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
- LSA = Large surviving American chestnut (potentially has some 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)
- SE = somatic embryogenesis or somatic embryo
- SS = somatic seedling
- CG = candidate gene for blight and/or Phytophthora resistance
- Va Tech = Hokies

FHI Phase 2 Objectives - UGA

1. Complete production of populations of LSA and B3F3 somatic seedlings for clonal testing
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. Work with Clemson and Carolinas-TACF cooperators to produce trees engineered with Phytophthora resistance candidate genes and test them for resistance

Deliverable: Complete production of populations of LSA somatic seedlings for clonal testing

- Seeds from crosses between ACCF LSAs Ragged Mountain (RM) x Thompson (TH) cultured in 2010
- RM x TH somatic seedlings transferred to ACCF Cooperator Carol Croy (USDA Forest Service) in October 2013; will be planted out on Jefferson National Forest this season (see table)

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**

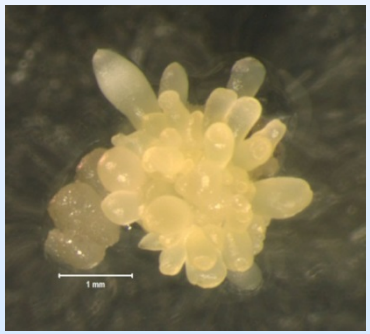
Gary Griffin with TH and RM

THxRM and RMxTH somatic seedlings in lath house

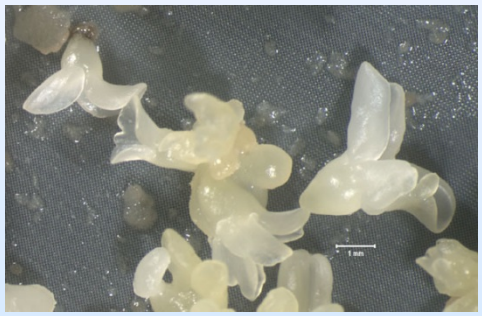
Carol Croy with RM x TH somatic seedling

Deliverable: Complete production of populations of B3F3 somatic seedlings for clonal testing

- TACF OP B3F3 embryogenic cultures started in 2010, 2011
  - Somatic seedlings already planted by Va Tech cooperators
- TACF CP B3F3 embryogenic cultures initiated in 2012
  - First somatic seedlings now in lath house--available for fall 2014 planting by Va Tech cooperators



First CP B3F3 culture initiated August 2012



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

First CP B3F3 somatic seedlings

Supplier	Mother Tree	Pollen Source	No. nuts	No. seeds	No. embryogenic cultures	Capture frequency
TACF	D5-17-130	W1-31-7	35	555	3	0.5%
TACF	W1-31-7	D3-23-53	42	621	5	0.8%
TACF	D3-21-53	W1-31-7	52	682	5	0.7%
TACF	D3-21-53	W1-30-6	34	473	7	1.5%
TACF	D4-17-5	D3-23-53	36	447	1	0.2%
TACF	D4-17-5	D4-27-78	33	476	2	0.4%
TACF	W1-31-7	D4-28-132	34	470	0	0.0%
TACF	D4-27-78	W1-30-6	24	358	1	0.3%
TACF	W1-31-7	D4-27-78	34	482	1	0.2%
Total			324	4564	25	0.5%

Deliverable: Produce B3F3 and other hybrid somatic seedlings for Powell River and Kentland plantings

VDF 76-5xOP somatic seedlings

Over 100 somatic seedlings (VDF, TACF OP B3F3) supplied to Va Tech cooperators for planting in 2013

TACF OP B3F3 somatic seedlings

Deliverable: Produce B3F3 and other hybrid somatic seedlings for Powell River and Kentland plantings

Trees Planted in May and October 2013

Genotype	Number
TACF B3F3 Trees	
D6-26-2	3
D6-26-3A	1
D6-26-9C	6
D1-26-19-1B	2
D3-18-61-2	1
D4-10-49-10	6
D4-10-49-5	8
W1-30-6-3A	1
W1-30-6-3C	1
W1-30-6-5	5
W1-30-63-2	7
W1-31-63-7A	1
W1-31-63-13A	1
W3-32-68-2	1
W1-31-144-11B	1
W1-31-144-1B	1
W1-31-144-3	3
Total	**49**

