**Summary overview for August 1, 2009 to January 20, 2011**
**Forest Health Initiative at University of Georgia**
**Clonal Testing / Gene Transfer Project**

**Scott Merkle (PI) and Joe Nairn (Co-PI)**
**Athens, GA**

**Abbreviations used:** AC = American chestnut; OP = open pollinated; CP = control-pollinated; SE = somatic embryo; SS = somatic seedling; CG = candidate gene; LSA = Large surviving American chestnut parent tree; BC1 = first backcross generation; B3F3 = Backcross 3-F3 generation

Project Objectives

1. Coordinate with Germplasm/Breeding Group to collect germplasm and initiate new embryogenic cultures.
2. Test somatic embryo/somatic seedling production abilities of new cultures.
3. Cryostore copies of all embryogenic cultures.
4. Construct vectors for cloning and expression of candidate resistance genes.
5. Establish field (nursery) sites in Georgia with current "first generation" transgenic lines.
6. Molecular screening of regenerated transgenic American chestnut.

**Year 1 (August 2009 – July 2010) Deliverables Summary**

1. Embryogenic culture initiation from full-sib American chestnut & hybrid germplasm
   - Over 9000 seeds cultured
   - 64 new embryogenic cultures initiated from 2 CP families & 1 OP family from ACCF and VDF
   - Embryogenic culture capture rate = 0.7%
   - 38 (1.5%) ACCF LSA cultures
   - 26 (5.2%) VDF OP hybrid (ACxCCxJC) cultures
2. Screening for somatic embryo (SE) & somatic seedling (SS) production
   - Some 2009 lines produced > 100 well-formed embryos per 0.5 g of starting material
   - Very high (>50%) conversion in some lines
3. Cryostorage of embryogenic cultures
   - > 3 copies of each line
   - All (64) 2009 embryogenic lines cryostored
   - All (276 so far) FHI transclones cryostored
4. Construct 1st generation vectors.
5. Characterization of *pFHI-03*-based reporter vectors.
6. Held a "Vector" workshop for science and science advisory groups.

**Year 2 (August 2010 – July 2011) Deliverables Summary**

1. Collect full-sib AC and TACF B3F3 germplasm and initiate new embryogenic cultures
   - Over 8500 seeds cultured
   - 107 new embryogenic cultures initiated from 19 different families
   - Capture rate averaged 1.23%
   - New embryogenic cultures were captured for multiple OP American chestnut families
   - First ever embryogenic cultures captured for B3F3 families representing both the Clapper and Graves lines of blight resistance

- Copies of all 2010 cultures cryostored.

2. Regenerate and establish somatic seedlings from ACCF (or other American chestnut) lines and TACF B3F3 lines
   - Six 2009 clones produced more than 10 somatic seedlings and six more clones produced 7-9 somatic seedlings for clonal testing.
   - Somatic seedlings hardened off, moved to the greenhouse in March 2011 and moved to lathe house in June 2011
3. Screen AC and B3F3 embryogenic lines for transformation competence to identify small set of "workhorse" lines.
   - One lead AC "workhorse" line (used in every transformation run with a CG) and rotating group of 2-4 backup "workhorse" lines (both AC and hybrid) transformed with each CG during Year 2
4. Assemble Candidate Gene vectors from comparative transcriptome analysis of Chinese and American chestnut
   - 16 Chinese chestnut CG vectors completed
   - 2 heterologous CG vectors completed
   - 3 reporter gene vectors constructed
5. Transform Chinese chestnut CG vectors into American chestnut lines
   - 16 Chinese chestnut CGs transformed
   - 2 heterologous genes and 3 reporter gene constructs transformed
6. Increase chestnut transformation productivity
   - Implementation of air-lift bioreactor accelerated rate of transformation runs up about 2 per month
   - 12-fold increase in transformation productivity over 2009
7. Molecular characterization of transformed American chestnut lines and regenerated plants
   - Over 150 plants containing the pTACF6 vector confirmed as stably transformed
   - Over 300 culture lines containing the first CG vectors confirmed
   - Regenerated American chestnut plants containing pTACF6 positive for transgene expression
8. Establish field (nursery) site in Georgia with current "first generation" transgenic lines
   - The UGA Group planted over 100 trees representing 10 different pTACF6 events and 10 transgenic controls in May 2011
9. Coordinate screening of somatic seedlings from transgenic lines for *Phytophthora* resistance with Clemson cooperators
   - Somatic seedlings screened for *Phytophthora* resistance on Joe James' farm during July and August 2011

**Year 3 (August 2011 – July 2012) Deliverables Reported at 6 months**

**1. Collect full-sib AC and TACF B3F3 germplasm and initiate a third round of new embryogenic cultures**

- Over 21,000 seeds cultured, resulting in 295 embryogenic cultures representing 30 different families; capture rates for different families ranged from 0% to over 10% (Table 1).
- New embryogenic cultures were captured for multiple OP American chestnut families, mainly from New York

- Embryogenic cultures were captured for all 11 of the TACF B3F3 families cultured, representing both the Clapper and Graves lines of blight resistance

