Agricultural

Social/historic

Keystone

Forest Species

Wood products

"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^{nd} generation (equal to, or possibly higher, resistance that Chinese chestnut controls)

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

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

Are there other options? Maybe.
Combinations? Maybe.

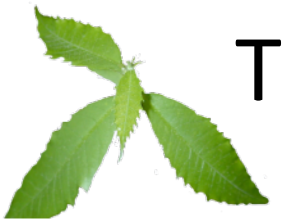
Goals/objectives of research

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

ESF

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.

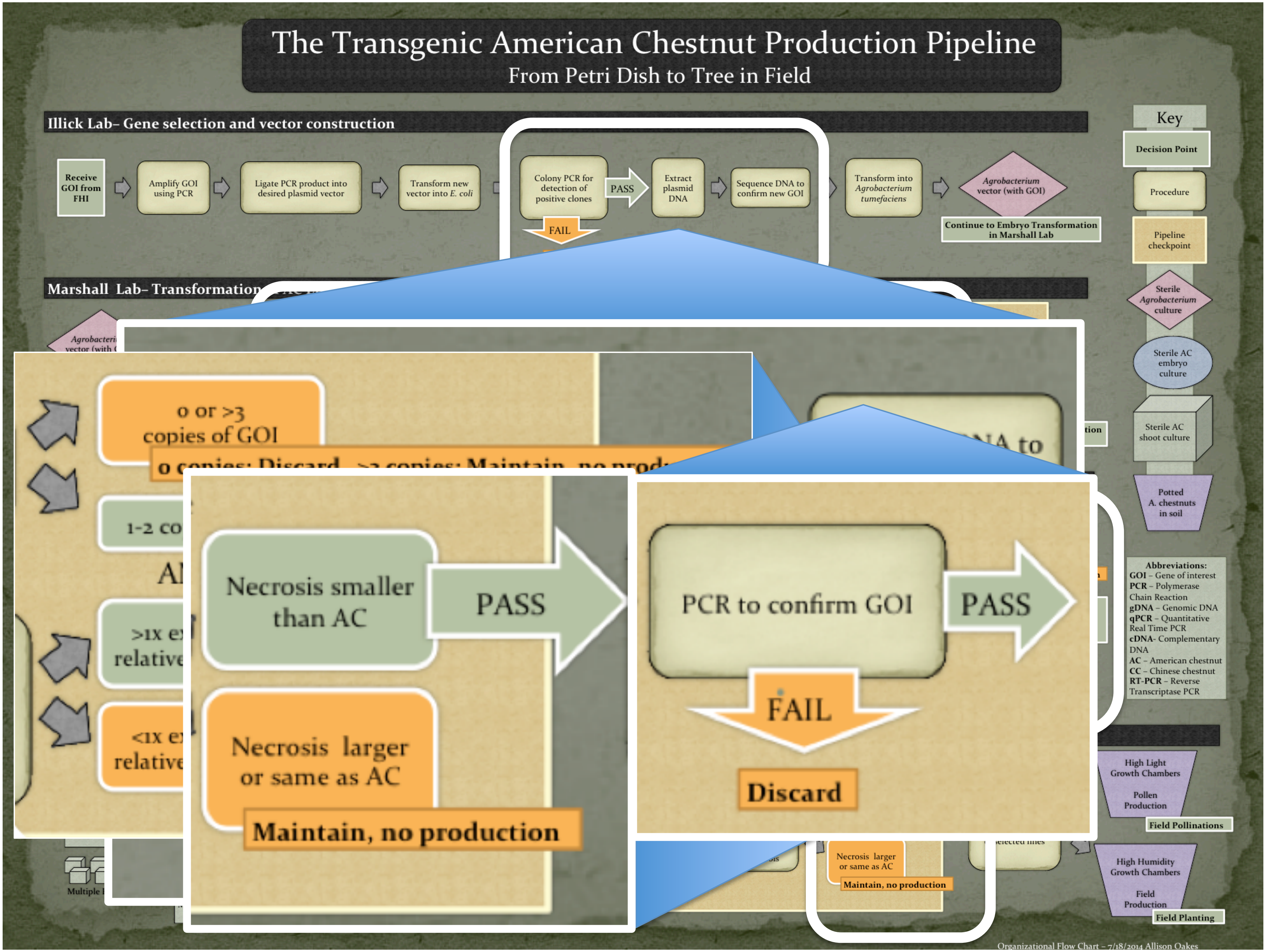
Therefore, no single gene is expected to provided full blight resistance.

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

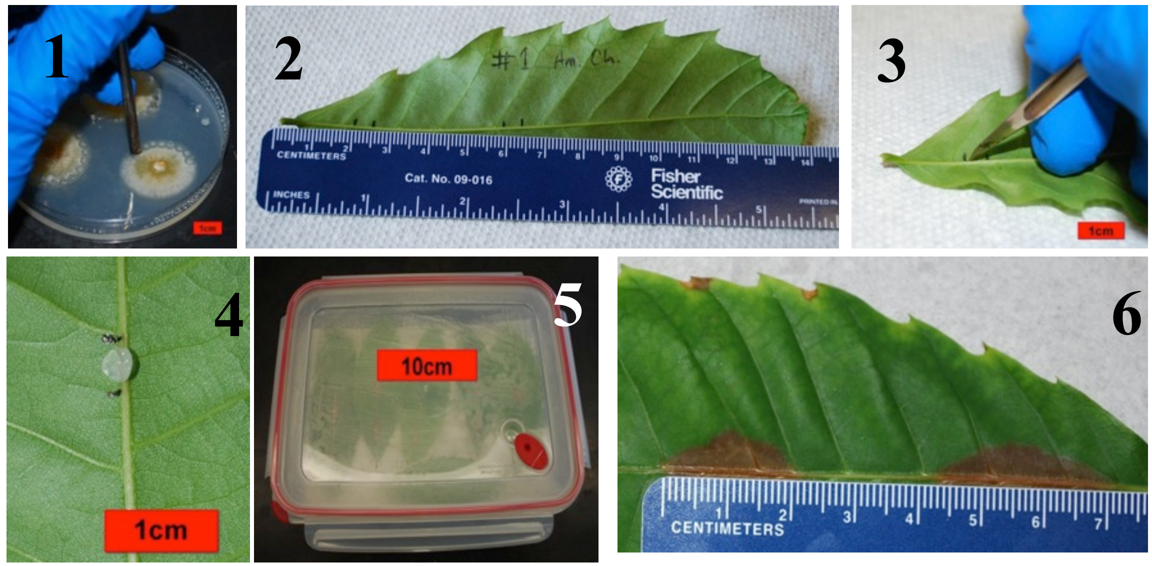
- **Orthologs** are genes in different species that evolved from a common ancestral gene by speciation.
- Normally, **orthologs** retain the same function in the course of evolution.
- Identification of **orthologs** is critical for reliable prediction of gene function in newly sequenced genomes.