Genotype	Number
VDF Hybrid Trees	
76-5xOP-1	2
76-5xOP-2B	28
76-5xOP-3A	8
76-5xOP-3B	1
76-5xOP-5D	5
76-5xOP-6	1
76-5xOP-7A	4
76-5xOP-7B	13
76-5xOP-7C	2
76-5xOP-7G	1
76-5xOP-8	4
76-5xOP-9A	1
76-5xOP-9C	3
76-5xOP-9D	1
76-5xOP-9F	1
76-5xOP-10A	5
76-5xOP-10B	7
76-5xOP-12	2
Total	**89**

Deliverable: Produce B3F3 and other hybrid somatic seedlings for Powell River and Kentland plantings

Trees for fall 2014 planting

Clone	Number
Open-pollinated	
D3-18-61-2	2
W1-30-6-3A	1
W1-31-144-3	1
W1-31-144-9B	6
Control-pollinated	
D3-21-53 x W1-31-7-2	1
D4-17-5 x D3-23-53-1	3
D5-17-130 x W1-31-7-2	8
D5-17-130 x W1-31-7-3	7
Total	**29**

Somatic seedlings in 4 inch pots in lighted chamber

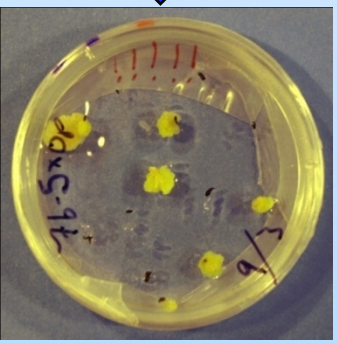
Breeding programs, SE and cryostorage are a powerful combination for chestnut restoration…and elite varietals

Selected B3F2 trees crossed to get B3F3 seeds

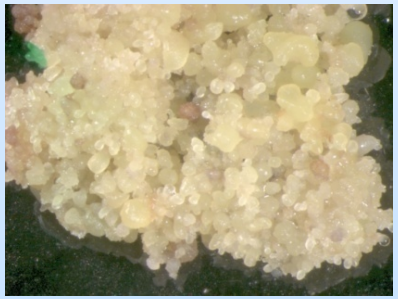
Somatic seedling production

Somatic seedlings screened/field tested

Screening Results



B3F3 seeds used to start SE cultures



"Captured" SE cultures

Copies of all cultures cryostored

Best clones recovered from cryo and mass propagated for planting

Deliverable: Produce transgenic trees with candidate genes for Kentland and Powell River plantings

About 180 transgenic somatic seedlings in UGA lath house 08/29/13, prior to transfer to Va Tech cooperators

Five years of work, 36 gene constructs, 100,000 somatic embryos picked, 122 transgenic events in trees

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

Construct	Genotypes transformed	Events on plates	Somatic embryos harvested	Number of events with trees	Planting Location(s)
pFHI-GUSi	3	65	4074	6	PR, K, JJ, UGA
pFHI-GUSiYFP	4	360	1345	6	PR, K, JJ, UGA
pFHI-NPR1	6	570	4467	5	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	2	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	1	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
pFHI-MIP	3	205	2292	2	UGA
pFHI-VST	4	287	3395	2	UGA
pFHI-SKDH	3	242	3311		
pFHI-CAD	3	194	3101	6	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	1	Just potted
pFHI-MAE	3	35	364	2	Just potted
pFHI-PAL	3	71	3164	1	Just potted
pFHI-RGAF	3	78	3150	3	Just potted
pFHI-23RN	3	173	1618	2	JJ
pFHI-33RNG	3	131	1017		Just potted
Total		**11034**	**104,076**	**122**	

Deliverable: Produce transgenic trees with candidate genes for Kentland and Powell River plantings

Trees planted fall 2013

Vector	Candidate gene	Gene source	# events*	Total trees
pFHI-THAUM	Thaumatin-like protein	Chinese chestnut (*Castanea mollissima*)	12	33
pFHI-BGLUC	β-glucanase	Chinese chestnut	4	9
pFHI-CBS	CBS domain-containing protein	Chinese chestnut	6	24
pFHI-LAC	Laccase	Chinese chestnut	7	41
pFHI-PRP	Proline-rich protein	Chinese chestnut	15	55
pFHI-GUSi (TG control)	β-glucuronidase	*E. coli*	4	9
pFHI-GUSiYFP (TG control)	β-glucuronidase plus yellow fluorescent protein	*E. coli and Aequorea victoria*	4	8
			Total trees:	**179**

