After 110 years of fighting the blight, plantable resistant American chestnut trees have been produced

1. Genes that enhance blight resistance
2. The oxalate oxidase transgene
3. Variation in cisgenes
4. The laccase-like cisgene
5. Stacking genes for sustainable resistance
6. Developing the best field trees

Deliverables

- **Deliverable 1:** cryopreservation
- **Deliverable 2:** clearing the pipeline
- **Deliverable 3:** field planting & resistance assays

To date, which genes have been able to enhance blight resistance?

-High level of enhancement (≥ Chinese chestnut)

-Transgene:

-Oxalate oxidase (OxO)

-Intermediate levels of enhancement (Chinese > I > American)

- Cisgenes:
- **Laccase-like protein (Lac)**
- Proline-rich protein (PRP), maybe
- Lipid transfer protein (LTP1), maybe

Oxalate oxidase from wheat

Detoxifies oxalate (oxalic acid)

$$1)\quad \underset{\text{Oxalic acid}}{\text{H-O-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-}\overset{\overset{\text{O}}{|}}{\text{C}}\text{-O-H}} + 2\,O_2 \xrightarrow{\text{Oxalate oxidase}} H_2O_2 + 2CO_2$$

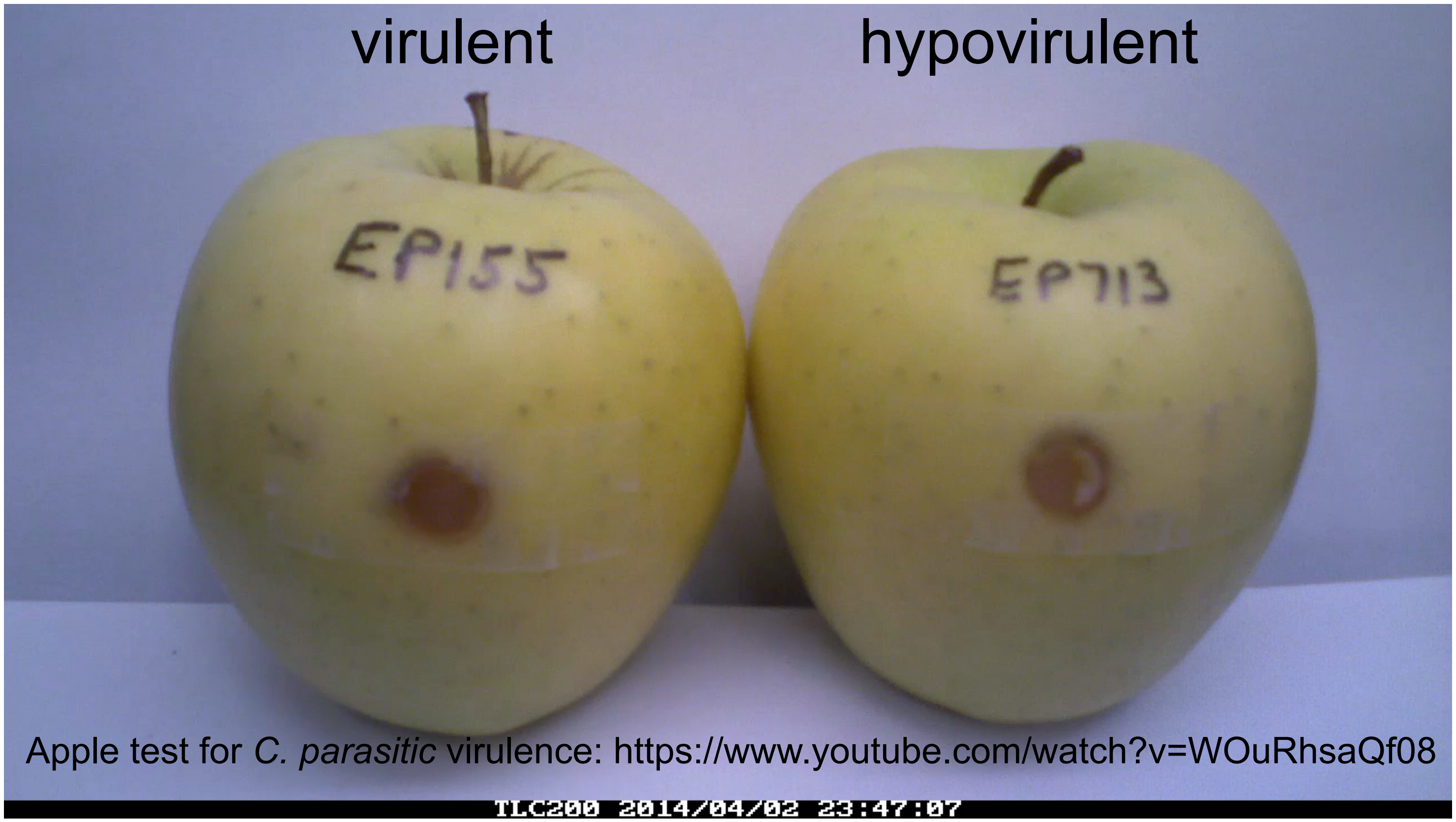
Not a pesticide, but is a plant protectant.

Does not kill the fungus, no ‘cidal’ activity.

Reduces the chance of the fungus developing resistance to OxO.

On transgenic chestnut, can change the fungal lifestyle from a pathogen to a saprophyte.

Where did this idea come from?
1983

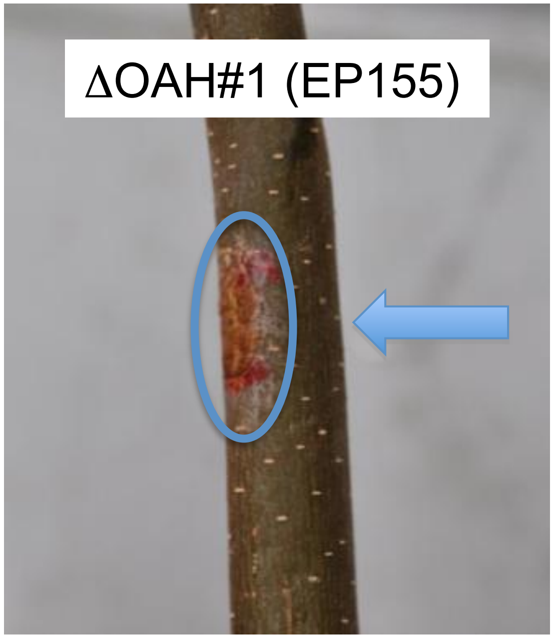


Oxalate associated with virulence in chestnut blight

Havir, E. A., & Anagnostakis, S. L. (1983). **Oxalate production by virulent but not by hypovirulent strains of *Endothia para*sitica**. *Physiological Plant Pathology*, *23*(3), 369–376

C. parasitica with & without OA production

Chen, C., Sun, Q., Narayanan, B., Nuss, D. L., & Herzberg, O. (2010). Structure of **Oxalacetate Acetylhydrolase, a Virulence Factor of the Chestnut Blight Fungus**. *Journal of Biological Chemistry*, *285*(34), 26685–26696