2. **Make second attempt to establish pure Chinese chestnut, F1 and BC1 embryogenic cultures**
   - Standard AC embryogenesis induction treatment failed to give any embryogenic cultures from pure Chinese, F1 or BC1 seed explants for second consecutive year, indicating that pure Chinese chestnut and hybrids with large proportions of CC genome do not respond to our standard induction protocol.
   - An alternative induction treatment using plant growth regulator treatments reported to be effective for induction of embryogenesis from European chestnut (*Castanea sativa*; Vieitez 1995) resulted in the initiation of 6 embryogenic cultures from immature seed explants of two of three Chinese chestnut trees tested (Fig. 1). Somatic embryos produced by 3 of these cultures have already been harvested and are undergoing a pre-germination cold treatment. Somatic embryogenesis of Chinese chestnut has not been previously reported in the literature.

3. Demonstrate regeneration of TACF B3F3 somatic seedlings for "clonal testing" of conventionally bred chestnut material
   - First B3F3 somatic embryos have recently completed pre-germination cold treatment and have been moved to lighted incubators to complete germination. See Table 2 for final numbers of somatic embryos produced in screens of B3F3 cultures and other 2010 cultures for SE embryo production potential.
   - First somatic seedlings regenerated from 2010 cultures from the Thoroughfare Gap Tree (16 somatic seedlings from 7 clones; Fig. 2A) from seeds provided by the Virginia Department of Forestry and the Nagle Tree (13 somatic seedlings from 2 clones) from seeds provided by Herb Darling (NY-TACF).

4. Continue to transform additional Chinese chestnut candidate genes from comparative transcriptome analysis into "workhorse" AC lines with goal of transforming with 30 total CC CGs by end of Year 3
   - As of January 2012, 21 Chinese chestnut candidate genes had been cloned into the pFHI expression vector and transformed into an average of 3 American chestnut embryogenic culture lines; we are on track to complete transformation with the target of 30 CGs by the end of July 2012 (Table 3)
   - In addition, 4 heterologous anti-fungal CGs and 3 reporter gene constructs have been transformed into American chestnut lines (Table 3)

5. Continue molecular characterization of expression and delivery vectors in transformed AC lines
   - Screened over 900 transgenic events for CG integration (Fig. 3)
   - Confirmed over 860 transgenic lines each containing one of 16 CGs or 3 reporter genes
   - Confirmed transcription and translation of the GUS-intron reporter gene in AC embryogenic cultures by GUS assay
   - Confirmed transcription and translation of GFP and GUSiYFP in embryogenic cultures via imaging in live tissues (Fig. 4)

- Confirmed gene expression of ESF39A in roots and stems of 12 transgenic American chestnut plants prior to screening plants for *Phytophthora* resistance (Fig. 5)
- Standardized protocol to screen for CG expression and confirmed CG expression in embryogenic cultures at 6-8 weeks post-transformation (Fig. 6)

**6.** Continue to move transgenic cultures, somatic embryos and somatic seedlings through the "pipeline" from lab to greenhouse to nursery/field

- Table 4 shows the most current update of the movement of the different CGs through the "pipeline" as of January 20, 2012. Highlights of Table 4 include:
  - Over 160 somatic seedlings in soil have already been regenerated for 2 of the heterologous candidate genes, 1 of the Chinese chestnut genes and 1 of the reporter gene constructs; another 260 transgenic somatic seedlings are growing in vitro
    - Somatic seedlings transformed with the first FHI candidate gene (GAFP1) already grew for one season in the greenhouse overwintering (dormant)
    - Somatic seedlings transformed with the next two FHI CGs (Thaumatin-like protein and NPR1) are in the hardening-off chamber (Fig. 2B).
  - Somatic embryos have been harvested for an additional 4 Chinese chestnut candidate genes and are currently in pre-germination cold treatment

**7.** Begin screening transgenic chestnuts for blight resistance using standard resistance assay and new leaf-based assay, once it is standardized

- Trees in first field test are due to be inoculated with blight fungus in June 2012; we do not know if the leaf-based assay being developed by the SUNY-ESF group is ready to be applied yet on an operational basis.

**8.** Continue collaborating with Clemson scientists to screen additional transformed chestnuts for *Phytophthora* resistance

- Results of *Phytophthora* screen conducted by Steve Jeffers and Joe James on pTACF6 and pTACF7 somatic seedlings during the summer of 2011 indicated that these constructs did not prevent *Phytophthora* from killing the trees
- Somatic seedlings transformed with the Gastrodia anti-fungal protein (GAFP) gene are currently in the greenhouse overwintering and are scheduled to be screened for *Phytophthora* resistance at the James Farm in July 2012

