Forest Health Initiative
Phase 2
Research at UGA

Clonal Testing/Gene Transfer Project

Scott Merkle

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FHI Jargon

- TACF = The American Chestnut Foundation
- ACCF = American Chestnut Cooperators Foundation
- VDF = Virginia Department of Forestry
- LSA = Large surviving American chestnut (potentially has some blight resistance)
- B3F3 (or BC3F3) = advanced generation hybrid backcross tree from TACF’s breeding program
  - OP = open-pollinated (half-sib)
  - CP = control-pollinated (full-sib)
- SE = somatic embryogenesis or somatic embryo
- SS = somatic seedling
- CG = candidate gene for blight and/or Phytophthora resistance
- Va Tech = Hokies
FHI Phase 2 Objectives - UGA

1. Complete production of populations of LSA and B3F3 somatic seedlings for clonal testing
2. Produce transgenic chestnut somatic seedlings for all CGs that are not yet represented in field tests (and for which we have insufficient numbers of events or trees per event)
3. Work with Clemson and Carolinas-TACF cooperators to produce trees engineered with Phytophthora resistance candidate genes and test them for resistance
Deliverable: Complete production of populations of LSA somatic seedlings for clonal testing

- Seeds from crosses between ACCF LSAs Ragged Mountain (RM) x Thompson (TH) cultured in 2010
- RM x TH somatic seedlings transferred to ACCF Cooperator Carol Croy (USDA Forest Service) in October 2013; will be planted out on Jefferson National Forest this season (see table)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM x TH-10A</td>
<td>2</td>
</tr>
<tr>
<td>RM x TH-10E</td>
<td>2</td>
</tr>
<tr>
<td>RM xTH-12A</td>
<td>6</td>
</tr>
<tr>
<td>RM xTH-29A</td>
<td>3</td>
</tr>
<tr>
<td>RM x TH-29B</td>
<td>6</td>
</tr>
<tr>
<td>RM x TH-32</td>
<td>5</td>
</tr>
<tr>
<td>RM x TH-5B</td>
<td>3</td>
</tr>
<tr>
<td>RM x TH-6B</td>
<td>5</td>
</tr>
<tr>
<td>RM x TH-8</td>
<td>7</td>
</tr>
<tr>
<td>TH x RM-5</td>
<td>3</td>
</tr>
<tr>
<td>TH x RM-7B</td>
<td>9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>51</strong></td>
</tr>
</tbody>
</table>
Deliverable: Complete production of populations of B3F3 somatic seedlings for clonal testing

- TACF OP B3F3 embryogenic cultures started in 2010, 2011
  - Somatic seedlings already planted by Va Tech cooperators

- TACF CP B3F3 embryogenic cultures initiated in 2012
  - First somatic seedlings now in lath house--available for fall 2014 planting by Va Tech cooperators

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Mother Tree</th>
<th>Pollen Source</th>
<th>No. nuts</th>
<th>No. seeds</th>
<th>No. embryogenic cultures</th>
<th>Capture frequency</th>
</tr>
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<tbody>
<tr>
<td>TACF</td>
<td>D5-17-130</td>
<td>W1-31-7</td>
<td>35</td>
<td>555</td>
<td>3</td>
<td>0.5%</td>
</tr>
<tr>
<td>TACF</td>
<td>W1-31-7</td>
<td>D3-23-53</td>
<td>42</td>
<td>621</td>
<td>5</td>
<td>0.8%</td>
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<tr>
<td>TACF</td>
<td>D3-21-53</td>
<td>W1-31-7</td>
<td>52</td>
<td>682</td>
<td>5</td>
<td>0.7%</td>
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<tr>
<td>TACF</td>
<td>D3-21-53</td>
<td>W1-30-6</td>
<td>34</td>
<td>473</td>
<td>7</td>
<td>1.5%</td>
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<tr>
<td>TACF</td>
<td>D4-17-5</td>
<td>D3-23-53</td>
<td>36</td>
<td>447</td>
<td>1</td>
<td>0.2%</td>
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<tr>
<td>TACF</td>
<td>D4-17-5</td>
<td>D4-27-78</td>
<td>33</td>
<td>476</td>
<td>2</td>
<td>0.4%</td>
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<tr>
<td>TACF</td>
<td>W1-31-7</td>
<td>D4-28-132</td>
<td>34</td>
<td>470</td>
<td>0</td>
<td>0.0%</td>
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<td>TACF</td>
<td>D4-27-78</td>
<td>W1-30-6</td>
<td>24</td>
<td>358</td>
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<td>0.3%</td>
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<tr>
<td>TACF</td>
<td>W1-31-7</td>
<td>D4-27-78</td>
<td>34</td>
<td>482</td>
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<td>0.2%</td>
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<tr>
<td>Total</td>
<td></td>
<td></td>
<td>324</td>
<td>4564</td>
<td>25</td>
<td>0.5%</td>
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Deliverable: Produce B3F3 and other hybrid somatic seedlings for Powell River and Kentland plantings

