Forest Health Initiative Phase 2 Research at UGA

Clonal Testing/ Gene Transfer Project

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FHI Jargon

- TACF = The American Chestnut Foundation
- ACCF = American Chestnut Cooperators Foundation
- VDF = Virginia Department of Forestry
- AC = American chestnut; CC = Chinese chestnut; JC = Japanese chestnut
- LSA = Large surviving American chestnut (potentially has some level of 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)
- PRR = Phytophthora root rot
- SE = somatic embryogenesis or somatic embryo
- SS = somatic seedling
- CG = candidate gene for blight and/or PRR resistance
- Hokies = Virginia Tech

Background/Rationale for Phase 2 UGA Work

- Large numbers of vigorous somatic seedlings still need to be produced and established in field tests for:
  - Clonal testing of LSA crosses (ACCF), ACxCCxJC hybrids (VDF) and B3F3 (TACF) germplasm
  - Testing of over 30 candidate genes for blight and PRR that were transformed into American chestnut cultures during Phase 2
- PRR remains a barrier to restoration of American chestnut in the southern half of the range
  - Trees engineered with PRR-resistance CGs need to be screened for resistance
  - An *in vitro* screen for PRR-resistance would greatly accelerate testing of CGs

FHI Phase 2 Goals and Objectives – UGA

Base Project

1. Complete production of populations of LSA and B3F3 somatic seedlings for clonal testing and establish in field tests
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. Produce trees engineered with Phytophthora resistance candidate genes and test them for resistance

Supplemental Project

4. Develop *in vitro* screen for PRR resistance/susceptibility
5. Improve chestnut somatic seedling quality and production efficiency

Objective 1: Clonal testing – Produce LSA somatic seedlings and establish them in field tests

- Seeds from crosses between ACCF LSAs Ragged Mountain (RM) x Thompson (TH) cultured in 2010
- Last of 51 RM x TH somatic seedlings planted out by ACCF Cooperator Carol Croy (USDA Forest Service) on George Washington and Jefferson National Forests, November 2014

Genotype	Number
RM x TH-10A	2
RM x TH-10E	2
RM xTH-12A	6
RM xTH-29A	3
RM x TH-29B	6
RM x TH-32	5
RM x TH-5B	3
RM x TH-6B	5
RM x TH-8	7
TH x RM-5	3
TH x RM -7B	9
Total	**51**

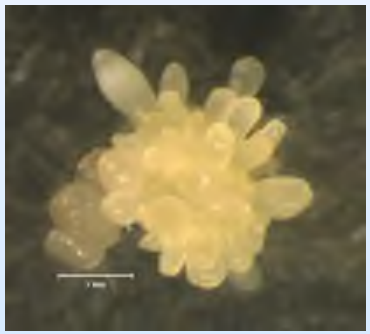
Gary Griffin with TH and RM

THxRM and RMxTH somatic seedlings in lath house

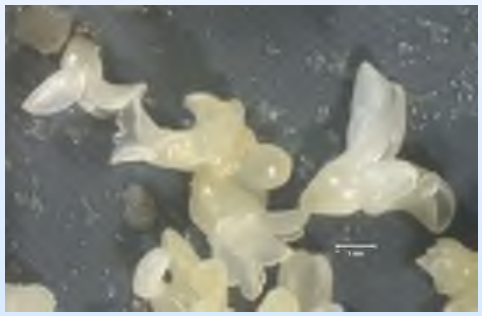
Carol Croy with RM x TH somatic seedling. None have died of blight yet.

Objective 1: Clonal Testing—Produce B3F3 and other hybrid somatic seedlings and establish them in field tests

- TACF OP B3F3 and VDF hybrid (ACxCCxJC) embryogenic cultures started in 2010, 2011
  - 138 somatic seedlings already planted by Va Tech cooperators in 2013
- TACF CP B3F3 embryogenic cultures initiated in 2012
  - 32 somatic seedlings picked up by TACF cooperators for planting at Meadowview, June 30, 2015



First CP B3F3 culture initiated August 2012



D3-21-53 x W1-31-7-1 somatic embryos

First CP B3F3 somatic seedlings

Genotype	# Plants
D3-21-53 x W1-30-6-1	6
D3-23-53 x W1-31-7-2	2
D4-17-5 x D3-23-53-1	9
D5-17-130 x W1-31-7-3	12
D5-17-130 x W1-31-7-2	3
Total	**32**

B3F3 somatic seedlings just prior to pick-up by Jared Westbrook (TACF)

How we hope to enhance TACF's breeding program using SE and cryostorage

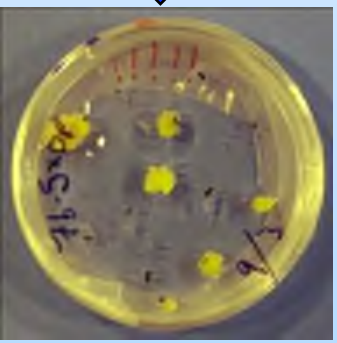
Selected B3F2 trees crossed to get B3F3 seeds

Somatic seedling production

Somatic seedlings screened/field tested

Screening Results

"Captured" SE cultures



B3F3 seeds used to start SE cultures

Copies of all cultures cryostored

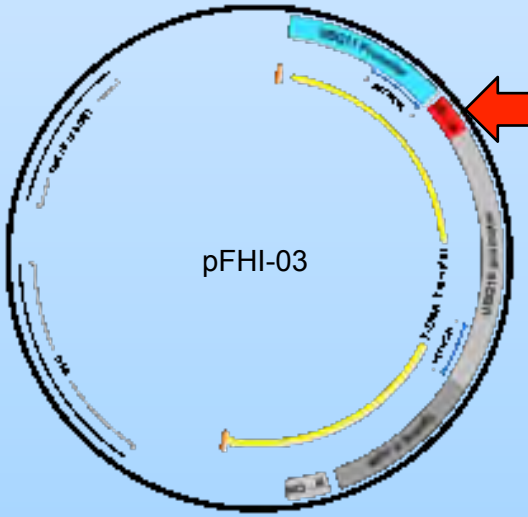
Best clones recovered from cryo and mass propagated for planting