**Other progress**

We have been dealing with a continuing problem of low somatic seedling quality for the first two years of the project and have been testing various modifications to our handling of the trees to try to overcome this. Generally, following potting, the somatic seedlings grew slowly and leaves showed chlorosis and curling, puckering and eventually browning at the tips. This recalcitrance began in the hardening-off chamber and continued in the greenhouse. In addition, somatic seedlings often grew plagiotropically, with very short internodes, in the pots and this continued following planting in the field. To try to improve somatic seedling quality, we have taken the following actions: (1) treatment of potting mix with Knockout Gnats™ to control fungus gnats in the peat-based potting mix, (2) changing from a peat-based potting mix, which tended to remain waterlogged, to a pine bark-based mix that drained more rapidly, (3) Using wet perlite

sand in somatic seedling trays to maintain consistent high relative humidity during hardening off, (4) moving somatic seedlings to high-intensity lighted growth chambers, (5) Growing somatic seedlings in a different greenhouse, (6) growing somatic seedlings in a lathe house instead of a greenhouse during the growing season, (7) removing original, plagiotropic main stems of some somatic seedlings to allow elongation of "stump sprouts" from root collar.

We believe at least some of these actions are having a positive impact on the quality of the somatic seedlings. Fungus gnat populations have been controlled in the hardening-off chamber and new leaves expanding on somatic seedlings in the hardening-off chamber appear to be less chlorotic and less prone to tip puckering and browning. Somatic seedlings also appeared to grow more rapidly, with healthier leaves in both the alternative greenhouse (Fig. 7A) and in the lathe house (Fig. 7B) during the summer of 2011. A few plagiotropic somatic seedlings that had their original stems removed rapidly elongated new shoots from the root collar that appear to be much higher quality than the original stems (Fig. 7C).

**Table 1.** Final 2011 embryogenic culture initiation summary

| Supplier | Mother Tree/Cross | Genotype | No. of Nuts | No. of Seeds | No. of Embryogenic Cultures | Capture frequency |
|---|---|---|---|---|---|---|
| TACF | D1-26-19 | OP B3F3 | 29 | 320 | 13 | 4.06% |
| TACF | D3-18-61 | OP B3F3 | 33 | 235 | 1 | 0.43% |
| TACF | D3-29-1 | OP B3F3 | 28 | 307 | 5 | 1.63% |
| TACF | D4-9-105 | OP B3F3 | 30 | 408 | 2 | 0.49% |
| TACF | D4-10-49 | OP B3F3 | 33 | 380 | 6 | 1.58% |
| TACF | D5-26-45 | OP B3F3 | 30 | 308 | 4 | 1.30% |
| TACF | W1-28-60 | OP B3F3 | 33 | 397 | 1 | 0.25% |
| TACF | W1-29-8 | OP B3F3 | 33 | 418 | 2 | 0.48% |
| TACF | W1-31-7 | OP B3F3 | 30 | 460 | 4 | 0.87% |
| TACF | W1-31-63 | OP B3F3 | 36 | 461 | 7 | 1.52% |
| TACF | W1-31-144 | OP B3F3 | 31 | 429 | 23 | 5.36% |
| TACF | A1349 X ML135 | CP LSA | 35 | 374 | 0 | 0.00% |
| TACF | GR119 X SM43 | CP F1 | 30 | 446 | 0 | 0.00% |
| Leffel (PA) | BR97-161 | OP BC1 | 22 | 210 | 0 | 0.00% |
| Leffel (PA) | BR04-12 | OP BC1 | 36 | 490 | 0 | 0.00% |
| Leffel (PA) | BR04-21 | OP BC1 | 34 | 442 | 2 | 0.45% |
| Leffel (PA) | BR04-37 | OP BC1 | 32 | 468 | 7 | 1.50% |
| Leffel (PA) | BR04-56 | OP BC1 | 17 | 172 | 4 | 2.33% |
| Leffel (PA) | BR04-75 | OP BC1 | 22 | 133 | 0 | 0.00% |
| Leffel (PA) | BR05-25 | OP BC1 | 29 | 53 | 0 | 0.00% |
| NY-TACF | Alessi 1 | OP AC | 30 | 402 | 42 | 10.45% |
| NY-TACF | Alessi 2 | OP AC | 30 | 404 | 8 | 1.98% |
| NY-TACF | Bedient 1 | OP AC | 27 | 371 | 3 | 0.81% |
| NY-TACF | Bedient 2 | OP AC | 28 | 214 | 0 | 0.00% |
| NY-TACF | Big Daddy | OP AC | 32 | 467 | 33 | 7.07% |
| NY-TACF | Spring Hole | OP AC | 32 | 420 | 44 | 10.48% |
| NY-TACF | Wishing Well | OP AC | 30 | 347 | 5 | 1.44% |
| NY-TACF | Kurz | OP AC | 30 | 352 | 0 | 0.00% |
| NY-TACF | Bass Mtn | OP AC | 27 | 308 | 3 | 0.97% |
| NY-TACF | Windbreak | OP AC | 33 | 474 | 25 | 5.27% |
| NY-TACF | 1-6-30006 | OP AC | 32 | 180 | 1 | 0.56% |
| NY-TACF | 2-2-90005-1 | OP AC | 10 | 15 | 0 | 0.00% |
| NY-TACF | 3-9-90005-2 | OP AC | 30 | 258 | 0 | 0.00% |
| NY-TACF | 6-13-90005-3 | OP AC | 10 | 30 | 0 | 0.00% |
| NY-TACF | 7-12-90042-1 | OP AC | 33 | 289 | 0 | 0.00% |
| NY-TACF | 7-16-90042-2 | OP AC | 37 | 398 | 18 | 4.52% |
| NY-TACF | 10-15-90042-3 | OP AC | 33 | 397 | 0 | 0.00% |
| NY-TACF | 8-14-30006-1 | OP AC | 30 | 483 | 0 | 0.00% |
| NY-TACF | 16-8-30006-2 | OP AC | 25 | 301 | 5 | 1.66% |

