“The Initiative will initially focus on restoring a test species and icon of the eastern U.S. forests – the American chestnut.”

FHI website: http://foresthealthinitiative.org/index.html

2015 Science update

Forest Health Initiative,
The American Chestnut Foundation,
& the New York State
American Chestnut Research & Restoration Project

SUNY College of Environmental Science & Forestry
William A. Powell & Charles A. Maynard
Successes to date:

‘Darling 4’ American chestnut – “proof of concept” tree (intermediate resistance)

‘Darling 215’ & ‘311’ American chestnut – 2\textsuperscript{nd} generation (equal to, or possibly higher, resistance that Chinese chestnut controls)

‘Darling 58’ American chestnut – 3\textsuperscript{rd} generation elite line developed specifically for regulatory review

All use the detoxifying enzyme, oxalate oxidase, common to many plant species

Goals/objectives of research

Production & testing of transgenic American chestnut

1. Brief description of candidate cisgene search method
2. Brief description production, screening, and culling
3. Current results from leaf assays
4. Next steps with cisgenes
   A. Objectives and budgets
How do you find genes involved in blight resistance?

- ~45,000 genes in the chestnut genome
The search for cisgenes to enhance blight resistance

**Source:** Blight resistant Chinese chestnut species, *Castanea mollissima* or *C. seguinii*. Used cDNA clones attached to a constitutive promoter for overexpression.

**Known:** Quantitative resistance in *C. mollissima* with 3 major blight resistance loci accounting for ~40% of blight resistance and with other minor genes contributing.

Therefore, no single gene is expected to provided full blight resistance.

**Selection criteria** (one or more):
1. Differential gene expression between canker margins on *C. mollissima* vs. canker margins on *C. dentata*. (previous NSF grant)
2. It or a similar gene located within a known blight resistance locus or at least on same linkage group
3. Orthologs (similar genes) shown to be involved in defense response or formation of lignin barriers in other plant species.
Definition of ortholog

- **Orthologs** are genes in different species that evolved from a common ancestral gene by speciation.
- Normally, **orthologs** retain the same function in the course of evolution.
- Identification of **orthologs** is critical for reliable prediction of gene function in newly sequenced genomes.
4-year-old tree, 1 1/2 inches (3.8cm) in diameter, inoculated with the chestnut blight to evaluate their resistance level after several months.

Leaf assays can be done with 6 or more leaves per plant or clonal event, results in 3 to 7 days.

Savings of up to 4 years!

(Andy Newhouse)

PA-TACF http://www.patacf.org/patacfactivities.htm
More enzyme = higher blight resistance

Amount of OxO produced

RT-qPCR relative OxO expression in TC shoots

Necrosis area

Leaf assays

3 transgenic events
Leaf assay data
(7 to 10 leaves per experiment, most need repeating)

Leaf Assay Results (SG2-3)

Necrosis relative to American chestnut control

Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut

OxO detoxifying enzyme
Chinese chestnut cisgenes
Acid Phosphatase

Source: *C. mollissima*
Differentially expressed, CC vs. AC: **Yes**
Linkage: **Cbr3**
Possible functions from published orthologs:
phosphorus acquisition and biomass production

Leaf Assay AP Results (SG2-3)

- **Intermediate, = CC, ≥ CC**
- **Error bars** represent +/- 1 Standard Error of the mean.
  *statistically different from American Chestnut

**comparisons**
Laccase-like protein

Source: *C. mollissima*
Differentially expressed, CC vs. AC: **Yes**
Linkage: LG-A, Cbr1; Cbr3
Possible functions from published orthologs:
flavanoid biosynthesis, lignification.

*statistically different from American Chestnut

Leaf Assay LC Results (SG2-3)

Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut
Lipid transfer protein

Source: *C. mollissima*
Differentially expressed, CC vs. AC: **Yes**
Possible functions from published orthologs:
  signaling plant defense & antimicrobial

Leaf Assay Results (SG2-3)

Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut
Cystatin, cysteine protease inhibitor

Source: *C. mollissima*
Linkage: LG-E
Possible functions from published orthologs:
  antifungal, antiviral, inhibits nematodes and insects,

Effect from gene or insertion site? Need more events to be sure.

Leaf Assay CY Results (SG2-3)

Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut
Glutathione s–transferase

Source: *C. mollissima*

Linkage: LG-E

Possible functions from published orthologs:

glutathione S-transferases (GST) represent a major group of detoxification enzymes.

Effect from gene or insertion site? Need more events to be sure.

Leaf Assay GT Results (SG2-3)

Error bars represent +/- 1 Standard Error of the mean.

*statistically different from American Chestnut
Deoxy-arabino-heptulosonate phosphate synthase or DAPH (DP)

Source: *C. mollissima*
Differentially expressed, CC vs. AC: **Yes**
Linkage: Cbr3
Possible functions from published orthologs:
  Shikimate pathway, wound and pathogen response.