[redacted]

27 Chestnut CGs, 5 Heterologous CGs, 3 Reporter Genes and 3 Multi-Gene Vectors

Vector Construct (*pFHI-*)	Chestnut Candidate Gene
BGLUC	Beta 1,3 Glucanase
CBS	CBS domain containing protein
DAHP	Deoxy-arabino-heptulosonate phosphate synthase
ACPHOS	Acid Phosphatase
UDPGT	UDP-glycosyltransferase
LAC	Laccase
PRP	Proline Rich Protein
THAUM	Thaumatin-like protein
ETF	Ethylene Transcription Factor
CYST	Cystatin, cysteine protease inhibitor
LTP	Lipid Transfer Protein, protease inhibitor
RPH	Resistance to *Phytophthora*
SKDH	Shikimate dehydrogenase
ACOX	ACC oxidase
TAGL	Triacylglycerol lipase
MIP	Myo inositol phosphate synthase
CAD	Cinnamyl alcohol dehydrogenase-like protein
PROX	Peroxidase
CCAOMT	Caffeoyl-CoA-O-methyltransferase
GLUC2	Glucanase; Glycoside Hydrolase Family 17
GST7	Glutathione s-transferase
LTP2	Lipid Transfer Protein 2
NPR34	Non-expressor of Pathogen Response 3/4
SBTL	Subtilisin
MAE	Malic Enzyme
PAL	Phenylalanine ammonia lyase
AOS	Allene Oxide Synthase

Vector Construct (*pFHI-*)	Heterologous Candidate Gene
GAFP	Gastrodia Anti-Fungal Protein
NPR1	Non-expressor of Pathogen Response
CAMP	Capsicum Anti-Microbial Peptide
VST	Vitis Stilbene synthase
OXO	Wheat Oxalate Oxidase
	Reporter Genes
GUSi	GUS intron
GUSiYFP	GUS intron-Yellow Fluorescent Protein fusion
GFP	Green Fluorescent Protein
	Multi-gene vectors
RGAF	RPH1 + GAFP
23RN	RPH1 + NPR34
33RNG	RPH1 + NPR34 + GAFP



Candidate Gene

FHI vectors built by Dr. Joe Nairn, UGA

Objective 2: Produce transgenic trees with candidate genes for field testing—Powell River and Kentland plantings

2015 Transgenic Trees

Gene/Construct	Genotype	Event	# Plants
23RN	TG-8A	1	4
23RN	TG-8A	10	4
23RN	TG-8A	15	4
CAD	RMxTH-22B	13	1
CAD	RMxTH-22B	15	1
CAD	WB484-3	33	1
DAPH1	WB484-3	19	1
DAPH1	WB484-3	25	1
ETF	WB484-3	23	1
ETF	WB484-3	25	1
GFP	AW3-46B	3	1
GFP	AW3-46B	8	1
GFP	AW3-46B	10	1
GFP	WB484-3	20	1
GFP	WB484-3	21	1
GFP	WB484-3	23	2
GFP	WB484-3	25	1
GUSi	TG-8A	9	13
GUSi	TG-8A	10	7
GUSi	Nagle-1E	5	1
GUSi	Nagle-1E	8	1
GUSi	RMxTH-22B	26	1
PRP	WB484-3	24	3
UDPGT1	RMxTH-22B	12	2
YFP-GUSi	WB484-3	11	3
	Total	**25**	**58**

2015 B3F3 and Wildtype Trees

Genotype	Number
76-5 x OP-2B	2
AW3-46B	8
D3-18-61-2	7
D6-26-9C	1
Nagle-1E	2
W1-31-144-9B	3
Total	**23**

58 transgenic somatic seedlings representing 25 events from 5 CGs, 3 transgenic controls and 23 B3F3 and wildtype trees were picked-up by Sara Klopf for planting at Powell River 5/16/15

PR = Powell River
K = Kentland
JJ = Joe James' Farm
UGA = Whitehall Forest (below)
UGA GH = UGA Greenhouse

Objective 2 output as of July 2015: 41 gene constructs, >124,000 somatic embryos picked,148 transgenic events in trees

Construct	Genotypes	Events on plates	SEs picked	Events w/ trees	Planting Location(s)
pFHI-GUSi	5	65	4074	13	PR, K, JJ, UGA
pFHI-GUSiYFP	4	360	1345	6	PR, K, JJ, UGA
pFHI-GFP	3	35	2696	7	PR, UGA
pFHI-NPR1	6	570	5060	6	PR, K, UGA
pFHI-THAUM	6	1,360	9812	29	PR, K, UGA
pFHI-ACPHOS	6	308	844	1	UGA
pFHI-UDPGT	7	335	4456	3	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	2	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	3	JJ
pFHI-CYST	5	334	5074	6	PR, K, UGA
pFHI-LTP1	4	171	3654	2	UGA
pFHI-RPH	3	138	5669	12	JJ
pFHI-ACOX	3	175	681	2	UGA GH
pFHI-MIP	3	205	2292	1	UGA GH
pFHI-VST	4	287	3395	1	UGA GH
pFHI-SKDH	3	242	3311		
pFHI-CAD	3	194	3101	9	PR, K, UGA
pFHI-PROX	3	282	1998	6	PR, K, UGA
pFHI-CCAOMT	5	458	272		
pFHI-GST7	4	355	0		
pFHI-CAMP	4	301	2087		
pFHI-GLUC2	4	362	663		
pFHI-TAGL	3	191	3608	3	PR, K, UGA
pFHI-SBTL	3	300	322		
pFHI-NPR34	3	318	1239		
pFHI-LTP2	3	357	509		
pFHI-AOS	3	129	2065		
pFHI-MAE	3	35	364	1	
pFHI-PAL	3	71	3164	1	UGA
pFHI-OXO	2	12	195		
pFHI-RG2	1	2	0		
pFHI-GAG	1	14	395	1	
pFHI-GUG	1	18	295		
pFHI-RGAF	3	78	3150	3	UGA GH
pFHI-23RN	3	173	1618	5	UGA, JJ
pFHI-33RNG	3	131	1017		
Total		**11638**	**124,426**	**148**	