*Events with at least 2 somatic seedlings

Deliverable: Produce transgenic trees with candidate genes for Kentland and Powell River plantings

Trees planted spring 2014

Vector	Candidate gene	Gene source	# events*	Total trees
pFHI-CAD	Cinnamyl alcohol dehydrogenase	Chinese chestnut	4	25
pFHI-CYST	Cystatin	Chinese chestnut	1	2
pFHI-ETF1	Ethylene transcription factor	Chinese chestnut	2	4
pFHI-NPR1	Non-expresser of pathogen response	Arabidopsis thaliana	2	5
pFHI-PROX	Peroxidase	Chinese chestnut	4	18
pFHI-TAGL	Triacylglycerol lipase	Chinese chestnut	1	3
			Total trees:	**57**

*Events with at least 2 somatic seedlings

Deliverable: Produce transgenic trees with candidate genes for Kentland and Powell River plantings

Trees available for transfer to Va Tech cooperators fall 2014

Vector	Candidate gene	Gene source	# events*	Total trees
pFHI-CBS	CBS domain-containing protein	Chinese chestnut	1	5
pFHI-DAPH	Deoxy-arabino-heptulosonate phosphate synthase	Chinese chestnut	1	4
pFHI-THAUM	Thaumatin-like protein	Chinese chestnut	1	2
pFHI-GUSi			5	15
pFHI-GFP			1	6
			Total trees	32

Deliverable: Produce transgenic trees with candidate genes for field testing in Georgia

Single tree events to be planted at Whitehall Forest Nursery fall 2014

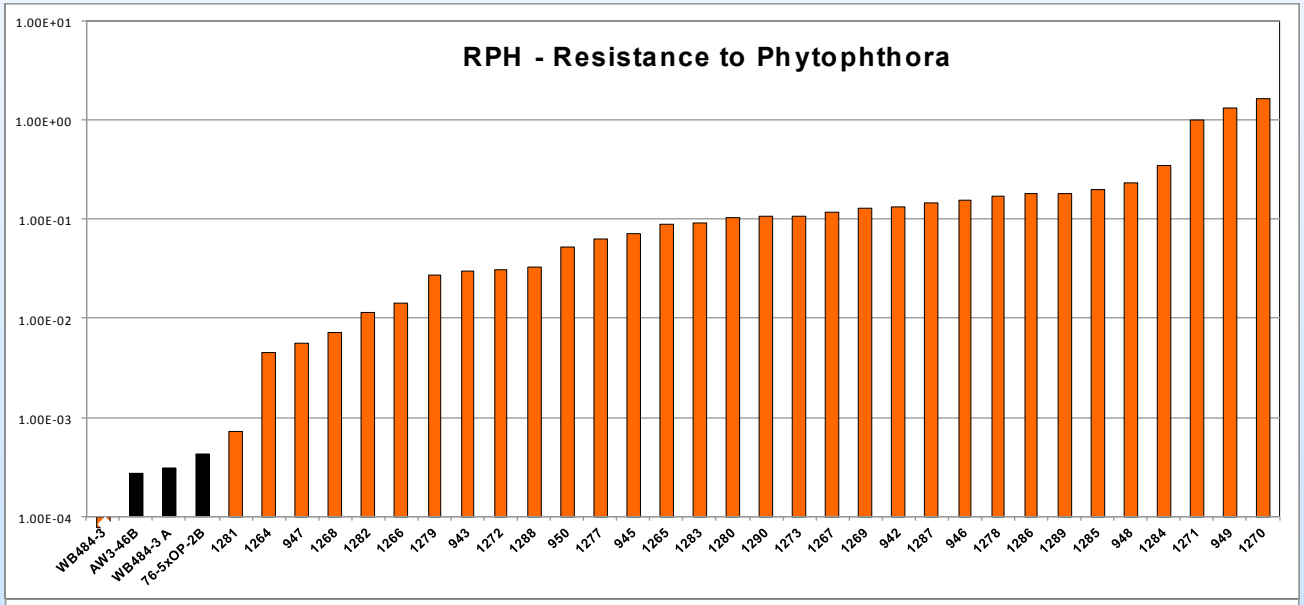
Gene	Genotype	Events
ACOX	AW3-46B	2
AcPHoS	RxT-22B	1
B-Gluc	WB484-3	2
CAD	76-5xOP-2B	1
CBS	76-5xOP-2B	2
Cyst	AW3-46B	3
ETF	WB484-3	2
GUSi	76-5xOP-2B	5
Lac	76-5xOP-2B	1
Lac	WB484-3	4
LTP1	AW3-46B	1
MIP	AW3-46B	1
NPR1	RxT-22B	5
Prox	76-5xOP-2B	2
PRP	RxT-22B	3
PRP	WB484-3	6
Thaum	AM54-1	2
Thaum	RxT-22B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