| | | | | | | |
|---|---|---|---|---|---|---|
| NY-TACF | 17-5-30006-3 | OP AC | 21 | 281 | 0 | 0.00% |
| NY-TACF | 18-16-30006-4 | OP AC | 19 | 214 | 8 | 3.74% |
| NY-TACF | 9-6-40011 | OP AC | 7 | 56 | 0 | 0.00% |
| NY-TACF | Van Dusen 90025 E-49 | OP AC | 30 | 312 | 0 | 0.00% |
| NY-TACF | Cadillac-Wexford, MI 90021 G-62 | OP AC | 25 | 163 | 0 | 0.00% |
| NY-TACF | Krall 90017 F-51 | OP AC | 36 | 484 | 1 | 0.21% |
| NY-TACF | Sodus 90010 B-37 | OP AC | 31 | 389 | 1 | 0.26% |
| NY-TACF | Bebolyer 90005 E-43 | OP AC | 37 | 455 | 3 | 0.66% |
| NY-TACF | Wilson-8 90009 A-33 | OP AC | 30 | 386 | 0 | 0.00% |
| NY-TACF | Rand 90020 F-48 | OP AC | 28 | 423 | 0 | 0.00% |
| NY-TACF | Gordon Hybrid 90038 D-38 | OP AC | 31 | 357 | 0 | 0.00% |
| NY-TACF | Zoar 90041 A-2 | OP AC | 29 | 249 | 0 | 0.00% |
| NY-TACF | Wells 90043 H-63 | OP AC | 25 | 142 | 0 | 0.00% |
| NY-TACF | Monroe 90026 G-41 | OP AC | 38 | 534 | 0 | 0.00% |
| NY-TACF | Bockenhauer 90012 E-35 | OP AC | 39 | 359 | 0 | 0.00% |
| NY-TACF | Moss Lake 90016 C-21 | OP AC | 30 | 213 | 0 | 0.00% |
| NY-TACF | 70036 H-61 | OP AC | 24 | 265 | 0 | 0.00% |
| NY-TACF | R-49 | OP AC | 29 | 352 | 0 | 0.00% |
| NY-TACF | Nagel-1  90003 | OP AC | 30 | 510 | 6 | 1.18% |
| NY-TACF | Nickols1 - White Hill | OP AC | 44 | 549 | 2 | 0.36% |
| NY-TACF | Nichols-2 - Crumhorn Mtn. | OP AC | 30 | 418 | 0 | 0.00% |
| NY-TACF | Pisconski-1 Larger Tree | OP AC | 31 | 319 | 0 | 0.00% |
| NY-TACF | Pisconski-1 Small Tree | OP AC | 6 | 57 | 0 | 0.00% |
| UGA | CS1 | OP CC | 32 | 350 | 5 | 1.43% |
| UGA | CS2 | OP CC | 32 | 350 | 0 | 0.00% |
| UGA | CS3 | OP CC | 32 | 350 | 1 | 0.29% |
| **Total/Avg** | | | 1888 | 21558 | 295 | 1.16% |

**Table 2.** Somatic embryo production from 2010 cultures as of January 20, 2012

| Genotype | No. Embryos Produced |
|---|---|
| **TACF B3F3 clones** | |
| D6-26-2 | 59 |
| D6-26-3A | 100 |
| D6-26-3B | 97 |
| D6-26-3C | 8 |
| D6-26-4A | 12 |
| D6-26-7 | 64 |
| D6-26-9C | 106 |
| W1-30-6-1A | 15 |
| W1-30-6-1B | 65 |
| W1-30-6-2A | 29 |
| W1-30-6-2B | 0 |
| W1-30-6-2C | 23 |
| W1-30-6-3A | 11 |
| W1-30-6-3B | 52 |
| W1-30-6-3C | 36 |
| W1-30-6-4 | 0 |
| W1-30-6-5 | 135 |
| W1-31-60-1A | 100 |
| W1-31-60-1B | 35 |
| W1-31-60-2A | 20 |
| W1-31-60-2D | 3 |
| W1-31-63-12B | 51 |
| W1-31-63-13A | 106 |
| W1-31-63-13B | 52 |
| W1-31-63-1B | 65 |
| W1-31-63-2 | 110 |
| W1-31-63-5 | 51 |
| W1-31-63-7A | 53 |
| W1-31-63-7B | 87 |
| W1-31-63-8B | 102 |
| W2-32-52-1 | 0 |
| W3-32-123-1 | 40 |
| W3-32-68-1 | 30 |
| W3-32-68-2 | 93 |
| W3-32-97 | 94 |
| W5-24-74-10 | 0 |
| W5-24-74-12A | 27 |