Objective 2: Produce transgenic trees with candidate genes for field testing—Georgia planting

Gene	Genotype	Events
ACOX	AW3-46B	2
AcPHoS	RxT-22B	1
B-Gluc	WB484-3	2
CAD	76-5xOP-2B	1
CBS	76-5xOP-2B	2
Cyst	AW3-46B	3
ETF	WB484-3	2
GUSi	76-5xOP-2B	5
Lac	76-5xOP-2B	1
Lac	WB484-3	4
LTP1	AW3-46B	1
MIP	AW3-46B	1
NPR1	RxT-22B	5
Prox	76-5xOP-2B	2
PRP	RxT-22B	3
PRP	WB484-3	6
Thaum	AM54-1	2
Thaum	RxT-22B	6
Thaum	WB484-3	9
TagL	AW3-46B	2
Total		**60**

60 single tree events representing 15 CGs will be planted at UGA's Whitehall Forest in September

Objective 3: Produce trees engineered with *Phytophthora* resistance candidate genes and test them for resistance

Effect of *Phytophthora cinnamomi* infection on chestnut

Collaboration with Steve Jeffers (Clemson University) and Joe James (Carolinas-TACF) to screen transgenic somatic seedlings for *Phytophthora* root rot resistance

Dr. James with one of his screening tubs

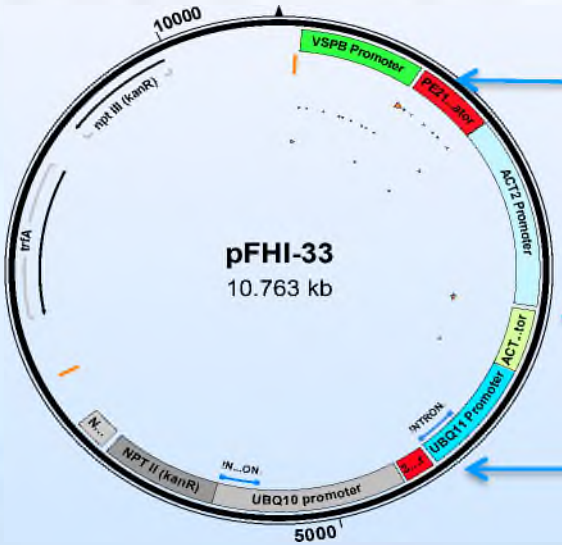
Transgenic chestnut lines with genes of interest for *Phytophthora* resistance

- *Castanea* gene constructs
  - pFHI-RPH (Resistance to *Phytophthora*)
  - pFHI-NPR3/4 (Non-expresser of pathogen response)
- Heterologous genes
  - pFHI-GAFP (*Gastrodia* anti-fungal protein)
  - pFHI-VST1 (*Vitis* stilbene synthase)
- Multi-gene constructs
  - pFHI-RGAF (RPH + GAFP)
  - pFHI-23RN (RPH + NPR3/4)
  - pFHI-33RNG (RPH + NPR3/4 + GAFP)

Only multi-gene events with high expression of all 3 *Phytophthora* resistance CGs are chosen for plantlet production

Multi-gene construct for "stacking" resistance genes

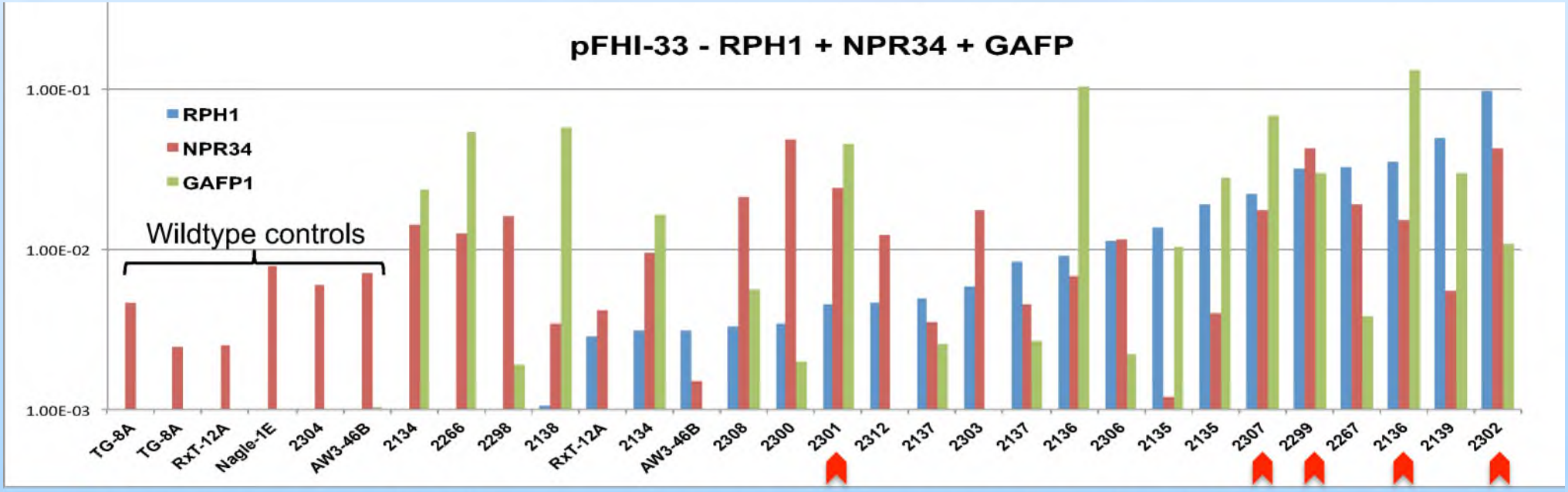
Highly variable expression from different promoters