VDF 76-5xOP somatic seedlings

TACF OP B3F3 somatic seedlings

Over 100 somatic seedlings (VDF, TACF OP B3F3) supplied to Va Tech cooperators for planting in 2013
Deliverable: Produce B3F3 and other hybrid somatic seedlings for Powell River and Kentland plantings

Trees Planted in May and October 2013

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TACF B3F3 Trees</strong></td>
<td></td>
</tr>
<tr>
<td>D6-26-2</td>
<td>3</td>
</tr>
<tr>
<td>D6-26-3A</td>
<td>1</td>
</tr>
<tr>
<td>D6-26-9C</td>
<td>6</td>
</tr>
<tr>
<td>D1-26-19-1B</td>
<td>2</td>
</tr>
<tr>
<td>D3-18-61-2</td>
<td>1</td>
</tr>
<tr>
<td>D4-10-49-10</td>
<td>6</td>
</tr>
<tr>
<td>D4-10-49-5</td>
<td>8</td>
</tr>
<tr>
<td>W1-30-6-3A</td>
<td>1</td>
</tr>
<tr>
<td>W1-30-6-3C</td>
<td>1</td>
</tr>
<tr>
<td>W1-30-6-5</td>
<td>5</td>
</tr>
<tr>
<td>W1-30-63-2</td>
<td>7</td>
</tr>
<tr>
<td>W1-31-63-7A</td>
<td>1</td>
</tr>
<tr>
<td>W1-31-63-13A</td>
<td>1</td>
</tr>
<tr>
<td>W3-32-68-2</td>
<td>1</td>
</tr>
<tr>
<td>W1-31-144-11B</td>
<td>1</td>
</tr>
<tr>
<td>W1-31-144-1B</td>
<td>1</td>
</tr>
<tr>
<td>W1-31-144-3</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>49</strong></td>
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<table>
<thead>
<tr>
<th>Genotype</th>
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</thead>
<tbody>
<tr>
<td><strong>VDF Hybrid Trees</strong></td>
<td></td>
</tr>
<tr>
<td>76-5xOP-1</td>
<td>2</td>
</tr>
<tr>
<td>76-5xOP-2B</td>
<td>28</td>
</tr>
<tr>
<td>76-5xOP-3A</td>
<td>8</td>
</tr>
<tr>
<td>76-5xOP-3B</td>
<td>1</td>
</tr>
<tr>
<td>76-5xOP-5D</td>
<td>5</td>
</tr>
<tr>
<td>76-5xOP-6</td>
<td>1</td>
</tr>
<tr>
<td>76-5xOP-7A</td>
<td>4</td>
</tr>
<tr>
<td>76-5xOP-7B</td>
<td>13</td>
</tr>
<tr>
<td>76-5xOP-7C</td>
<td>2</td>
</tr>
<tr>
<td>76-5xOP-7G</td>
<td>1</td>
</tr>
<tr>
<td>76-5xOP-8</td>
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<tr>
<td>76-5xOP-9A</td>
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</tr>
<tr>
<td>76-5xOP-9C</td>
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</tr>
<tr>
<td>76-5xOP-9D</td>
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<tr>
<td>76-5xOP-9F</td>
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<tr>
<td>76-5xOP-10A</td>
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<tr>
<td>76-5xOP-10B</td>
<td>7</td>
</tr>
<tr>
<td>76-5xOP-12</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>89</strong></td>
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</table>
**Deliverable:** Produce B3F3 and other hybrid somatic seedlings for Powell River and Kentland plantings

Trees for fall 2014 planting

<table>
<thead>
<tr>
<th>Clone</th>
<th>Number</th>
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<tbody>
<tr>
<td><strong>Open-pollinated</strong></td>
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</tr>
<tr>
<td>D3-18-61-2</td>
<td>2</td>
</tr>
<tr>
<td>W1-30-6-3A</td>
<td>1</td>
</tr>
<tr>
<td>W1-31-144-3</td>
<td>1</td>
</tr>
<tr>
<td>W1-31-144-9B</td>
<td>6</td>
</tr>
<tr>
<td><strong>Control-pollinated</strong></td>
<td></td>
</tr>
<tr>
<td>D3-21-53 x W1-31-7-2</td>
<td>1</td>
</tr>
<tr>
<td>D4-17-5 x D3-23-53-1</td>
<td>3</td>
</tr>
<tr>
<td>D5-17-130 x W1-31-7-2</td>
<td>8</td>
</tr>
<tr>
<td>D5-17-130 x W1-31-7-3</td>
<td>7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>29</strong></td>
</tr>
</tbody>
</table>