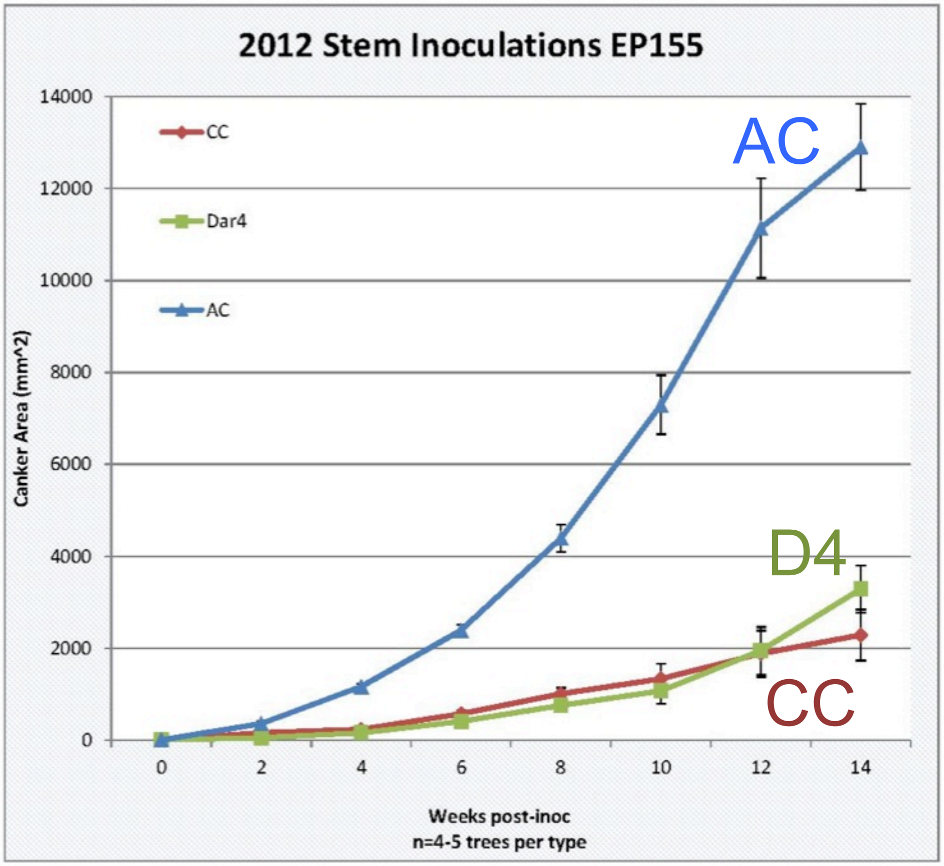


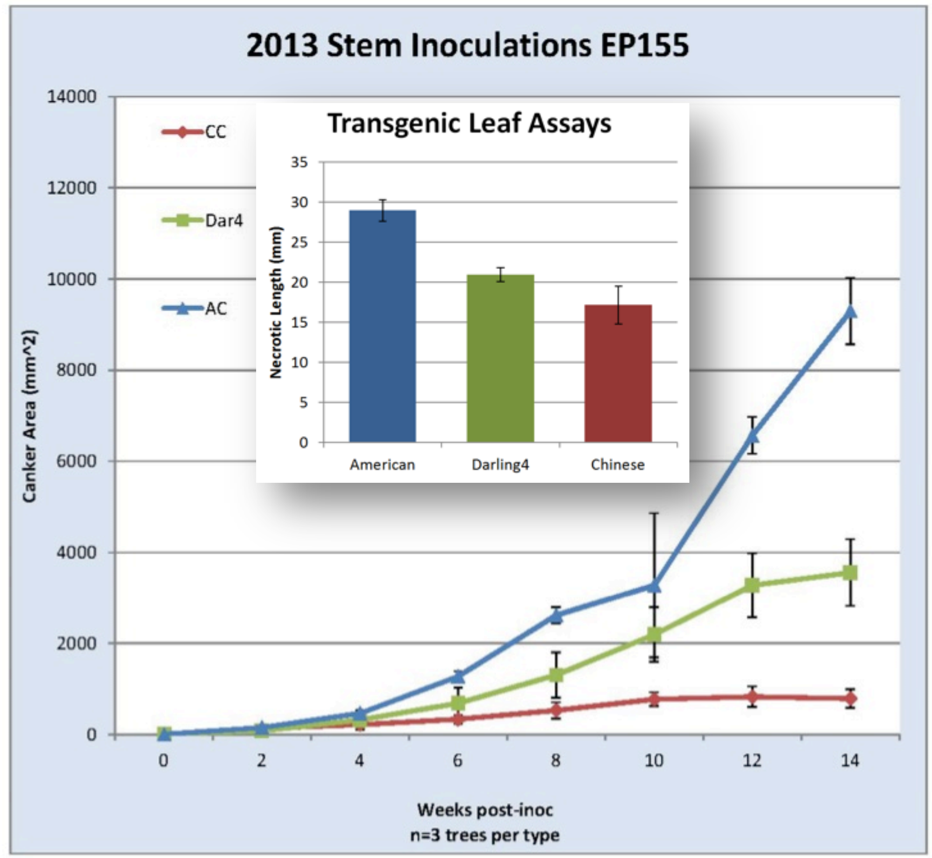
Fungus unable to make Oxalate (oxalic acid)

Therefore, can we design a resistance mechanism that removes oxalate? Yes, with oxalate oxidase. Mimics what happens naturally in hypovirulence.

Field Inoculations demonstrate enhanced blight resistance & validity of leaf assays predictions

(Blight strain EP155 on stems of the Darling 4 event)
2012 vs 2013





From TEDx talk

2013 much more & consistent rain than 2012

(Andy Newhouse *et al.*)

Representative photos of SG2 cankers on each tree type, **8-30-12 (~11 weeks)**.
Scale was equalized according to ruler in photo.