VSPB Promoter - GAFP1

ACT2 Promoter – NPR34

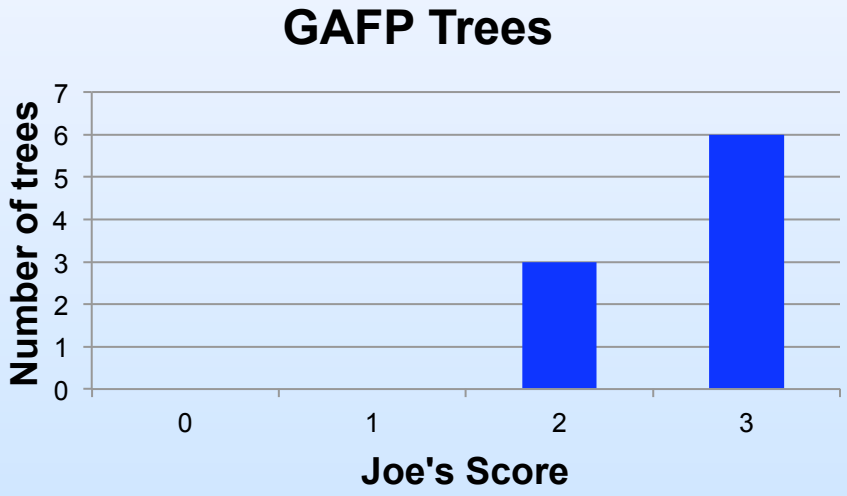
UBQ11 Promoter – RPH1

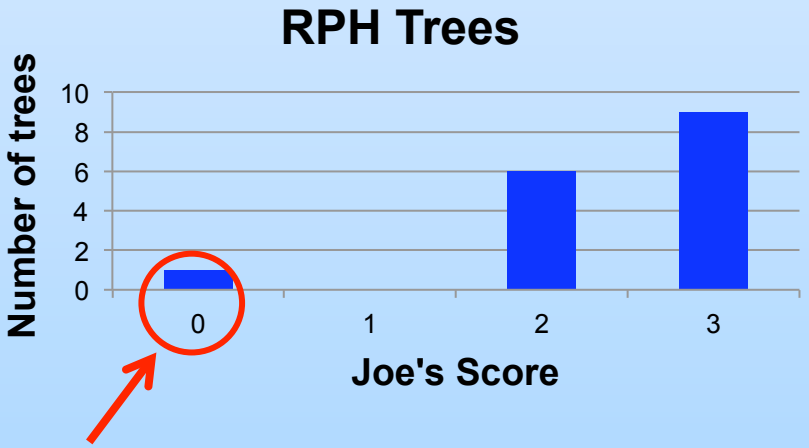


indicates event with high expression of all 3 genes we chose for plantlet production

Results of 2013 screen of transgenic somatic seedlings for Phytophthora resistance

- 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





One RPH tree showed no symptoms in 2013, but subsequently died in 2014.

Results of 2014 screen of transgenic somatic seedlings for Phytophthora resistance

Vector	Candidate gene(s)	# events	Total trees	Surviving as of 01/15
pFHI-RPH	Resistance to Phytophthora	3	3	1
pFHI-23RN	RPH and NPR3/4	2	5	2
pFHI-GUSi		2	2	2
pFHI-GUSiYFP		2	2	1
		Total trees	**12**	**6**

July 2014

December 2014

- Trees planted at James Farm 07/3/14
- Tub inoculated with *P. cinnamomi* 9/11/14
- Planting inspected by APHIS 10/22/14
- Joe supplied survival data 1/9/15

pFHI-23RN (RPH/NPR3/4) transgenics and controls available for 2015 PRR-resistance screening

Event	No. of Trees
TG-8A-1::23RN	38
TG-8A-10::23RN	22
TG-8A-15::23RN	23
TG-8A-3::23RN	7
TG-8A-9::GUSi	17
TG-8A-10::GUSi	13

Transgenic chestnuts with PRR-resistance candidate genes in lath house

Objective 4: Develop *in vitro* screen for PRR resistance/susceptibility

- 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 while resistant *C. mollissima* 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, so…
- Subsequent trials used 48-well-plates to restrict *P. cinnamomi* access to medium, and Petri plates with water agar, which does not support *P. cinnamomi* growth.

Steve Jeffers

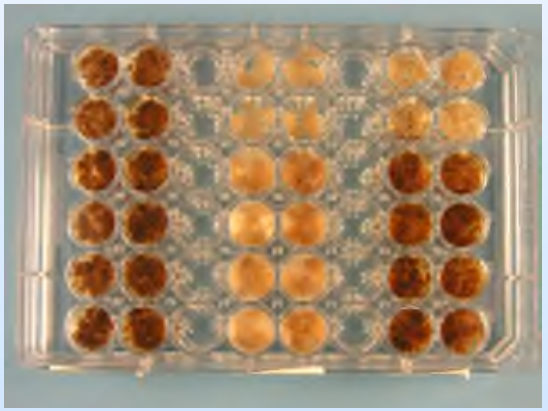
January 2015 *Phytophthora* resistance screen results using 48-well plates