Somatic seedlings in 4 inch pots in lighted chamber
Breeding programs, SE and cryostorage are a powerful combination for chestnut restoration…and elite varietals

- Selected B3F2 trees crossed to get B3F3 seeds
- B3F3 seeds used to start SE cultures
- Copies of all cultures cryostored
- "Captured" SE cultures
- Somatic seedling production
- Screening Results
- Somatic seedlings screened/field tested
- Best clones recovered from cryo and mass propagated for planting
Deliverable: Produce transgenic trees with candidate genes for Kentland and Powell River plantings

About 180 transgenic somatic seedlings in UGA lath house 08/29/13, prior to transfer to Va Tech cooperators
Construct | Genotypes transformed | Events on plates | Somatic embryos harvested | Number of events with trees | Planting Location(s)
--- | --- | --- | --- | --- | ---
pFHI-GUSi | 3 | 65 | 4074 | 6 | PR, K, JJ, UGA
pFHI-GUSiYFP | 4 | 360 | 1345 | 6 | PR, K, JJ, UGA
pFHI-NPR1 | 6 | 570 | 4467 | 5 | PR, K, UGA
pFHI-THAUM | 6 | 1,360 | 9812 | 29 | PR, K, UGA
pFHI-ACPHOS | 6 | 308 | 844 | 1 | UGA
pFHI-UDP GT | 7 | 335 | 4456 | 2 | UGA
pFHI-PRP | 4 | 1,307 | 4163 | 21 | PR, K, UGA
pFHI-LAC | 4 | 367 | 4380 | 10 | PR, K, UGA
pFHI-BGLUC | 3 | 109 | 3831 | 7 | PR, K, UGA
pFHI-DAPH | 4 | 556 | 3407 | 1 | PR, K
pFHI-CBS | 4 | 312 | 4467 | 7 | PR, K, UGA
pFHI-ETF | 4 | 366 | 3213 | 7 | PR, K, UGA
pFHI-GAFP | 4 | 255 | 2783 | 7 | PR, K, UGA
pFHI-CYST | 5 | 334 | 5074 | 6 | PR, K, UGA
pFHI-LTP1 | 4 | 171 | 3654 | 2 | UGA
pFHI-RPH | 3 | 138 | 5669 | 12 | UGA
pFHI-ACOX | 3 | 175 | 681 | 2 | UGA
pFHI-MIP | 3 | 205 | 2292 | 2 | UGA
pFHI-VST | 4 | 287 | 3395 | 2 | UGA
pFHI-SKDH | 3 | 242 | 3311 | 2 | UGA
pFHI-CAD | 3 | 194 | 3101 | 6 | PR, K, UGA
pFHI-PROX | 3 | 282 | 1998 | 6 | PR, K, UGA
pFHI-CCAOMT | 5 | 458 | 272 | 0 | UGA
pFHI-GST7 | 4 | 355 | 2087 | 0 | UGA
pFHI-GLUC2 | 4 | 362 | 663 | 0 | UGA
pFHI-TAGL | 3 | 191 | 3608 | 3 | PR, K, UGA
pFHI-SBTC | 3 | 300 | 322 | 1 | UGA
pFHI-NPR34 | 3 | 310 | 1239 | 1 | UGA
pFHI-LTP2 | 3 | 357 | 509 | 1 | UGA
pFHI-AOS | 3 | 129 | 2065 | 1 | UGA
pFHI-MAE | 3 | 35 | 364 | 2 | UGA
pFHI-PAL | 3 | 71 | 3164 | 1 | UGA
pFHI-RGAF | 3 | 78 | 3150 | 3 | UGA
pFHI-23RN | 3 | 173 | 1618 | 2 | UGA
pFHI-33RNG | 3 | 131 | 1017 | 3 | UGA
Total | 11034 | 104,076 | 122 |

Five years of work, 36 gene constructs, 100,000 somatic embryos picked, 122 transgenic events in trees

PR = Powell River
K = Kentland
JJ = Joe James’ Farm
UGA = Whitehall Forest (above)
### Deliverable: Produce transgenic trees with candidate genes for Kentland and Powell River plantings