The Transgenic American Chestnut Production Pipeline
From Petri Dish to Tree in Field
Illick Lab– Gene selection and vector construction
Receive GOI from FHI
Amplify GOI using PCR
Ligate PCR product into desired plasmid vector
Transform new vector into E. coli
Colony PCR for detection of positive clones
PASS
FAIL
Extract plasmid DNA
Sequence DNA to confirm new GOI
Transform into Agrobacterium tumefaciens
Agrobacterium vector (with GOI)
Continue to Embryo Transformation in Marshall Lab
Marshall Lab– Transformation
Key
Decision Point
Procedure
Pipeline checkpoint
Sterile Agrobacterium culture
Sterile AC embryo culture
Sterile AC shoot culture
Potted A. chestnuts in soil
0 or >3 copies of GOI
0 copies: Discard >3 copies: Maintain, no prod
1-2 co
>1x e
relative
<1x e
relative
Necrosis smaller than AC
PASS
Necrosis larger or same as AC
Maintain, no production
PCR to confirm GOI
PASS
FAIL
Discard
Abbreviations:
GOI – Gene of interest
PCR – Polymerase Chain Reaction
gDNA – Genomic DNA
qPCR – Quantitative Real Time PCR
cDNA- Complementary DNA
AC – American chestnut
CC – Chinese chestnut
RT-PCR – Reverse Transcriptase PCR
High Light Growth Chambers
Pollen Production
Field Pollinations
High Humidity Growth Chambers
Field Production
Field Planting
Necrosis larger or same as AC
Maintain, no production
Organizational Flow Chart – 7/18/2014 Allison Oakes

Traditional stem assay vs. leaf assay

Traditional assays:



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

(Andy Newhouse)

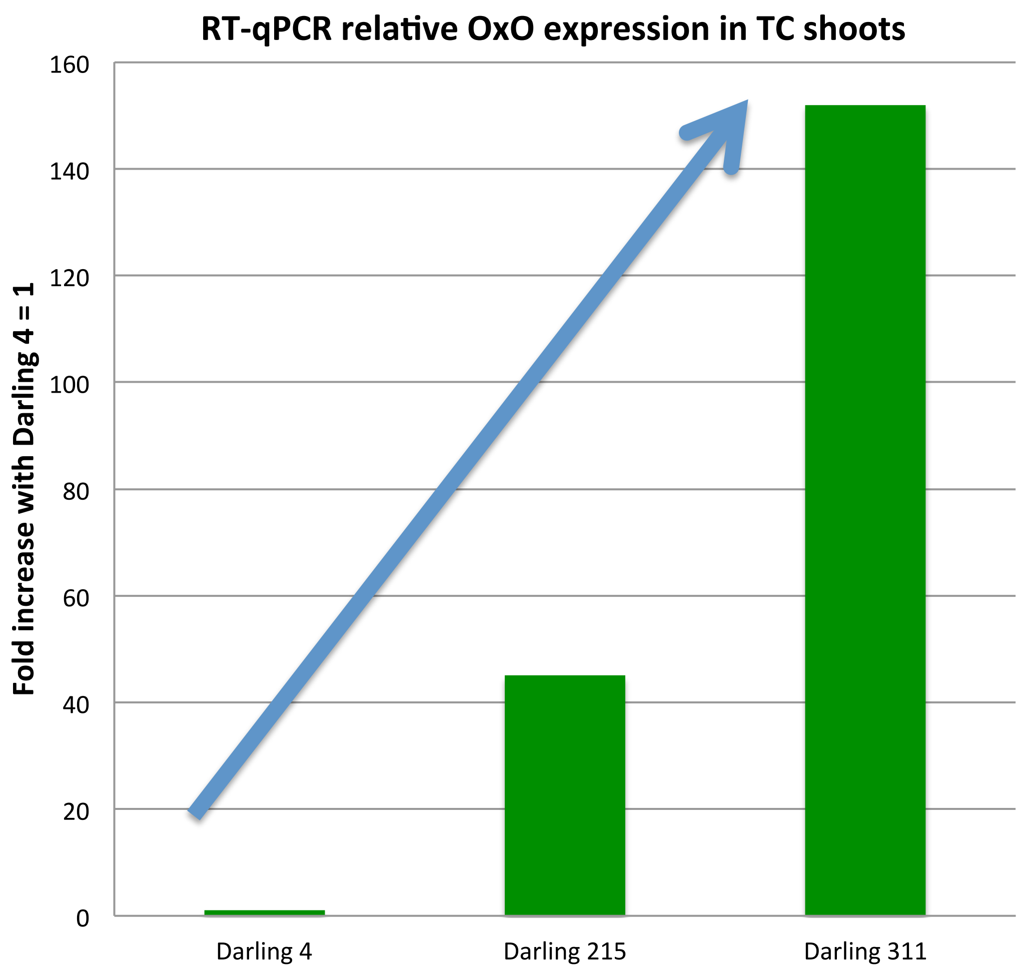
4-year-old tree, 1 1/2 inches (3.8cm) in diameter, inoculated with the chestnut blight to evaluate their resistance level after several months.