| | |
|---|---|
| W5-24-74-14 | 10 |
| W5-24-74-4B | 34 |
| W5-24-74-8B | 7 |
| **OP NY-TACF clones** | |
| Donowick-1 | 104 |
| Donowick-10 | 114 |
| Donowick-11 | 29 |
| Donowick-2A | 93 |
| Donowick-2B | 32 |
| Donowick-2C | 0 |
| Donowick-2D | 64 |
| Donowick-2E | 121 |
| Donowick-3A | 97 |
| Donowick-4A | 45 |
| Donowick-4B | 35 |
| Donowick-4C | 0 |
| Donowick-4D | 13 |
| Donowick-5B | 5 |
| Donowick-5C | 0 |
| Donowick-5D | 55 |
| Donowick-6B | 59 |
| Donowick-7A | 58 |
| Donowick-7B | 27 |
| Donowick-8A | 0 |
| Donowick-8B | 68 |
| Donowick-8C | 0 |
| Donowick-9 | 117 |
| Haun Orch.-2 Row1, Tree12 | 104 |
| Moss Lake 15-C-11 | 11 |
| Moss Lake 15-C-4 | 0 |
| Moss Lake 15-C-6 | 20 |
| Moss Lake 15-C-7 | 43 |
| Nagle-1B | 101 |
| Nagle-1C | 18 |
| Nagle-1D | 65 |
| Nagle-1E | 100 |
| Nagle-1F | 0 |
| Nagle-6B | 0 |
| Nagle-6C | 20 |
| Nagle-6D | 21 |
| P-38-11 | 12 |
| P-38-12 | 12 |
| P-38-14 | 0 |

| | |
|---|---|
| P-38-15 | 0 |
| P-38-16 | 0 |
| P-38-2 | 25 |
| P-38-21 | 45 |
| **OP VDF clones** | |
| TG-11 | 107 |
| TG-12 | 98 |
| TG-13B | 132 |
| TG-1C | 56 |
| TG-2A | 0 |
| TG-3A | 101 |
| TG-4 | 0 |
| TG-5A | 103 |
| TG-5B | 100 |
| TG-5C | 0 |
| TG-6B | 0 |
| TG-7A | 0 |
| TG-7B | 100 |
| TG-7C | 100 |
| TG-7D | 77 |
| TG-8A | 65 |
| **OP Vermont Clones** | |
| VT-CC040-1D | 0 |
| VT-CC040-3A | 0 |
| **Total** | 4754 |

**Table 3.** Chinese chestnut candidate gene constructs, heterologous candidate gene constructs and reporter gene constructs generated as of January 20, 2012

| Vector Construct (*pFHI-*) | Candidate Gene in *pFHI-03* | Status |
|---|---|---|
| **Chinese Chestnut Genes** | | |
| *B13Gluc* | Beta 1,3 Glucanase | Transformed |
| *CBS1* | CBS domain containing protein | Transformed |
| *DAHP* | Deoxy-arabino-heptulosonate phosphate synthase | Transformed |
| *AcPhos1* | Acid Phosphatase | Transformed |
| *UDPGT1* | UDP-glycosyltransferase | Transformed |
| *Lac1* | Laccase | Transformed |
| *PRP1* | Proline Rich Protein | Transformed |
| *Thaum* | Thaumatin-like protein | Transformed |
| *ETF1* | Ethylene Transcription Factor | Transformed |
| *Cyst1* | Cystatin, cysteine protease inhibitor | Transformed |
| *LTP1* | Lipid Transfer Protein, protease inhibitor | Transformed |
| *RPH1* | Resistance to *Phytophthora* | Transformed |
| *SKDH1* | Shikamate dehydrogenase | Transformed |
| *ACOX1* | ACC oxidase | Transformed |
| *TAGL1* | Triacylglycerol lipase | Transformed |
| *MIP* | Myo inositol phosphate synthase | Transformed |
| *CAD1* | Cinnamyl alcohol dehydrogenase-like protein | Transformed |
| *PrOx1* | Peroxidase | Transformed |
| *CcoAOMT* | Caffeoyl-CoA-O-methyltransferase | Transformed |
| *Gluc2* | Glucanase; Glycoside Hydrolase Family 17 | Transformed |
| *GST7* | Glutathione s-transferase | Transformed |
| **Heterologous Genes** | | |
| *GAFP1* | Gastrodia Anti-Fungal Protein | Transformed |
| *NPR1* | Non-expressor of Pathogen Response | Transformed |
| *CAMP1* | Capsicum Anti-Microbial Peptide | Transformed |
| *Vst1* | Vitis Stilbene synthase | Transformed |
| **Reporter Genes** | | |
| *GUSi* | GUS-intron | Transformed |
| *GUSiYFP* | GUSintron-Yellow Fluorescent Protein fusion | Transformed |
| *GFP* | Green Fluorescent Protein | Transformed |