**Trees planted fall 2013**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Candidate gene</th>
<th>Gene source</th>
<th># events*</th>
<th>Total trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFHI-THAUM</td>
<td>Thaumatin-like protein</td>
<td>Chinese chestnut <em>(Castanea mollissima)</em></td>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td>pFHI-BGLUC</td>
<td>β-glucanase</td>
<td>Chinese chestnut</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>pFHI-CBS</td>
<td>CBS domain-containing protein</td>
<td>Chinese chestnut</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>pFHI-LAC</td>
<td>Laccase</td>
<td>Chinese chestnut</td>
<td>7</td>
<td>41</td>
</tr>
<tr>
<td>pFHI-PRP</td>
<td>Proline-rich protein</td>
<td>Chinese chestnut</td>
<td>15</td>
<td>55</td>
</tr>
<tr>
<td>pFHI-GUSi (TG control)</td>
<td>β-glucuronidase</td>
<td>E. coli</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>pFHI-GUSiYFP (TG control)</td>
<td>β-glucuronidase plus yellow fluorescent protein</td>
<td>E. coli and Aequorea victoria</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

*Total trees: 179

*Events with at least 2 somatic seedlings*
Deliverable: Produce transgenic trees with candidate genes for Kentland and Powell River plantings

Trees planted spring 2014

<table>
<thead>
<tr>
<th>Vector</th>
<th>Candidate gene</th>
<th>Gene source</th>
<th># events*</th>
<th>Total trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFHI-CAD</td>
<td>Cinnamyl alcohol dehydrogenase</td>
<td>Chinese chestnut</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>pFHI-CYST</td>
<td>Cystatin</td>
<td>Chinese chestnut</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>pFHI-ETF1</td>
<td>Ethylene transcription factor</td>
<td>Chinese chestnut</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>pFHI-NPR1</td>
<td>Non-expressor of pathogen</td>
<td>Arabidopsis</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>response</td>
<td>thaliana</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFHI-PROX</td>
<td>Peroxidase</td>
<td>Chinese chestnut</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>pFHI-TAGL</td>
<td>Triacylglycerol lipase</td>
<td>Chinese chestnut</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total trees:</td>
<td>57</td>
</tr>
</tbody>
</table>

*Events with at least 2 somatic seedlings
Deliverable: Produce transgenic trees with candidate genes for Kentland and Powell River plantings

Trees available for transfer to Va Tech cooperators fall 2014

<table>
<thead>
<tr>
<th>Vector</th>
<th>Candidate gene</th>
<th>Gene source</th>
<th># events*</th>
<th>Total trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFHI-CBS</td>
<td>CBS domain-containing protein</td>
<td>Chinese chestnut</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>pFHI-DAPH</td>
<td>Deoxy-arabino-heptulosonate phosphate synthase</td>
<td>Chinese chestnut</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>pFHI-THAUM</td>
<td>Thaumatin-like protein</td>
<td>Chinese chestnut</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>pFHI-GUSi</td>
<td></td>
<td></td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>pFHI-GFP</td>
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<td>6</td>
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<td></td>
<td></td>
<td><strong>32</strong></td>
</tr>
</tbody>
</table>
**Deliverable:** Produce transgenic trees with candidate genes for field testing in Georgia

Single tree events to be planted at Whitehall Forest Nursery fall 2014

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Events</th>
</tr>
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<tbody>
<tr>
<td>ACOX</td>
<td>AW3-46B</td>
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</tr>
<tr>
<td>AcPHoS</td>
<td>RxT-22B</td>
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</tr>
<tr>
<td>B-Gluc</td>
<td>WB484-3</td>
<td>2</td>
</tr>
<tr>
<td>CAD</td>
<td>76-5xOP-2B</td>
<td>1</td>
</tr>
<tr>
<td>CBS</td>
<td>76-5xOP-2B</td>
<td>2</td>
</tr>
<tr>
<td>Cyst</td>
<td>AW3-46B</td>
<td>3</td>
</tr>
<tr>
<td>ETF</td>
<td>WB484-3</td>
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</tr>
<tr>
<td>GUSi</td>
<td>76-5xOP-2B</td>
<td>5</td>
</tr>
<tr>
<td>Lac</td>
<td>76-5xOP-2B</td>
<td>1</td>
</tr>
<tr>
<td>Lac</td>
<td>WB484-3</td>
<td>4</td>
</tr>
<tr>
<td>LTP1</td>
<td>AW3-46B</td>
<td>1</td>
</tr>
<tr>
<td>MIP</td>
<td>AW3-46B</td>
<td>1</td>
</tr>
<tr>
<td>NPR1</td>
<td>RxT-22B</td>
<td>5</td>
</tr>
<tr>
<td>Prox</td>
<td>76-5xOP-2B</td>
<td>2</td>
</tr>
<tr>
<td>PRP</td>
<td>RxT-22B</td>
<td>3</td>
</tr>
<tr>
<td>PRP</td>
<td>WB484-3</td>
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<tr>
<td>Thaum</td>
<td>AM54-1</td>
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<tr>
<td>Thaum</td>
<td>RxT-22B</td>
<td>6</td>
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<td>Thaum</td>
<td>WB484-3</td>
<td>9</td>
</tr>
<tr>
<td>TagL</td>
<td>AW3-46B</td>
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<tr>
<td><strong>Total</strong></td>
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<td><strong>60</strong></td>
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Deliverable: Work with Steve Jeffers (Clemson University) and Joe James (Carolinas-TACF) to screen transgenic somatic seedlings for Phytophthora resistance
Transgenic chestnut lines with genes of interest for Phytophthora resistance

