Modifying Trees To Meet The Sustainability and Economic Challenges Of the Bioproducts Industry

PAG 2013
Maud Hinchee
July 23, 2012

The Southeastern US is the nation’s “wood basket”

- 29% of the forest land of the US

Southern US forests are “working forests”

- Produce 18% of world’s pulp and paper products (although only 2% of the world’s forests)*

Requirements for continued economic viability of the Southeastern US forest industry

- Increased profitability in staying a forest landowner by increasing the capability to produce more wood per acre per year
- Shortened rotation times (i.e. harvesting pine in 18 years instead of 23 years)
- Improved genetics that increases the value of wood in end-product processing
- Accessible, consistent and inexpensive hardwood supplies
- Additional end use markets for wood which expands on traditional markets

Eucalyptus could address southeast U.S. forest industry’s hardwood availability and cost challenges

- *E. grandis x E. urophylla* hybrid in south Florida
  - Grows 15-20 ft/yr
  - 20-30 green tons/ac/yr at 2.5 - 3 year rotation

Where Eucalyptus can be grown

- *E. benthamii*
- *Biotech E. urograndis*
- *E. urograndis*
**AGEH427 Freeze Tolerant Eucalyptus**

- Base clone – *E. grandis* x *urophylla* EH1 variety
- CBF (dreb) gene and mechanism
  - Cold responsive transcription factor in plants
  - Induces cold protection pathway
  - Found across all plant species, but plants adapted to tropical climates do not properly express pathway
  - First demonstrated in transgenic *Arabidopsis* that overexpression of inserted CBF genes can promote cold and freeze tolerance
- Expression can be controlled by a cold inducible promoter
  - Rd29a – *Arabidopsis* promoter that drives gene expression in response to cold and other abiotic stress

**Freeze Tolerant Eucalyptus (AGEH427)**

- Rd29a:CBF2 confers freeze tolerance in chamber tests
  - Artificial acclimation
  - Cold chamber for 12-24 hrs at different below freezing temperatures
  - Recovery in greenhouse

**Field Trial Examples of Freeze Tolerance (Line AGEH427)**

- Results from first winter in South Carolina
- Results from second winter in Alabama

**Our Freeze Tolerant Eucalyptus Contains Pollen Control**

- Utilizes an anther/male cone-specific promoter from pine and modified barnase with reduced RNase activities
  - *Pinus radiata* anther specific promoter
  - PrMC2
  - Modified *Bacillus amyloliquefaciens* barnase coding sequences
    - Single amino acid substitutions were generated: barnaseH102E, barnaseK27A, barnaseE73G and barnaseF106S.
    - Relative activity estimated by *E. coli* modified barnase transformants (w/o barstar) colony growth characteristics (colony size and number)
      - strongest to weakest (no colonies up to 1.0mm colonies after 24hr)
      - barnaseK27A > barnaseF106S > barnaseE73G = barnaseH102E = barnaseH102Y

**First Demonstrated in Tobacco**

- All 18 barnase:H102E tobacco transgenic lines containing the construct did not produce pollen.
- All 12 GUS tobacco lines produced normal pollen.
**Demonstrated in Eucalyptus**

- Eucalyptus occidentalis lines produced
- Flowering occurred 4 months after planting in greenhouse
- 22 of the 23 lines had completed pollen control; all wild-type trees produced pollen.

**Microscopic view of material in anthers prior to anthesis**

- 23 Eucalyptus occidentalis lines produced
- Flowering occurred 4 months after planting in greenhouse
- 22 of the 23 lines had completed pollen control; all wild-type trees produced pollen.

**Microscopic images of contents from dissected anther of barnaseH102E flowers and control flowers**

- Aborted Pollen Grains
- Normal Pollen Grains
- Anther Tissue Debris

**Results from Field Grown Eucalyptus**

<table>
<thead>
<tr>
<th>Field Location</th>
<th>Construct</th>
<th># of Lines / Total # of Trees Studied</th>
<th># of Lines with Pollenless Phenotype</th>
<th>Did Pollenless Lines Show Stable Expression?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central Florida</td>
<td>pARBS8</td>
<td>19 / 67</td>
<td>27</td>
<td>Yes</td>
</tr>
<tr>
<td>Southern Alabama</td>
<td>pARBS8 or pARB599</td>
<td>12 / 36</td>
<td>12</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Pollen control in freeze tolerant Eucalyptus**

- Microscopic pollen ablation analyses in >4,500 flowers collected from transgenic lines and untransformed control trees from two different field trials

**Location** | Tree Age at flower collection | # Lines | Number of trees sampled | Pollen production |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Southern Alabama</td>
<td>2 yr</td>
<td>control</td>
<td>8</td>
<td>Yes</td>
</tr>
<tr>
<td>Central Florida</td>
<td>4 yr</td>
<td>control</td>
<td>12</td>
<td>96</td>
</tr>
</tbody>
</table>

**Demonstrating Pollen Control in Pine**

- Top graft transgenic scions onto 7 year old trees
- Each scion labeled with a metal tag and a colored flag; the graft union was painted.

**Pollen Control in Field Grown Pine**

- 17 barnaseH102E lines, 6 GUS lines and wild-type loblolly
- 16 of the 17 barnaseH102E lines tested produced no pollen, with one barnaseH102E line producing a small quantity of abnormal pollen
- 6 GUS lines and wild-type loblolly grafts produce pollen.
Excellent Repeatable Pollen Control in Pine Across Multiple Lines, Constructs, Locations

<table>
<thead>
<tr>
<th>Test Field Locations</th>
<th># of Years Data was Collected</th>
<th>Experimental Materials</th>
<th>Number of Constructs</th>
<th># of Transgenic Line Studied</th>
<th># of Lines with Pollen-less Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southern Georgia</td>
<td>5</td>
<td>Pinus rigida $\times$ Pinus taeda grafts</td>
<td>1</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Southern Georgia</td>
<td>2</td>
<td>Pinus taeda grafts</td>
<td>5</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Eastern South Carolina</td>
<td>2</td>
<td>Pinus taeda grafts</td>
<td>5</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

All constructs in the table contain the pollen ablation cassette of PrMC2::BarnaseH102E

Ablated tapetum is likely cause of pollen abortion in barnaseH102E flowers and male cones

- Pollen development was arrested at the tetrad stage.
- No clearly defined quadrant boundaries can be seen.
- Microspore nuclei DNA appears disorganized and degraded.
- Tetrads were disrupted before the formation of individual pollen grains.

Summary

- ArborGen's freeze tolerant Eucalyptus demonstrated excellent biomass production in many, multiple year field trials in the lower Southeast U.S. A valuable plantation hardwood for the pulp, paper and bioenergy/biofuels industries
- Pollen control can be useful in aiding in gaining regulatory approvals for long term field trials of perennial tree species
- PrMC2::barnaseH102E confers essentially complete pollen control in multiple species: Nicotiana tabacum, Eucalyptus occidentalis, E. grandis $\times$ E. urophylla, Pinus taeda, P. rigida $\times$ P. taeda.
  - Stable performance when tested over multiple locations.
  - Consistent performance over multiple years.
  - Works when stacked with other genes of interest.

This research was the work of:

Dr. Chunsheng Zhang  
Dr. William Rottmann  
Kimberly Norris-Caneda  
Dr. Shujun Chang  
J. Eric Gulledge  
Brian Kwan  
Dr. Les Pearson  
Dr. Michael Cunningham  
and many others!