PA-TACF http://www.patacf.org/patacfactivities.htm

Savings of up to 4 years!

More enzyme = higher blight resistance

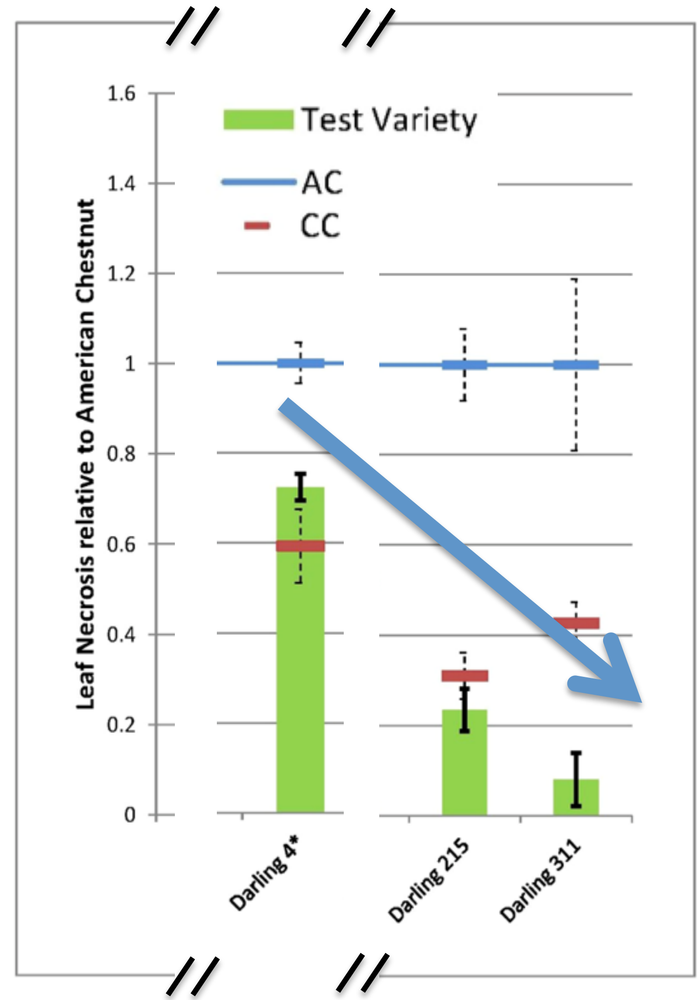
Amount of OxO produced