- **Castanea** gene constructs
  - pFHI-RPH (Resistance to *Phytophthora*)
  - pFHI-NPR3/4 (Non-expressor of pathogen response)

- **Heterologous genes**
  - pFHI-GAFP (*Gastrodia* anti-fungal protein)
  - pFHI-VST1 (*Vitis* stilbene synthase)

- **Stacked constructs**
  - pFHI-23RN (RPH + NPR3/4)
  - pFHI-33RNG (RPH + NPR3/4 + GAFP)
Expression analysis of transgenic lines with single Phytophthora resistance candidate genes

Dark bars are wildtype controls
Expression analysis of transgenic lines with stacked Phytophthora resistance candidate genes

Multi-gene construct for “stacking” resistance genes

Highly variable expression from different promoters
Deliverable: Work with Clemson and TACF-NC collaborators to screen transgenic somatic seedlings for Phytophthora resistance – 2013 screen

- 9 GAFP and 16 RPH transgenic somatic seedlings planted at Joe James’ Farm 07/06/13
- Tubs inoculated with *P. cinnamomi* 07/30/13
- Planting inspected by APHIS 8/16/13
- Joe scored for symptoms in early December 2013:
  - 0 = no symptoms
  - 1 = slight infection of roots
  - 2 = moderate infection
  - 3 = severe infection
Deliverable: Work with Clemson and TACF-NC collaborators to screen transgenic somatic seedlings for Phytophthora resistance – 2014 screen

<table>
<thead>
<tr>
<th>Vector</th>
<th>Candidate gene(s)</th>
<th>Gene source</th>
<th># events</th>
<th>Total trees</th>
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<tbody>
<tr>
<td>pFHI-RPH</td>
<td>Resistance to Phytophthora</td>
<td>Chinese chestnut</td>
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<td>pFHI-23RN</td>
<td>RPH and NPR3/4</td>
<td>Chinese chestnut</td>
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<td>5</td>
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<td>pFHI-GUSi</td>
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<tr>
<td></td>
<td>Total trees</td>
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<td>12</td>
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</tbody>
</table>

Trees planted at James Farm 07/3/14
New Phytophthora Work

• Chestnut cambium-derived callus-based screen described by Vieitez (1961) and Grente (1961)
• Plus/minus screen where susceptible *C. sativa* callus turned black within 3 days following inoculation and resistant *C. chinensis* callus did not change color
• First trial with Steve Jeffers (Clemson Univ.) in June 2013 failed due to rapid *P. cinnamomi* spread to tissue culture medium in Petri plates
• Second trial using 48-well-plates to restrict *P. cinnamomi* access to medium is underway
Bottlenecks and plans for improvement

- **Problem:** Plenty of events and somatic embryos, but persisting problems with low somatic embryo conversion % and low somatic seedling quality

- Testing temporary immersion bioreactors (TIBs) for production of more vigorous shoots that may root better
- Testing misting bench for rooting and hardening off
- Personnel change

RITA® TIBs with chestnut shoots
Specific objectives for coming year

- Continue to thaw cryostored cultures to “fill-in” somatic seedling production for B3F3s and CGs/events with insufficient numbers of somatic seedlings for replicated field tests
- Apply SUNY-ESF leaf assay and begin inoculations of transgenic trees at Whitehall Forest
- Expand work to improve somatic seedling quality (previous slide)
- Produce more somatic seedlings with Phytophthora resistance CGs (single and stacked) for larger screen at Joe James’ farm in 2015
- Continue work to develop *in vitro* screen for Phytophthora resistance
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Candidate Gene vector construction
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Steve Strauss (OSU/FHI advisor)
Steve Jeffers (Clemson)
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Meg Staton (Univ. of Tennessee)
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