American chestnut lines

Non-inoc.
Isol. #1
Isol. #52

top view – 0 DAI

top view – 7 DAI



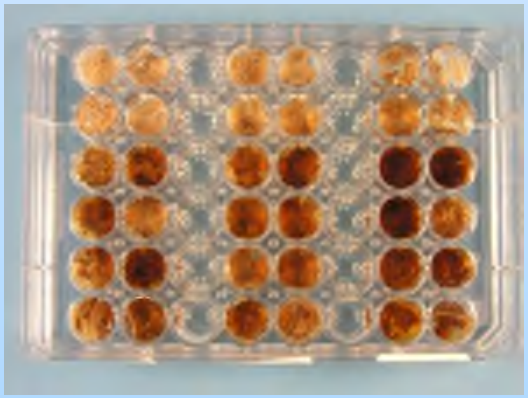
bottom view – 7 DAI

Chinese chestnut lines

Non-inoc.
Isol. #1
Isol. #52

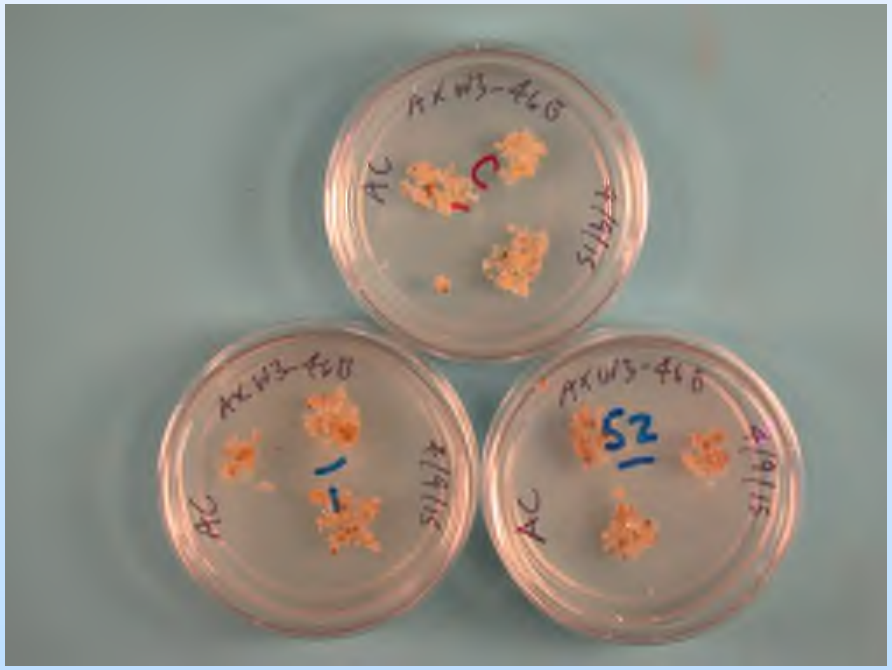
top view – 0 DAI

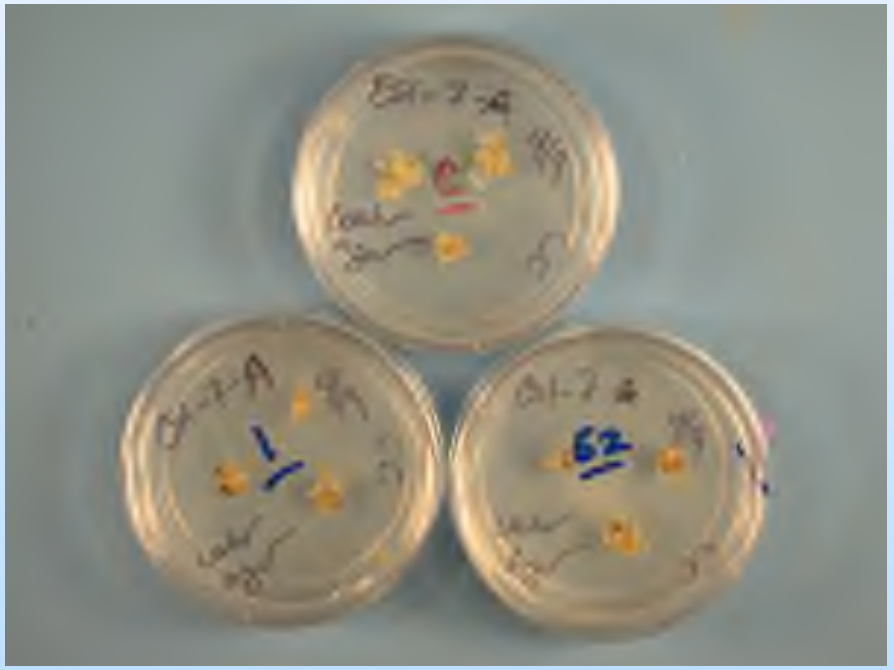
top view – 7 DAI



bottom view – 7 DAI

April 2015 *Phytophthora* resistance screen results using Petri plates with water agar



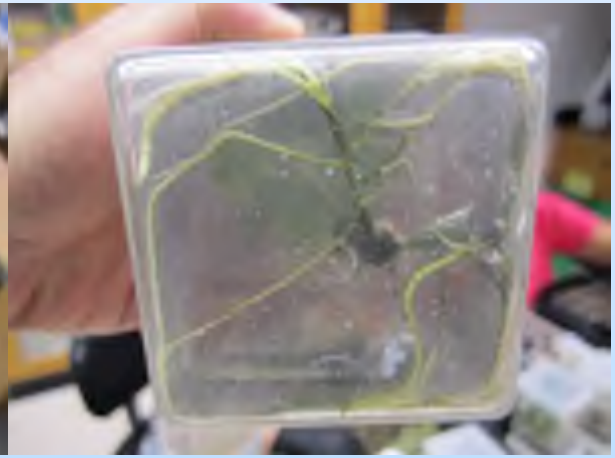


Conclusion: We were unable to distinguish PRR-resistant from PRR-susceptible chestnuts using embryogenic callus using any of the approaches we tested