3 transgenic events

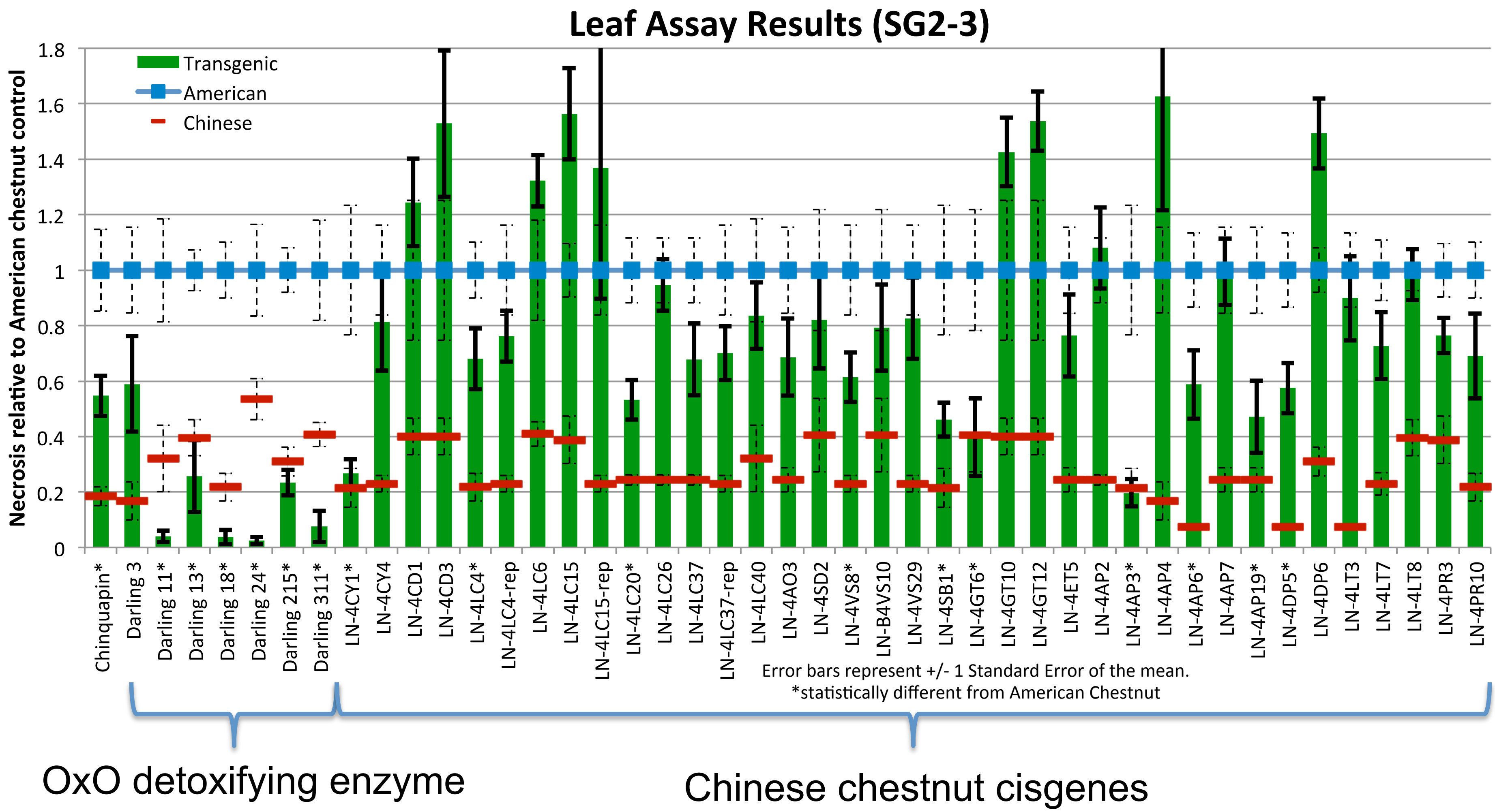
Necrosis area

Leaf assays



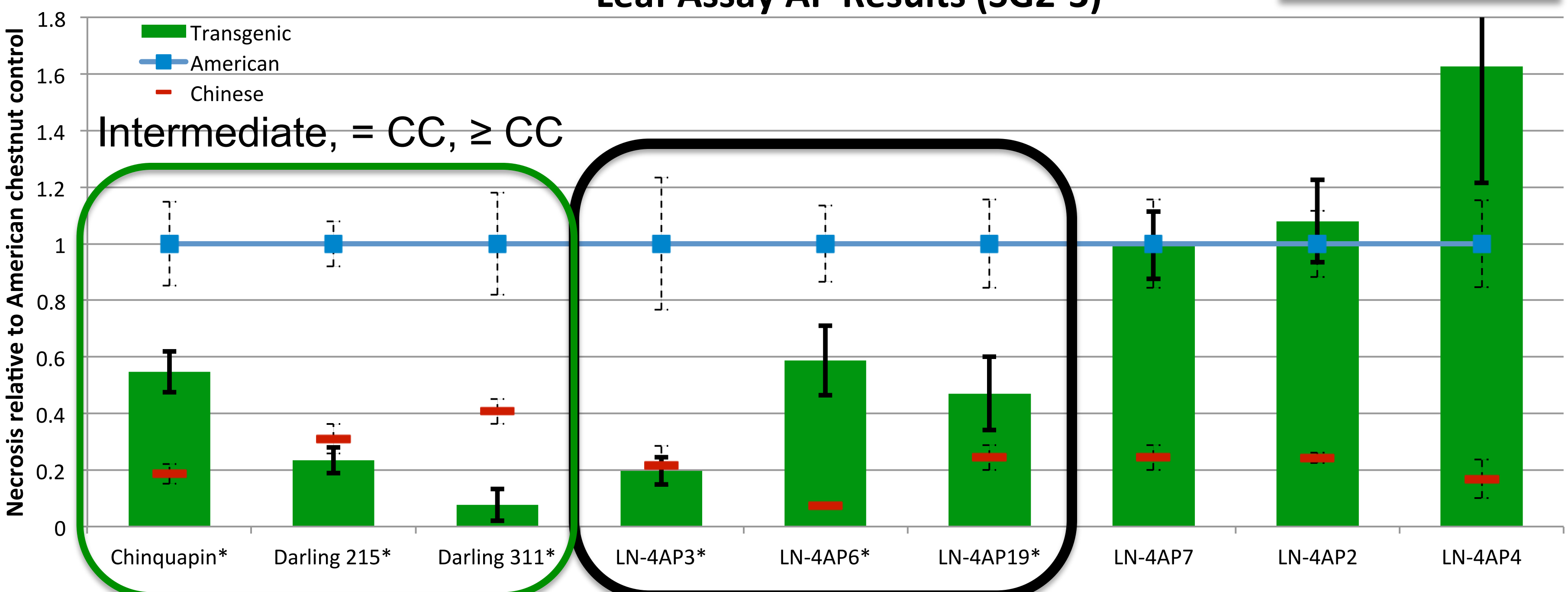
Leaf assay data

(7 to 10 leaves per experiment, most need repeating)