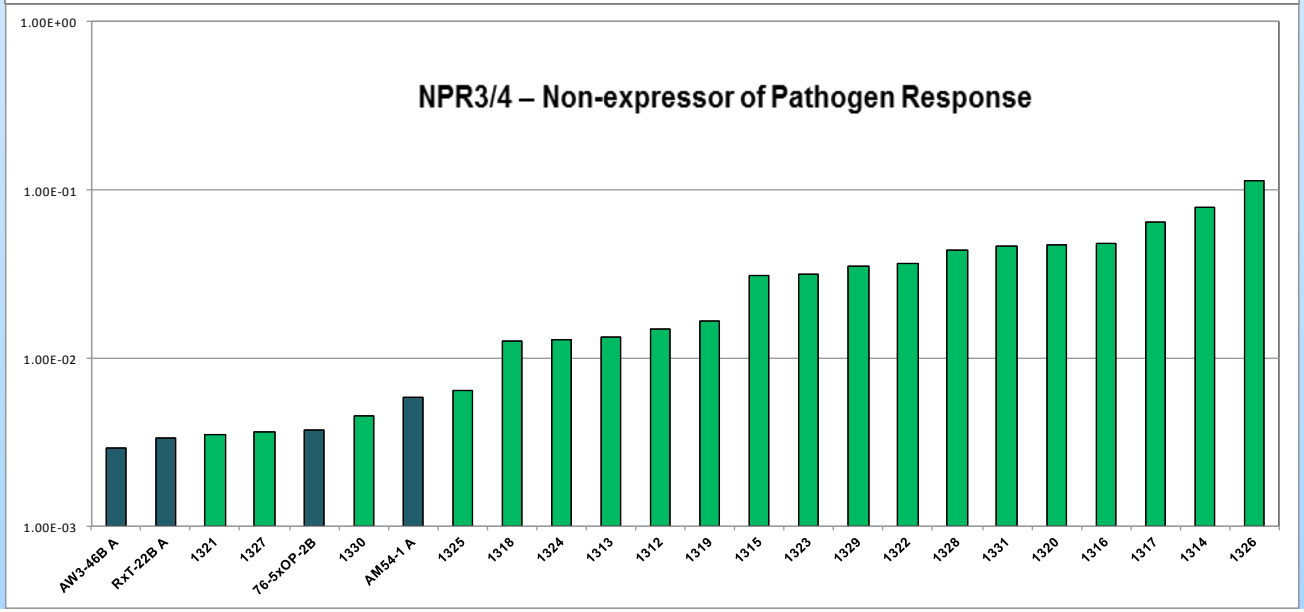
Deliverable: Work with Steve Jeffers (Clemson University) and Joe James (Carolinas-TACF) to screen transgenic somatic seedlings for Phytophthora resistance

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)
- Stacked constructs
  - pFHI-23RN (RPH + NPR3/4)
  - pFHI-33RNG (RPH + NPR3/4 + GAFP)

Expression analysis of transgenic lines with single Phytophthora resistance candidate genes



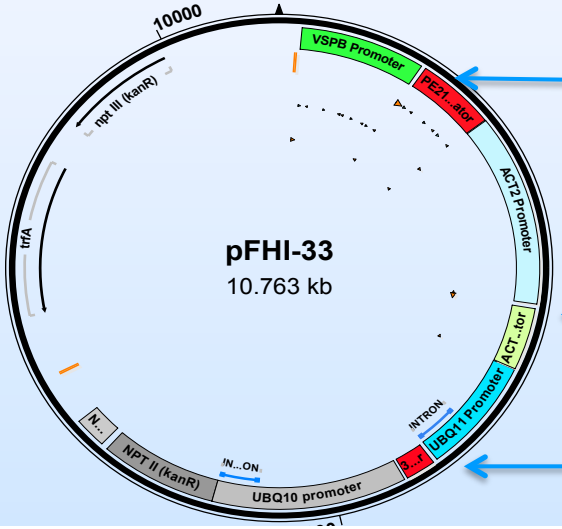


Dark bars are wildtype controls

Expression analysis of transgenic lines with stacked Phytophthora resistance candidate genes