AC: Avg ht 89mm

CC: Avg ht 31mm

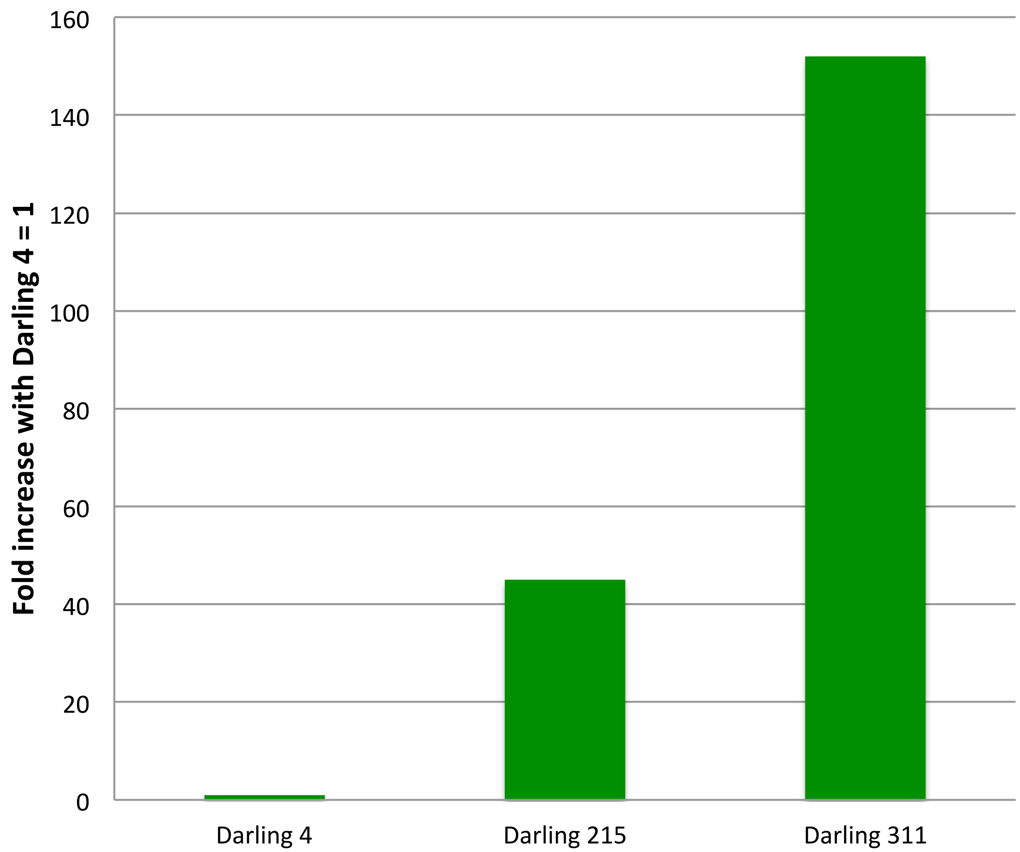
Darling4: Avg ht 48mm

OxO gene expression vs. relative resistance

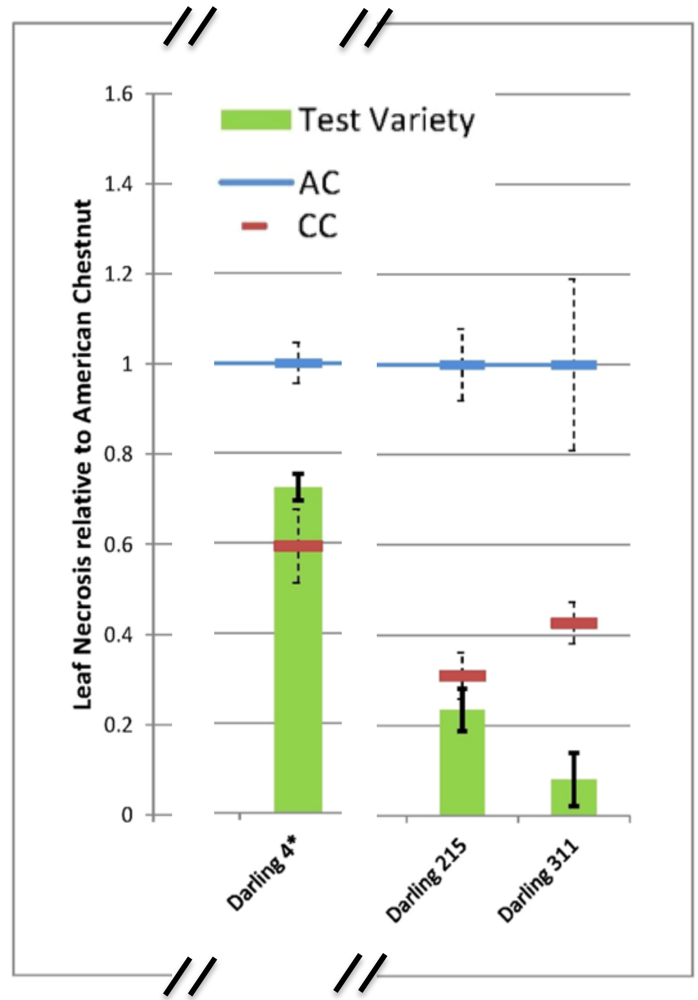
'Darling4' – VspB promoter

'Darling215' & '311' – CaMV 35S promoter

RT-qPCR relative OxO expression in TC shoots



Leaf assays



10 single insertion events ≥ Darling 215

Goal, blight resistance ≥ Chinese
Small stem assay (2.5-3mm dia.)

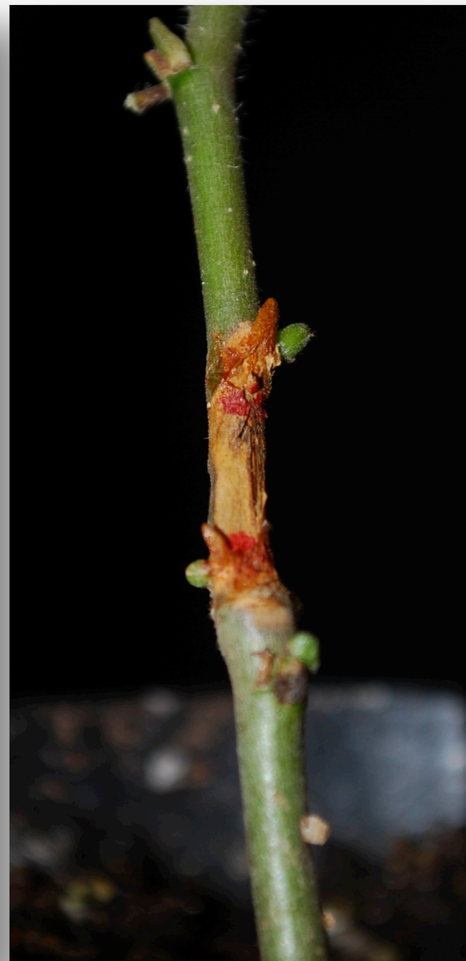
http://www.esf.edu/chestnut/resistance.htm