Acid Phosphatase

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

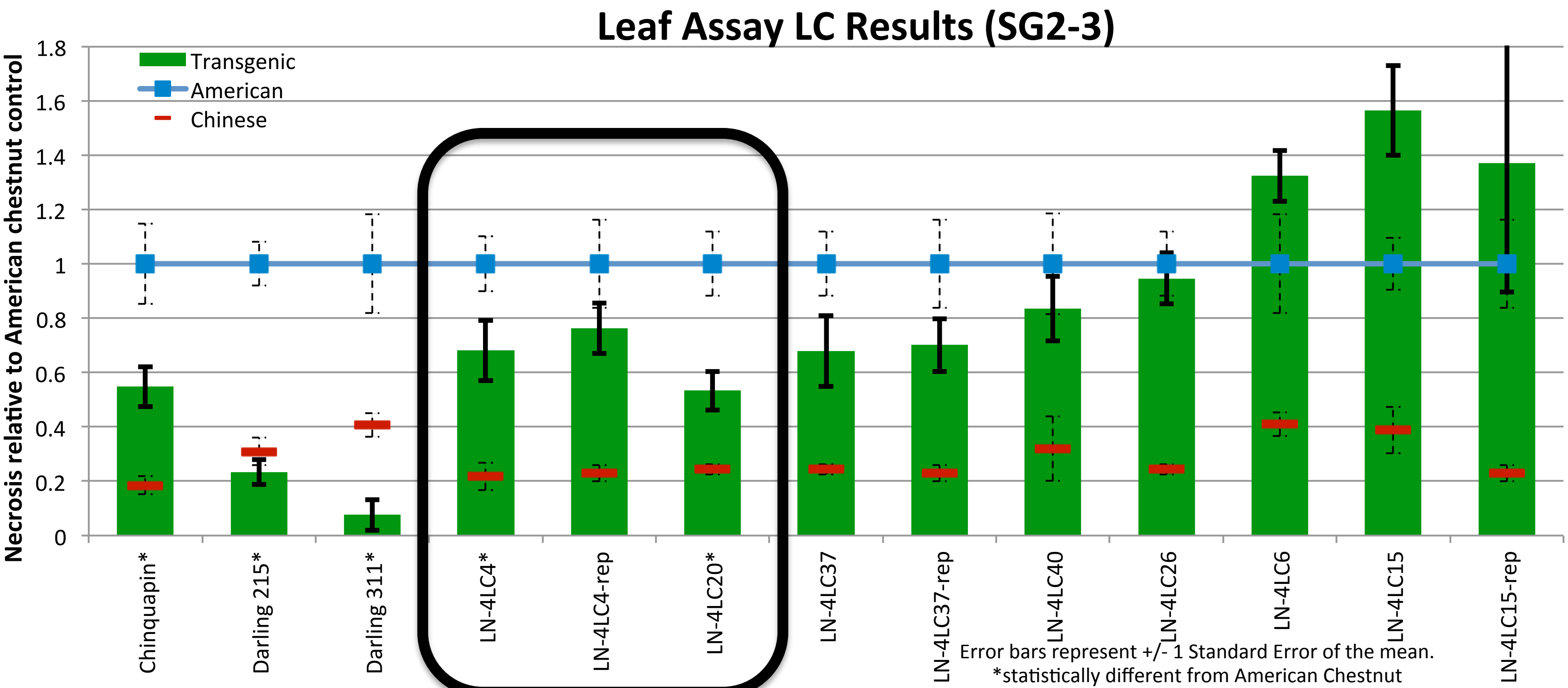


comparisons

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

Laccase-like protein

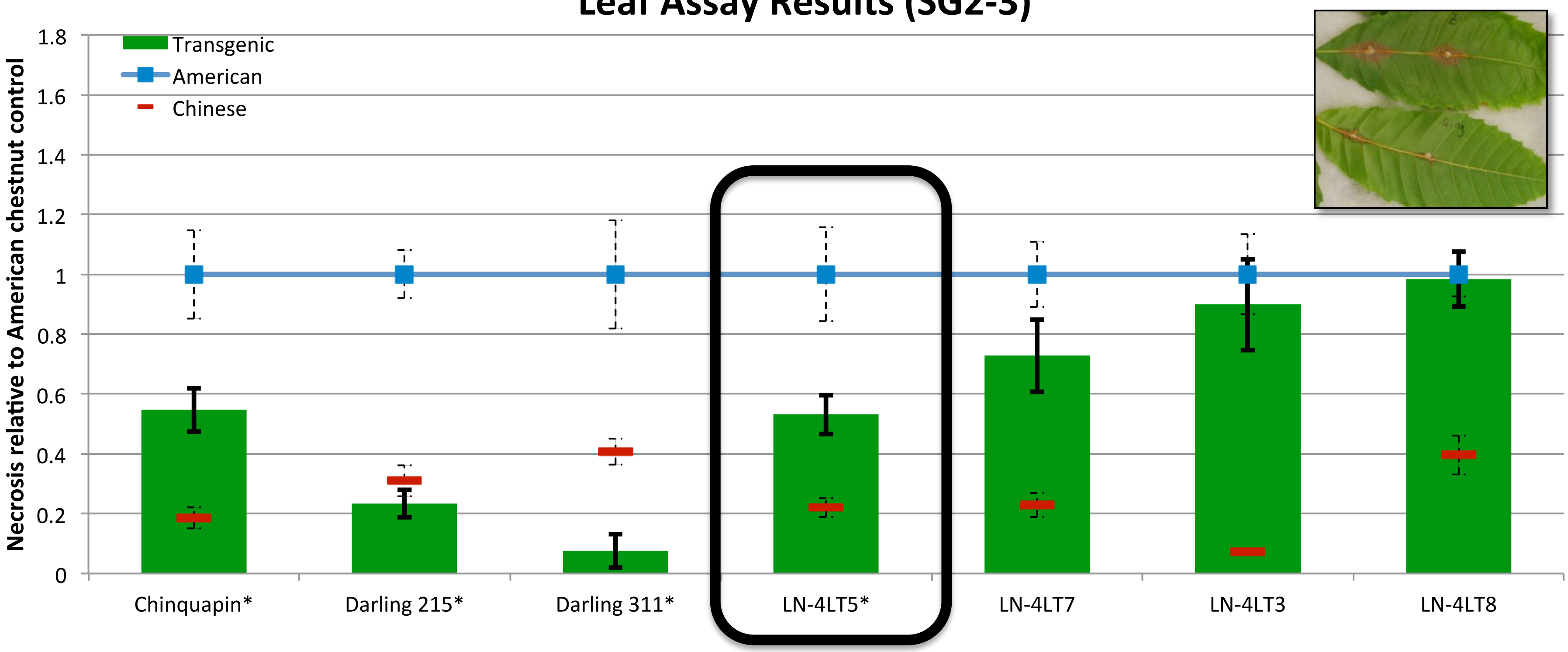
Source: *C. mollissima*
Differentially expressed, CC vs. AC: **Yes**
Linkage: LG-A, Cbr1; Cbr3
Possible functions from published orthologs:
flavanoid biosythesis, ligninfication.



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)