Multi-gene construct for "stacking" resistance genes

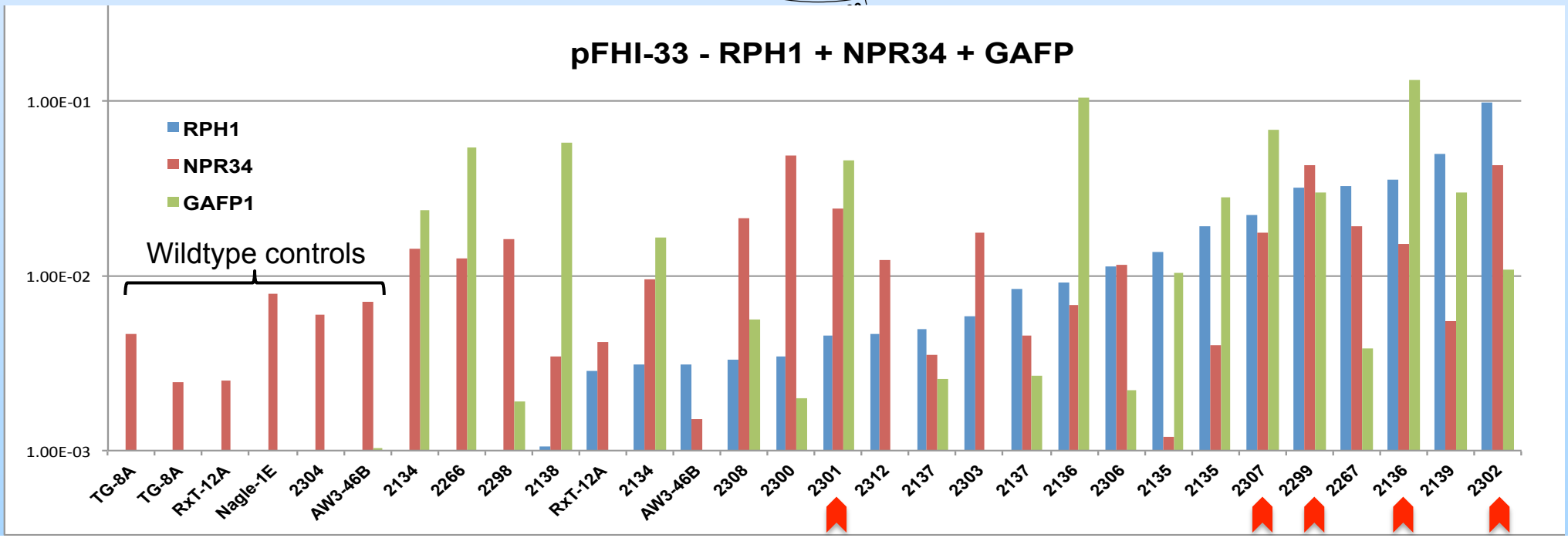
Highly variable expression from different promoters