**Table 4.** Status of Chinese chestnut candidate genes, heterologous anti-microbial candidate genes and reporter genes transformed into American chestnut embryogenic cultures as of January 20, 2012

| Construct | Target lines | Transformed lines | Geneticin resistant | Transclones on plates | Transclones in flasks | Transclones in SE production | Somatic embryos harvested | Plants in vitro | Plants in soil |
|---|---|---|---|---|---|---|---|---|---|
| pFHI-GAFP-1* | 2 | ND | ND | ND | ND | ND | ND | 0 | 29 |
| pFHI-GUSi* | 2 | ND | ND | ND | ND | ND | ND | 0 | 17 |
| pFHI-GUSi | 4 | 3 | >65 | 65 | 42 | 42 | 914 | 6 | 9 |
| pFHI-YFPGUSi | 6 | 4 | 360 | 360 | 16 | 16 | 679 | 11 | 0 |
| pFHI-NPR1 | 4 | 4 | 132 | 132 | 59 | 47 | 2,113 | 27 | 34 |
| pFHI-Thaum | 8 | 5 | > 1,360 | 1,360 | 201 | 172 | 8,855 | 218 | 75 |
| pFHI-ACPHOS | 4 | 4 | >708 | 708 | 41 | | | | |
| pFHI-UDPGTI | 4 | 3 | | | 16 | | | | |
| pFHI-cmPRP | 4 | 4 | >1,307 | 1,307 | 59 | 40 | 3,221 | | |
| pFHI-cmLac | 3 | 2 | >500 | 367 | 69 | 12 | 1,335 | | |
| pFHI-B-Gluc | 4 | 3 | >109 | 109 | 40 | 11 | 891 | | |
| pFHI-DapHi | 4 | 4 | >800 | 556 | 24 | | | | |
| pFHI-NPR1 (R1) | 4 | 4 | >550 | 438 | 10 | 10 | | | |
| pFHI-CBS1 | 4 | 4 | >600 | 312 | 71 | 10 | | | |
| pFHI-ETF1 | 4 | 4 | >600 | 366 | 79 | 45 | 2,880 | | |
| pFHI-GAFP1 (R1) | 5 | 3 | > 42 | 42 | 53 | 1 | 104 | | |
| pFHI-Cyst1 | 4 | 4 | >497 | 334 | 30 | | | | |
| pFHI-LTP1 | 4 | 3 | >201 | 171 | 56 | 4 | | | |
| pFHI-GFP | 4 | 3 | >100 | 94 | | | | | |
| pFHI-GAFP1 (R2) | 3 | 2 | >330 | 213 | | | | | |
| pFHI-ACPHOS (R1) | 4 | 4 | >1062 | 308 | | | | | |
| pFHI-UDPGTI (R1) | 4 | 4 | >1030 | 335 | | | | | |
| pFHI-RPH1 | 4 | 3 | >230 | 138 | 25 | | | | |
| pFHI-AcOX | 4 | 3 | >394 | 175 | 11 | | | | |
| pFHI-MIP | 4 | 3 | >420 | 205 | | | | | |
| pFHI-VST1 | 4 | 4 | >1159 | 287 | 10 | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| pFHI-SKDH1 | 3 | 3 | >495 | 242 | | | | | |
| pFHI-CAD | 3 | 3 | >520 | 194 | | | | | |
| pFHI-PROX | 3 | 3 | >615 | 282 | | | | | |
| pFHI-CcAOMT | 5 | 5 | >1541 | 458 | | | | | |
| pFHI-GST7 | 4 | 4 | >555 | 355 | | | | | |
| pFHI-CAMP1 | 4 | 4 | >1101 | 301 | | | | | |
| pFHI-Gluc2 | 4 | 4 | >615 | 362 | | | | | |
| pFHI-TagL (TL) | 4 | 3 | >202 | 191 | | | | | |
| **Totals** | | | | **10767** | **912** | **410** | **20,992** | **262** | 164 |

**Fig. 1.** Somatic embryogenesis from Chinese chestnut. (**A**) Chinese chestnut source tree CS1. (**B**) Proliferating Chinese chestnut embryogenic culture. (**C**) Early cotyledonary stage Chinese chestnut somatic embryos.