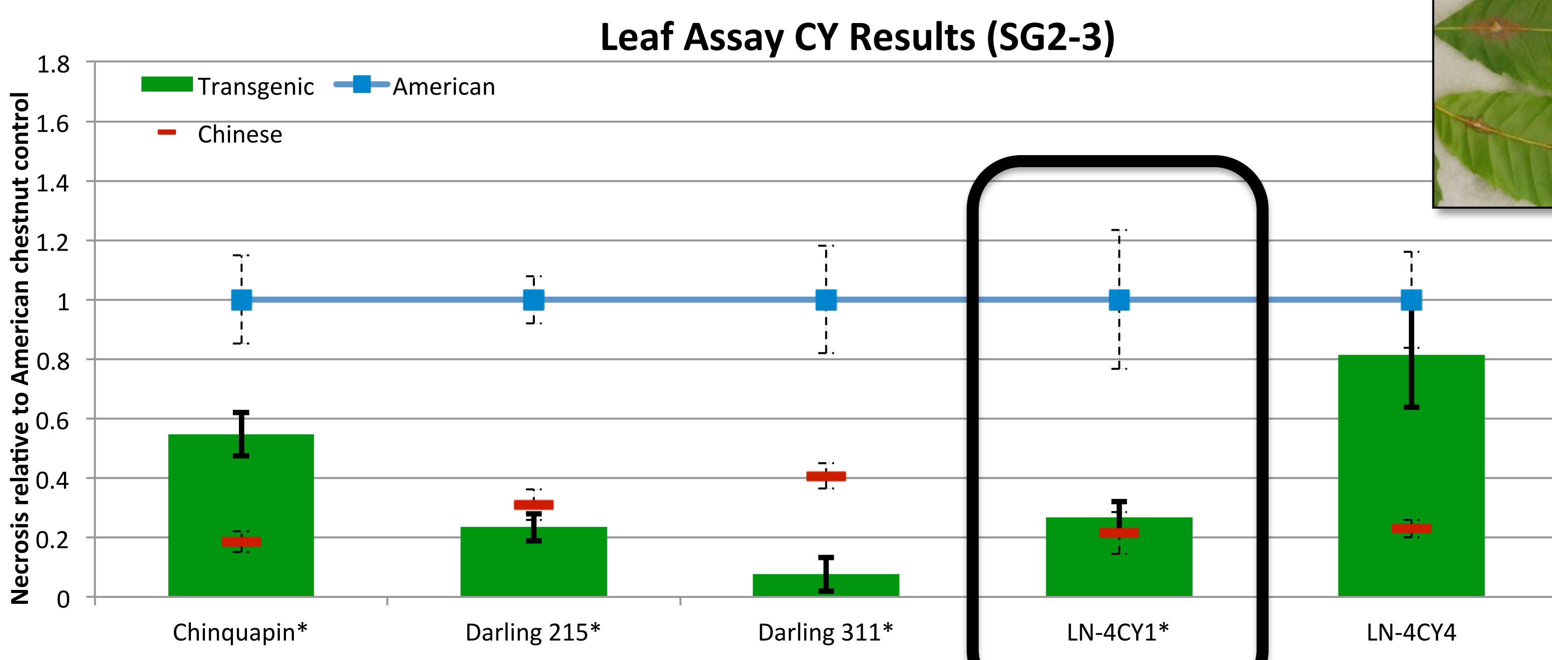


Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut

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.

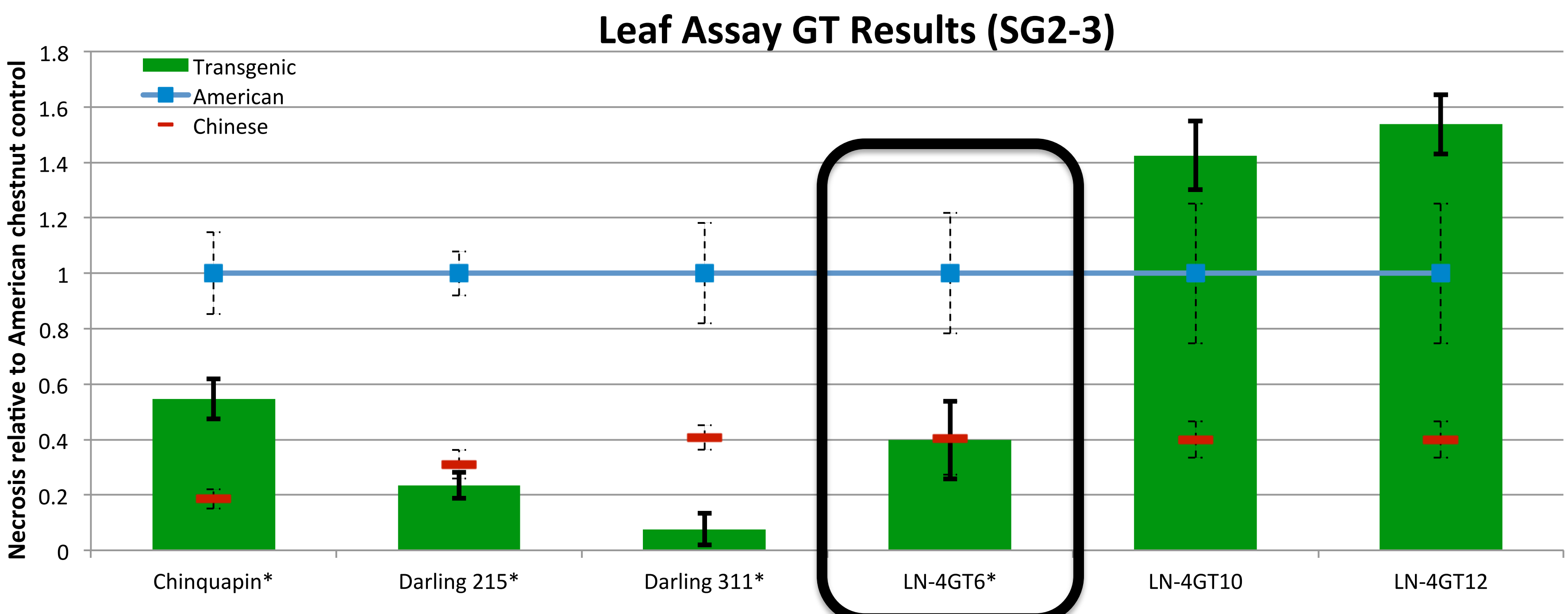


Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut

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.