Chestnut blight
susceptible & resistant controls

Blight resistance enhanced
transgenic American chestnut

**American chestnut
Ellis 1**

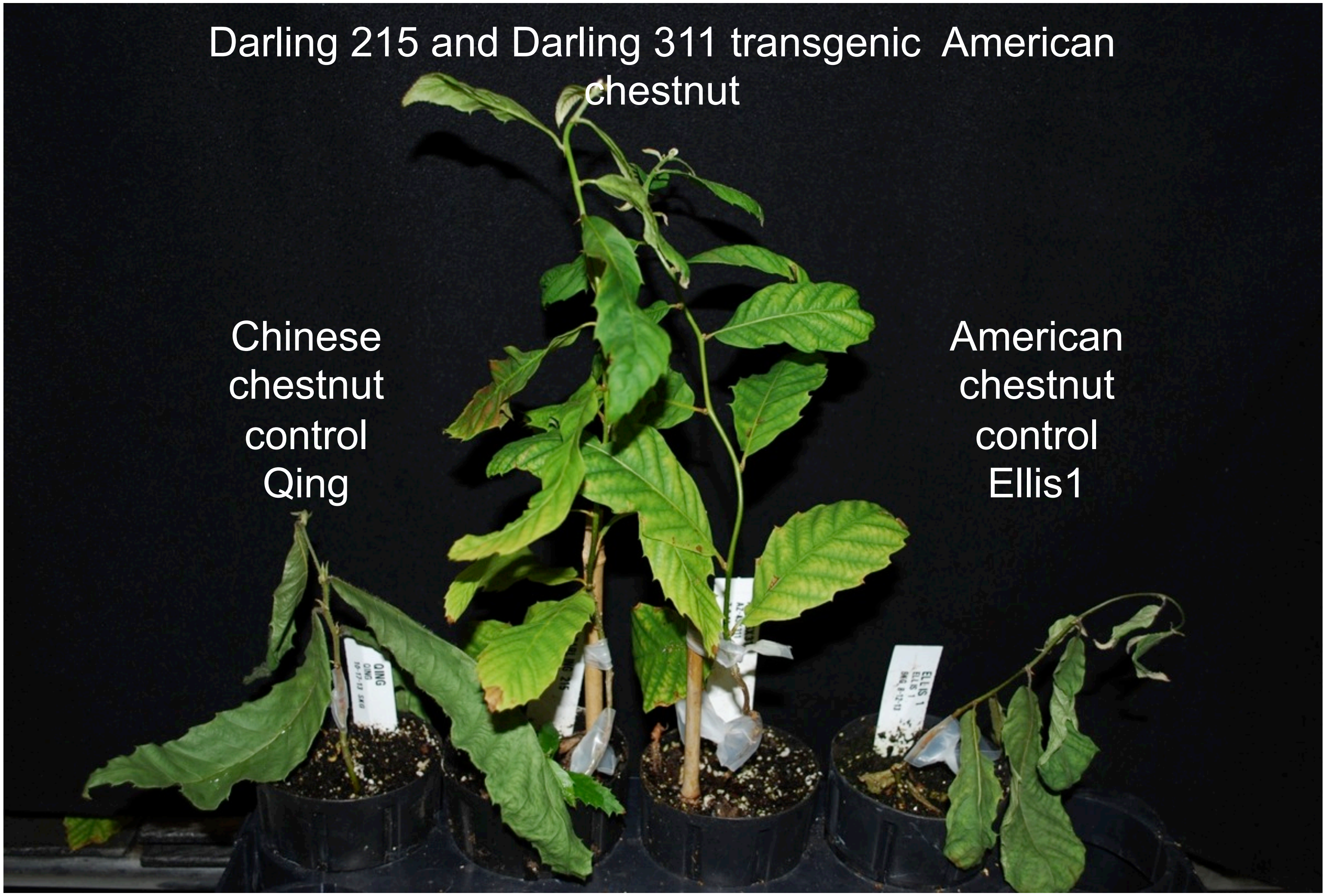
**Chinese chestnut
Qing**



**American chestnut
Darling 215**

**American chestnut
Darling 311**

Previous very small stem (≤ 2.0 mm dia.) assay using EP155, 8 Days Post Infection



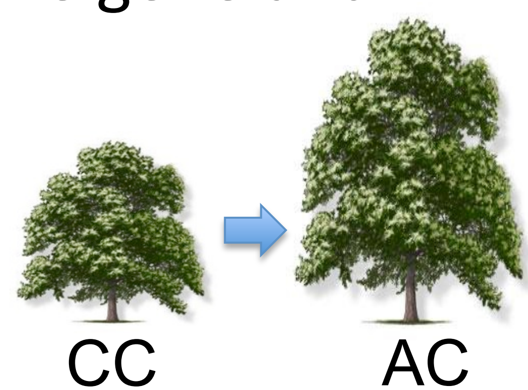
Conclusions

The oxalate oxidase transgene can confer dominant, single gene resistance to chestnut blight.

Level of resistance is linked to level of OxO expression.

Variability among cisgenes in transformations

- Using the same UBQ11 constitutive promoter (genetic switch) in the chestnut cisgene vectors:
  - Some give many events with broad ranges of gene overexpression
  - Some give fewer events with narrow ranges of gene overexpression
  - Some appear to down regulate even the native gene
- Remember these events have their native gene and the added cisgene.



	CG from chestnut: name	PCR pos events	# events wt >2 fold overexpression	Range of fold increase in TC shoots	source_species	CmQTL	SSH_Diff Exp
1	Laccase / diphenol oxidase	40	35 (all tested) 14 selected	2.6 - 12.5	*C. mollissima*	Cbr1; Cbr3	CC1.2-E04
2	Ethylene Transcription Factor	5	2	2.2 - 5.7	*C. mollissima*	Cbr2	CC2.2-DO6
3	Proline-Rich Protein	11	1	5.2 (as low as 0.01)	*C. mollissima*	Cbr3	CC3.2-F07
4	Deoxy-arabino-heptulosonate phosphate synthase (DAPH)	7	2	2 -2.6	*C. mollissima*	Cbr3	CC1.2-D06
5	Thaumatin-like protein	5	4	4 - 10.5	*C. mollissima*	Cbr3	
6	Lipid transfer protein SSH (LT)	8	5	2.6 - 10.7	*C. mollissima*		CC1.2-H07
7	Acid Phosphatase	19	7	5.9 - 12	*C. mollissima*	Cbr3	CC1.2-A11
8	CBS domain containing protein (unknown)	0	NA	NA	*C. mollissima*		CC2.2-E07
9	Beta 1,3 Glucanase	0	NA	NA	*C. mollissima*		CC1.2-DO3
10	Myo inositol phosphate synthase	5	0	0.1 -0.2	*C. mollissima*	Cbr1	CC1.2 – B07
11	Lipid transfer protein / proteinase inhibitor (LP)	28	(7) 5	9.3 - 142	*C. seguinii*	Cbr3	
12	Caffeoyl-CoA-O-methyltransferase	6	1	4.6	*C. mollissima*		CC1.2-G10
13	Cystatin, cysteine protease inhibitor	5	5	3.4 - 75	*C. mollissima*	LG-E	
14	Resistance to *Phytophthora (RPH1)*	0	NA	NA	*C. mollissima*	LG-E	
15	Non-expressor of Pathogen Response 3/4	15	2	7.5 - 9.3	*C. seguinii*	LG-E	
16	Peroxidase	0	NA	NA	*C. mollissima*	Cbr1	CC1.2-F11
17	Subtilisin-like protease	22	3	2.2 - 2.3	*C. seguinii*	Cbr1; LG-E	CC2.2 – G01
18	UDP-glycosyltransferase	0	NA	NA	*C. mollissima*	Cbr1; Cbr3	CC2.2-C08
19	Malic enzyme	5	0	1 - 1.3	*C. seguinii*	LG-E	CC1.2-A02
20	Cinnamyl alcohol dehydrogenase-like protein	5	3	6.5 - 61	*C. mollissima*	Cbr1	CC1.2 - C04
21	Shikimate dehydrogenase	4	1	2.1	*C. mollissima*		CC1.2 - F02
22	Phenylalanine ammonia-lyase	5	0	1.2 - 1.9	*C. seguinii*		CC1.2-E08
23	ACC oxidase	6	1	2.2	*C. mollissima*	LG-E	
24	Glucan endo-1,3-glucosidase	4	1	9.7	*C. mollissima*	Cbr1	
25	Cytochrome P450 allene oxide synthase	5	3	12.7 - 36	*C. seguinii*	LG-E	
26	Glutathione s-transferase	17	6	2 - 16.7	*C. mollissima*	LG-E	
27	Triacylglycerol lipase	1	1	4.8	*C. mollissima*	LG-E	

Take home message

- PCR is a good first screen but can't be relied upon by itself
- Gene expression must be compared to background levels of non-transgenic controls
- Gene expression must be done early in the process to prevent wasted resources on producing plants

Why do we expect to get only partial resistance with cisgene transformation?

CC

R_1R_1 R_2R_2 R_3R_3

AC

r_1r_1 r_2r_2 r_3r_3

CC has **quantitative resistance** to blight
3 major resistance loci + additional minor
(how many ?)