VSPB Promoter - GAFP1

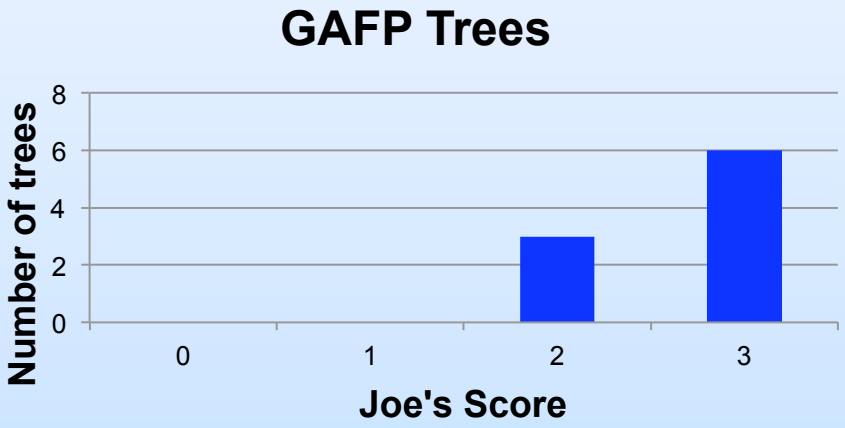
ACT2 Promoter – NPR34

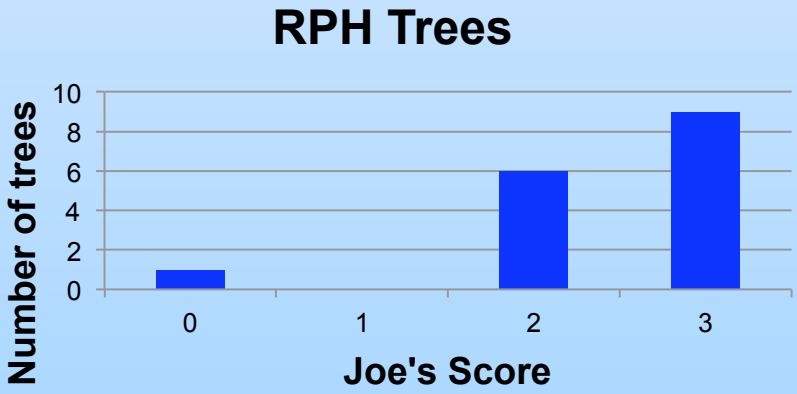
UBQ11 Promoter – RPH1



Deliverable: Work with Clemson and TACF-NC collaborators to screen transgenic somatic seedlings for Phytophthora resistance – 2013 screen

- 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





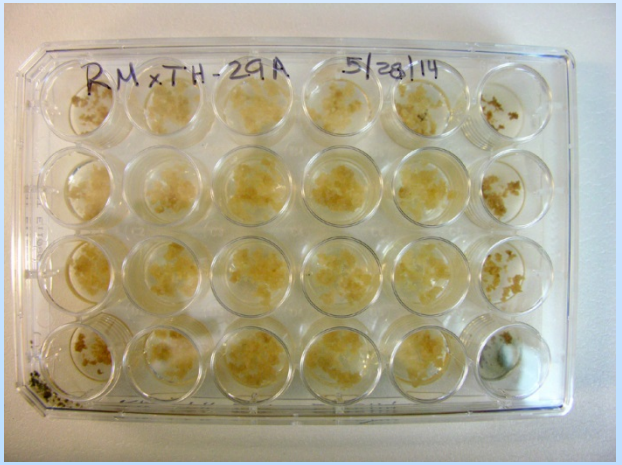
Deliverable: Work with Clemson and TACF-NC collaborators to screen transgenic somatic seedlings for Phytophthora resistance – 2014 screen

Vector	Candidate gene(s)	Gene source	# events	Total trees
pFHI-RPH	Resistance to Phytophthora	Chinese chestnut	3	3
pFHI-23RN	RPH and NPR3/4	Chinese chestnut	2	5
pFHI-GUSi			2	2
pFHI-GUSiYFP			2	2
			Total trees	**12**

Trees planted at James Farm 07/3/14

New Phytophthora Work

- 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 and resistant *C. chinensis* 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
- Second trial using 48-well-plates to restrict *P. cinnamomi* access to medium is underway



Bottlenecks and plans for improvement

- **Problem:** Plenty of events and somatic embryos, but persisting problems with low somatic embryo conversion % and low somatic seedling quality

- Testing temporary immersion bioreactors (TIBs) for production of more vigorous shoots that may root better
- Testing misting bench for rooting and hardening off
- Personnel change

RITA® TIBs with chestnut shoots

Specific objectives for coming year

- Continue to thaw cryostored cultures to “fill-in” somatic seedling production for B3F3s and CGs/ events with insufficient numbers of somatic seedlings for replicated field tests
- Apply SUNY-ESF leaf assay and begin inoculations of transgenic trees at Whitehall Forest
- Expand work to improve somatic seedling quality (previous slide)
- Produce more somatic seedlings with Phytophthora resistance CGs (single and stacked) for larger screen at Joe James’ farm in 2015
- Continue work to develop *in vitro* screen for Phytophthora resistance

UGA Group Personnel

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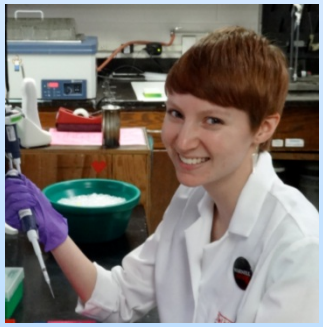

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Research Technician III
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Candidate Gene vector construction
Transgenic/transgene screening on Phase 1

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Tatyana Zhebentyayeva (Clemson)

Joe James (Carolinas -TACF)