Objective 5: Improve chestnut somatic seedling quality and production efficiency

- K-IBA dips of shoot-germinated embryos
- Shoot cultures to supplement germinated embryos
- RITA temporary immersion bioreactors for shoots
- ABA and GA treatments for embryo germination
- Alternative carbohydrates to sucrose
- Alternative hardening-off treatments
- Greenhouse changes

K-IBA dips improve root production from shoot-germinated somatic embryos



Shoot cultures to supplement germinated embryos

Gene/Construct	Genotype	# Events
33RNG	AW3-46B	2
	Nagle-1E	4
	TG-8A	1
23RN	AW3-46B	1
	Nagle-1E	1
	RMxTH-8	2
	TG-8A	4
	WB484-3	1
AOS	WB484-3	3
DAPH1	WB484-3	2
ETF	WB484-3	1
GAFP	WB484-3	4
GFP	AW3-46B	1
	RMxTH-22B	1
	WB484-3	2
GUSi	AW3-25B	6
	AW3-46B	3
	Nagle-1E	4
	TG-8A	2
	WB484-3	5
NPR	WB484-3	1
PAL	AW3-46B	8
	WB484-3	9
RGAF	AW3-25B	4
	WB484-3	12
RPH	WB484-3	10
TAGL	AW3-46B	1
UDPGT1	WB484-3	2
VST	WB484-3	1
YFP-GUSi	AW3-46B	3
	WB484-3	4
17	**Total**	**105**

Warnell Undergrad Researcher Cara Eck

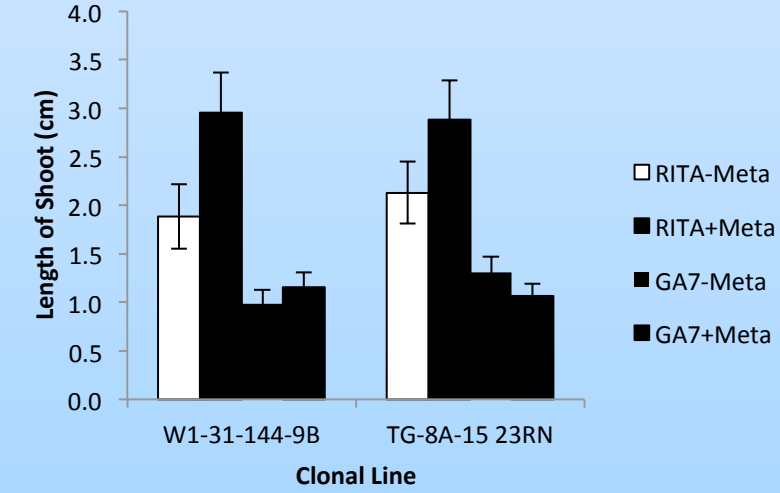
Experiment to test different GA7 closures (solid, vented, Suncap) for chestnut shoot elongation

Shoot cultures representing 17 genes/constructs and 105 events are being used to supplement plantlet production

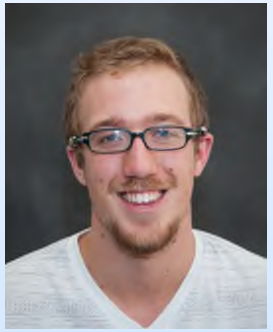
Shoot production and germination treatment experiments

RITA temporary immersion bioreactor experiment

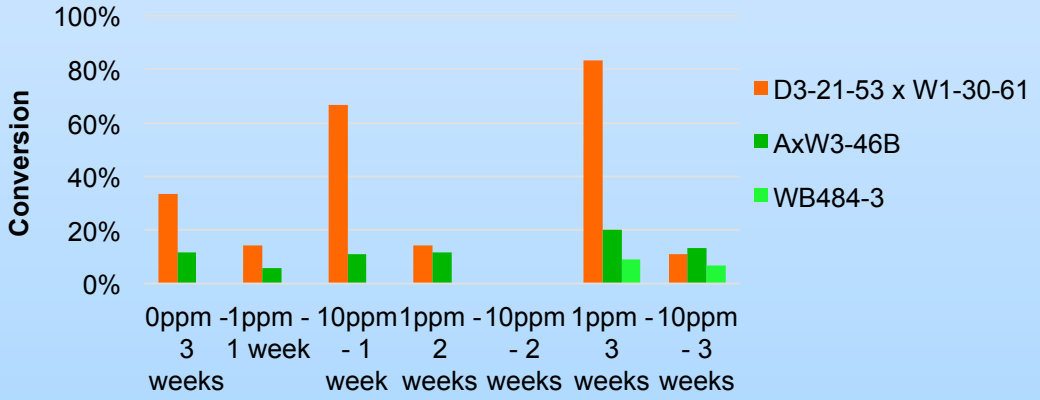
Warnell Undergrad Researcher Serenia Larrison



ABA experiment



Warnell Undergrad Researcher Nathan Polley



Greenhouse changes and alternative hardening-off treatments

UGA Hort Farm Director and PhD student Ryan McNeill

Using *Phytoseiulus persimilis* and other predatory mites for biocontrol of plant-damaging mites allows us to now grow somatic seedlings in the greenhouse through the winter

Hardening off somatic seedlings on the mist bench is being compared to hardening off in the incubator

Proposed work/deliverables for coming year

- Continue production of trees to “fill-in” somatic seedling production for B3F3s and CGs/events with insufficient numbers of somatic seedlings for replicated field tests
- Additional experiments to improve quality of plantlets produced via SE germination and shoot cultures
- Expand screening of somatic seedlings with PRR-resistance CGs (single and stacked) with Steve Jeffers and Joe James
- Test shoot-based *in vitro* screen for Phytophthora resistance by Cuenca et al. (2009) with Steve Jeffers
- Collaborate with Jeff Donahue (TACF) to initiate new B3F3 cultures from high-value crosses
- Collaborate with Joe James to initiate embryogenic cultures from some of his blight- and PRR-resistant parents

New ideas and challenges looking forward

- UGA needs help to conduct SUNY-ESF leaf assays, stem inoculations
- Do we need to engineer (not cross) *OXO* into regionally adapted American chestnut genotypes?
- Extend SE and transgenic work to Ozark chinkapin?