If transformation produces overexpression of cisgenic R_1 = R_1R_1

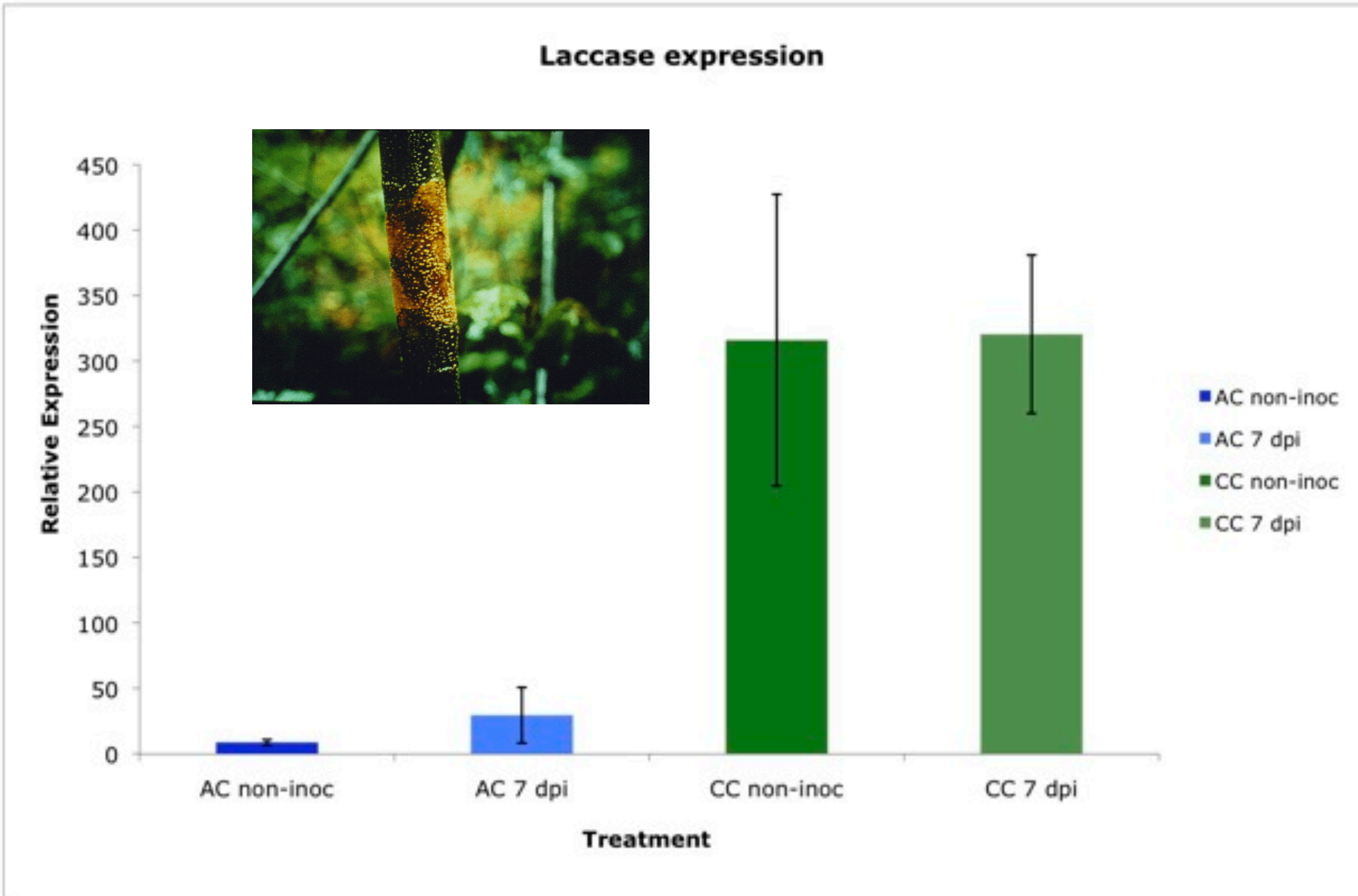
But you still have r_2r_2 r_3r_3

Therefore, best you can expect is
intermediate resistance (CC > I > AC)

For full resistance need stacking or a more potent gene

Laccase-like gene from Chinese chestnut (cisgene)

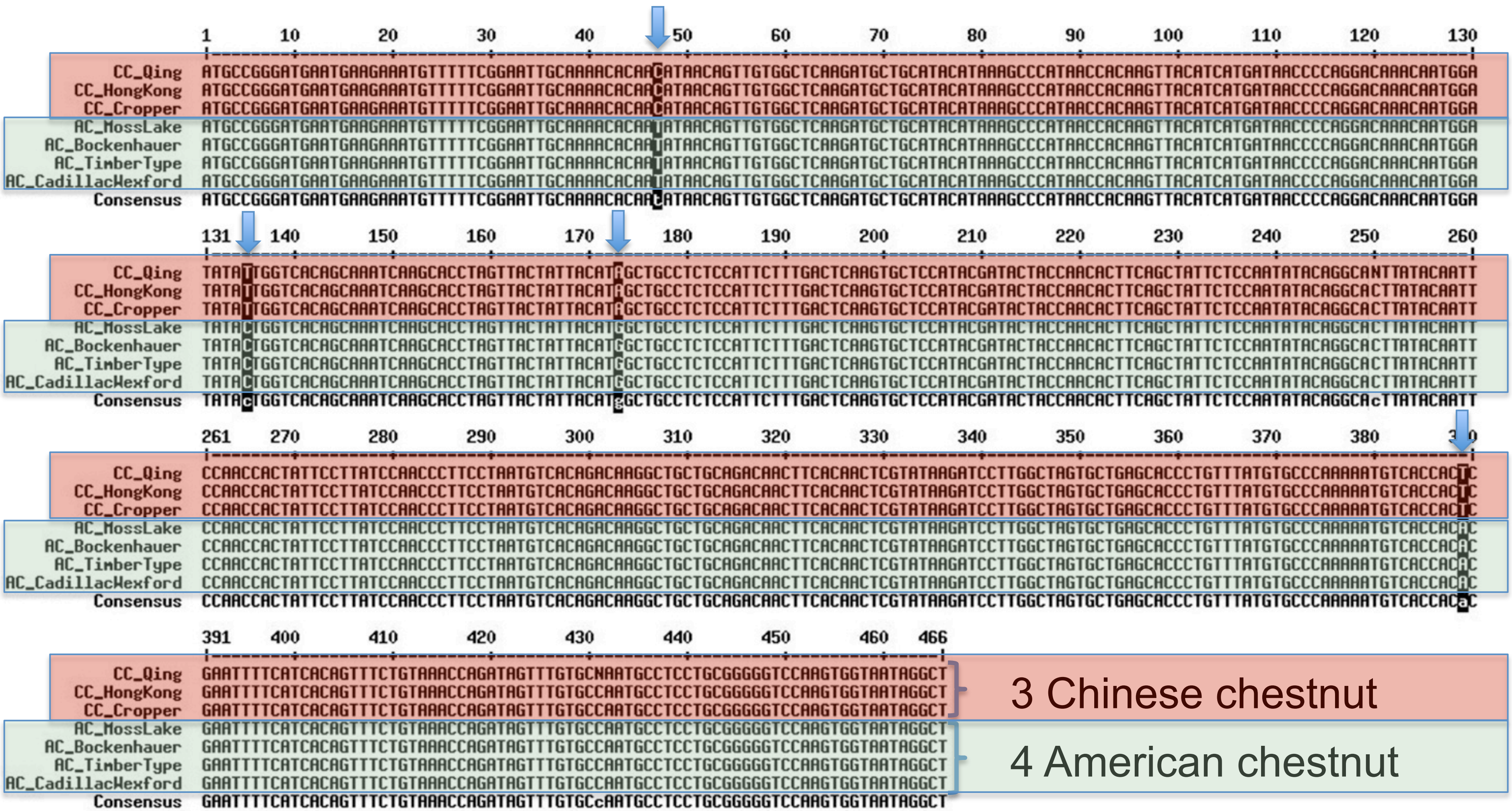
Laccase-like gene expression up to 300-fold higher in resistant Chinese chestnut vs. the susceptible American chestnut