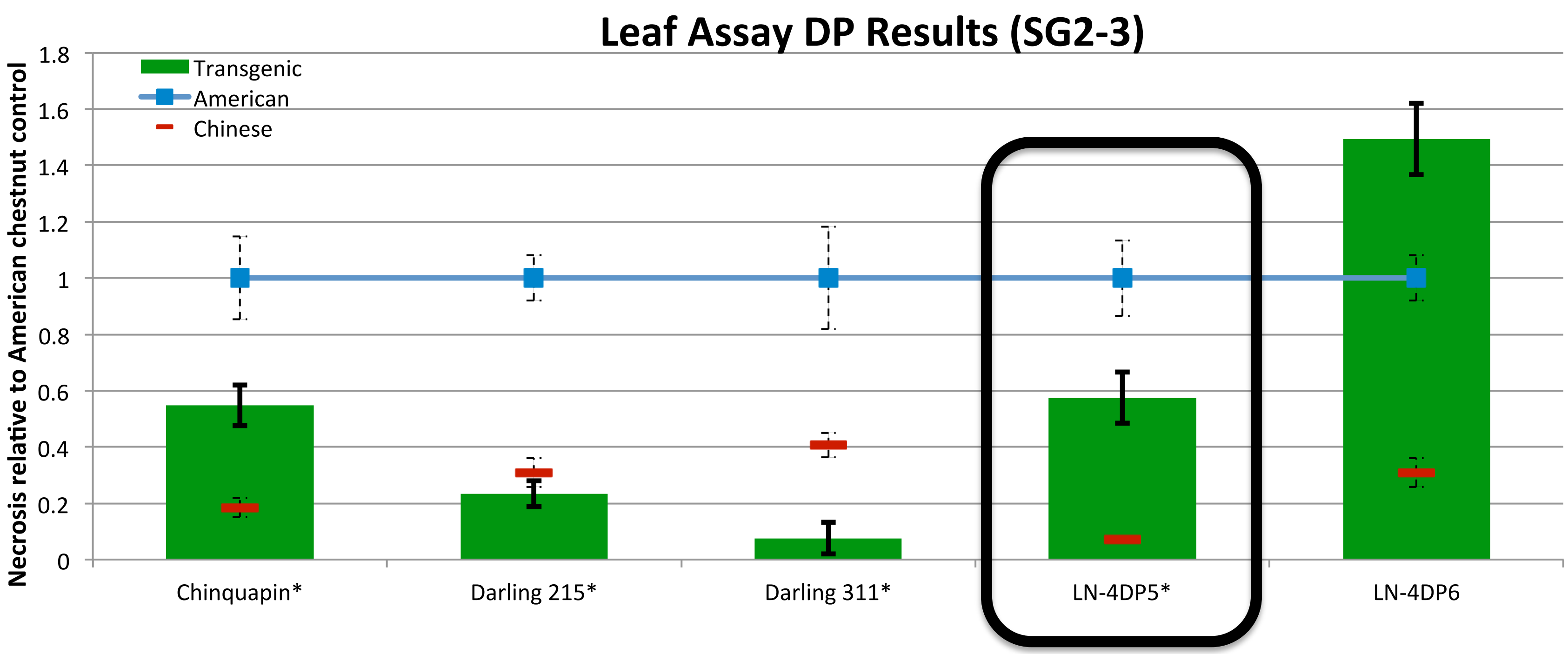


Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut

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.

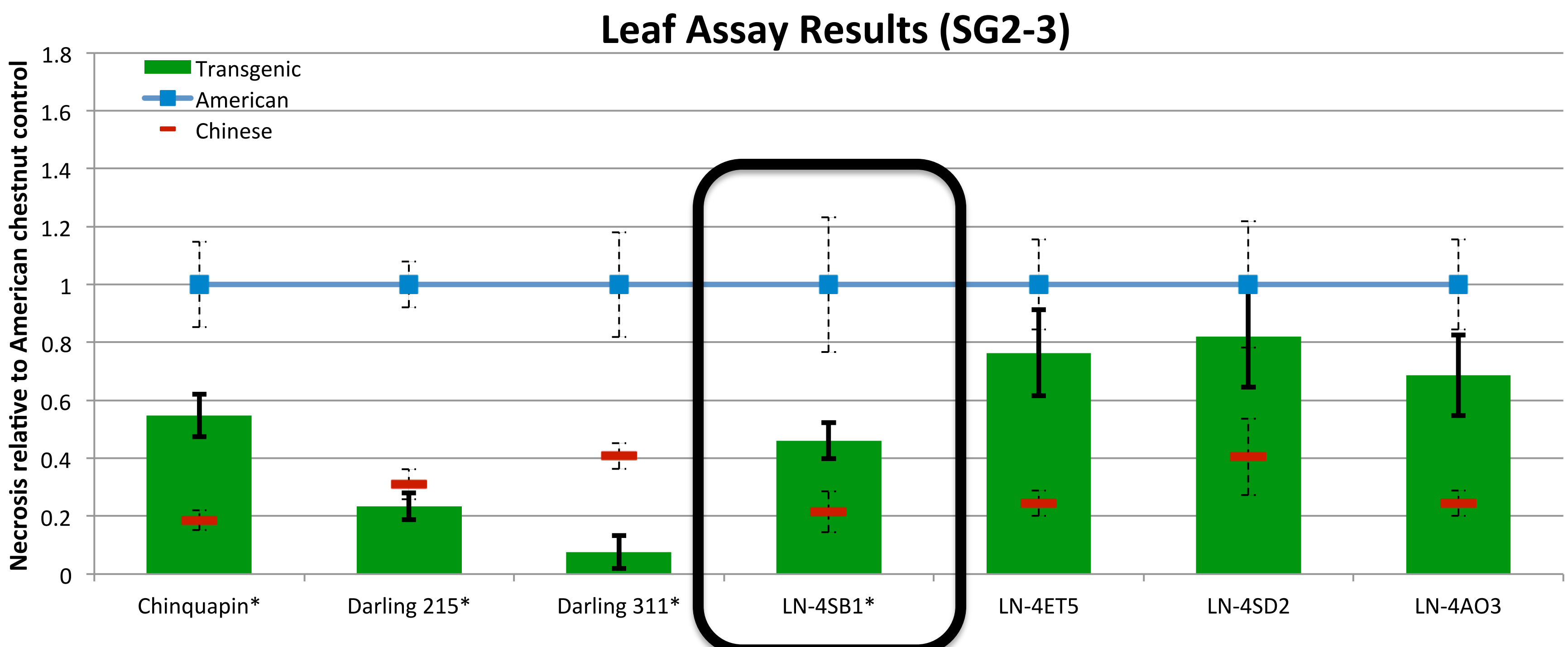


Error bars represent +/- 1 Standard Error of the mean.
*statistically different from American Chestnut

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.