Effect from gene or insertion site? Need more events to be sure.

**Leaf Assay DP Results (SG2-3)**

Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut
Assorted events: **Subtilisin-like protease (SB)**, Ethylene Transcription Factor (ET), Shikimate dehydrogenase (SD), & ACC oxidase (AO)

Source: *C. seguinii*
Differentially expressed, CC vs. AC: **Yes**
Linkage: Cbr1; LG-E
Possible functions from published orthologs: protein turnover, defense signaling

**Effect from gene or insertion site? Need more events to be sure.**

**Leaf Assay Results (SG2-3)**

Source: *C. seguinii*
Differentially expressed, CC vs. AC: **Yes**
Linkage: Cbr1; LG-E
Possible functions from published orthologs: protein turnover, defense signaling

Effect from gene or insertion site? Need more events to be sure.

Leaf Assay Results (SG2-3)

Error bars represent +/- 1 Standard Error of the mean. *statistically different from American Chestnut*
Best over expression of Subtilisin–like protease cisgene was 2.3X background Compared to Lipid transfer protein gene (C. seguini, Cbr3) with up to 142X background
Another transgene: stilbene synthase (grape) 
(from Joe Nairn’s lab)

Mediates resistance to pathogens by enhancing the production of phytoalexins (example: resveratrol). Confers resistance to *Phytophthora palmivora* when expressed in papaya.

Leaf Assay VS Results (SG2-3)

Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut
Other cisgenes and events

<table>
<thead>
<tr>
<th>event</th>
<th>gene</th>
<th>significant resistance (lower necrosis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN-4PR3</td>
<td>Proline-Rich Protein</td>
<td>No (but lower necrosis, error bars don’t overlap)</td>
</tr>
<tr>
<td>LN-4PR10</td>
<td>Proline-Rich Protein</td>
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<td>LN-4LT7</td>
<td>Lipid transfer protein</td>
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<td>LN-4LT3</td>
<td>Lipid transfer protein</td>
<td>No</td>
</tr>
<tr>
<td>LN-4LT8</td>
<td>Lipid transfer protein</td>
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</tr>
<tr>
<td>LN-4CD1</td>
<td>Cinnamyl alcohol dehydrogenase-like protein</td>
<td>No</td>
</tr>
<tr>
<td>LN-4CD3</td>
<td>Cinnamyl alcohol dehydrogenase-like protein</td>
<td>No</td>
</tr>
</tbody>
</table>

More testing should be done.
Proposed work and deliverables for coming year
(with expected funding level - $87K)

• Reboot cisgene plant production
  – Estimated 3 to 6 months to get back to levels to production levels

• Test more events already in pipeline as they become available
  – no new cisgene transformations unless funding is significantly increased

• Provide trees of events that pass all tests for field trials and Phytophthora testing
  – Example 11 events from 8 gene constructs shown in leaf assay data reported today
New ideas and challenges looking forward

• Need to produce and test more events from genes showing blight resistance enhancing ability
  – To determine if the gene is causing the enhancement

• Need to test different promoters (genetic switches) to regulate expression of resistance enhancing genes
  – Example: we have events with a wound-inducible promoter (win3.12) attached to OxO
  – Can do the same for cisgenes (example: Subtilisin-like protease)

• Need to **stack** promising genes to determine if resistance is additive
  – Can be done by transformations or...
  – Can be done by breeding

• Help with breeding program by crossing transgenic trees with backcross trees to add levels of resistance from both sources
Under the spreading “American” chestnut tree photo in MI, 1980s by Alan D. Hart

Thank You!
Many supporters over the past 25 years
Estimated $6M grants & more from SUNY-ESF

The Forest Health Initiative

Camp Fire Clubs
NYSTAR
TACFNY/Monsanto Fund
Wild Turkey Federation
Mississippi State Univ.
Crowd Funding & other public donations
Unger Vetlesen Foundation
The National Hardwood Lumber Association
Northern Nut Growers Association
New York State legislative grants
Other grants in 2015
(focused on oxalate oxidase gene)

• MSU collaboration supporting PhD student - $60K/year – 4 years
• New York State legislative grant $100K/yr – duration?
  – NYS American Chestnut Research & Restoration Program
• USDA IR-4 project (priority project) - $15K
  – But more important is their help with the regulatory process
• Crowd funding campaign to propagate trees - $104K
• National TACF - $50K/year – 4 years
  – Working to raise more – estimated $100K per year
• TACFNY – just ended previous grant and working on next
  – Might help College Foundation below
• Exemplary Researchers award (Maynard & Powell) $3K ea = $6K
• ESF College Foundation – working to raise funds for production & regulatory process
  – Goal: $3M
Background/rationale of project:

“focus on restoring a test species and icon of the eastern U.S. forest”

First we need a plantable blight resistant American chestnut tree