American & Chinese chestnut laccase-like
cisgene products are identical

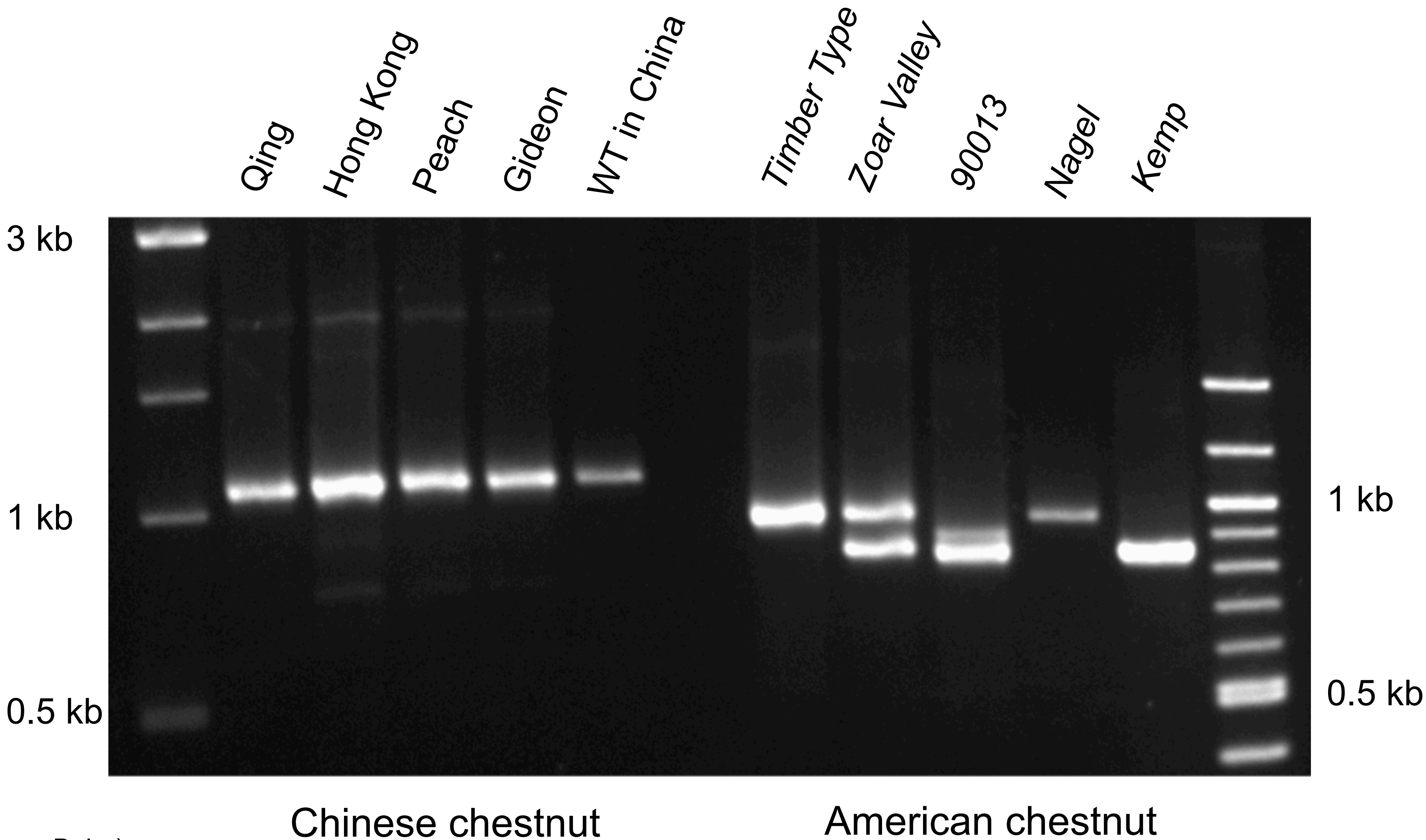
amino acid sequence 100% identical

Nucleotide sequences 99% identical



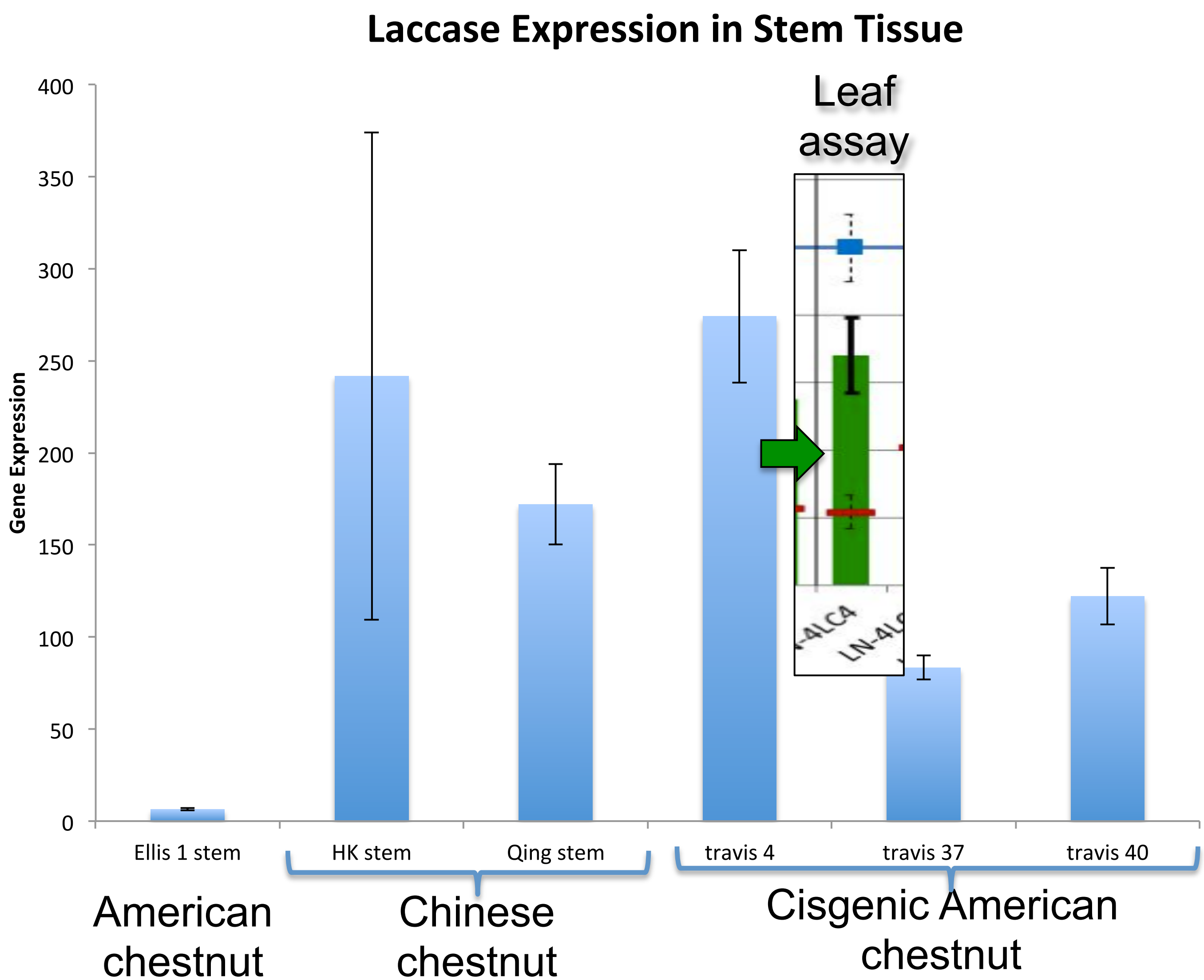
Gene products the same but the promoters (genetic switches) are different