UGA Group Personnel

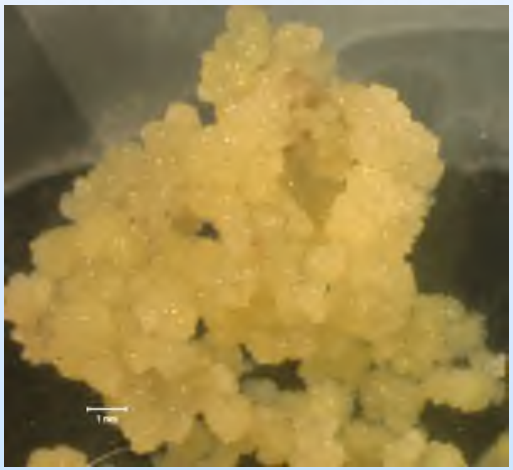
Ryan Tull
Research
Technician III
SE culture initiation
& screening

Heather Gladfelter
Research
Professional II
Transformation &
SE technology

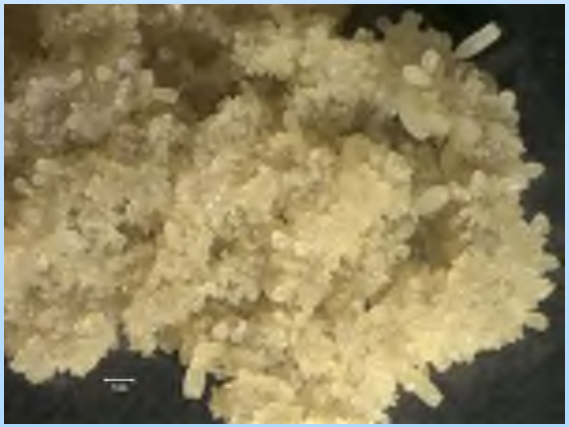
Development of ash (*Fraxinus*) SE technology for production of emerald ash borer-tolerant trees

Collaboration with Dan Herms (Ohio State University)

Seeds and zygotic embryos used as explants



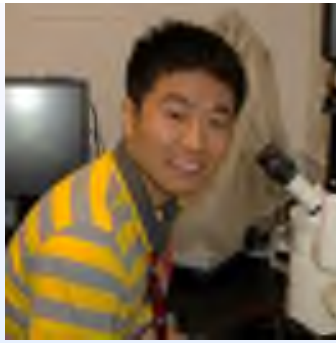
Proembryogenic masses (PEMs)



Somatic embryo production from PEMs

"Lingering ash" somatic seedlings in greenhouse

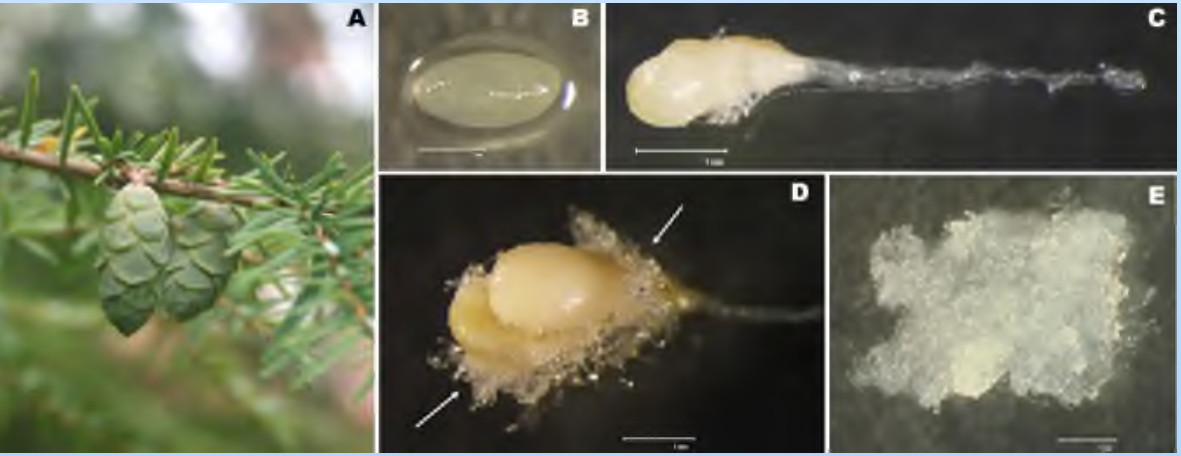
Combination of breeding and somatic embryogenesis could help restore hemlocks, too