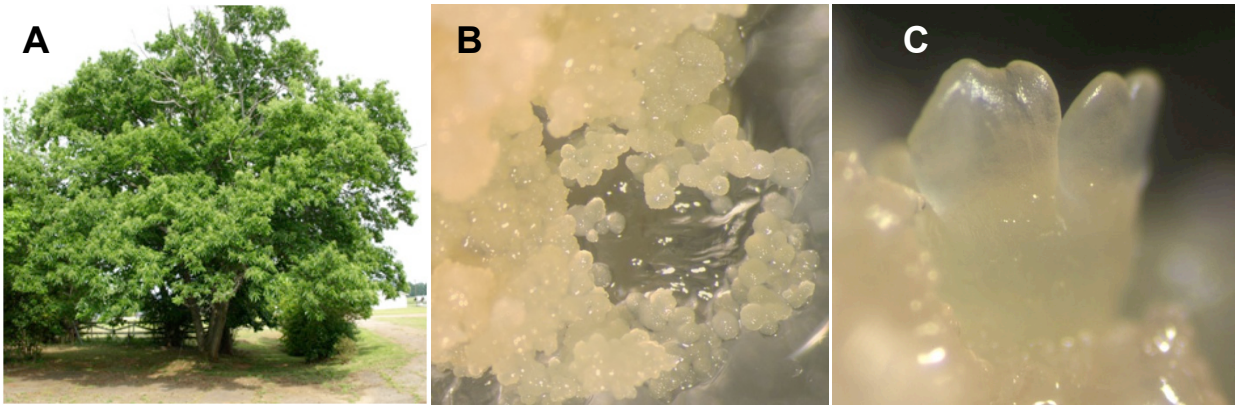


**Fig. 2.** Somatic seedlings in hardening-off chamber. **A.** Somatic seedling derived from Thoroughfare Gap tree culture (2010 culture initiation) in hardening-off chamber. **B.** Transgenic somatic seedlings transformed with the Thaumatin-like protein gene and the NPR1 gene in the hardening-off chamber.

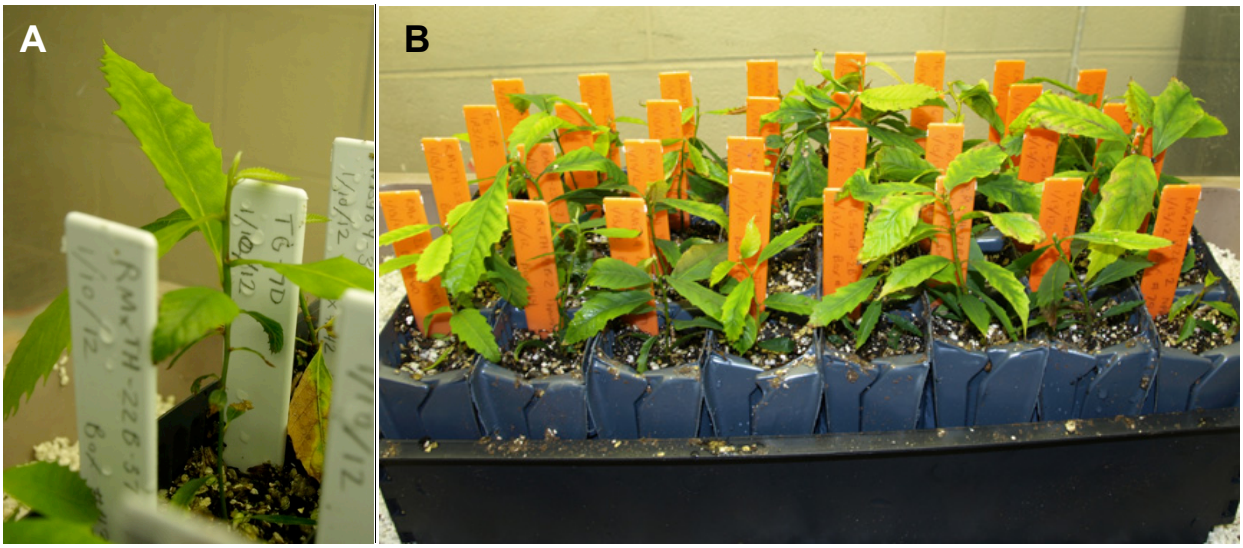

**Fig. 3.** Molecular screening of putative transgenic embryogenic tissue. Example showing event numbers 884 through 890. Lanes labeled A represent the RPH1 transgene and B lanes represent the endogenous American chestnut control gene. The wild type (WT) control is negative for the RPH1 transgene and positive for the control gene.

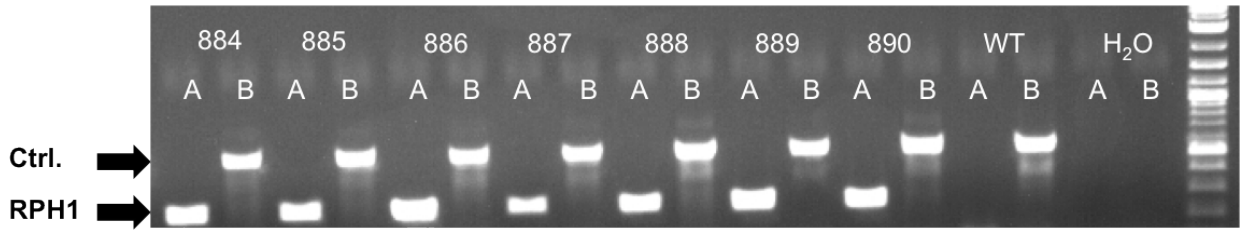


**Fig. 4.** Green fluorescent protein (GFP) expression in live American chestnut embryogenic cultures. **(A)** Map of the *pFHI-GFP* expression vector. **(B)** Visible light image of embryogenic tissue showing lighter colored transgenic and darker colored non-transgenic tissue on selection medium. **(C)** Fluorescence of the GFP corresponding to the lighter colored transgenic tissue in panel B demonstrates transcription and translation of the introduced gene in transgenic chestnut tissue.