PCR analysis comparing size variations within the **promoter** of a laccase-like gene in Chinese and American chestnut



(Kathleen Baier)

Cisgenic American chestnut mimics laccase-like gene expression in Chinese chestnut



14 Transgene vectors (heterologous genes)
Single & pyramid

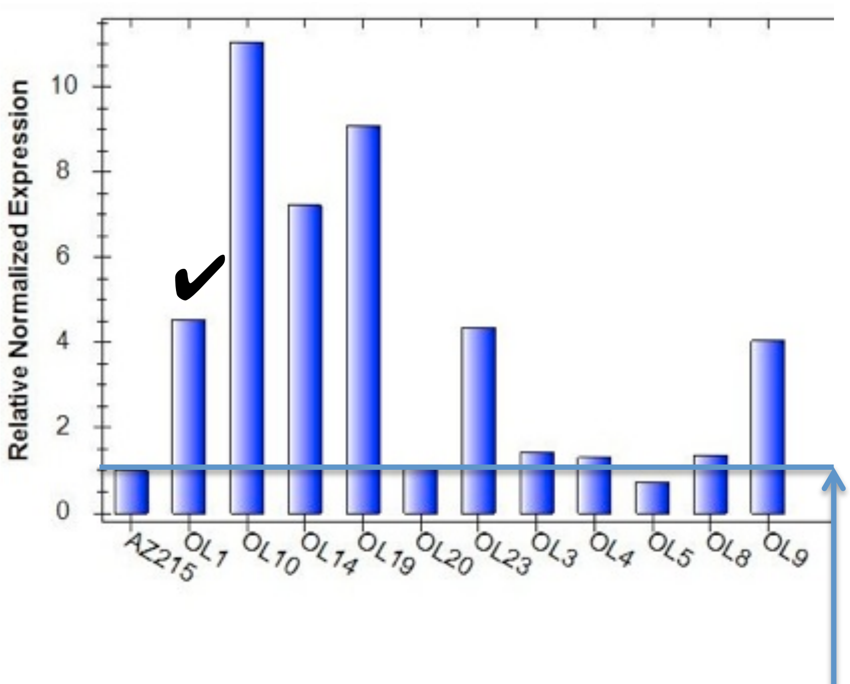
Newest to oldest

SUNY-ESF
UGA

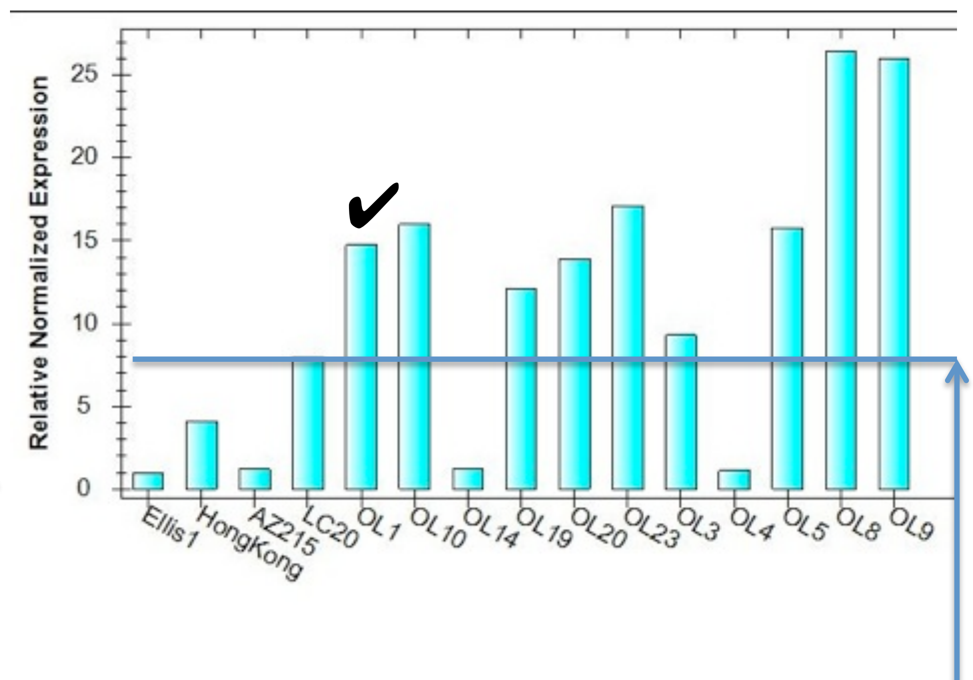
- pOV – CaMV 35S promoter - Oxalate oxidase + VST1
- pOR - CaMV 35S promoter - Oxalate oxidase + RPH1
- pFHI33-RNG (Rph1 + NPR34 + GAFP1)
- pFHI23-RN (Rph1 + NPR34)
- pFHI23-RGAF (Rph1 + GAFP1)
- pOL, 35S- oxalate oxidase + UBQ11-lacccase (wheat & chestnut)
- *pFHI-CaAMP, UBQ11 - capsicum antimicrobial peptide*
- *pFHI-VST1, UBQ11 promoter - stilbene synthase gene (grape)*
- *pFHI-NPR1 - Non-expressor of Pathogen Response 1 (Arabidopsis)*
- *pFHI-GAFP - Gastrodia anti-fungal peptide*
- ***p35S-OxO, CaMV 35S promoter - Oxalate oxidase (Wheat)***
- pTACF7, VspB promoter - Oxalate oxidase + ACS2-ESF39 (Wheat, des.)
- *pTACF6, ACS2 promoter- ESF39 antimicrobial peptide (designed)*
- *pTACF3, VspB promoter - Oxalate oxidase (Wheat)*

OxO + Lac (OL) events have been produced

OxO expression (TC shoots)



Lac expression (TC shoots)



OxO expression threshold to match Chinese resistance (in leaf assays)

Lac expression threshold to match intermediate resistance (in leaf assays)

