPG event 165-1

2014

2015

2016

2017
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

WT CTR

MPG 211
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) 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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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

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

USDA
TBGRC