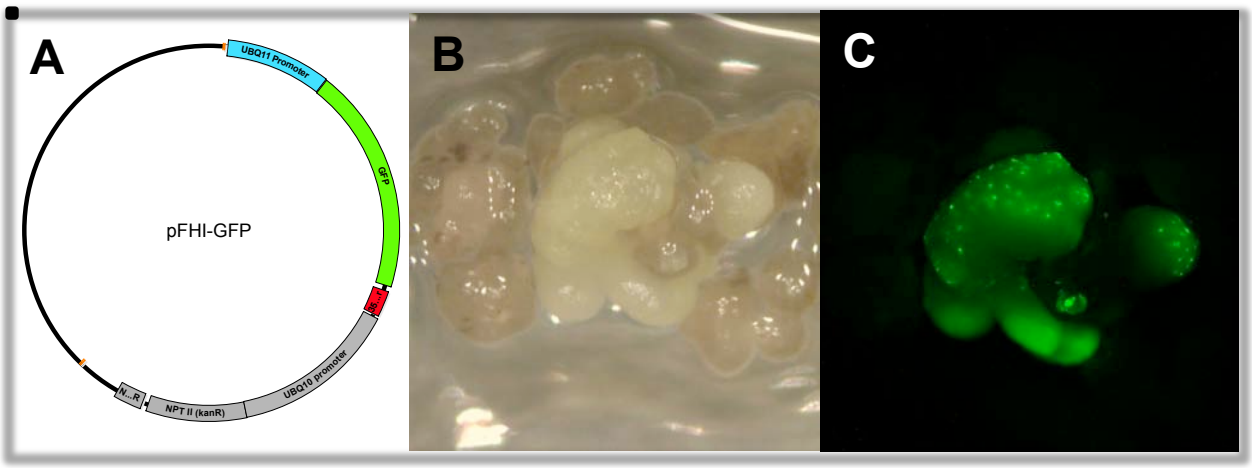

**Fig. 5.** An example of reverse transcription PCR (RT-PCR) screening results for expression of the ESF39A transgene in stems (S) and roots (R) of regenerated pTACF6 transgenic American chestnut plants. These pTACF6-positive plants were tested for *Phytophthora* resistance by Joe James and Steve Jeffers, but failed to demonstrate resistance.

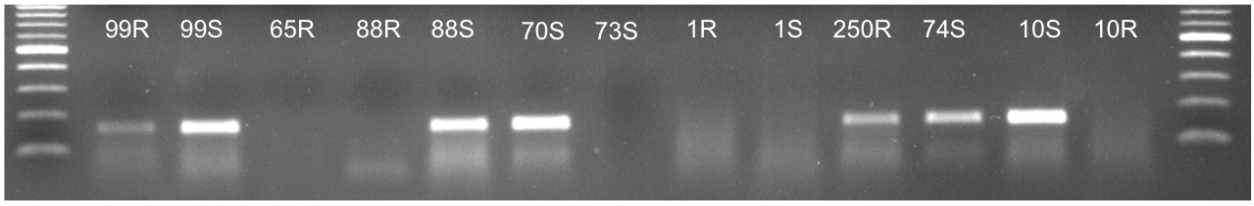


**Fig. 6.** An example of results from RT-PCR screens for expression of the Chinese chestnut proline rich protein (PRP) and laccase genes in transgenic embryogenic lines at 6-8 weeks post-transformation. Wild type (WT) controls are negative for gene expression.

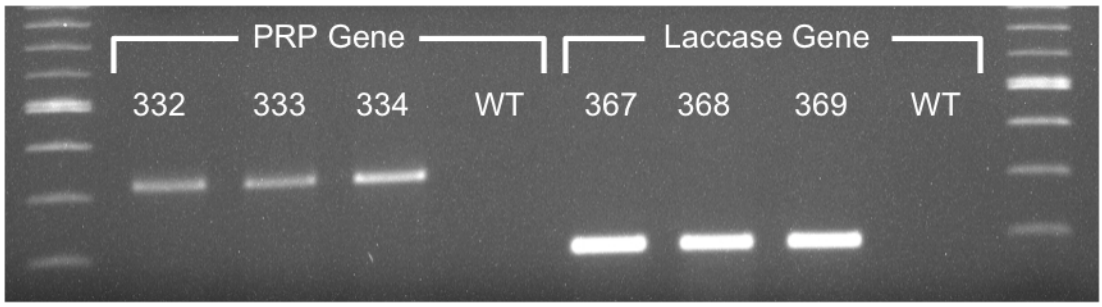


**Fig. 7.** Improvements in somatic seedling quality. **(A)** Vigorous somatic seedlings in the Plant Biology Department Greenhouse. **(B)** Vigorous somatic seedlings in the lathe house. **(C)** Rapidly-elongating "stump sprout" from somatic seedling following removal of original stem (arrow).