Conclusion

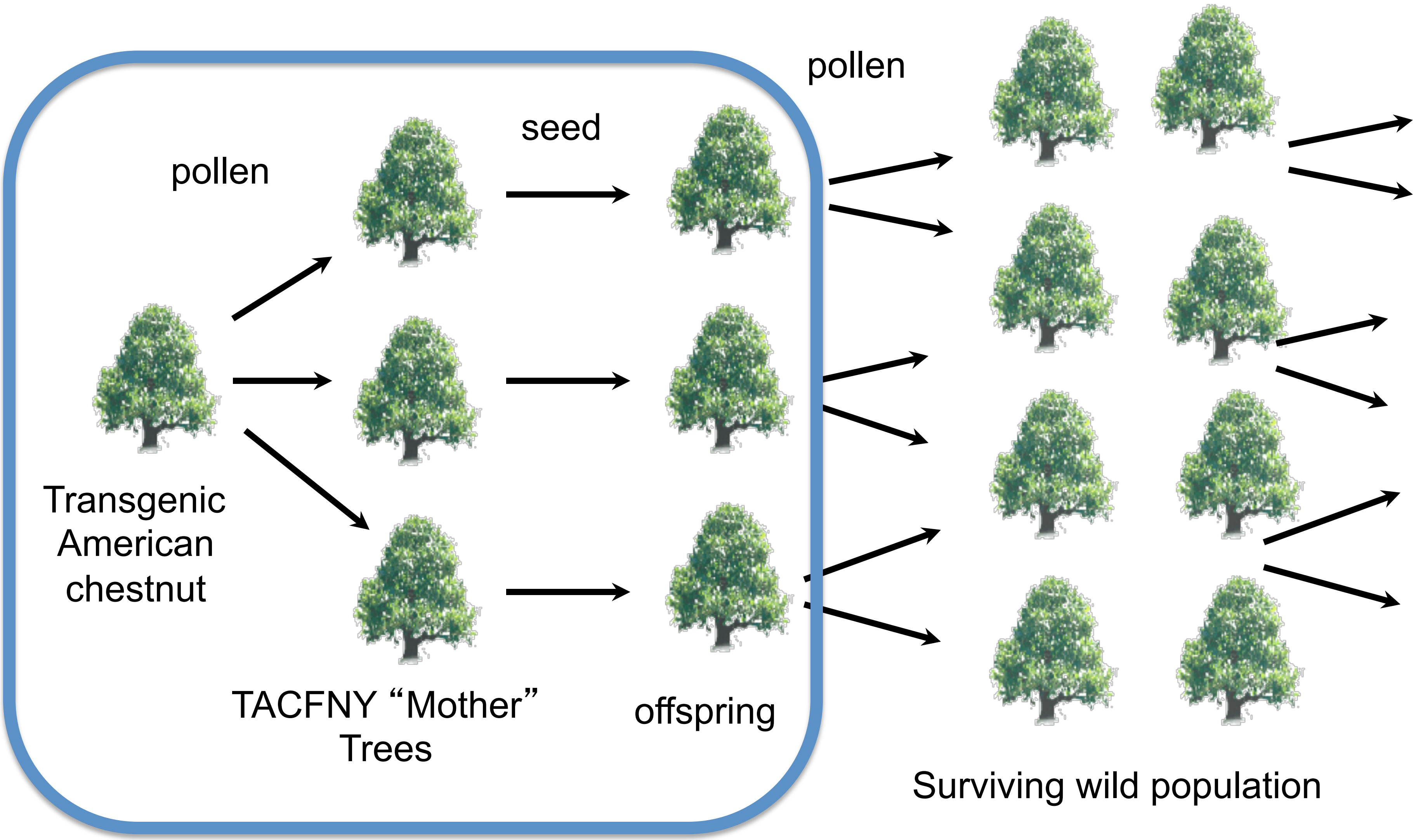
The Laccase-like gene enhances blight resistance at least part way.

Pyramiding the OxO with the Laccase-like gene should provide even more sustainable blight resistance.

Pollination to increase genetic diversity & produce healthier seedlings

Goal: resistance & diversity

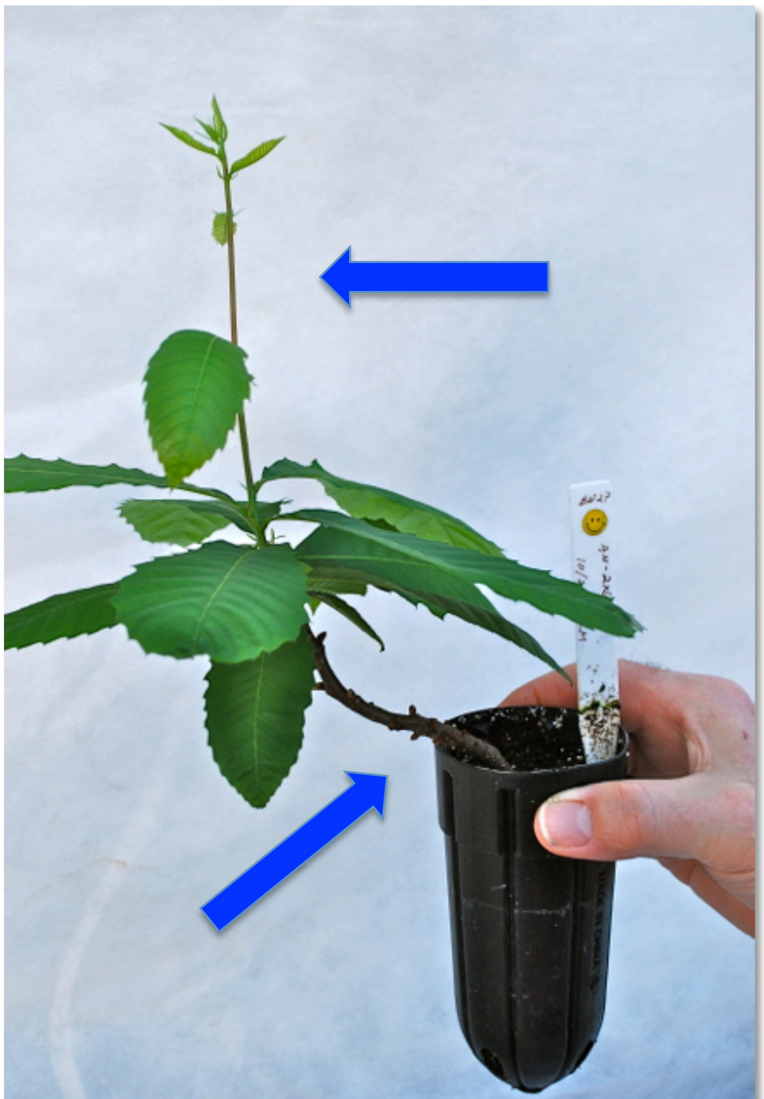
Maximize out-crossing



Tissue culture vs. seedling first year growth

Need open pollination to produce the best trees.

Tissue culture plantlets sometimes think they are branches



“As the twig is bent, so grows the tree.”

Coppicing to bring back timber type growth.

> 7 ft

One season

American chestnut Research & Restoration team at SUNY-ESF

Co-Directors: Charles Maynard & William Powell

Researchers: Andy Newhouse, Kathleen Baier, Linda McGuigan

Graduate students: Allison Oakes, Kristen Stewart

100’s of people (collaborators, visiting scientists, postdocs, graduate students, undergraduate students, High School students, and volunteers) contributed to this research over the past 24 years.


Thank you!
Questions?