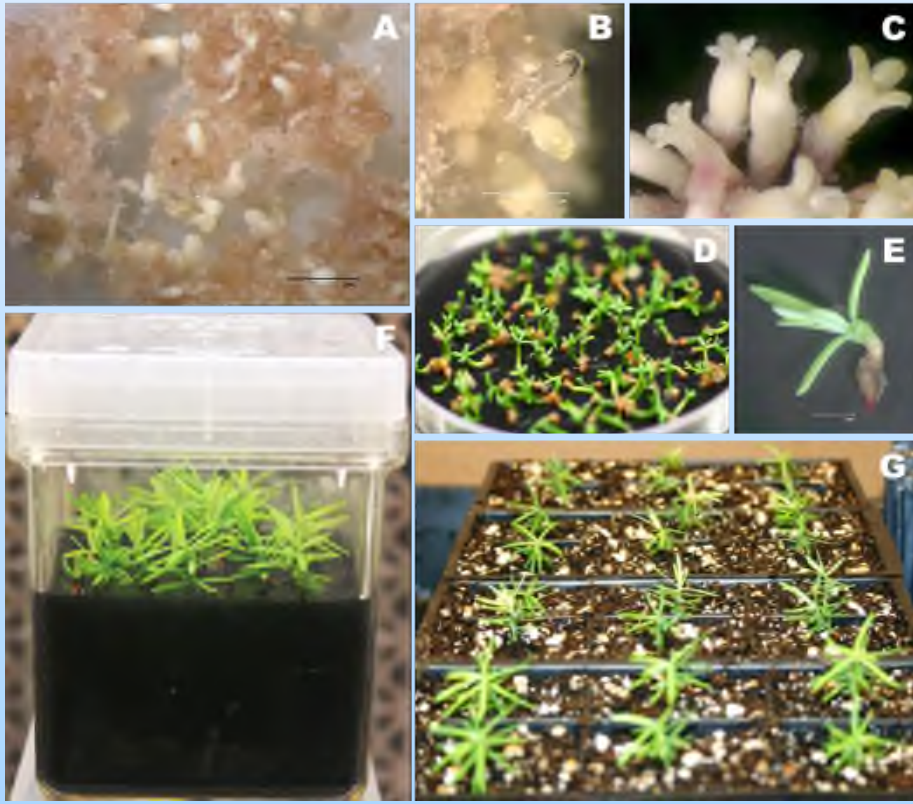


PhD student Changho Ahn

- Hemlock woolly adelgid (HWA)-tolerant hemlock clones could be produced by crossing North American and Asian hemlocks (*T. caroliniana* x *T. chinensis* and *T. caroliniana* x *T. sieboldii*) and starting embryogenic cultures from the hybrid seeds (see photos)
- HWA-tolerant hemlock native hemlock clones could be produced by starting cultures from putatively HWA-tolerant hemlocks, since they have a high level of self-compatibility (almost like cloning the mother tree)

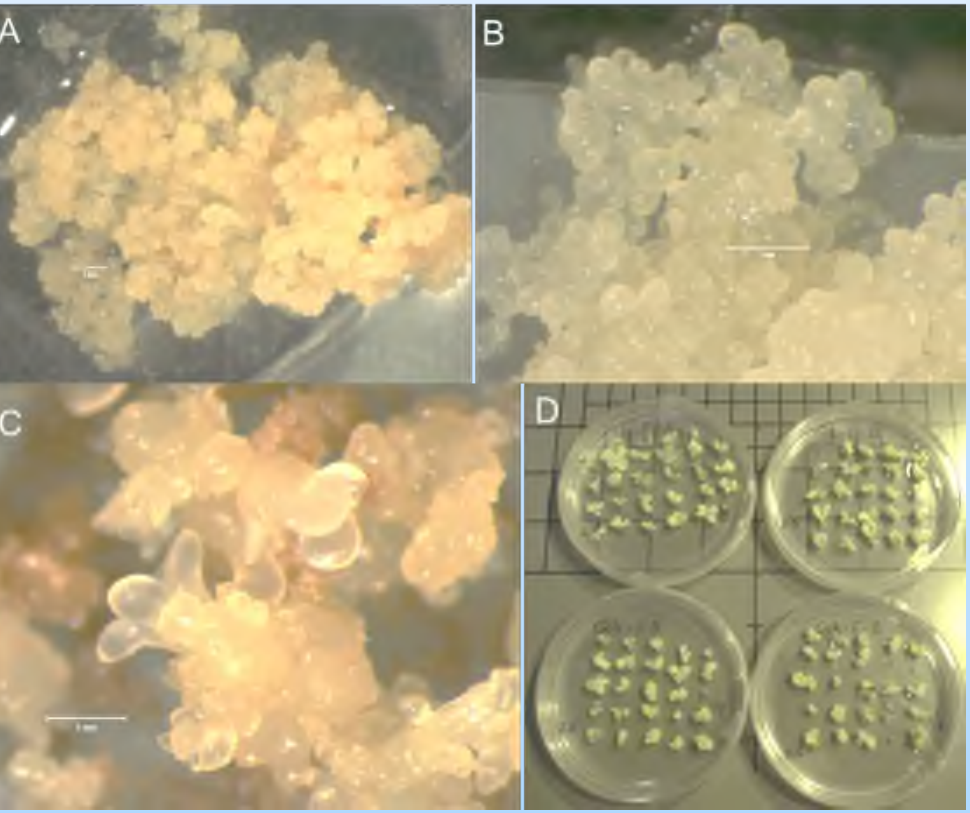
Collaboration with Alliance for Saving Threatened Forests



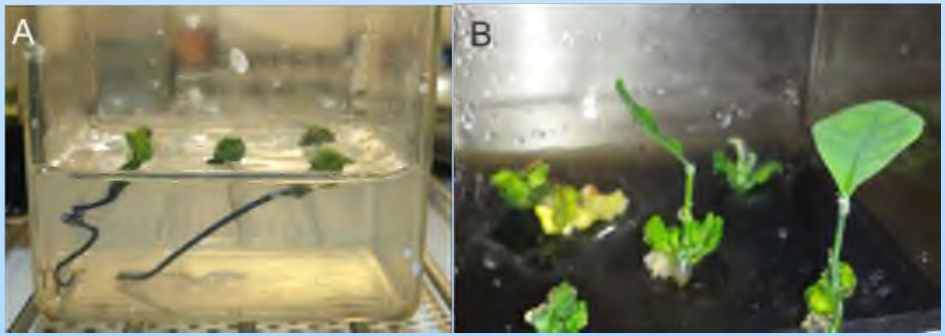


FHI collaboration has led to new collaborative proposal that includes white oak SE:

"Rapid cycle breedomics: A next generation approach to sustaining and improving fruit and nut tree crops" (Bert Abbott, PI)



White oak embryogenic cultures (initiated summer 2014) will provide transformable material for rapid cycle breeding



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