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

Expression screening of SB events

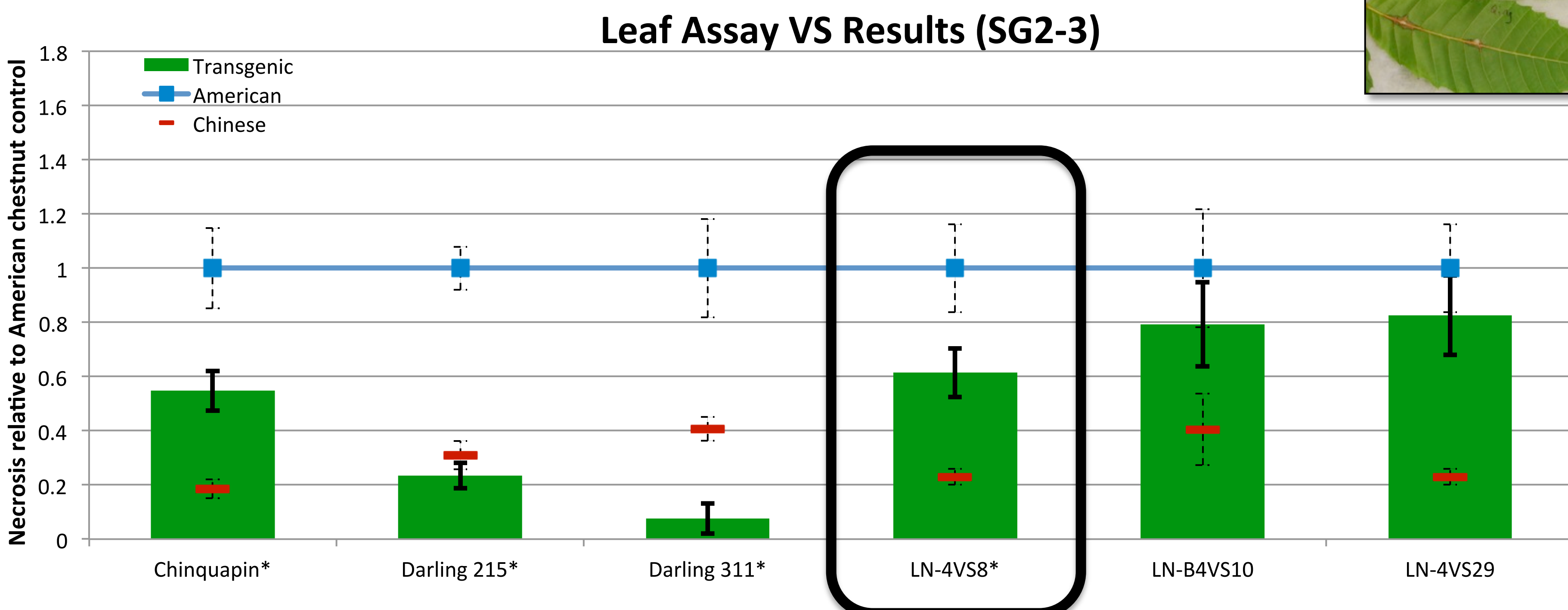
Best over expression of Subtilisin-like protease cisgene was 2.3X background

Compared to Lipid transfer protein gene
(*C. seguini*, Cbr3) with up to 142X background

pFHI-SBTLI	Event name	Embryo cell line	Embryo PCR (SBTLI)	Shoots	Shoot PCR SBTL1	insert# pFHI-SBTLI	RT-qPCR relative to Ellis1 xpn	I M A r
	Ellis 1						1	
1	LN-4SB1	Ellis 1	pos	y		2	2.2	
2	LN-4SB2	Ellis 1	pos	Missing				
3	LN-4SB3	Ellis 1	pos	y		2	2.3	
4	LN-4SB4	Ellis 1	pos			2	1.6	
7	LN-4SB5	Ellis1	pos				1.4 (12-30-13)	
8	LN-4SB6	Ellis1	pos	Missing				
9	LN-4SB7	Ellis1	pos				1.4 (12-30-13)	
10	LN-4SB8	Ellis1	pos				0.9 (4-17-14, kb)	
11	LN-4SB9	Ellis1	pos				1.0 (4-17-14, kb)	
12	LN-4SB10	Ellis1	pos				1.3 (12-30-13)	
13	LN-4SB11	Ellis1	pos		yes		2.5 (11-2014, RB)	c
	LN-4SB12	Ellis 1			yes		1.1 (7-11-14, kb)	
	LN-4SB13	Ellis 1			yes		0.8 (7-11-14, kb)	
14	LN-4SB14	Ellis1	pos				1.1 (4-17-14, kb)	
15	LN-4SB15	Ellis1	pos	Missing				
16	LN-4SB16	Ellis1	pos				1.2 (12-30-13)	
17	LN-4SB17	Ellis1	pos				1.0 (5-23-14, kb)	
18	LN-4SB18	Ellis1	pos				1.2 (12-30-13)	
5	LN-B4SB19	Ellis 1	pos	Missing				
6	LN-B4SB20	Ellis 1	pos	y		1 (1-7-14)	2.2 (12-30-13)	
19	LN-4SB21	Ellis1	pos				1.2 (12-30-13)	
20	LN-4SB22	Ellis1	pos					
21	LN-4SB23	Ellis1	pos				1.1 (4-17-14, kb)	
22	LN-4SB24	Ellis1	pos				0.9 (4-17-14, kb)	

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.



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

Other cisgenes and events

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

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 with breeding program by crossing transgenic trees with backcross trees to add levels of resistance from both sources

Giants of the Eastern Forests

ESF

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

Biotechnology Risk Assessment Grants

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